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2010

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ADVANCES IN CHROMATOGRAPHY AND ELECTROPHORESIS

&

CHIRANAL 2010

Advances in Chromatography and Electrophoresis & Chiranal 2010

Olomouc, February, 8 – 11, 2010

CHIRANAL

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EDITORIAL

In despite of rapid development in separation science and (illusory) all-power of modern instrumentation, still a great number of non-resolved tasks appears ahead. Although many of them seem to be trivial at a first glance, a real solution is troublesome after all and requires a large knowledge together with many experiments.

We believe that the forthcoming conference, Chiranal & Advances in Chromatography and Electrophoresis, has a potential to get together experts from industry with people from research sphere and help to uncover burning issues of today separation theory and practice and outline possible approaches for their solution.

Olomouc is from regional point of view natural place for organization of Central European specialized meetings. Enclosed list of participants fully reflects the situation - majority of participants is comprised by Czechs and Slovaks, some attendants come from Austria and Poland. It is pleasant that also colleagues from Italy, United States and Turkey decided to extend the number of participants. Hopefully the symposium will be a friendly platform for exchange of experiencies and evaluation of assorted ideas without borders.

It is our great pleasure to dedicate this year symposium to one of founders of Czech and Slovak chromatographic community, professor Jaroslav Janák, in occassion of his latter 85th birthdays. We would like to wish him sound health and many indulgence with achievements of modern chromatography.

Dear colleagues, friends,

Welcome in Olomouc

Petr Bednář
Karel Lemr
Juraj Ševčík
on behalf of Organizing committee.

LECTURES

Recent advances in two-dimensional LC-LC techniques

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Introduction

The main objectives of two-dimensional (2D) LC-LC separation techniques are: 1/ significant increase in the peak capacity, i.e., in the number of sample compounds that can be separated in a single run in complex samples, and 2/ possibilities of sample separation into distinct classes on the basis of structural similarities. In addition to classical off-line and heart-cutting two-dimensional separation techniques, in-line "comprehensive" LCxLC has experienced rapid development during the past few years, mainly because - once correctly set-up - it allows automatic performance and significantly increased number of compounds separated per the time unit in comparison to single-dimension HPLC separations.

Comprehensive LCxLC: experimental consideration, data processing and presentation.

In comprehensive 2D systems, equal proportions (100% or less) of all sample components pass through different separation systems in the first dimension and in the second dimension [1]. Every part (fraction) of the sample is subjected to separation on the second column in the real analysis time [2], while the next effluent fraction from the first dimension is being collected in the interface between the first and the second dimension columns, usually an eight-port or a ten-port switching valve with two identical fraction collecting loops or two short trapping columns. All information from a two-dimensional LCxLC separation is contained in the record of the detector coupled to the outlet from the second-dimension column, where the subsequent second-dimension chromatograms are stacked side-by-side. Using appropriate software, the individual compounds in the consecutive chromatograms are identified and distinguished from each other and overlapping peaks in the fractions from the first dimension are deconvoluted. The original set of stacked chromatograms is arranged into a data matrix file, from which the peaks and valleys are transferred onto a retention plane with the separation times in the first and in the second dimension as the coordinates, where a single maximum is assigned to each peak. 2D data are finally presented in a two-dimensional plane in the form of a contour or peak apex plots.

Peak capacity, phase system selectivity and orthogonality, combinations of LC modes.

Best peak capacity is achieved in "orthogonal" comprehensive LCxLC separation systems, which do not show significantly correlated retention and separation selectivity in the first and in the second dimension [3]. This can be principally achieved by combining different LC separation mechanisms, such as reversed-phase (RP), ion-exchange (IE), size-exclusion (SEC) and normal-phase (NP) chromatography on polar adsorbents.

To avoid excessively long analysis runs, the separation time in the second dimension is limited to a rather short period (2 min or less), in which the next fraction from the first dimension is collected. Consequently, the peak capacity is considerably lower in the second than in the first dimension and the second-dimension column is usually a short one, packed with fine or superficially porous particles, or a monolithic column, to allow a high second-dimension flow-rate, which may be enhanced at a high-pressure or a high-temperature.

A longer efficient column, usually narrow-bore, to avoid excessive sample dilution and decrease in detection sensitivity, is employed in the first-dimension and is operated at a low flow rate to allow the collection of several fractions of each sample band [4]. Gradient elution is preferred in both dimensions wherever possible, as it provides significantly higher peak capacity in comparison to isocratic conditions. Matching gradients in the first and in the second dimension can significantly improve the regularity of the coverage of the available 2D retention space [5].

Development and optimization of LCxLC methods, fraction transfer modulation.
Development and optimization of 2D LCxLC separations is more complex than in the one-dimensional HPLC, as the operation conditions in the two dimensions should match to provide maximum two-dimensional peak capacity in the time available for the analysis. Column dimensions, particle size, the chemistry of the stationary phase and the composition of the mobile phase should be carefully selected to provide a matching 2D system. A peak eluting from the first dimension should be sampled in at least three fractions for the transfer into the second dimension, to maintain the resolution obtained in the first dimension [4].
The mobile phases usual in the NP and in the RP systems are often incompatible due to limited solubility and (or) to large differences in the elution strength, which seriously hinders possibilities for real-time operation of principally highly orthogonal RPxNP 2D systems. Hence, reversed phase systems with different bonded functionalities are often used in comprehensive RPxRP setups [6]. In this arrangement, it is important that a column providing higher retention be employed in the second rather than in the first dimension, which offers the advantage of on-column pre-concentration (focusing) of the analytes transferred from the first to the second dimension. Various sample transfer modulation techniques were suggested to suppress broadening of the transferred bands and a loss of resolution such as using short trapping columns instead of the sampling loop in the interface switching valve [7, 8].

Conclusion
Even though the main application domain of comprehensive LCxLC is still the analysis of biopolymers, the applications for small molecules are steadily increasing in the analysis of drugs, pharmaceuticals, natural compounds, food, metabolites, environmental pollutants, etc.

Acknowledgements
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nano-Liquid Chromatography: a very promising and useful tool in food analysis

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Introduction

Recently, it has been amply demonstrated that the use of miniaturized analytical techniques offers obvious advantages over classical one. Among them, fast separations, high sensitivity, high efficiency, limited consumption of both samples and mobile phases seems to be the most important features appreciated by a large number of those working in both research and application areas. Among miniaturized techniques, capillary electrophoresis (CE), with its several separation modes, plays a very important role in analytical chemistry since a long time. Here the separation is performed in capillaries as well as in chips making use of relatively high electric fields and assisted by a high electroosmotic flow (EOF). The flat flow profile of EOF contributes to the achievement of the high efficiency and consequently the good resolution even with very low differences in migration/retention times [1, 2].

Although the basic principles of nano-liquid chromatography (nano-LC) have been introduced in 1988 [3] this miniaturized technique has been estabilished more recently as complementary or alternative to classical LC [3, 4]. The nano-LC name has been defined in literature considering the capillary internal diameter (ID) [5]. Consequently the flow rate can be different (nano-liter or micro-liter) depending on the capillary type (empty, packed or containing monolith material). Therefore in nano-LC packed capillaries of 10-100 μm ID with flow rates of 50-800 nL/min are currently used. When higher ID are employed, e.g., 150-500 □m ID, the technique is named capillary-LC (CLC). Although theoretically nano-LC has higher sensitivity than classical technique, this is one of the drawbacks mainly due to the low sample injected volume (10-100 nL). It has been reported that decreasing the capillary ID, due to the lower flow rate, chromatographic dilution is reduced, therefore high sensitivity should be achieved [6]. Thus, in order to obtain higher sensitivities, some approaches have been experimented, e.g., i) use of sensitive detectors such as laser induced fluorescence (LIF), mass spectrometry (MS) ii) on-column focusing iii) 2D separations etc. [4]. Hyphenation of nano-LC with MS, easy to perform due to the low flow rates, offers advantages not only considering the sensitivity but also for the characterization of analytes. The last possibility is very important when analyzing real samples with complex matrices. As above briefly illustrated, nano-LC employing conventional UV or more expensive MS detectors, can be considered a powerful tool to be used for a wide number of applications in different areas. Although the applicability in the field of proteomic has been largely demonstrated [7], other

areas such as drug, environment and food analysis seems to be particularly attractive [4, 8]. A large number of foodstuff are daily consumed by human in the world because necessary to provide active compounds necessary for their life. Among them peptides, sugars, proteins, lipids etc. are the most important. The knowledge of food composition is very important in order to discover the presence of harmful compounds that can be present for several reasons, e.g., fraudulently added to the food, originated by contaminations etc. Adulteration is another important issue not only for safety but also for economy because offering product of lower quality. Miniaturized techniques with their demonstrated capabilities can be advantageously used in food analysis. As an example, in this communication, we report the analysis of phytosterols in extra-virgin olive oil by means nano-LC.

Experimental

nano-LC experiments were carried out in a laboratory assembled instrument utilizing an HPLC pump equipped with a splitting device to obtain flows at nL levels. The split system consisted of a stainless steel tee connected with the pump (peek tube 50 cm x 130 μm); another entrance was connected to the waste using a silica capillary (50 cm x 50 μm). The third entrance was connected with the injection valve (stainless steel tube, 3 cm x 500 μm). Fused silica capillaries (100 μm ID, total and effective length, 25 and 20 cm, respectively) were slurry packed (15 cm) with RP18 sub 2 μm (hydride-silica particles). UV detection was on-column at 195 nm. Optimum conditions for the separation of brassicasterol, cholesterol, stigmasterol, campesterol and β-sitosterol were obtained using isocratic elution with a mobile phase containing only methanol. Coupling nano-LC with MS, the same mobile phase also contained 15 mM of ammonium acetate. Confirmation of separated compounds present in real samples (extra-virgin olive oil) was done using an ESI-Ion Trap mass spectrometer (Thermo-Finnigan). The column was connected to a nano-electrospray interface with a capillary tip (25 μm ID) positioned just in front of MS orifice at about 2 mm. Sample preparation was done following previously published methods employing liquid-liquid extraction after saponification [9].

Results

Figure 1 shows a scheme of the nano-LC instrument, laboratory assembled, used for the analysis of phytosterols in extra-virgin olive oil. The system is very simple and allows to perform analytical separation mainly using isocratic elution mode with good repeatability. Attention must be paid to avoid band broadening due to: i) any dead-volume, e.g., optimizing connections and capillaries (ID); ii) injection (appropriate volumes, 10-100 nL) and iii) detection cell of low volume. Concerning the last point is noteworthy mentioning that the best detection to obtain the highest efficiency and resolution is obtained using on-column detection realized preparing the detector window closely to the second frit after removing the polyimide layer of the capillary for about 0.5 cm. The described instrumentation was used for the analysis of sterols such as brassicasterol, cholesterol, stigmasterol, campesterol and β-sitosterol that can be found in vegetal oils and in the so called “novel food”, e.g., yougurt. Analytes are relatively hydrophobic and therefore capillaries were packed with RP8, -phenyl, -cholesteryl, -CN or RP18 silica particles of different size (5, 3 and sub-2 μm). All columns had the same length (15 cm packed). Mobile phases based on methanol (MeOH), acetonitrile (ACN) alone or mixed with water were evaluated in order to separate the four sterols. Best separation was obtained using the RP18 sub-2 μm particles eluting with MeOH. Indeed the two structural related compounds, namely stigmasterol and campesterol were not baseline

resolved. However their separation was not achieved in HPLC [10]. The optimized method was validated achieving good results, e.g., intra-day and inter-day precision of retention time were in the range 0.93-1.18 and 3.22-4.15 %, respectively; limit of detection (LOD) and limit of quantification (LOQ) were 0.78 and 1.56 μg/mL, respectively. Recovery was very good in the range 90-103 %. Encouraged by the above described results, the method was applied to the analysis of sterols present in two samples of extra-virgin olive oils. The chromatograms revealed the presence of all four sterols in the case of Algerian oil while the commercial product did not contain brassicasterol. In addition some peaks, eluting before the sterols were observed and, tentatively, corresponded to δ-, γ-, α-tocopherol. The confirmation of sterols present in olive oil samples was done running experiments with the nano-LC coupled with MS. Good mass spectra signals were observed for all analytes with a main signal peak corresponding to the fragment ion resulting from loss of one water molecule $[M+H-H_2O]^+$. Several stationary phases were tested for the nano-LC method optimization.

Conclusion

The obtained results demonstrate the potentiality of nano-LC in food analysis. A laboratory assembled instrument equipped with UV and MS detectors allowed the separation of phytosterols using packed capillary columns containing RP18 particles of low size. Good results (qualitative and quantitative) were obtained in short analysis time (about 20 min).

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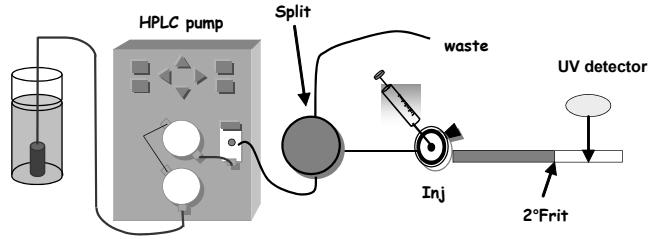

Fig. 1: Scheme of the nano-LC instrumentation used for the analysis of phytosterols.

Mixed-mode liquid chromatography: Potential and applications

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Introduction

Mixed-mode liquid chromatography makes use of separation materials that combine at least two distinct orthogonal or complementary separation principles in one column. Such materials have reached considerable popularity in solid-phase extraction and are also well known from capillary electrochromatography where ionizable groups have been attached on reversed-phase (RP)-like stationary phase surfaces for sake of EOF generation. Mixed-mode materials attracted significant attention recently also for the separation of proteins e.g. antibody purification.

Only recently, attempts were made by several researchers to use mixed-mode materials for preparative and analytical separations of small polar molecules and peptides. We were focussing our interests on the development of mixed-mode reversed-phase/weak anion-exchange (RP/WAX) type stationary phases which turned out to be very versatile adsorbents for peptide separations and analysis of hydrophilic metabolites [1-3].

In this presentation, the potential of these mixed-mode materials which combine hydrophobic and ion-exchange type interaction principles in a single chromatographic ligand will be illustrated. Such stationary phases can, in dependence of the solutes' structures and the utilized elution conditions, be employed in multiple modes (reversed-phase, anion-exchange, ion-exclusion, hydrophobic interaction chromatography, and hydrophilic interaction chromatography mode). Solutes may be separated by lipophilicity or charge differences, or a mixture of both. Thus, they offer a great versatility during method development and optimization. This enhances the chance for obtaining complementary methods to RP which may be valuable for the control of assay specificity of routine RP analysis methods. Moreover, mixed-mode RP/AX type stationary phases have shown to possess enhanced loading capacities for synthetic peptide purification as compared to RP materials. These major advantages will be outlined in this presentation.

Experimental

A RP/WAX stationary phase with structure as shown in Fig. 1a was employed. The material was based on 100Å 5 µm spherical silica particles and the column dimension was 250 x 4 mm ID. For the separation of the tetrapeptide a mobile phase consisting of 10% (v/v) acetonitril in acetic acid buffer (175 mM) (pH 4.5 adjusted with ammonia) was used (Fig. 1c). The oclumn temperature was 25°C and the flow rate 1 mL/min. The chromatogram was monitored by UV detection at 316 nm. For comparison a Beckman Ultrasphere (ODS) (100Å, 5 m) column was employed with dimension 150 x 4,6 mm ID. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in water (A) and 0.1% TFA in acetonitrile (B) and a linear gradient from 5 to 60% B was run in 20 min (Fig. 1b).

Results

The potential of this "mixed-mode" RP/WAX material for synthetic peptide separation is shown in Fig. 1. The N- and C-terminally protected diagnostic tetrapeptide, *N*-Acetyl-Ile-Glu-Gly-Arg-p-nitroanilide, is prepared in a 9-step synthesis and chromatographically purified. In RP-HPLC one of the major impurities closely elutes on the tailing edge of the main peak (Fig. 1b). In preparative separations the resolution is lost due to overloading the purification is therefore very inefficient and unproductive. In contrast, the RP/WAX column has complementary retention and selectivity profiles and the impurity is resolved with much better resolution. Moreover, it elutes close to the solvent front while the main component is strongly retained. Comparative loadability studies on RP/WAX and RP showed that the loadability on RP/WAX is much higher and the productivity could be improved by a factor of about 13 as compared to RPLC purification.

Another aspect is that common strategy in peptide synthesis is to perform both preparative purification and analyticl quality control on the same RP stationary phase. Thus, the chance of detecting impurities that coeluted in preparative run is negligible. If quality control is performed on a complementary separation material such as RP/WAX, the probability to detect impurities is significantly raised. This is in particular of relevance for very hydrophilic peptides that elute early in RPLC with usually low selectivity for impurities. The presented RP/WAX column has turned out as a valuable complementary tool for preparative peptide purification and analytical quality control which we recommend as a second generic analysis method for synthetic peptides in support to RPLC.

Conclusion

The mixed-mode RP/WAX column presented in this communication is a flexible tool for preparative separation and analysis of synthetic peptides and polar compounds.

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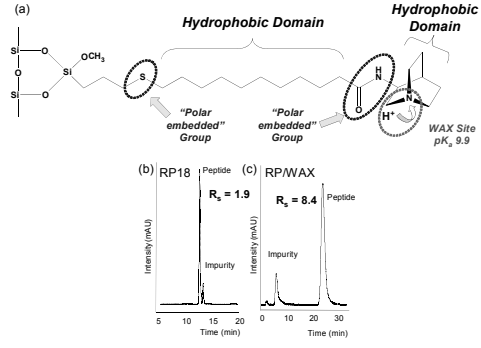

Fig. 1: Structure of RP/WAX material (a) and comparison of separations of synthetic tetrapeptide *N*-Acetyl-Ile-Glu-Gly-Arg-p-nitroanilide on RP18 (b) and RP/WAX (c).

Recent applications of electromigration techniques in forensic drug analysis

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Introduction

Nowadays, the use of illicit drugs still represents an important social and medical problem all over the world. Among the illicit drugs, heroin, cocaine, marihuana and amphetamine and its analogues are the most used. It is well-known that the use of these compounds, in addition to determine a psycho-physical harm, can be dangerous for the healthy, causing cardiac arrhythmias, cardiovascular and respiratory failures [1]. On this basis, the determination of these compounds either in seized materials or in biological fluids is relevant in both forensic and clinical analysis.

In forensic drug testing, two independent methods are required to report a sample as positive. Immunoassays methods are generally used because they are fast and offer adequate sensitivity in most cases, but they lack the specificity to distinguish particular drugs within the drug classes. For this reason, confirmation separation methods are usually required. Chromatographic techniques such as gas chromatography (GC) and high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) have been employed for the determination of illicit drugs. GC-MS, which is often the method of choice for screening of drugs of abuse, requires a derivatization step, which is time consuming and may contribute to sample loss [2]. Therefore HPLC and HPLC-MS are currently the separation techniques mostly used in that area. In fact, they can analyze polar, thermally labile and non volatile compounds [3]. In recent years, capillary electrophoretic (CE) techniques also coupled to MS have become popular in the analysis of seized drugs in illicit preparations and in biological samples [4].

Aim of the overview

The high power of separation and resolution, rapid analysis time, low consumption of reagents and minimum sample requirements, high level of automation make CE techniques an attractive methodology for forensic analysis and a routine tool for the analysis of seized drugs. Applications in forensic drug analysis include the use of capillary zone electrophoresis (CZE), chiral separations, micellar electrokinetic capillary chromatography (MEKC), and more recently capillary electrochromatography (CEC) [5].

Aim of this communication is to overview the recent results obtained using CE and CEC for the screening and determination of illicit and abused drugs in urine and hair samples [6].

In latest years, CEC, an emerging analytical separation technique, which combines the high peak efficiency of CE with stationary phase selectivity of LC, has given valuable results in the separation of drugs of abuse [7, 8].

The hyphenation of the CE techniques with mass spectrometry will be also shown. Indeed, a significant enhancement in the analysis of drugs in biological samples has been achieved coupling the electromigration techniques with mass spectrometers as detectors, mainly because in forensic analysis high specificity and sensitivity are requested [9,10].

Conclusion

These methods have proved to be suitable for rapid, sensitive and unequivocal confirmation of the presence of drugs of abuse in both clandestine preparations and biological samples of abusers. These analytical tools are simpler and more versatile than the traditional methods based on GC, requiring derivatization procedure, or based on HPLC. CE methods are complementary to existing analytical methods in forensic drug analysis, owing to several advantages such as low costs, requirement of small sample volumes, use of small amounts of solvents and/or expensive chemicals and short analysis time.

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Antimony(III)-Tartrate: Chiral Selector, Therapeutic, and New Insight on the Electrospray Ionization Process

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Introduction

Antimony(III)-tartrate has been known since medieval times as an emetic drug, and since then has had a turbulent history as an indication for the treatment of bacterial and parasite infections [1]. To date, little is known about its mechanism of action in biological systems. Reports of severe side effects have limited its use, and presumably investigations of medicinal activity. More recently, antimony(III)-tartrate has been shown to be useful as a chiral selector in liquid phase separations and for co-crystallization by diastereomeric complexation [2, 3]. These findings have sparked our interest in the use of mass spectrometric techniques to further investigate the enantioselective recognition behavior of the compound. A variety of experiments, along with theoretical studies, have allowed us to gain a better understanding of the behavior of this versatile compound [4]. The data have provided new insight, and often unexpected results, which have allowed us to develop novel hypotheses regarding its therapeutic use and physical binding characteristics. Furthermore, our most recent studies have revealed the ability of antimony(III)-tartrate to trap unusual solvent reaction intermediates, presumably formed during negative ionization mode electrospray ionization (ESI), which provide an unprecedented view of ESI processes.

Experimental

Antimony(III)-*D*- and *L*-tartrates were synthesized and purified in-house. Enantioselective recognition behavior towards a variety of enantiomerically-pure natural amino acids was studied using straightforward competitive binding ESI-MS and collision threshold dissociation MS/MS techniques [5] in an LCQ Deca XP ion trap mass spectrometer. Experimental work was supported by theoretical calculations using density functional theory and *ab initio* (MP2) computations. Additional studies, using low temperature ^{1}H and ^{13}C NMR have also been performed to help confirm findings. In the study of electrospray-induced solvent reaction products, the response of antimony(III)-tartrate adducts has been investigated in a variety of standard hydroorganic solvent mixtures using normal and deuterated solvents.

Results

Enantioselective recognition experiments, using ESI-MS, and supported by theoretical calculations, showed that antimony(III)-tartrates exhibit proton-assisted enantioselective binding behavior. Diastereomeric complexes formed with deuterated and non-deuterated amino acids are enantioselective in the 1- charge state and show no discrimination in the 2-charge state (antimony(III)-tartrate is a dianionic compound) [4]. Interestingly, when

antimony(III)-tartrate was analyzed alone by ESI-MS, the appearance of a number of unexpected adduct ions drew our interest. By varying the composition and nature of the solvent system, the abundance and location (m/z) of the adduct ions changed. Through this systematic variation, the adducts have been tentatively assigned as radical recombination products, produced as a result of homolytic cleavage of solvent molecules exposed to the high voltage supplied in the negative ionization mode. We hypothesize that antimony(III)-tartrate, particularly the dianion form, acts as a "chemical trap" for solvent reaction products produced during negative ionization mode ESI, and allows their visualization in the mass spectrum. Figure 1A shows a representative mass spectrum for this study.

Conclusion

Our investigations of antimony(III)-tartrate through negative ionization mode ESI-MS and MS/MS have revealed some interesting results that first, have allowed us to gain insight into the physical binding characteristics, particularly enantioselective recognition behavior, of this compound. Such studies are believed to be relevant to its therapeutic application. Additionally, close inspection of ESI mass spectra have shown that this compound may also provide a unique window into some of the most fundamental aspects of the ESI process. This talk will provide an overview of our studies performed to date on this very interesting compound.

Acknowledgements

Support for this work by the National Science Foundation (CHE-0846310), the University of the Texas at Arlington, and the Welch Foundation (Y-0026) is gratefully acknowledged.

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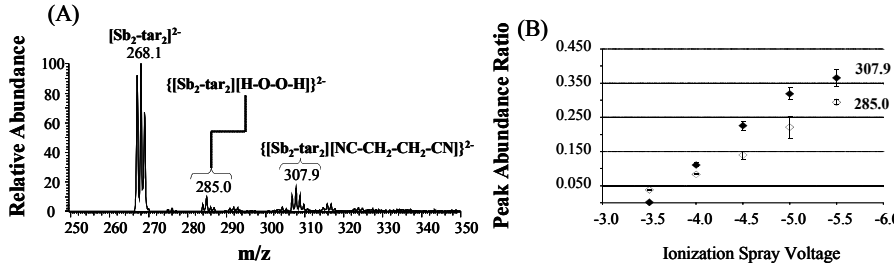

Fig. 1: (A) Negative mode ESI-mass spectrum of antimony(III)-*L*-tartrate (100 µM) in H_2O/ACN (50/50 v/v) produced by applying a - 4.0 kV ionization spray voltage (relevant solvent reaction products are labeled as assigned based on systematic studies). (B) Plots indicating the change of peak abundance ratios (calculated with respect to the peak at 268.1 Th) for peaks at 307.9 Th (♦) and 285.0 Th ( ) with the change in applied spray capillary voltage.

Zwitterionic Chiral Ion exchangers: Synthesis and Chromatographic Evaluation for the Enantiomer Separation of Chiral Acids, Bases, Amino Acids and Peptides

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Introduction

Powerful analytical and preparative methods for enantiomer separations in chromatography, especially for chargeable chiral analytes (selectands, AAs), are connected to the concept of chiral ion exchangers; in concrete: chiral anion exchangers (1) chiral cation exchangers (2), and chiral zwitterionic ion exchangers (3).

Chiral weak anion exchangers (AX type stationary phases, CSPs) based on Cinchona alkaloid derivatives have already been extensively investigated by us for the separation of chiral acidic compounds of a broad structural range [1]. Also complementary enantioselectively weak and strong cation exchangers (CX) type CSPs have been developed by us and studied for the enantiomer separation of chiral amines [2]. And as newest developments we concentrate our efforts on zwitterionic CSPs [3].

Experimental

By the chemical fusion of characteristic structural elements of chiral CX and AX to one selector (SO) entity one can create zwitterionic low molecular mass chiral selectors (SOs) to be immobilized onto silica thus creating zwitterionic CSPs. Conceptual and experimental details will be presented together with the chromatographic evaluation of the resulted CSPs with respect to enantiomer separations of the title analytes.

Results

In any case ion exchange mechanisms dominate globally the process of retention together with stereodiscrimination aspect derived form a spectrum of intermolecular interactions between the SO and SA molecules. This holds for aqueous but also non-aqueous polar organic elution conditions for all three classes of chiral analytes: acids, bases, and zwitterions. In this context the effects of acid-to-base ratios of mobile phase additives to the mobile phase as well as the type and nature of co- and counterions were examined for the various enantiomer separations.

An inherent characteristic feature of zwitterionic selectors and CSPs was found to be the intramolecular counterion sites that guide and modulate the elution behavior of ionizable analytes. Most interesting, however, was the observation that these novel zwitterionic CSPs bind particularly well zwitterionic analytes due to cooperative effect of the ionizable sites of SAs and SO. This becomes particularly obvious for the separation of chiral peptides and peptide like compounds.

Conclusion

The concept of fusing purposely a chiral acidic structure motif with a chiral basic molecular motif to one chiral selector (SO) moiety with multiple chiral centers opens up a novel way of

optimizing specifically the enantioselectivity of a target compound via a combinatori approach. In parallel also the search for more broadly applicable ion exchanger type CSPs can be envisioned.

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Capillary zone electrophoresis on-line coupled with capillary isotachophoresis offers very significant analytical benefits

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CE with a hydrodynamically opened separation system, as favored by Jorgenson, currently dominates in (bio)analytical practice (the single-column with 75-100 ěm I.D.s or so). However, such a CE concept is limiting for some relevant CE runs. For example, in this context, it should be stressed: (a) relatively high concentration limits of detection (cLOD) for the analytes, (b) restrictions for very differing concentrations of the analytes and (c) minimized resolutions for highly complex sample matrices. Apparently, the capillary with a higher I.D., while using a restrictor at the end of the capillary (e.g., CE for a 180 μm I.D. capillary as ending with a 50 μm I.D. restrictor [1]), is solving for some of the mentioned limits.

Further, the opened system, while operating for an EOF transport, might be restrictive in using ITP (see, a standstill operation for ITP [2, 3]). Obviously, such a restriction is eliminated by using the hydrodynamically closed separation system with a suppressed EOF (see, as clearly recognizable on reading the fundamental chapters by Vacík as included for the electrophoresis book [4]). Apparently, both the opened and closed separation systems are analytically rather restrictive for the separation as based on the single-column technology, especially, for multicomponent mixtures of constituents. On the other hand, it is analytically very beneficial by using the column-coupling technology while including the column-switching operation (CC-CE).

Undoubtedly, the opened system, electrophoresis with the hydrodynamic and EOF transports, it is very complex to join the all transport processes, especially, as these might have very significant velocity fluctuations [5]. Using CC-CE with the closed separation system (eliminating the hydrodynamic and EOF transports) is analytically combined, for example, ITP with CZE. In this context, it should be stressed highly reproducible for the migration velocities of the sample constituents by ITP-CZE and, in addition, offering specific benefits as using for the ITP and CZE stages.

Very currently, it was developed ITP-CZE with using discrete spacers (DS) as these were operating for the ITP(DS)-CZE combination. Such an approach is significantly enhancing for the CZE separation, especially, as joined with the use of ITP. It is very important to note, as the CE simulation, based for the concept by Gaš et al., documents clear analytical benefits by operating the ITP(DS)-CZE combination.

Acknowledgements

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Conducting polymers: Application in chemical analysis

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Introduction

Organic conducting polymers have been extensively studied for various technological applications over the last 25 years. They have applications in various areas of chemical analysis. These include in particular chemical sensors [1,2], enzymatic biosensors and immunosensors [3-4]. Conducting polymer modified electrodes can also be employed for amperometric or potentiometric detection of some biomaterials. In this communication, some interesting applications of conducting polymers such as polyporphyrine, polyaniline/chitosan and polythiophene/TiO_2 composites will be discussed from the viewpoint of their use as sensors and potential materials for separation techniques.

Experimental

Conducting polymers were synthesized by using chemical or electrochemical polymerization methods. The polymers were characterized using FTIR, SEM analysis methods and electrochemical methods. Conducting polymer modified electrodes were immobilized with enzyme or ODN using physical sorption. Sensor response was investigated by amperometric, potentiometric or fluorescence methods.

Results

Figure 1 shows amperometric response of substituted polyaniline/chitosan based sensor to glucose. The steady-state current was measured in 10 ml of 0.10 M phosphate buffered solution (pH 7.4) under a polarizing potential of 0.6 V versus SCE at 25°C. Then, an increase in time-dependent current with the oxidation of hydrogen peroxide was recorded as shown in Figure 1. For determination of glucose concentrations, the reaction with glucose oxidase (GOD) enzyme electrode can be written:

$$\text{glucose} + O_2 \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2 \quad (1)$$

$$H_2O_2 \longrightarrow O_2 + 2\,H^+ + 2\,e^- \quad (2)$$

The amount of hydrogen peroxide produced in equation (1) is usually determined by the amperometric method by oxidation at the working electrode according to equation (2). The response current, time and concentration of all samples were compared.

For polyporphyrine, a biological recognition can be monitored by comparision with electrochemical signal (CV) of single and double strand state oligonucleotide. The oxidation

current of double strand state oligonucleotide is lower than that of single strand, that corresponds to decrease of electroactivity of porphyrin with increase of stiffness of polymer structure (Figure 2).

Conclusion

Amperometric and potentiometric sensors with electrodes modified by conducting polymers offer interesting detection properties as proven for sugars and oligonucleotides. These devices can be easily applied in separation techniques.

Acknowledgements

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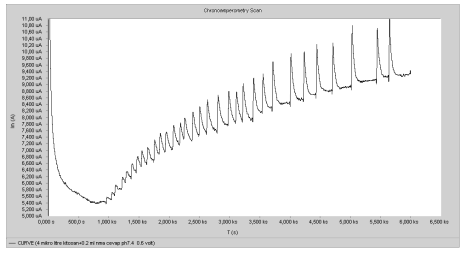

Fig. 1: Amperometric response of PNMANI/Ch/GOD enzyme electrode applying a potential step to +0.60V in 0.10M phosphate buffer solution (pH 7.4) at 25 ^{o}C.

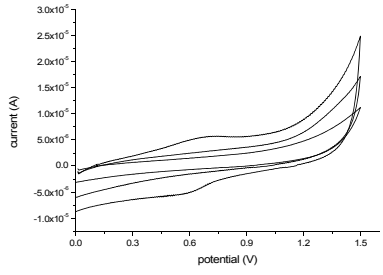

Fig. 2: Cyclic voltammograms of the (a) PAPADPP, (b) PAPADPP/ODN and (c) ODN hybridized PAPADPP/ODN electrodes in acetonitrile/$LiClO_4$ (Scan rate 100mV/s).

Comparison of anthocyanin profile of 17 red wine cultivars using μLC/ESI-MS

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Introduction

Anthocyanins represent a wide group of natural colorants which are recently thoroughly studied for their positive effects on human health [1, 2]. Content and composition of these pigments with protective antioxidant activity are specific for each coloured plant tissue and can be used for identification and verification of authenticity of fruit products [3]. Combination of high efficient separation techniques with mass spectrometry quickly became a common analytical method for fulfilling these demands [4]. The aim of this communication is to evaluate and compare anthocyanin profile in 17 red wine cultivars over three years by means of micro-column liquid chromatography with electrospray ionization-mass spectrometric detection.

Experimental

Samples of red 17 red wine cultivars, vintage 2005, 2007, 2008 were obtained from Central Institute for Supervising and Testing in Agriculture, Oblekovice, Czech Republic. Anthocyanins in red wine samples were refined using SPE (Strata SDB-L Tubes, Phenomenex). Analyses were performed using mLC/MS method (CapLC XE System and Micromass Q-Tof Premier Mass Spectrometer, both Waters, Milford, USA). Chromatographic separation was performed using a micro-column Gemini C-18 (150x0.3 mm, Phenomenex). Gradient elution was utilized with mobile phase A – 0.12% (v/v) of trifluoroacetic acid, 5% (v/v) of acetonitrile in water; mobile phase B – 0.12% (v/v) of trifluoroacetic acid in acetonitrile; gradients: 0-5 min 10% B, 5-10 min 10-20% B, 10-30 min 20-30% B, 30-40 min 30-50% B, 40-50 min 50-70% B, 50-54 min 70-100% B, 54-59 min 100% B; flow rate of mobile phase 5mL/min. The electro spray (ESI+, Z-spray) was used as an ion source for the mass spectrometer.

Results

It is well known that the elution order of anthocyanins when using C-18 type column is closely related to their polarity, with more polar compounds eluting first [5]. According to this fact the first in elution order was delphinidin followed by cyanidin (both minor), petunidin, peonidin and finally the most abundant malvidin derivatives. Diglucosides are more polar than related monoglucosides. Acylation on the other hand leads to further loss of polarity (Fig. 1). The optimized mLC/MS method was used for identification and quantification of anthocyanin content in 17 red wine cultivars of 2005, 2007 and 2008 vintage. Obviously, the content of particular anthocyanins strongly depends on seasoning of grapes (weather conditions) and therefore the direct comparison of particular cultivars for fast

identification conditions) and therefore the direct comparison of particular cultivars for fast identification is impossible. However, when relative data are utilized (e.g. a ratio of monoglycosylated and acylated derivatives for each cultivar) a certain specific differences among cultivars are revealed. For example, Blaufrankish, Rubinet and Pinot Noir exhibit substantially higher monoglycosides/acylmonoglycosides ratio with respect to other cultivars (Saint Laurent, Cabernet Sauvignon, etc.). Also, some cultivars form specific anthocyanins (malvidin-3-p-coumaroylglucoside-5-glucoside and corresponding peonidin derivative). Significant content of those less usual dyes was observed in Rubinet and only a trace amounts in Agni and Alibernet. This fact can be used to identify and confirm this kind of teinturier and possibly its use for detection of a wine color enhancement. A detailed statistical evaluation is now in progress.

Conclusion

Three years mLC/MS study of anthocyanin dyes in complete set of Moravian red wine varieties proved a number of well known glycosylated and acylated anthocyanins but some less common dyes were found. As results showed, the direct comparison of content of identified dyes does not allow distinguishing among varieties. The only important exception was observed in case of Rubinet (all three vintages), where a significant amount of 3-p-coumaroylglucoside-5-glucoside of malvidin and peonidin was proven. Beside, some interesting dependences among relative values coming from anthocyanin contents were observed, allowing sorting varieties to several groups. As can be expected, the total content of anthocyanins depends on weather conditions over the year. Though, several cultivars showed to have more stable anthocyanin production (Ariana, Saint Laurent) than others (Alibernet, Cabernet Sauvignon, etc.). mLC/MS proved to be an excellent tool for study of anthocyanin profile in wines in the long term.

Acknowledgements

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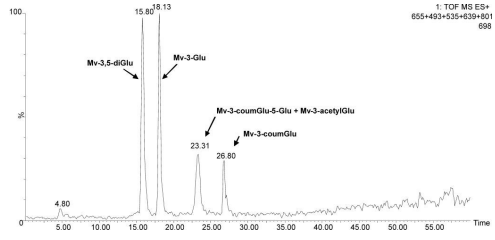

Fig. 1: Chromatogram displaying separation of malvidin derivatives, Rubinet, 2005.

Determination of Antidiabetic Drugs by Capillary Electrophoresis with Mass Spectrometry

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Introduction

The International Diabetes Federation calls the diabetes as a epidemic of 21st centry and alarming that we should take some action against it to avoid the high economic and human costs. This disease takes a long time and we can not cure it yet. The only way to cost-saving treatment for diabetes is a good treatment. Concerning that all pharmaceutical investigetions, therapeutic drug monitoring or pharmacokinetics studies are important.

Various therapeutic agents are available for management of patients with 2 type diabetes (85-90 % the main type of diabetes in the population). There are now five classes of oral medications which are available in therapy: sulfonylureas, glinides, biguanides, thiazolidinediones and alpha-glucosidase inhibitors. Rosiglitazone belongs to thiazolidinediones and might be used in monotherapy or in combination with metformin (biguanide) or a sulfonylurea (glitazone). The commercially available combination 1000 mg of metformin hydrochloride and 2 mg of rosiglitazone was used in our study.

Drugs levels were monitored individually both in biological samples and pharmaceutical formulations [1-17]. Analytical procedures included potentiometric, spectrophotometric determination, capillary electrophoresis (MEKC, CZE), and high performance liquid chromatography with different detection techniques (fluorescence, UV or MS). The study concerning both drugs determination was carried out by the liquid chromatography with UV [19] or MS/MS detection [18, 20].

The hypnethation of capillary electrophoresis with mass spectrometry allows the quantitative and qualitative drugs determination in blood plasma and could be a great alternative to HPLC analyses.

Experimental

The CE measurements were carried out using the CE instrument HP ^{3D}CE, (Agilent technologies, Waldbronn, Germany) equipped with diode array detector and connected to an Agilent G6130 single quadrupole mass-selective detector. Uncoated fused-silica capillary (MicroSolv Technology Corporation, NJ, USA) of 50 ěm id, with 21.5 cm of the effective length was used. Injection was performed by pressure 50 mbar for 5 s. The applied voltage was +25 kV for experiments carried out on standards and +20kV for blood plasma due to the current leakage which appeared in a few experiments. All of the measurements were performed five times unless stated otherwise.

Results

The proposed method demonstrates the potential use of capillary electrophoresis with mass spectrometry (CE-MS) for pharmaceutical analysis. It was developed for separation and simultaneous determination of rosiglitazone and metformin in plasma. To obtain good and stable ionization and sensitivity a few different parameters were studied. The choice of the

sheath liquid (SL) composition, flow rate, the effect of drying gas temperature and the voltage applied on the spraying capillary were discussed. The method allowed drugs separation within 11 minutes, using the 50 mM formic acid as a background electrolyte at +20 kV. The sheath liquid was composed as 1:1 methanol:water mixture with addition of 0.5% formic acid at 0.2 mL/min flow rate. The blood sample was collected from patient suffering on diabetes mellitus and prepared by simple precipitation method with acetonitrile.
The calibration curves were linear over the concentration range 10-1000 ng/mL for rosiglitazone and 2-400 ng/mL for metformin, with correlation coefficient that exceeded 0.997. The limit of quantification (LOQ) was based on the standard deviation of the response and the slope and was 10.21 ng/mL for rosiglitazone and 5.66 ng/mL for metformin.

Conclusion

This simple method could be a good alternative for HPLC analyses for the routine separation, identification and determination of metformin and rosiglitazone in plasma.

Acknowledgements

The financial support of the research by the Research Project MSM6198959216 is gratefully acknowledged.

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Spolupráce se soudními znalci – rozhodující nástroj k objasňování a vyšetřování násilné trestné činnosti

Luboš Valerián

Krajské ředitelství Policie Moravskoslezského kraje, služba kriminální policie a vyšetřování, Ostrava, Česká Republika

Souhrn

Příspěvek provádí rozbor celkem dvou případů, kdy různí byli pachatelé, různý byl způsob spáchání a zakrývaní činu a rozdílný byl i motiv činu. Co však uvedené případy spojovalo, byla spolupráce se soudními znalci a využití rozmanitých vědních oborů. Právě tato spolupráce se ukázala jako jeden z rozhodujících nástrojů k objasnění závažných násilných trestných činů a usvědčení jejich pachatelů. Maximální úsilí kriminalistů, využití celé škály kriminalisticko-taktických metod a úzká spolupráce se soudními znalci z různých oborů a odvětví přispěly k tomu, že všechny případy právně kvalifikované nakonec jako trestné činy vraždy byly objasněny a pachatelé těchto činů byli postaveni před soud a spravedlivě potrestáni.

Klíčová slova

Vražda, soudní znalci, znalecké posudky, spolupráce se znalci.

Úvod

Odhalování a vyšetřování násilné trestné činnosti patří mezi jedny z nejsložitějších činností policie. Nejen proto, že objektem těchto trestných činů je lidský život a zdraví, ale zejména proto, že policie musí velmi pozorně obstarávat důkazy, později sloužící k usvědčení pachatele. Vyšetřování takovýchto případů je složitější o to, že v popisovaných případech, ke kterým došlo na území Moravskoslezského kraje, bylo k objasnění skutečností, důležitých pro trestní řízení, potřeba i odborných znalostí. Bez úzké spolupráce se soudními znalci z různých oborů a odvětví, jakož i bez možnosti využít různých vědních oborů, by se nepodařilo usvědčit pachatele těchto činů, což byl předpoklad k jejich spravedlivému potrestání.

Materiál a metodika

Při zpracování příspěvku bylo využito v praxi zpracovaných vyšetřovacích spisů, včetně podkladů státních zástupců, kteří v jednotlivých případech podávali obžaloby. V neposlední řadě se autor opíral o pravomocné rozsudky příslušných soudů. Použita byla stávající metodika vyšetřování vražd a podezřelých úmrtí tak, jak je zpracována současnými autory. Bylo využito doporučení optimalizace kriminalistických metod při vyšetřování násilné trestné činnosti. V daných případech šlo o trestnou činnost pachatelů, a to ať už po přípravě na čin či bez ní, ale zejména se snahou o utajení svého násilného jednání. Dalšími kritérii byl mechanismus usmrcení, způsob provedení činu, jeho utajení a motivace pachatelů. Vycházelo se z počátečních a následných vyšetřovacích situací.

Výsledky
V této části je nutno se zmínit o stručné charakteristice jednotlivých případů.

1. **Neobvyklý nález cizího tělesa při pitvě-klíč k odhalení nebezpečného sexuálního devianta**

S nástupem roku 1991 šetřila ostravská policie případ nálezu mrtvoly 33-ti leté ženy, která ležela na dně terénní úpravy (tzv. francouzský dvorek) jedné ze základních škol ve Frýdku-Místku. Tělo bylo ve spodní části obnaženo a prvotním ohledáním soudním lékařem byla zjištěna mnohočetná tržně-zhmožděná poranění obličeje, hlavy a bodnořezné rány na krku. Šlo jednoznačně o smrt násilnou způsobenou cizí osobou. Po zjištění totožnosti zemřelé a sestavení tzv. časového snímku jejího pohybu bylo potvrzeno, že poté, co dne 31.12.1990 ukončila pracovní směnu v Rehabilitačním ústavu Hrabyně, kde pracovala, se ve večerních hodinách zúčastnila silvestrovské zábavy v místním kulturním domě. Úsilí kriminalistů směřovalo ke zjištění, s kým se na této oslavě setkala, event. s kým kulturní zařízení opustila. Silvestrovské oslavy se zúčastnilo více než 400 hostů, přičemž neexistoval žádný jmenný přehled hostů, příp. evidence rezervací.
Zlom v pátrání přinesly závěry soudních znalců, obor zdravotnictví, odvětví soudního lékařství. Mimo závažných poranění hlavy, krku, horních i dolních končetin byla zjištěna rozsáhlá devastující poranění oblasti malé pánve. V pochvě zemřelé pak znalci nalezli kruhovitou čepičku, jak se později ukázalo, zátku výbušného prostředku zn. PETARD. Příčinou smrti bylo udušení vdechnutím krve při současném epidurálním krvácení, za souběhu dalších smrtících poranění. Znalci konstatovali celkem tři smrtící mechanismy souběžně. Chronologicky popsali fáze útoku jako aktivní tupé násilí proti obličeji, původně neozbrojenou rukou, kopání obutou nohou, útok proti obličeji a hlavě lahví vína, řezná poranění krku střepy a barotrauma s poraněním orgánů malé pánve a pochvy, způsobené tlakovou vlnou po explozi petardy, založené v pochvě.
Výbušný prostředek sloužil k vyvolání zvukového efektu. Tento je vyvolán výbuchem pyrotechnické slože v uzavřené kartonové trubičce, uzavřené právě nalezenou čepičkou. Chemická a pyrotechnická expertiza potvrdila, že petardy obsahují 1,2 g pyrotechnické slože citlivé na tepelný podnět. Složobsahuje jako hořlaviny hliník a hořčík, jako okysličovadlo slouží chloristany a chlorečnany sodné nebo draselné. Ke zkoumání biologického materiálu zemřelé (zmineralizované vzorky pochvy) bylo využito metody atomové absorpční spektrometrie (atomový absorpční spektrometr AAS, fy Varian Spektra 30/40). Metoda prokázala, že sledovaná část pochvy vykazuje o dva řády vyšší obsah hliníku než srovnávací část, tedy oproti 0,013 ug/g to bylo 3,6 ug/g, což je téměř třistanásobné zvýšení oproti normálu. I obsah hořčíku byl nesrovnatelně vyšší (35 ug/g proti srovnávacímu vzorku, kde byl obsah pod 1 ug/g).
Sdělení o použití petardy při útoku na zavražděnou byla rozhodující pro tipování pachatele. Podařilo se zjistit výskyt 24 letého recidivisty, který petardy na silvestrovské oslavě používal a který byl viděn v přítomnosti zavražděné. Tohoto se později podařilo zadržet v jednom z místních hotelů v okamžiku, kdy se pokusil o sebevraždu podřezáním žil. V dopisech na rozloučenou vinu za uvedený čin popíral. Po předestření důkazů však usmrcení ženy doznal, popíral však sexuální motiv. Ten objasnili soudní znalci z oboru zdravotnictví, odvětví psychiatrie, psychologie, kteří konstatovali přítomnost sexuální deviace-sadismu jako motivující prvek jeho jednání. Odbrzďujícím mechanismem jeho jednání byl vliv alkoholu při nedodržování nařízených pravidel ambulantní sexuální léčby. Zarážející na tomto případu je, že pachatel činu byl již v roce 1987 odsouzen za opakovaná napadení žen, která prováděl

s nožem v ruce a která byla sexuálně motivována. Vše vyvrcholilo sexuálním útokem na 9-ti letou dívku. Poté, co byl odsouzen k nepodmíněnému trestu odnětí svobody v délce 4,5 roku s uložením ústavní psychiatricko-sexuologické léčby, mu byl na základě amnestie prezidenta republiky trest zkrácen. Po propuštění na svobodu v lednu 1990 došlo dokonce ke změně ústavní léčby na ambulantní, jejíž podmínky stejně nedodržoval. To vše umožnilo nebezpečnému sexuálnímu deviantovi spáchat zločin nejtěžší.

1. Nešťastná náhoda, nebo vražda?

Policie ČR se v praxi setkává s případy, které se zpočátku přinejmenším jeví jako nešťastná náhoda. Ve skutečnosti však může jít o předem promyšlenou vraždu. Dne 8. ledna 2002 začala policie v Novém Jičíně prošetřovat případ úmrtí 30-ti letého muže z České Třebové, který byl nalezen mrtev v nákladním prostoru skříňové nadstavby nákladního vozidla zn. Avia. To v době šetření stálo u benzinové stanice u obce Libhošť. Zemřelý vozidlem cestoval společně s dalšími spolujezdci, kteří však seděli v kabině řidiče. Přivolaná lékařka konstatovala, že jde o otravu oxidem uhelnatým spojenou s hypoxickou zástavou srdeční. V nákladovém prostoru nadstavby vozidla byly totiž instalovány čtyři teplometové hořáky napojené na propan-butanovou plynovou láhev. Bylo provedeno ohledání místa nálezu mrtvoly s dokumentací stavu mrtvoly a zejména stavu vnitřního vybavení nadstavby vozidla, zejm. teplometů, plynové láhve, ventilů apod. Soudní znalci z oboru zdravotnictví, odvětví soudního lékařství při soudní pitvě konstatovali jako příčinu smrti akutní otravu oxidem uhelnatým, jež se začal vyvíjet v důsledku nedokonalého hoření a spalování plynových hořáků těles, které hořely za výrazné spotřeby kyslíku v malém prostoru uzavřené a nevětrané nadstavby nákladního vozidla. Chemicko-toxikologické vyšetření stanovilo v krvi zemřelého 88,5% karboxyhemoglobinu.

Další šetření potvrdilo, že uvedené vozidlo řídil 27-mi letý Pavel Š. bez řidičského oprávnění, vozidlo nemělo platnou technickou prohlídku. I přesto policisté umožnili dotyčnému po vysvětlení situace z místa odjet. Na pozdější výzvy k zastavení již nijak nereagoval a zmíněné vozidlo nebylo do ukončení vyšetřování nalezeno. Týž den se ozvala matka zemřelého s vyslovením podezření, že v případě smrti jejího syna nejde o nešťastnou náhodu. Své tvrzení opírala o skutečnost, že syn měl uzavřeno několik životních pojistek pro případ smrti s částkou plnění převyšující několik milionů korun. Oprávněnou osobou k plnění pojistných nároků byl 28-mi letý Radek Š. (bratr Pavla Š.), který byl současně i plátcem pojistek. Potvrdilo se, že na zemřelého byly uzavřeny u 6-ti institucí pojistné smlouvy, kdy v případě jeho smrti „úrazem“ by celková částka plnění přesahovala 30 mil. korun. Oba bratři s další ženou tvořili v kritickou dobu osádku vozidla, sedící v kabině vozidla tak, aby zemřelý musel do nadstavby vozidla.

Podrobné šetření policie přineslo úspěch v podobě získání důkazů, že zemřelý byl úmyslně zavražděn oběma bratry shora popsaným způsobem. Bylo potvrzeno, že o jeho vraždu se pokoušeli již dříve, a to prostřednictvím „zásahu elektrickým proudem“ poté, co provedli technické úpravy na elektrickém vedení chladničky, kterou jeho prostřednictvím stěhovali. On však zásah elektrickým proudem tehdy přežil. Další pokus o jeho likvidaci měl z iniciativy bratrů Š. proběhnout na Slovensku, kde jej měl zastřelit najatý vrah. K uvedenému jednání však nedošlo.

Důležité okolnosti byly potvrzovány znaleckými posudky. Znalec z oboru požární ochrany, z oboru vyhrazených plynových a tlakových zařízení, z oboru rozvod plynu a plynových spotřebičů a z oboru toxikologie, popsal stav zařízení a další okolnosti související s otravou. Byl proveden vyšetřovací pokus ke stanovení schopnosti hoření hořáků, délky pobytu osoby

v zamořeném prostoru potřebné ke smrti člověka. Soudní znalec z oboru dopravy posoudil technický stav nákladního vozidla s konstatováním, že po případné úpravě podlahy nadstavby je pravděpodobné, že s ohledem na zjištění znalce-toxikologa pachatelé použili mimo hořáky ještě jeden zdroj produkující CO (např.vyvedení výfukového potrubí z motoru do nadstavbového prostoru, což zvýšilo hladinu CO). Materiály týkající se návodu na výrobu výbušnin, nalezené při domovních prohlídkách, posoudila i Univerzita Pardubice, Fakulta chemicko-technologická, Katedra teorie a technologie výbušnin. Chladničku, která měla sloužit k usmrcení oběti, posoudil znalec z oboru elektrotechnika s konstatováním úmyslné záměny vodičů a zjištění fázového napětí na krytu chladničky. Neméně důležitý byl znalecký posudek z oboru počítačové expertizy, který posoudil mobilní telefony pachatelů a jejich média. Duševní stav pachatelů a jejich osobnost pak posuzovali znalci z oboru zdravotnictví, odvětví psychiatrie a klinická psychologie. Bez této spolupráce se soudními znalci by se nepodařilo objasnit skutečnosti, k nímž bylo potřeba odborných znalostí.
Uzavřený a ucelený řetězec získaných důkazů vedl ke konečnému podání obžaloby a k pravomocnému a spravedlivému odsouzení pachatelů.

Diskuze

Uvedené případy byly na dokazování velmi obtížné. Rozhodující byl organizovaný a plánovitý postup policie včetně stanovení reálných vyšetřovacích verzí, které byly postupně vyvraceny či potvrzovány. Policie dospěla k úspěšnému závěru (dopadení a usvědčení pachatelů) poté, co důsledně dodržovala a využila celé škály kriminalisticko-taktických metod a praxí osvědčených postupů, spolupráci se soudními znalci nevyjímaje. Právě precizní a správná činnosti policistů přispěla k úspěšnému a rychlému objasnění a uzavření obou případů.

Závěr

Ve svém příspěvku jsem popsal případy vraždy, ke kterým došlo v rozdílných podmínkách, odlišným způsobem a z rozdílné motivace pachatelů. K objasnění případu nehledě na mravenčí práci policistů přispěla příkladná spolupráce se soudními znalci z různých oborů a odvětví. Využití různorodých vědních oborů bylo rozhodující pro objasnění okolností, k jejichž posouzení bylo potřeba odborných znalostí.

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Collaboration with forensic experts – a decisive tool in investigation and elucidation of violent crimes

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Keywords: murder, forensic experts, expert opinions, collaboration with experts

Summary
Investigation of violent crimes counts among the most complex police activities. In order to reveal and convict perpetrators, it is imperative for the investigators to gather evidences very carefully which must be often subsequently examined by forensic experts with scientific or technical background. In this report, the importance of expert opinions in the process of investigation is demonstrated on two unrelated murder cases.
The victim in the first case was a 33 year old woman whose corpse was found in Frýdek-Místek in 1991. The woman was killed after leaving a New Year's Eve party in a club where there was approximately 400 visitors. Among other injuries, the forensic doctors revealed a devastating injury in the region of lesser pelvis and found a small cap in the vagina that was later proven to be a part of a firecracker. Using atomic absorption spectrometry, heavily increased amounts of aluminium and magnesium, common components of the firecracker mixture, were found in the vaginal tissue. This evidence directed the police to the perpetrator as he had been seen both with the victim and using firecrackers at the club.

In the second case, a passenger travelling in the enclosed cargo space of a lorry driven by his acquaintances was found dead in 2002. Four heating burners and a gas cylinder were found installed in the cargo space. Medical examination and chemical analysis showed a severe intoxication with carbon monoxide. Since the dead had several life insurance policies, the driver and his two fellow passengers became suspect of murder. Expert opinions regarding operation of the heating burners in a closed space, technical condition of the vehicle and psychological profiles of the suspects helped to convict them of murder that was camouflaged as an accident in order to collect the money from insurance.

Capillary electrophoresis employed for quantitative characterization of peptide interactions with small ions and cyclodextrins

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Introduction

Capillary electrophoresis (CE) has developed into a high-performance separation method widely used not only for analysis and physicochemical characterization of peptides but also for investigation of non-covalent peptide interactions with other (bio)molecules [1]. These peptide interactions are of great importance as follows from their participation in vitally relevant processes, such as e.g. signal transduction via peptide hormone-receptor binding, regulation of enzyme activity by enzyme-substrate or enzyme-inhibitor binding, and ion transport performed via ion-ionophore complexation. The effects of peptide drugs can be also often explained in terms of binding to receptors, enzymes or ion channels. Strength of these non-covalent peptide interactions can be quantitatively characterized by the binding (stability, association, complexation) constant, K_b, of peptide complexes, which can be advantageously determined by affinity mode of CE (ACE) [2-4].

Experimental

The experiments were performed in i) home CE analyzer [5] equipped with bare fused silica capillary (total/effective length 300/200 mm, id/od 50/375 m) and UV-absorption detector operating at 206 nm; or ii) commercial CE analyzer P/ACE MDQ (Beckman-Coulter, Fullerton, CA, USA) using bare fused silica capillary (total/effective length 295/397 mm. id/od 50/375μm) and UV-vis photodiode array detector set at 206 nm.

Results and discussion

ACE has been applied to investigate quantitatively two types of non-covalent peptide interactions: i) complexes of an electroneutral pseudopeptide valinomycin (Val), antibiotic macrocyclic dodecadepsipeptide ionophore, with small cations, ammonium ion NH_4^+ and alkali metal ions, Li^+, Na^+, K^+, Rb^+ and Cs^+, in methanolic background electrolytes (BGEs), and ii) complexes of enantiomers of a charged short linear dipeptide -alanyl-D,L-tyrosine and its *N*-acetylated or *C*-amidated derivatives (*N*-Ac-β-Ala-D,L-Tyr-OH and H-β-Ala-D,L-Tyr-NH_2) with an electroneutral oligosaccharidic chiral selector, 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CD), in aqueous BGEs.

The investigation of Val complexes involved measurement of effective electrophoretic mobility of Val at increasing concentrations of the above cations (added in the form of chlorides) in methanolic BGEs (BGE I: 0.1 M tris, 0.05 M acetic acid, pH_{MeOH} 10.2; BGE II: 50 mM chloroacetic acid, 25 mM Tris, pH_{MeOH} 7.8). The problem of ACE determination of binding constant is that due to the Joule heating and addition of interacting cations into the BGE, the analyses are performed at different temperature and variable ionic strength. For that reason, the measured effective mobilities of Val at actual temperature and ionic strength of

the BGE have to be recalculated to reference temperature of 25°C and to constant ionic strength of the BGE free of the cationic additives [6]. Finally, from the dependence of corrected Val effective mobility on the cation concentration in the BGE, the apparent binding constant K_b was determined by nonlinear regression analysis according to equation (1), describing the dependence of Val effective mobility, $m_{Val,eff}$, on the concentration of cation C^+, $[C^+]$, in the BGE:

$$m_{Val,eff} = \frac{K_b[C^+]}{1 + K_b[C^+]} m_{Val-C^+} \qquad (1)$$

where is the mobility of Val-C^+ complex.

The decadic logarithmic form of the apparent binding constant (log K_b) of the Val-NH_4^+ complex in methanol was found to be equal to 1.57 0.18, which is in a very good agreement with the earlier determined value (1.67) obtained from spectrophotometric measurements [7]. In addition, it was confirmed that K_b of Val-NH_4^+ complex is approximately of the same magnitude as K_b of Val complexes with small alkali metal ions, Li^+ and Na^+ (log K_b = 1.45-1.54) and about two to three orders lower than K_b of Val complexes with large alkali metal ions, Cs^+, K^+ and Rb^+ (log K_b = 3.81-4.63), which fit by their size much better into the Val cavity than the Li^+, Na^+ and NH_4^+ ions [8, 9].

In an analogous way, binding constants of the complexes of enantiomers of a charged antimicrobial dipeptide H-β-Ala-D,L-Tyr-OH and its derivatives (*N*-Ac-β-Ala-D,L-Tyr-OH and H-β-Ala-D,L-Tyr-NH_2) with an electroneutral oligosaccharidic chiral selector, 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CD), have been determined by nonlinear fitting of the mobility data to regression model describing the dependence of the effective mobility of these peptides on cyclodextrin concentration in the BGE. Binding constants of these complexes were found to be in the range log K_b = 1.44–1.89. (2-HP-β-CD) formed somewhat stronger complexes with L-isomers of these peptides than with their D-isomers.

Conclusion

ACE proved to be a suitable method for investigation of both weak and medium-strong non-covalent interactions of charged as well as non-charged peptides with ions and molecules of different character and size.

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Preparative capillary isotachophoresis as a sample pretreatment technique for mass spectrometry

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Bioanalytically important compounds, e.g., xenobiotics and/or their metabolites, are often needed to be determined in biological samples at (ultra)trace concentration levels. Apparently, complex characters of biological samples are challenging for several analytical chemistry technologies. For example, consider several hundreds of different constituents as these still spanning various chemical/biological properties and, in addition, concentrations of analytes as differing several orders of magnitude. Due to these facts, a combination of a powerful separation technique is required, especially, to reflect for an adequately sensitive and/or selective detection method. Capillary electrophoresis (CE) techniques offer some favorable properties, like very high efficiency separations along with very low consumption of both the samples and the operating solutions. MS detection provides an excellent selectivity while keeping sufficient detection sensitivity.

Micropreparative capillary isotachophoresis (pITP) experiments were performed using a hydrodynamically closed CE separation system. Urine samples (urine served as a model matrix) were spiked with busereline (a peptidic model analyte). pITP served as a tool for isolating busereline while accompanied only by minimum interferents from the urine matrix. Such an isolation procedure was performed as aiding at a fractionation valve. pITP was included for discrete spacer constituents as added to the sample solutions. The isolated fractions were lyophilized and then reconstituted for final mass spectrometric analyses: (i) a direct injection mode, (ii) UPLC/MS and (iii) CE/MS combinations.

This work deals with different approaches as regarding busereline containing samples (a short peptide as present in urine). A very low concentration level of the analyte by using pITP and, subsequently, as loading to chromatography and electrophoresis techniques as coupled to MS. MS and MS/MS spectra were obtained from the reconstructed fractions as included both the pITP clean-up effect and its ITP concentration power as regarding the analyte as present in the complex sample at very low concentration levels. This study is showing high potentialities and compatibility of pITP as the sample pretreatment technique before several modes of MS analysis.

Acknowledgement

This work was supported by: the Slovak Research and Development Agency (the project No. VVCE-0070-07) and the Slovak Grant Agency (the project No. 1/0882/09).

Evaluation of ion-exchange TLC chromatograms of humic substances by simple means of common scanner and data processing

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Introduction

Humic substances (HSs) are ubiquitous natural materials occurring in huge amounts in soils, sediments and waters as a product of the chemical and biological transformation of animal and plant residua. Due to their ability to interact with various components of environmental compartments HSs play an important role in soil and aquatic chemistry and therefore they attract the attention of researches for many years. Despite the great effort resulting in an enormous number of published papers, the structure and function of HSs are not yet fully understood [1]

In general, HSs are amorphous, brown or black acidic and poly-disperse substances and they have molecular masses in range from several hundreds to tens of thousands. They may be considered as consisting of substituted aromatic rings linked together by aliphatic chains (polyols, peptides, sugars etc). Various simple organic compounds are considered to be building blocks, from which the complex structure of HSs is composed, e.g. salicylic acid, phthalic acids and the others [2]. Many studies have shown that humic substances possess both aromatic and aliphatic characteristics. The dominant functional groups which contribute to their surface charge and reactivity are phenolic hydroxylic and carboxylic groups. Humic substances may chelate multivalent cations such as Mg^{2+}, Ca^{2+}, and Fe^{2+}. By chelating the ions, they increase the availability of these cations to organisms, including plants [3].

From the literature results that the most frequently used separation methods for analysis and characterization of humic substances are column chromatographic methods (mainly RP-HPLC, SEC and their combination HPLC-SEC) [e.g. 4-6] and electroseparation methods (especially CZE, PAGE etc.) [7-10].

Among the chromatographic methods, techniques based on a size-exclusion effect appear to be most useful, as they allow us to relate elution data to the molecular mass distribution of humic substances. For their study almost all analytical methods were used, from liquid chromatography techniques mainly size-exclusion and reversed-phase mechanisms were extensively evaluated. In spite of direct evidence for potential of ion-exchange mechanisms, there is lack of articles on this topic. Therefore we decided to evaluate potential and figures-of-merit of both anion-exchange and cation-exchange thin-layer chromatography (TLC) for characterization of humic acids (of various origin) chromatographic profiles combining image processing and data processing

Experimental

We used TLC plates FIXION 50X 8 (strongly acidic catex in sodium cycle) and POLYGRAM IONEX 25 SB-Ac, (strongly basic annex in acetate cycle). Various types of humic acids (e.g. Aldrich, Ecohum etc.) were chromatographed. The TLCs were developed in closed chambers, using several types of aqueous solutions and buffers (water, sodium chloride, sodium sulphate, sodium citrate) with different ionic strength and pH. We also compared two sample deposition modes – on dry and on pre-wetted TLC plates.

For quantitative data acquirement from thin layers we utilized a PC equipped with a common digital imaging device (scanner). The obtained images were processed to chromatograms (retardation factor versus intensity charts) using Microcal® ORIGIN® Pro 8.

Results

IEX TLC of humic acids processed by proper digital image processing and digital data conversion into 2D domain intensity versus retardation factor is useful tool for evaluation of large data files obtained by simple means of TLC run under wide range of conditions. IEX TLC enables to get reliable and reproducible data for HAs characterization. Due to defined and restricted separation space IEX TLC is acceptable as a fractionation or sample treatment technique and enables also archival backup of physically separated HAs. Another possibility is either transfer obtained data to IEX HPLC column techniques or further analyse TLC fractions by complementary techniques.

Conclusion

The selected approach is useful simple tool for various humic acids characterization. Its potential is further investigated.

Acknowledgements

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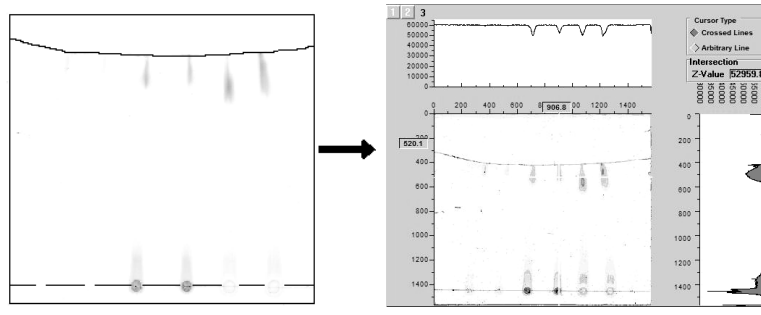

Fig. 1: Principle of processing developed IEX TLC plates for humic acids characterization.

A comparison of sub-2-microne particles with porous shell particles in UHPLC analysis of estrogen steroids

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Introduction

UHPLC has recently become a widespread analytical technique in many laboratories which focus on fast and sensitive assays. The key advantages of UHPLC are the increased speed of analysis, higher separation efficiency and resolution, higher sensitivity and much lower solvent consumption as compared to other analytical approaches. This is all enabled by specially-designed instruments and sub-2-microne particle packed analytical columns. Newly, porous shell particles demonstrated many advantages when used in UHPLC system. The aim of this study was to compare analytical performance of sub-2-microne particles with porous shell particles in terms of efficiency, resolution, analysis time and system back-pressure. The influence of temperature on selectivity of was also studied.

Experimental

Acquity UPLC chromatographic system was used for the purposes of this study. Three analytical columns were evaluated: BEH Phenyl (100 x 2.1 mm, 1.7 μm, Waters), Kinetex 2.6 PFP (100 x 2.1 mm, Phenomenex) and Ascentis Express Phenyl-Hexyl (100 x 2.1 mm, 2.7 μm, Sigma-Aldrich). Phenyl functional group should provide special selectivity for compounds possessing phenolic ring, therefore the group of seven estrogen steroids was chosen for the purpose of this study (estriol, α-estradiol, ®-estradiol, estrone, estradiol-methlyether, estradiol acetate and ethynylestradiol). The elution was enabled by gradient elution using generic gradient profile from 5% of organic component up to 95% within 5.5 minutes. The flow-rate was adjusted individually depending on column back-pressure (in the range 0.35 – 0.7 ml/min). The mobile phase was formed by water and acetonitrile. The influence of temperature was measured in the range 20-90 °C for BEH Phenyl column and in the range 20-60 °C for for Kinetex 2.6 and Ascentis Express analytical columns.

Results

The separation of steroids on BEH Phenyl was influenced by temperature - see Fig. 1. Up to 50 °C the critical pair of peaks (estradiol methylether and estradiol acetate) was separated, while higher temperature worse the separation leading to co-elution at 70°C. Similarly, resolution between the two peaks decreased with temperature on Ascentis Express. Kinetex 2.6 demonstrated no separation at all for critical pair of peaks.

Separation efficiency was quite comparable on BEH Phenyl and Ascentis Express - see Table 1 - about 200.000 generated for selected peak of estrone. Slightly better results were obtained on sub-2-microne column. Much lower efficiency, about 120.000 was obtained on Kinetex 2.6 analytical column.Concerning column back-pressure, it is more advantageous to use Ascentis Express porous shell column, as it demonstrates substantially lower back-pressure, especially at low temperatures - see Table 1. Both BEH Phenyl and Kinetex 2.6 analytical columns demonstrated much higher back-pressure. This was inconvenient mainly for Kinetex 2.6 because of its low limit of back-pressure 8700 Psi.

Conclusion

Comparable results in separation of estrogen steroids were obtained on Ascentis Express, which might be more convenient for its low back pressure and BEH Phenyl, which might be more convenient for its stability at high temperature and slightly higher efficiency.

Acknowledgements

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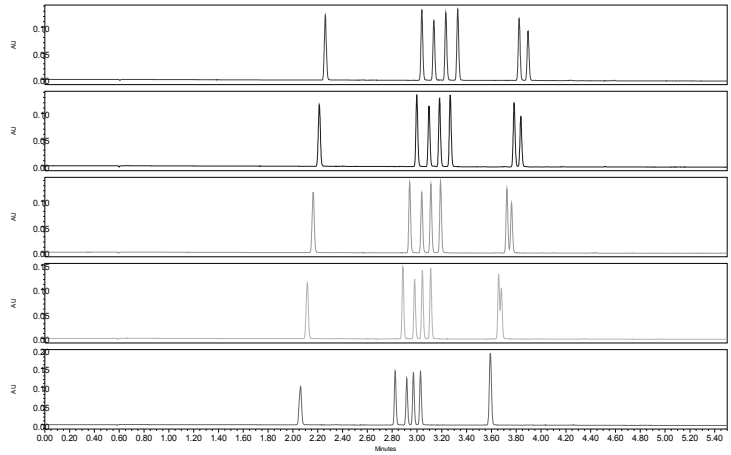

Fig. 1: Chromatogram of analysis of 7 steroids – 30 (upper), 40, 50, 60 and 70 °C (lower).

Table 1: A comparison of column efficiency (expressed as numer of theoretical plates N) and column back-pressure (P in Psi) for Ascentis Express and BEH Phenyl analytical column at various temperatures for peak of estrone in analysis of estrogen steroids.

	Ascentis Express		**BEH Phenyl**	
Flow-rate	*0.5 ml/min*		*0.5 ml/min*	
Temperature	**N**	**P**	**N**	**P**
20°C	206034	12150	194644	6900
30°C	219195	10400	189921	6400
40°C	219136	9050	196359	5900
50°C	219773	7950	195701	5460
60°C	221491	7070	-	-
70°C	215534	6450	-	-
80°C	202510	5900	-	-

Application of short-end injection capillary electrophoresis mode for assessment of drug stability against human liver microsomes

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Introduction

Since pharmacological profile of a drug highly depends on its metabolism by cytochromes P450 (CYP), stability against these enzymes belongs to the important drug parameters. During a new drug development, the pharmaceutical companies have to usually decide between analysis of a new drug candidate with chosen CYP isoforms mixture, which has limited evaluation value about processes in a real human body. Or eventually analysis of a designed molecule on the liver slices but it is laborious and time-consuming procedure. In this manner, human liver microsomes (HLM) represent a certain trade-off enabling a good simulation of conditions inside the liver and fast analysis.

The goal of this study was to introduce a new generic method based on capillary electrophoresis (CE) allowing determination of drug stability against HLM.

Experimental

Work with HLM carries the onus of their membrane proteins content given by preparation technique nevertheless. These HLM mixture components have a tendency to adsorb onto inner capillary wall. Therefore, we proposed a method based on the short-end injection mode to reduce the time spend by HLM inside the capillary. The second lay-out leading towards higher reproducibility represents utilization of background electrolyte (BGE) containing replaceable polymeric gel.

Method optimization and validation was performed with samples containing 0.1 $mmol.l^{-1}$ bufuralol as the probe drug, 1 mg/ml HLM, 0.1$mmol.l^{-1}$ NADPH, 0.1 $mmol.l^{-1}$ $NADP^+$ and 0.2 mg/ml phtalic acid prepared in 10 $mmol.l^{-1}$ phosphate buffer pH 7.4. Samples were injected by application of a pressure 0.5 psi to the outlet vial for 3 s. 10 % (v/v) linear polyacrylamide in 50 $mmol.l^{-1}$ disodium hydrogen phosphate was used as a BGE. An uncoated fused silica capillary with total length of 50 cm (effective length 10 cm) and internal diameter 75 μm was thermostated at 37 °C. Separations were accomplished by application of 22 kV (positive polarity). All experiments were conducted on the P/ACE™ MDQ CE system.

Results

As the result we introduced the method based on $NADP^+$ production monitoring. Following summary shows the results obtained by method validation: RSD of 1.77 % for migration time (n = 6), RSD of 2.04 % for relative peak area (n = 6), LOD of 6.5 $\mu mol.l^{-1}$ (S/N = 3), LOQ of 20 $\mu mol.l^{-1}$ (S/N = 10) and recovery of 100.63-103.91 % (n = 6).

The fast screening of 12 chosen probe drugs was carried out to prove method's capabilities as the versatile tool for a drug stability determination.

Conclusion

The new method enabling a fast drug stability assessment was established. Utilization of HLM provides the balance between requirements of fast analysis and proper view of drug stability in a human body. The principle based on $NADP^+$ production monitoring guarantees its generic applicability.

Acknowledgements

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Influence of chiral selector coverage of silica gel support on enantioseparative properties of chiral stationary phases

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Introduction

Chiral separation of analytes in HPLC with chiral stationary phases (CSPs) can be influenced by many factors, such as chiral selector (CS) type and carrier type, spacer, chiral selector coverage, mobile phase composition, temperature, mobile phase flow rate, etc. Macrocyclic antibiotics (MAs) are an important class of chiral selectors widely used in enantioseparation. Namely vancomycin and teicoplanin have been shown to successfully resolve a variety of enantiomers. This presentation will deal with the effect of the macrocyclic antibiotics coverage of the silica support.

Experimental

Chiral stationary phases with various chiral selector coverages, vancomycin-based Chirobiotic V and V2 and teicoplanin-based Chirobiotic T and T2 (V2 and T2 mean higher MA coverages of the silica gel support) were used to study the retention and enantioseparation behavior of structurally diverse analytes – profens, beta-blockers, amino acids, chlorophenoxypropionic acid derivatives in reversed-phase and polar-organic separation modes. Mobile phases were composed of methanol, water or triethylammonium acetate buffer and methanol with addition of small amounts of triethylamine and acetic acid, respectively.

Results

Concentration of a CS added to mobile phases can substantially influence enantioseparation. Variation of MAs concentration is easy to perform but certain restrictions arise from solubility of these CSs in some mobile phases and high absorbance of the MAs in UV region of spectra. Low amounts of vancomycin or teicoplanin chiral additives that are sufficient for successful enantioresolution easily solve these problems. However, the use of MA-based chiral stationary phases becomes still more popular. These stationary phases are easy to handle and can be used in various separation modes without loss of separation performance. In this work we compare the effect of vancomycin and teicoplanin chiral selectors contents in liquid chromatography separation systems with MA-based CSPs on enantioseparation of various drugs and drug constituents and other analytes of interest. Hereby we show and compare an ambiguous behavior of the various analytes on the CSPs Chirobiotic V *versus* V2 and Chirobiotic T *versus* T2 columns. Differences in the interaction mechanisms of different analytes on these chiral stationary phases with different macrocyclic antibiotics amount result in different retention and enantioselectivity that cannot be easily predicted. The results show that it is quite difficult to generalize the proper choice of the CSPs with different CS coverage.

Conclusion

Chiral stationary phases based on macrocyclic antibiotics, vancomycin and teicoplanin, exhibit rather different enantioresolution results depending on the chiral selector coverage of the silica support. The results show that the amount of the MAs on the silica gel and the chemistry of binding influence retention and chiral separation of structurally different analytes in different ways. Therefore, it is difficult to predict the success of enantioseparation on the CSPs with various CS coverages.

Acknowledgements

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Comparison of C_{18} silica-rod monolithic and C_{18} packed columns in HPLC separation of silybin and acetylsilybin diastereoisomers

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Introduction

Monolithic columns consisting of one piece of a porous material are a recent acquisition of analytical chromatography [1]. The porous material is characterized by a bimodal pore (macro-and mesopores) size distribution. From the technical point of view the monolithic column have two prominent advantages over the packed column: low resistance to mass transfer and high permeability (the external porosity is about 50% higher than that of packed columns). Of course, there is also some important drawback of monoliths, mainly higher HETP, because of high eddy diffusion.

Flavonolignan silybin (Fig. 1) (CAS No. 22888-70-6) is the major component of the silymarin complex extracted from the seeds of *Silybum marianum* (L.) Gaertn (milk thistle). Natural silybin is equimolar mixture of two diastereomers[2] silybin A and silybin B. Recently, silybin has received attention due to its anticancer and chemopreventive actions, as well as hypocholesterolemic, cardioprotective, and neuroprotective activities effects.

Experimental

The separations were performed by HPLC-DAD system (Prominence UFLC, Shimadzu). Two columns were tested: monolithic one Chromolith Performance RP-18e (Merck, 100 x 3 mm i.d.) and conventional particle-packed 5µm C_{18} column Nucleosil 100-5 AB (Macherey-Nagel, 125 x 3 mm i.d.). The mobile phase composition was optimized by changing type and amount of organic solvents (CH_3CN, CH_3OH). Finally an isocratic mobile phase was used consisting of CH_3OH/H_2O/TFA (45/55/0.1; v/v). Column temperature was set at 25 °C, detection at 285 nm. The flow rates for monolithic column were set at 0.76 ml/min and particle-packed at 0.3 ml/min respectively.

Results

The separation of given analytes (silybin and acetylsilybin diastereomers) on monolithic column under isocratic conditions was shorter. The separation of all analytes (silybin A, silybin B, acetylsilybin A, acetylsilybin B) was finished up to 12 minutes. In case of particle-packed column the analysis time was approximately 60 minutes. We found that the separation efficiency of both columns was very similar (HETP between 25-37 µm for all analytes).

Conclusion

The C_{18} silica-rod monolithic column is able to separate silybin and acetylsilybin diastereomers (Fig. 2) with separation efficiency similar to conventional 5µm C_{18} particle-packed column, but much shorter. This fact is important in case of both biotransformation monitoring and chemical modification of target analytes.

Acknowledgements:
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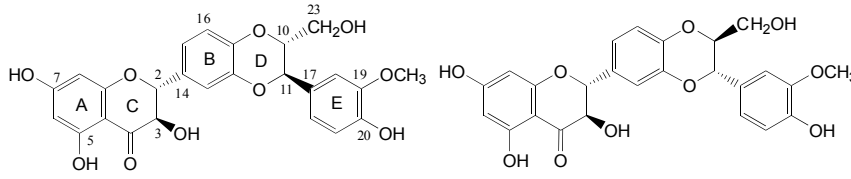

Silybin A (2*R*, 3*R*, 10R, 11*R*) Silybin B (2*R*, 3*R*, 10*S*, 11*S*)

Fig. 1: Silybin diastereomers.

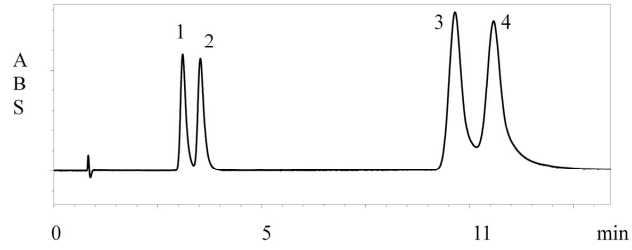

Fig. 2: Fast HPLC-DAD separation of silybin (1,2) and acetylsilybin (2,3) diastereomers on monolithic column.

Highly selective SRM (h-SRM) - application in food analysis

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Introduction

The TSQ Quantum series of Thermo Fisher Scientific mass spectrometers are the most advanced and powerful set of triple quadrupole mass spectrometers available in the world today, featuring the patented HyperQuad Quadrupole mass analyzer system, a rugged orthogonal source, and a completely redesigned analyzer geometry for maximum sensitivity, precision, and reliability. One of the key features of the TSQ Quantum which sets it apart from any other commercially available triple quadrupole instrument is the ability to carry out H-SRM for quantitative assays in food analysis.

H-SRM is an acronym for Highly-Selective Reaction Monitoring, which is a more advanced form of Selective Reaction Monitoring (SRM). SRM is a longstanding quantitative technique used on triple quadrupole mass spectrometers for quantitation. During the SRM experiment there are three distinct events which occur within the triple quadrupole mass spectrometer.

First, ions of a specific mass (precursor ion) are transmitted through the first quadrupole, while ions of different masses are filtered out.

Second, the selected ions collide with a neutral gas present in the second quadrupole (collision cell) where they undergo collisional induced decomposition (CID).

Third, product ions of specific mass are then transmitted through the third quadrupole, after which they are detected. In this way a specific signature can be set up for target analytes in a complex sample.The SRM experiment using most triple quadrupole instruments is usually conducted at unit (or higher) resolution for the precursor ion. However, with H-SRM, the precursor ion is selected at a higher resolution, typically at 0.1 FWHM without significant loss of transmission of your analyte. This is achievable due to the patented HyperQuad Quadrupole technology used on the TSQ Quantum series (TSQ Quantum Access Max, TSQ Quantum Ultra and TSQ Vantage). No other commercially available triple quadrupole system can currently achieve this.

Conclusion

Using H-SRM, interferences from the sample matrix background are substantially reduced and the LOQs improved. Similarly, no cross-talk issues were detected for any of the tested analytes. makes H-SRM the technique of choice for improving detection limits in complex matrices(see Figure 1). H-SRM can also eliminate chemical noise and reduce the likelihood of generating false positives.

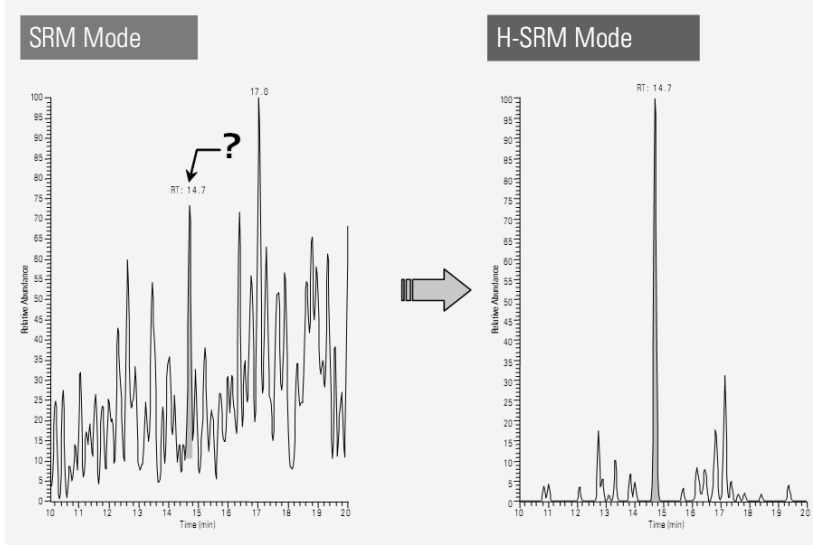

Fig.1: Comparison SRM a H-SRM mode for the analysis of iprodione.

Today's Ultra-High Performance Liquid Chromatography (UHPLC) for Complex Sample Analysis Needs Ultra-High Resolution Mass Spectrometer (UHR MS) Giving Best Combination of Mass Resolution, Accuracy and Data Acquisition Speed

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Introduction

QTOF are common mass spectrometers in LC-MS/MS applications because of their good mass accuracy and resolution. In particular the combination of accurate mass and isotope pattern match facilitates confident automated formula generation by MS and MS/MS. The trend to decrease chromatographic separation times by means of using ultrafast LC systems (UHPLC) further supports the use of a QTOF instrumentation since any the compromise between speed and mass resolution is largely avoided.

Best performance values of QTOF so far were ~15.000 for resolution and ~ 2ppm mass accuracy with internal calibration. However, applications on highly complex samples like target screening, metabolome or quantitative proteomic studies require even an increased mass resolution in the range of 60.000 at best possible acquisition rates. A mass accuracy below 1 ppm dramatically increases the confidence in search results, e.g. for elemental compositions or protein database hits.

Experimental

Presented here will be a novel mass spectrometer Bruker maXis based on QTOF technology that matches the above requirements without compromising other performance factors like sensitivity or dynamic range. Putting a QTOF up to this performance level is not trivial – the principal technical background will be illustrated and explained.

The instrument performance will be demonstrated with examples from various complex samples areas like proteomics, metabolomics and the analysis of multiple targets in matrices. E.g., common matrices from metabolomic studies are spiked with known analytes only differing slightly in their mass-to-charge ratio and separated by a fast UPLC method. Using a 5 cm column with a particle size of 1.8 µ and a 3 minute gradient, UPLC peaks at a width of < 1 sec. (FWHM) are observed with a spectral acquisition rate of 10 mass spectra per second – essential for good statistics on both isotope pattern as well as for quantitative requirements.

Conclusion

This presentation should show the ability of modern UHR-QTOF technology to be very effectively coupled with UHPLC chromatography in many areas of research. For protein ID, several hundreds of proteins can be confidentially identified from minor amounts of, e.g. 100 ng complete cell lysate, with false positive rates of < 1%. For label-free proteomic quantitation studies, the high resolution results in major benefits for the overall monitoring

of the analyzed complex proteome, namely in a highly increased number of readily identifiedand quantified regulated peptides and proteins. The limit of detection and quantitation as well as the effective dynamic range for quantitation set new standards for this application.

How to overcome the critical steps in analysis of biological materials

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Introduction

Currently the innovative technologies as MS, NMR and X-ray crystallography provide the sufficient capabilities for detection, identification, structural and functional characterization of clinically important biomolecules, e.g., proteins, glycoproteins, glycolipids, etc. Due to the high complexity and heterogeneity of original biological materials, the separation steps have to be involved. In spite of classical separation methods based on physico-chemical principles, the methods operated on molecular recognition called bioaffinity chromatography or their electrophoretic alternate provide an adequate separation strategy of highly complex mixtures of analytes in various biological matrices (blood, urine, tissue homogenate, and cell lysates). Only such approach enables to isolate the analyte in submicromolar concentration in desired amount and purity even in the presence of most abundant proteins. Target biomolecules create biospecific but reversible complexes with ligands as lectins, metal ions or specific mono(poly)clonal antibodies bound to solid phase.

Results

In order to overcome the limitations of current bioanalytical approaches (batch or column arrangement) as undesired dilution, loss of the material, low binding efficiency and instability of bioactive analytes due to the mechanical stress and partial denaturation, the micro/ nanoparticles with a magnetic core are gaining an increasing attention. Magnetic spherical particles enable routine highly efficient, rapid and gentle separation without any adverse effect to analytes. Magnetic microspheres typically consist of a superparamagnetic core embedded in a polymer shell protecting the affinant from the contact with metal oxide [1]. Magnetic particles can be easily manipulated using permanent magnets or electromagnets, independently of normal fluidic or biological processes. Similarly to chromatographic separations, analogous interactions are realized on magnetic particles starting from general nonspecific (ionic, hydrophobic, hydrogen bond), through group-specific (dye ligand, IMAC, chelating), up to specific affinity interactions (antigen-antibody, avidin-biotin, enzyme-inhibitor, lectin-glycans, etc.) [2-3]. Such systems offer sophisticated analysis, separation and purification of compounds starting from inorganic ions to biopolymers (enzymes, antibodies, nucleic acids) and cell populations. To improve sensitivity of detection, the biofunctionalized carriers can be employed as integral part of a microfluidic total analytical system (μTAS) [4-5]. This presentation aims to describe few examples based on such analytical

approaches combined with microfluidic analytical device and to focus on their benefits [6-9].

Conclusion

The miniaturization and integration of innovative detection technologies will greatly extend the sensitivity of biomarker detection, and thus improve the precocity of analysis. Important to say that analysis of biological material focused to predicting and validating of new clinically important biomolecules or to routine analysis in diagnostics slowly become a detached branch of analytical chemistry, requiring specialists well-educated not only in analytical chemistry and instrumentations but also in pre-clinical and clinical subjects. Only by this way we can forward the research on diagnostics and therapy of serious diseases.

Acknowledgements

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Study on oligosaccharide composition of wort and beer samples by liquid chromatography/electrospray ionization tandem mass spectrometry

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Introduction

Important roles of oligosaccharides in processes in living cells and in the products of brewing industry, as well the development in instrumental analytical equipment have gain attention to their separation and characterization. Due to polarity, hydrophilicity and low volatility, high performance liquid chromatography (HPLC) is technique of first choice for the separation of underivatized oligosaccharides [1]. Tandem techniques of mass spectrometry (MS), in particular in connection with the electrospray ionization (ESI) has been successfully applied to the structural elucidation of saccharides. Fragmentation of negative ions provides, in addition to sequence data obtained in positive-ion mode, information on linkage positions and branching [2].

Experimental

Samples of sweet wort, wort, and green beer (variety Bojos) were diluted with acetonitrile/ water and filtrated. HPLC-MS analyses and MS/MS experiments were performed with Esquire LC ion trap mass spectrometer (Bruker Daltonics) or Q-Tof Premier instrument (Waters), coupled with 1100 HPLC system (Agilent). An Prevail Carbohydrate ES column (150×2.1 mm, 5 um; Grace) was used. MS conditions were optimized with the solutions of standard maltooligosaccharides.

Results

In this study we examined the samples collected during the brewing procedure containing complex pool of (iso)maltooligosaccharides by the HPLC with MS detection. The LC conditions were adopted to separate oligosaccharides according to their degree of polymerization (DP). Changes in the composition and quantity of corresponding fractions were not observed, with the exception of considerably lower amount of di- and trisaccharides in green beer.

Optimization of separation conditions were accomplished using positive-ion mode, where abundant sodium adducts dominated. However, MS/MS spectra of $[M+Na]^+$ ions did not allow us to unambiguously differentiate between individual isomers of dextrins due to the absence of structural identifier of α-(1-4) linkages. On the other hand, MS spectra in negative-ion mode showed less stable $[M–H]^–$ ions accompanied by the in-source fragments and adducts with anions. The results of $[M–H]^–$ fragmentations were compared for both the spectrometers, ion trap and q-TOF instruments. They gave almost the same MS/MS pattern

containing $^{0,2}A$, $^{0,2}A–H_2O$, $^{0,3}A$, $^{2,4}A$, B, and C-type ions and differ only in the abundance of fragments. The assignment of molecular structure to the MS/MS spectra were deduced from Finally, using the similar analytical conditions reported for on-line LC-MS analysis, direct infusion of previously collected chromatographic fractions into mass spectrometers were examined. Also in this case, all mass spectra showed the presence of the deprotonated molecule and its fragmentation produced expected fragmentation pattern.

Conclusion

This analytical approach was successfully applied to the description of oligosaccharides mixtures in wort and beer samples. The study of the MS/MS spectra obtained from the deprotonated molecules pointed out important differences useful to establish the structural features of coeluted oligosaccharides.

Acknowledgements

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Determination of related substances in Cyclosporine final dosage forms with chemiluminescent nitrogen specific HPLC detector

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Introduction

Cyclosporine is an immunosuppressant drug widely used to prevent rejections of transplanted organs. Cyclosporine final dosage forms are usually based on solution of cyclosporine in placebo containing ethanol and mixture of other excipients, which are responsible for the cyclosporine bioavailability. Such excipients represents, however, usually very complex mixtures of UV absorbing compounds with quite wide range of hydrofobicity. It is therefore impossible to find conditions for confident determination of all cyclosporine related substances or potential unknown degradation products with HPLC instrumentation based on UV detection.

Experimental

Experimental HPLC conditions are

- Column: LUNA C18 (2) 3 µm, column dimension 300 x 2.0 mm (two columns with dimension 150 x 2.0 mm serially coupled).
- Mobile phase: A: Acetone-TBME-H_2O-TFA; 29:5:66:0.01
B: Acetone-TBME-H_2O-TFA; 49:5:46:0.01
- Flow rate: 0.15 mL/min
- Gradient: chanel B: 0 – 70 minutes: 0 – 100 %, linear gradient
15 minutes: 100 %
Column conditioning: 15 minutes with 100 % A
- Column temperature: 100 °C
- Detection: pyro-chemiluminiscent detector selective for nitrogen
- Injection volume: 7.5 µL
- Diluent: Acetone-TBME-H_2O; 40:6:54
- Analyzes time: least 85 minutes

Samples are prepared on concentration level of cyclosporine 10 mg/mL. Quantification is based on external standard method with cyclosporine standard on concentration 0.07 mg/mL.

Results

Application of nitrogen specific HPLC detector (NSD) simplifies the separation problem mentioned above, as cyclosporine and all his potential impurities are peptides (cyclic undecapeptides) whereas used excipients are nitrogen free. All detected peaks represent thus only cyclosporine impurities and degradation products. Main advantages of the proposed

method are:
- selectivity related to placebo (placebo chromatogram is without any interfering peak)
- sensitivity (LOD about 0.01 – 0.02 % related to cyclosporine)
- easy evaluation and distinct data interpretation (all found peaks represents cyclosporine impurity or a degradation product).

Conclusion

A method for determination of related substances in cyclosporine final dosage forms was developed. Based on the validation results and long-term method use it can be stated that method is suitable as a QC method for confident determination of cyclosporine related substances in final dossage forms containing cyclosporine.

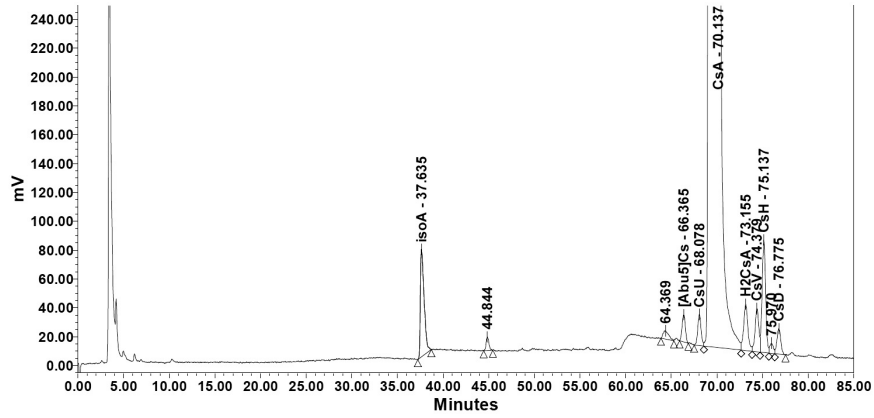

Fig. 1: Chromatogram of real sample (Cyclosporine capsules, 100 mg).

Table 1: The validation parametrs of presented method.

Cyclosporine impurity	LOD [%]	LOQ [%]	R
Cyclosporine U	0.01	0.03	0.9987
Dihydrocyclosporine A	0.02	0.05	0.9996
Cyclosporine H	0.01	0.03	0.9999
Cyclosporine D	0.01	0.04	0.9993
Isocyclosporine A	0.01	0.04	0.9998

The determination of Salvinorin A and Salvinorin B in body fluids by LCMS-IT-TOF

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Salvia divinorum is a sprawling perennial herb found in the Sierra Mazatec region of Mexico. The Salvia divinorum application can exihibit different dosage-dependent experience. It varies from subtle, just-off-basseline state to full-blown psychedelic one . Salvinorin A and B belongs to the many related compounds of the plant like divinatorines, salvidivines etc. But just Salvinorin A appears to be pharmacologically active. It is a potent, efficacious, and selective κ opioid receptor agonist.

The aim of this study was to develope the method for the determination of both Salvinorin A and Salvinorin B in biological materials (urine and blood) by LCMS-IT-TOF system.The MS^n experimental access was applied for basic pharmacokinetic and metabolic study related to different drug administration ways .

The results of $LCMS^n$ method development and the data of metabolic pathway investigations will be presented.

PLATINblue - optimized system for UHPLC and conventional HPLC columns

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Introduction

Knauer, the LC instrument manufacturer, launched new UHPLC system PLATINblue. High-quality PLATINblue components were designed to work together perfectly to enable accurate measurement with optimal reproducibility. The sharp and narrow peaks produced by fast chromatography in conjunction with low-noise and low-drift UV and PDA detectors ensure sensitive measurements.

System features

PLATINblue offers wide range with unsurpassed compatibility. The UHPLC system can be configured as either binary high pressure gradient or quaternary low pressure gradient system. UHPLC pump head offers flow rates of 2 ml/min with the maximum pressure up to 1000 bar and 5 ml/min with the maximum pressure up to 800 bar. Quick system response is driven by 110µl system volume (including mixer and autosampler).

Short cycle time is ensured by fast gradient possibility (up to 3% composition change rate per second) and high-performance autosampler with fast injection (15 s or <60 s with needle wash). The UHPLC system acquires data with a frequency up to 200Hz, which ensures high resolution and peak capacity, as well as a more accurate integration. The software includes new tool – *Method convertor* to downscale existing method to UHPLC method.

Results

The PLATINblue UHPLC system shows nice results in many applications. One of the applications is shown in figure 1. This is a separation of beta-blockers under following conditions:

Column:	50 x 2 mm Blue Orchid C18 1.8µm
Eluent: A:	25 mM NaH_2PO_4 pH 2.7
	B: Eluent A / ACN 40:60 (v/v)
Gradient:	0 – 0.6 min 11% B
	0.6 – 2 min 11% - 75% B
Flow:	1 ml/min
Volume:	0.5 µl
Temp.:	35°C
Detection:	PDA-1, 254 nm (10 mm 2 µl Cell), 100 Hz, 0.005 s
Pressure:	650 bar

Conclusion

Knauer Platin Blue is one of the best UHPLC instruments in the market and it would be used not only with PDA detector but it is full compatible with Thermo MSD or Grace NQAD. Therefore is open for many applications from different fields of natural sciences.

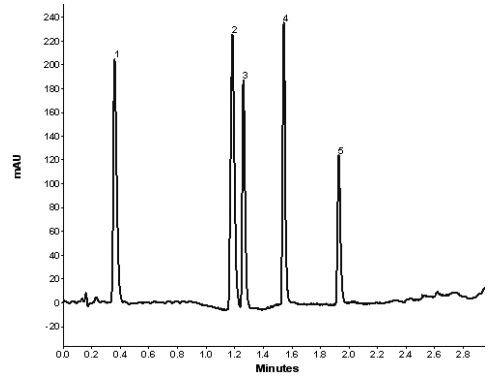

Fig. 1: Chromatogram of beta-blockers separation (1-atenolol, 2-pindolol, 3-nadolol, 4-metoprolol, 5-alprenolol).

Off-line combination of liquid chromatography methods HPLC-SEC for analysis and characterization of soil humic acids

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Introduction

Humic substances (HS) are created by a complex mixture of amorphous, yellow to black colored, hydrophilic, polyelectrolyte poly-disperse macromolecules and maybe no two molecules are identical. They have direct influence to various processes playing significant role in an environment [1]. Therefore, we could call them loosely defined or fuzzy chemical systems. This signal does not usually result in an exact numerical physical-chemical data, but is described also by their distribution function or range of validity.

Based on thorough study of available literature on the topic we concluded, that relatively few works solve problems of off-line combination of two or more chromatographic methods based on different principles and their application to the characterization of HS. This fact is surprising, because adoption of this approach could lead e.g. to more complex insight to their behavior in different chromatographic systems [2] and on-line combination SEC – RP-HPLC [3] for the characterization of HS in Bayer liquor during technology of aluminum production. The aim of this work is an introductory study of off-line combination RP-HPLC and SEC.

Experimental

Target group of humic acids was obtained by the procedures published by Kandráč et al. [4]. Solutions of humic substances were prepared daily fresh by dissolution of weighed HSs at cca 3 mg/ml concentration level in initial composition of mobile phase for RP-HPLC slightly adjusted by 0.05 mol.dm^{-3} solution of NaOH.

The devised stepwise gradient chromatographic [5] method with tandem DAD and fluorimetric detection was used for characterization and fractionation of HSs. SEC separation was carried out using a stainless-steel column 250 x 2.2 mm filled by Spheron HEMA 100 (metacrylate copolymer) sorbent, spherical particles diameter was below 25 μm using mobile phase mixture of 99% DMF / 1% phosphate buffer, pH = 3,00 (v/v). Void volume (V_0; 0.35 ml) and total permeation volume (V_i; 0.95 ml) of the column were determined using Blue Dextran 2000 and Styrene, respectively. The column system was calibrated using polystyrene standards with different nominal molar mass for estimating the averaged molar mass of injected fraction of Hss.

Results

Vertically located chromatograms (RP-HPLC profiles) in the Fig.1, show typical examples of the profiles as resulted from analysis of humic acid (isolated from soil, for specifications see in figure captions). From the drawing it is evident that the combination of chromatographic methods is capable to distinguish among the fractions of humic acids. The records show that each fractions from RP-HPLC we can find by SEC different peaks coding distribution of HA fractions with the highest signal in the region below relative molar masses value about 5000. It means that regardless the hydrophobicity and/or interaction HA-DMF ability of humic acids – their SEC profiles are similar but not identical under FLD detection

conditions (Ex. 470 nm, Em. 530 nm). This record data can be transformed to the plot of individual recognizable chromatographic peaks that represents two dimensional separation space of RP/HPLC and SEC separation mechanizms. To prove the non-existence of retention times (RP-HPLC/ SEC) correlation (its existence is defined as H0 hypothesis) we did statistical test by Spearman rank correlation and Pearson product-moment correlation coefficient.

Conclusion

The results suggest that the devised off-line 2D RP-HPLC and SEC method is highly reliable for characterisation and fractionation of soil HAs in a wide concentration range and also at trace concentration levels. Analysis of individual fractions obtained by the described RP-HPLC method by the method working on independent separation principles – described SEC method - provided data of even higher dimensionality needed for HSs investigation.

Acknowledgements

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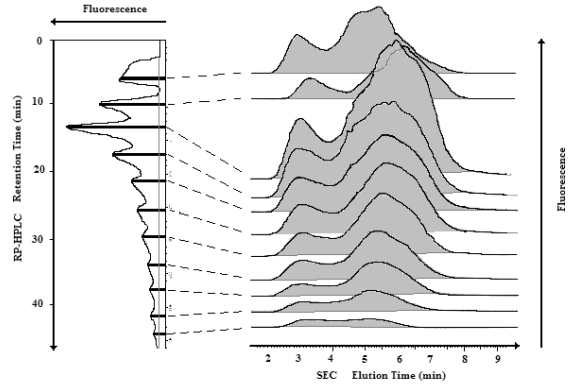

Fig. 1: Example multichromatogram of **HA J** obtained by aplication of combination of chromatographic methods RP-HPLC and SEC. Vertically located record: background corrected RP-HPLC profile obtained using FLD (Ex. 470 nm, Em. 530 nm), flowrate 1 ml/min, after injection of 100 µl sample volume. Horizontally located record: SEC profiles of **HA J** fractions (Ex. 470 nm, Em. 530 nm) at flowrate 0.2 ml/min. Injection volume was 50 µl.

Identification of muscarine in human urine by liquid chromatography-mass spectrometry

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Introduction

The alkaloid muscarine (for structure see Fig. 1) is found in a number of fungi of the genera *Amanita*, *Clitocybe*, and *Inocybe*. Muscarine is responsible for the parasympathomimetics effects. Muscarine poisoning is characterized by increased salivation, perspiration and lacrimation [1]. In the recent years, the number of people, who abuse fly agarics *Amanita muscaria* and *Amanita pantherina*, has notably increased. Although intoxications by these mushrooms are rarely lethal, it is important to determine the cause of poisoning soon and to initiate a medical treatment. The absence of a suitable analytical method for identification of muscarine in urine or blood is the main problem in the diagnostics of these intoxications. A few articles focused on isolation of muscarine from mushrooms [2,3,4] and only one article concerning its isolation from human urine [5] have been published. New LC/MS method for identification of muscarine in human urine was developed and two systems, equipped with a single quadrupole or an ion trap, were tested for this purpose.

Experimental

LC/MS conditions

The experiments were performed using an LC-MS-2010A quadrupole system (Shimadzu, Japan) and an LC-MS ion trap system (LC Shimadzu, Japan and MS Thermo Scientific, USA) equipped with APCI and ESI ion sources Intensive ion [M^+] was observed using ESI. Fragmentation of muscarine was evaluated by multistage mass spectra using an ion-trap. Chromatographic separation was performed on a Gemini C18 column (150 mm × 2.0 mm, 5 μm) maintained at 30 °C, 8 mmol.l^{-1} heptafluorobutyric acid was used as the mobile phase at flow rate 0.2 ml.min^{-1}.

Isolation step by SPE

Different SPE cartridges were tested for isolation of muscarine. The best results were obtained using Strata X-CW column. Strata X-CW column (60 mg, 3 ml) was washed with 2 ml of methanol and 2 ml of 0.1 mol.l^{-1} hydrochloric acid. A 1.0 ml aliquot of urine was loaded onto the SPE column. The column was rinsed with 2 ml of acetate buffer (pH 4.5).

Muscarine was eluted from the column by 2 ml 5% formic acid in methanol. The eluate was evaporated using nitrogen at the room temperature. The dry residue was dissolved in 0.1 ml of water and 5 μl were injected onto an LC/MS system.

Results

Isolation of muscarine by solid phase extraction on Strata X-CW was satisfactory for LC/MS analysis. Under the described chromatographic conditions, the retention time of muscarine was 14.2 min. An intensive ion at *m/z* 174 [M^+] represented a base peak in mass spectrum. Its fragmentation led to two main fragment ions at *m/z* 115 (100%) and *m/z* 97 (72%). They can be used for identification of muscarine.

The applicability of developed method was demonstrated by the analysis of the urine sample of old man, that consumed unknown amount of *Amanita muscaria* (see Fig. 1).

Conclusion

The LC/MS method for identification of muscarine in human urine using either a quadrupole or an ion trap was developed. The method is sufficiently specific and rapid, both tested mass spectrometric systems were adequately sensitive for real sample analysis.

Acknowledgements

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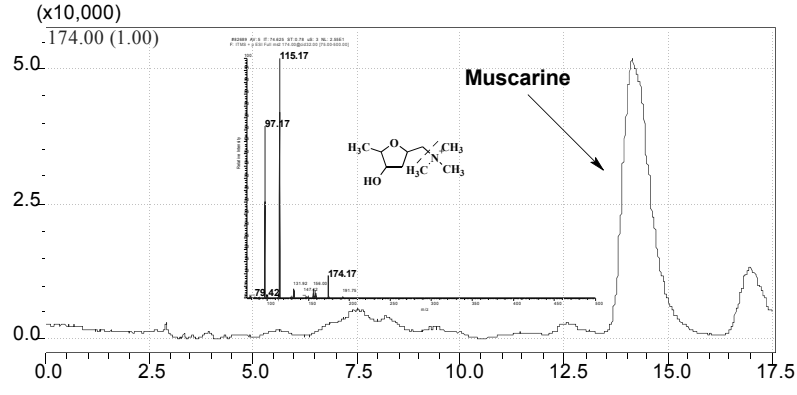

Fig. 1: Chromatogram of patient´s urine with MS/MS spectrum of ion m/z 174.

Application potential of electrophoretically mediated microanalysis

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Introduction

Today, electrophoretically mediated microanalysis (EMMA) attracts the attention for its prospective applications. EMMA method belongs to techniques of capillary electrophoresis (CE) and thus for the first time CE does not serve as a separation tool only. Thanks to the EMMA methodology it represents the technique which combines reaction and separation in the confines of capillary column. The first work, in which this in-line arrangement was used, was published by Bao and Regnier [1]. Subsequently the EMMA method has been utilized for a range of biochemical systems comprising assays of enzyme activity and kinetics and determination of substrates, inhibitors, Michaelis constants, and inhibition constants. Later on the EMMA methodology was introduced into non-enzymatic systems as derivatization reactions and examination of chemical reactions in microscale.

In the presentation the basic principles of the method, individual modes of mixing and solution of possible problems will be explained and clarified. Further, the examples of enzymatic reactions from the field of proteomics and drug metabolism studies will be shown.

Experimental

All measurements were conducted on Agilent ^{3D}CE instrument equipped with diode array detector. Capillary column with inner diameter of 50 μm was employed for all analyses. The temperature of capillary was set according to the temperature optimum of particular enzyme. Signals were detected at 200 nm and data were evaluated by Agilent ^{3D}CE ChemStation software.

Results

EMMA method was applied to a proteomic study in which trypsin was utilized as a digestion enzyme. A method was developed with usage of protein fragment, oxidized insulin B-chain and optimized for digestion of β-casein. Final conditions enabled to gain a complete peptide map within an hour as a total analysis time.

Drug metabolism studies are represented by the enzymatic reaction catalyzed by microsomes or a recombinant form of cytochrome P450 2D6 which convert dextromethorphan as a model substrate to dextrorphan and to others possible metabolites. Method conditions were adapted to a given system and again the whole analysis took about one hour.

Conclusion

EMMA methodology offers a lot of advantages over a traditional off-line format. It allows rapid execution of an examined reaction in homogenous assay with a minimal dilution and detection both the decrease of substrate concentration and the rise of product signal in the only analysis. Together with advantages of CE techniques EMMA method shows huge application potential in different fields of research. The main assets of this complex method are full integration of all reaction steps, minimal consumption of reagents and high degree of automation.

Acknowledgements
The authors thank for financial support Grant Agency of Czech Republic (grant No. P206/10/0057) and Czech Ministry of Education (research project No. MSM0021622413 and research center LC06023).

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Do we still measure precisely and accurately - new terminology in analytical chemistry?

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Introduction

Once upon a time there are new things coming into our lives. One of them that we usually do not like is new terminology. It has recently happened also in the branch of analytical chemistry. In 2007 the new version of International vocabulary of metrology (VIM3) [1] has been published as new ISO Guide. In February 2009 translation of VIM3 into Czech language has been published as Czech national standard (TNI 010115) [2]. The reason of this contribution is to emphasize important changes that has influenced or are going to influence us as analytical chemists in the Czech Republic. That is the reason why this contribution will be presented in Czech language.

Generalities

The translation heterogeneity of fundamental metrological terms from English into Czech has been causing difficulties for many years. The development of metrological terminology in VIM3 takes note of reality that fundamental metrological principles have been in increasing measure applied in alternative branches for metrology like analytical chemistry or clinical medicine. In a simplified way this new version of VIM was written not only for physics, representing typical metrological sector, but also for chemists. VIM3 itself brings new concept of measurement that wipes differences in fundamental measurement principles in physics and chemistry.

This contribution deals with both new terms and new way of translation into Czech. Experts from different fields successfully cooperated on the translation of VIM3 into Czech and brought out new Czech terminology for commonly used words describing quality of measurement – precision, accuracy and trueness (see Table 1).

Table 1: The new Czech terminology

English term	Czech equivalent from [2]	Traditional Czech equivalent used in chemistry
Measurement trueness	*pravdivost měření*	*pravdivost měření*
Measurement accuracy	***přesnost** měření*	***správnost** měření*
Measurement precision	***preciznost** měření*	***přesnost** měření*
Measurement uncertainty	*nejistota měření*	*nejistota měření*
Uncertainty budget	*bilance nejistot*	*rozpočet nejistot*

The linkage among precision, accuracy and trueness is self-explanatory shown in Figure 1 with a target as example.

New philosophy of measurement based on uncertainty evaluation was introduced in 1993 by Guide to the expression of uncertainty in measurement (GUM) by the International Organization of Standardization in the name of seven international organizations including IUPAC and ILAC. This concept has become an integral part of standards EN ISO/IEC 17025 and EN ISO 15189 that are mandatory for most of commercial laboratories. This uncertainty concept took place of traditional concept based on random and systematic errors. New

version of GUM [5] was published in 2008. VIM3 brings a little different terminology of uncertainty but with very similar meaning.

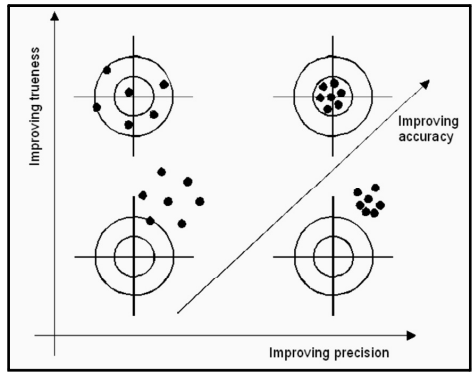

Fig. 1: Linkage among fundamental terms for chemists [4].

Conclusion

I have tried to convey some news dealing with up today Czech terminology that came into effect about a year ago. I am sure it will take some time to bring these new "Czech words" into general knowledge among chemists, but I am sure it will help us with communication with people from different fields. EURACHEM-CZ is a national organization of all-European organization EURACHEM that has "a focus for analytical chemistry in Europe". You can find details including freely accessible so-called methodical leaflet dealing with new Czech terminology on website http://www.eurachem.cz.

Acknowledgements

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POSTERS

Electrochemical Characterization of Repaglinide and its Determination in Human Plasma Using HPLC with Dual-channel Coulometric Detection

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Introduction

Type 2 diabetes, or non-insulin-dependent diabetes mellitus (NIDDM), is one of the most common metabolic disorders affecting humans. The disorder is characterized by depressed insulin secretion associated with increased resistance to the action of insulin, a hormone that regulates sugar absorption.

Peroral antidiabetics (PADs) are an integral part of NIDDM complex treatment. The occurrence of this metabolic disorder has been growing explosively over the last years in the population whereas even younger age groups are affected.

Recently, PADs have become, as a consequence of increasing amount of information about this metabolic disorder, subject of an intense development. Much effort has been devoted to the research and development of new, potentially more effective drugs. Especially for new generations of PADs, much lower dosages are commonly needed. At the same time, with the tendency to decrease therapeutic dosages, however, the demands on sufficiently selective analytical methods are constantly increasing. The determination of drugs in plasma at low concentration levels is a very challenging analytical task: only highly sensitive instrumental techniques can meet these requirements. For the clinical and metabolic studies as well as rational therapy, monitoring of PADs is of great importance. Since the free-drug blood levels are very low there is a logical need for highly sensitive analytical methods which could meet such requirements.

Repaglinide (PRANDIN®) is a synthetic PAD of the meglitinide class, used in the therapy of type 2 diabetes mellitus. Repaglinide (RPG) acts as an effective blood glucose-lowering drug - glucose concentrations are lowered via stimulating the release of insulin from the pancreas. The pharmacological effects of RPG occur very fast - the drug is rapidly eliminated from the blood stream with a half-life of approximately 1 hour. Moreover, the therapeutic dosed are typically much lower compared to those of other PADs. High-performance liquid chromatography coupled with electrochemical detection (HPLC/ED) represents a very powerful analytical tool: the technique is well known for its high sensitivity and selectivity towards substances exhibiting electrochemical activity. Thanks to these properties, some routine processes connected with the preliminary sample preparation (and thus with the risk of loss or even decomposition of the analyte) can be avoided or simplified. In addition, more straightforward and facile sampletreatment saves time and costs markedly. We developed a fast, simple and sensitive HPLC method employing dual-channel coulometric detection for the determination of repaglinide in human plasma.

Experimental

The chromatographic conditions used were as follows: Stationary phase – a Varian Polaris™ AQ 3 m 150 x 3 mm I.D. column, mobile phase – 50 mM sodium phosphate dibasic/ acetonitrile (58/42, v/v), adjusted with phosphoric acid, final pH 7.5. Flow rate 0.40 mL/min, manual injection, sample size 10 uL (0.02 mg/mL RPG + 0.02 mg/mL IS, 1:1). Detection –

coulometric, applied voltage +750 mV (vs. Pd/H_2) (first channel).

Results

A simultaneous voltammetric characterization of RPG has also been carried out to compare both hydrodynamic and static method. RPG undergoes electrochemical oxidation at carbon working electrode allowing its direct electrochemical detection.

Chromatographic behaviour of RPG is strongly pH-dependent due to its two weak acidic hydrogen atoms. At the same time, electrochemical response is also influenced. To evaluate this parameter, we tested different pH values and compared retention, peak symmetry and area. A pH value 7.5 was chosen, yielding in good peak shapes and oxidation efficiency for both RPG and internal standard.

In order to remove proteins from the plasma sample, we tested four different deproteination methods: direct deproteination using plasma centrifugation through the Microcon® filters, and three diverse precipitation/extraction procedures: precipitation using methanol or acetonitrile and liquid-liquid extraction by ethyl acetate. The most effective drug recovery for the latter method was observed, and on the contrary, very low recoveries using the ultrafiltration. The obtained results can be ascribed to the reported strong protein-binding affinity of RPG: in untreated plasma, the majority of analyte exists in a bonded-form whereas the addition of a less polar organic modifier breaks the formed associates resulting in higher extraction recoveries. Extraction by ethyl acetate followed by re-constitution is a rather more time-consuming procedure, however, it enables further pre-concentration of the analyte.

We used rosiglitazone as an internal standard for the quantitative analysis of RPG in plasma. Rosiglitazone, a synthetic thiazolidinedione (glitazone) derivative, also used for NIDDM treatment, was a good candidate as it is electroactive and during usual therapy, these two drugs are never used simultaneously. Plus, its chromatographic behavior and retention characteristics proved to be convenient for our purposes.

Conslusion

We demonstrated that the dual-channel coulometric detection is a very useful method for the determination of RPG in human plasma at physiological levels.

It offers excellent sensitivity and lacks typical electrode response instability, common with the use of amperometric electrodes. The developed HPLC/ECD method is well suitable for the either pharmacokinetic studies or therapeutic monitoring of the drug in targeted treatment of patients suffering from type 2 diabetes mellitus.

Acknowledgements

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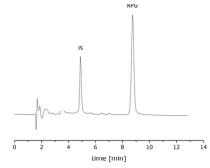

Fig. 1: A chromatogram showing separation of repaglinide and internal standard (rosiglitazone).

Monitoring of levels of vitamin B in a barley caryopsis and products of its processing

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Introduction

Barley caryopsis is a source of many natural, for health beneficial substances. Quantity and quality of these valuable substances change in the course of germination. Their identification and optimization of their content in the caryopsis in the course of its technological processing will enable to utilize a barley caryopsis as a material of a higher quality in the brewing and food industries. The optimized UPLC method of high performance liquid chromatography with fluorescence detection was used for the determination of vitamins B (Thiamine, Riboflavin, Pyridoxine) in barley products of its processing.

Experimental

Selected vitamins were determined in samples of two malting barley varieties Bojos and Sebastian during malting and brewing. The samples from the harvest year 2008 were collected from the growing station Hrubčice in the Czech Republic. The malting barley samples included both the fungicide treated and non treated variants. Malts were prepared in the micromalting plant of the Malting Institute of the RIBM in Brno using the procedure with short steeping and CO_2 extraction. Sweet wort, hopped wort and beer were prepared in Brewing Institute of the RIBM in Prague. Beer was prepared by a classical decoction method. Vitamins B were extracted from food matrix by acid hydrolysis and following enzymatic hydrolysis, which enabled to release vitamins from phosphorylated derivates. In vitamin B1, oxidation of thiamin to fluorescent thiochrom using derivatization agent was performed before chromatographic analysis.

LC chromatographic analyses were performed on Ascentis Express RP-amide column (3.0 x 150 mm and particle size 2.8 μm) using binary gradient methanol-phosphate buffer adjusted with H_3PO_4 to pH 7.0. The system equipped with a programmable fluorescence detector.

Results

Transmission of selected B vitamins during the technological process of beer production was analyzed in 2 samples of barley, malt, sweet wort and hopped wort and beer produced from them. The method for the extraction of B vitamins from barley and malt was optimized and the analytical UPLC method with fluorometric detection was developed and validated. Barley and products from it represent a very complicated matrix, therefore, sample preparation and extraction procedure prior to the analysis is very important. The use of selective fluorescence detector was the most suitable method for detection, because UV detection techniques suffer from a lack of sensitivity and from UV absorbing interferences, especially when the low amounts of B vitamins in nonenriched products have to be determined.

Conclusion

Selected water soluble vitamins were extracted from brewing materials and were identified and quantified employing the UPLC/FLR. The method is very quick and its sensitivity is comparable to the routinely used HPLC method.

Acknowledgements

Results were achieved in the framework of the Research Plan of the MEYS 6019369701, Research Centre 1M0570 and NAZV QH 91053.

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Monitoring of changes in ferulic acid content in the brewing materials and beer

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Introduction

Plant polyphenols are amorphous substances that are spread in various parts of plants – bark, wood, leaves, fruits and roots. Polyphenols contain one or more aromatic nuclei with attached hydroxylated groups 1, 2. Polyphenol substances contained in the brewing materials and beer play an important role in the process of ensuring and maintaining beer quality and stability. These substances, due to their antioxidant properties, affect sensory characters and total beer shelf life, they are significant in beer production technology and in a final phase they contribute to a positive assessment of beer as a source of natural antioxidants. Polyphenols occurring in the brewing process can be split into two big groups. The first one comprises flavonoids which are further classified into flavans, anthocyans and flavonols. The second group is formed by phenolic acids comprising derivatives of benzoic acid (salicylic, gentisic, p-hydroxybenzoic, protocatechuic, gallic, vanillic, and syringic acids) and derivatives of cinnamic acid (p-coumaric, caffeic, ferulic and sinapic acids). Ferulic acid (4-hydroxy-3-methoxycinnamic) belongs to bound low-molecular weight phenolic acids in a barley grain, it occurs especially in its outer layers. During malting content of this acid is even doubled. Its antioxidant activity plays an important role in brewing materials and biochemical process of beer production. Together with other polyphenolic substances, it contributes to beer stability and helps maintain beer quality parameters 1, 3, 5.

Our study is focused on monitoring the changes in ferulic acid content in the series barley – malt – sweet wort– hoped wort – beer.

Experimental

Ferulic acid was determined in the samples of two varieties of malting barley (Bojos and Sebastian) from harvest 2008. Malts were prepared in the micromalting plant of the malting Institute of the RIBM in Brno using the traditional EBC method 4. Sweet wort, hopped wort and beer were prepared with the classical decoction method in the Brewing Institute of the RIBM in Prague. Barley and malt samples were analyzed for the content of total and free ferulic acid. Preparation of the total ferulic acid was based on the extraction of the sample with alcalic hydrolysis and subsequent clean-up of the extract on the SPE column. Free ferulic acid was extracted from the sample with water and then cleaned-up on the SPE column. In liquid matrices only free ferulic acid was determined. Beer samples were degassed on the ultrasound device. Sweet wort, hopped wort and beer were cleaned up on the SPE column without prior extraction.

The samples were then analyzed using gradient elution on the UPLC WATERS ACQUITY liquid chromatograph equipped with WATERS 2996 PDA detector. Mobile phase was of 0.01M phosphate buffer (pH 3.5) and acetonitrile. Column used was ACQUITY UPLC HSS T3 1.8 µm 2.1x100mm.

Results

Content of total ferulic acid was determined in barley and malt in two malting varieties. Content of total ferulic acid in barley moved in the range of 547.7 – 799 $mg.kg^{-1}$ depending on the variety. Content of total ferulic acid in malt in the range of 617.3 – 976.8 $mg.kg^{-1}$ depending on the variety. Content of free ferulic acid was determined in the series barley – malt – sweet wort – hopped wort – beer in two malting varieties. Content of free ferulic acid moved in within 14.9 – 15.7 $mg.kg^{-1}$ in barley, 17.7 – 20.6 $mg.kg^{-1}$ in malt, 7.2 – 12.5 $mg.l^{-1}$ in sweet wort, 6.3 – 6.9 $mg.l^{-1}$ in hopped wort, and 14.7 – 20.2 $mg.l^{-1}$ in beer.

Conclusion

Calibration curve for the determination of total ferulic acid in solid matrices was linear within 170.0 – 2550.0 $mg.kg^{-1}$ (concentration of ferulic acid in a real sample) with correlation factor 0.9984, detection limit 30.0 $mg.kg^{-1}$. Calibration curve for the determination of free ferulic acid in solid matrices was linear within 5.0 – 426.0 $mg.kg^{-1}$ (concentration of ferulic acid in a real sample) with correlation factor 0.9973, detection limit 1.5 $mg.kg^{-1}$. Calibration curve for the determination of free ferulic acid in liquid matrices was linear within 1.7 – 25.5 $mg.l^{-1}$ (concentration of ferulic acid in a real sample) with correlation factor 0.9964, detection limit 0.5 $mg.l^{-1}$.

Content of total ferulic acid was determined in two malting varieties. In both the varieties, we found approximately 20% increase in total ferulic acid content in malt compared to barley. Further, concentration of free ferulic acid was assessed in these malting varieties in the series barley – malt – sweet wort – hoped wort – beer. All the samples exhibited the same content of free ferulic acid depending on the technological phase of the beer production.

Acknowledgements

Results were achieved in the framework of the Research Plan of the MEYS 6019369701 and Research Centre 1M0570.

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Analysis of T-rich oligonucleotides labeled with osmium tetroxide 2,2´-bipyridine by capillary zone electrophoresis

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Introduction

Labeling of nucleic acids offers broader option for its biochemical and physicochemical studies by the analytical separation methods such as capillary electrophoresis. Suitable labeling allows monitoring of DNA hybridization as well as following of DNA interactions with other biological and/or non-biological compounds.

Chemical DNA probe consisting of osmium tetroxide and 2,2'-bipyridine (Os,bipy) is well-known especially for its electrochemical activity [1,2]. In contrary to vanishing reactivity with purines, Os,bipy reagent creates covalent adducts with pyrimidine bases. The fastest adduct formation was observed namely with thymine [3].

In our work, we analyzed selected pentanucleotides with various number and position of thymine bases and we monitored its reactivity with Os,bipy reagent. Our interest was also taken to ageing of Os,bipy reagent and the role of reagent abundance in mixture with oligonucleotide was studied. Capillary zone electrophoresis as an analytical technique was chosen for the sufficient ability to monitor the changes of the modified oligonucleotides [4].

Experimental

3D CE Agilent Technologies equipment (Waldbronn, Germany) was used for all measurements. Analysis were carried out in PEI coated fused-silica capillaries (Composite Metal Services, The Chase, Hallow, UK), total length 32 cm (23.5 cm effective length) and 75 μm diameter. All experiments were done in TBE buffer (89 mM Tris, 89 mM boric acid and 5 mM EDTA) pH 9.4 with 0.5 % HEC. Hydrodynamic injection (50 mbar, 10 sec) and negative voltage -10 kV was applied in each analysis.

Results

In our experiments, we measured series of 10 pentanucleotides that differed in number of adenines, guanines and thymines in the molecule. We modified all samples with the Os,bipy reagent and we recorded the formation of new compounds (example on the Fig. 1.). It was shown that mentioned modification strongly depends on the position of thymine in the molecule. Terminal thymines react more willingly than those inside the oligonucleotide chain and the modification rate decreased with the higher number of potentially labeled thymines in the molecule.

However, we observed substantial changes in adduct peak heights and areas in dependence on the freshness of the Os,bipy reagent. The ageing of osmium tetroxide and 2,2´-bipyridine mixture plays significant role for the adduct formation. Further measurements confirmed time-instability and loss of efficiency of Os,bipy reagent. Based on that observations, the abundance of Os,bipy reagent and different molar ratios between oligonucleotide and Os,bipy

reagent were also tested.

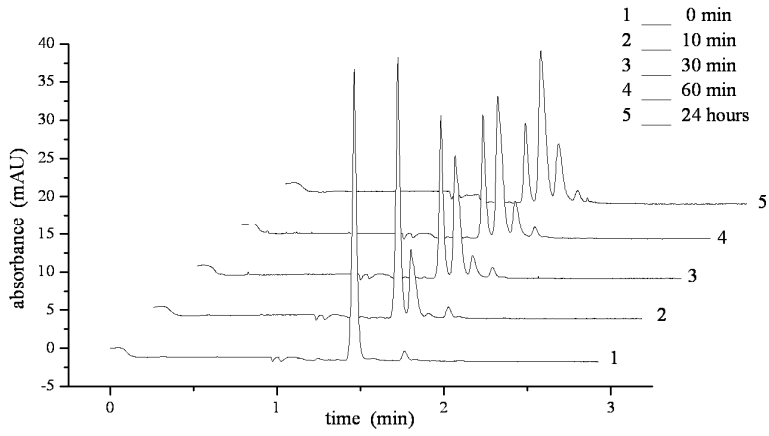

Fig. 1: Modification of AGTAT (0,1 M) with Os,bipy reagent (1M).Mixed in the ratio 1:1. 1 - oligonucleotide with no reagent, 2-5 - Os,bipy adducts formation during 24 hours.

Conclusion

It was shown that the modification rate related to the number and position of thymine in oligonuleotide chain as well as to the freshness of Os,bipy reagent. Terminal thymines react more willingly and earlier than those inside the oligonucleotide chain and the formation of Os,bipy adduct proceeds significantly slower in molecules with higher number of thymines. Also, the abundance of Os,bipy reagent causes faster reaction rate, but the same reaction between lower concentrated oligonucleotide and reagent proceeds slower again. However, the freshness of the Os,bipy reagent has fundamental influence on the oligonucleotide modification.

Acknowledgements

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Study of isolation methods of selected group of pesticides from soil sample before the RP-HPLC analysis

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Introduction

Soil is a heterogeneous system containing organic and inorganic matter. It contains solid particles, soil colloids and gases between which equilibrium is established [1]. Soil from this point-of-view is a complex matrix and the isolation of analytes from soil is a complex analytical problem [2]. The basis for successful analytical method for determining residues of pollutants in complex matrices at trace concentration levels is the use of selective isolation techniques [3,4] enabling isolation of analytes with high recovery and with minimum quantity of matrix co-extracts. Selection of proper treatment of soil sample depends on the sample matrix, its size, type, concentration of the analyte, separation and detection system. Two sample pretreatment methods, namely matrix solid phase dispersion (MSPD) and off-line flow-through solid-liquid extraction (FSSLE) for isolation eight common pesticides (atrazine, propazine, simazine, terbutrine, metoxuron, cloquintocet-mexyl, cypermethrin and permethrin) from soils before their RP-HPLC analysis were studied.

Experimental

Chromatographic conditions

The optimal conditions chosen for the separation of 8 pesticides were:
Purospher Star RP 18e (50x4 mm) analytical colum, with linear gradient elution from methanol:water 50:50 (v/v) to 100% methanol in 35 minutes. The injection volume was 0.02 ml. UV spectrometric detection at 235 nm wavelength was selected as a compromise between detection sensitivity and detection selectivity of the whole analytes group. The mobile phase flow rate was 0.5 ml/min.

Results

The work is presenting results of the study of several parameters affecting the extraction efficiency of pesticides mixture (atrazine, propazine, simazine, terbutrine, metoxuron, cloquintocet-mexyl, cypermethrin and permethrin) at concentration levels around 1-2.5 mg/ kg dry soil. HPLC separation conditions enabled separation of all studied pesticides with resolution higher than 1.2 for critical pair.

MSPD

For pretreatment of soil sample optimal conditions for given pesticides set were achieved by use of silica L 40/100. Experimental conditions for isolation of pesticides are as follows: desorption by 3.00 ml of 100% methanol, reduction of the extract volume to 0.50 ml by evaporation under dry air stream, make-up the volume to 1.00 ml. Extraction recovery for the mixture of 8 studied pesticides achieved at 2.5 mg/g dry soil was within the range 56-94%.

FSSLE

The optimal mass of silica bottomed in the column is 0.50 g. Experimental conditions for the isolation of pesticides are as follows: desorption by 3.00 ml of 100% methanol, flow rate

0.30 ml/min, reduction of the extract volume to 0.50 ml by evaporation under dry air stream, make-up the volume to 1.00 ml. Extraction recovery for the mixture of studied pesticides achieved at 2.5 mg/g dry soil was within the range 67-88% with exception of cypermethrine and permethrine where it was between 40-60%. Low recovery of pyrethroids was caused by their higher volatility under experimental conditions.

Conclusion

Both sample pretreatment methods proved to be practically useful for analysis of selected pesticides set even in small soil samples.

Acknowledgements

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Using of polar stationary phases in phytochemical analysis

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Introduction

At present, reversed phase liquid chromatography (RP-HPLC) is one of the most widely used tool for analysis of non-volatile organic compounds. A typical problem is how to create retention for compounds with no or low distribution toward packings with RP functionality [1]. Polar stationary phases (i.e. silica gel, diol phases, etc.) sound like strong tool to analyze polar and hydrophilic compounds. Among others, glycans, carbohydrates, peptides, polar drugs and their metabolites exhibit good chromatographic behavior on polar phases as recently reviewed [1-4]. On the other hand problems with clasical normal phase, such as solubility of analyte(s) or conection to MS detection, often complicate analysis. The aim of this communication is to discuss the potential of different HILIC systems for the separation of selected phytochemicals and compare it with RP-HPLC.

Experimental

Model mixture was prepared by mixing of standards of p-hydroxybenzoic, gallic, hippuric, caffeic, vanillic acids, catechin and quercetin (10 mg/l). Experiments were performed on an analytial HPLC system (Knauer) equipped with a UV/VIS DAD detector. Chromatographic separations were performed on several selected columns: Pinnacle DB Silica, 3µm (150 x 4.6 mm, Restek), LUNA 3u HILIC 200 A (150x2.0 mm, Phenomenex), TSKgel Amide-80, 3µm (150x2.0 mm, TOSOH Bioscience), Kinetex 2.6µ C18 100A (100 x 2.1 mm, Phenomenex), Gemini 5µ C18 110A (150 x 2.0 mm, Phenomenex). DAD spectra and addition of standards were used for identification of analytes in mixture. ESI-MS detection (Q-TOF Premier, Waters) was applied to study the suitability of chromatographic system for combination with mass spectrometry.

Results

First, we studied the effect of mobile phase on separation using unmodified *silica gel column* (normal phase mode without the addition of water). To date, the best results were obtained with hexane-methanol-ethyl acetate (8:2:1) at isocratic conditions. Mobile phase was further modified with acetic acid and trifluoroacetic acid (TFA). The best results provided mobile phase with 0.5% TFA (v/v). This analysis was finished in 10 minutes. A poor separation of hippuric, vanillic, p-hydroxybenzoic and caffeic acid was observed and optimization continues (study of the effect of buffer).

LUNA HILIC was the second tested column. A mixture of acetonitrile and water (silica based phase; forming of a water-enriched layer on its surface) was studied as mobile phase. Effect of water content, addition of acids or buffer (pH=7) was tested. Efficient retention of phenolic acids was achieved, however very bad peak shape of quercetin and catechin was achieved. A mixture of acetonitrile and 0.25M ammonium acetate buffer pH=7 (95:5) provided best results. Effect of light alcohols is now investigated.

HILIC stationary phase containing bonded non-ionic carbamoyl groups (*TSKgel Amide-80*) seems to be very promising. Separation of catechin, quercetin, caffeic and gallic acid was

achieved using isocratic elution with acetonitril:water (95:5) within 9 minutes. Fast separation of the other compounds using this column at buffered mobile phases is expected.
We compared the results obtained on polar stationary phases with two reversed phases Kinetex C18 and Gemini C18 and generic mobile phases (0.1% HCOOH:acetonitrile or methanol). Both RP´s provide separation of model mixture except the pair catechin-vanillic acid (analysis finished within 40 min). The reproducibility of retention was better using the latter column. Figure 1. compares the LC/MS analysis of model mixture using TSKgel column and Kinetex C18. It can be seen that elution order on TSKgel is not strictly opposite with respect to RP. The retention seems to be a combination of partitioning on the surface water layer with dipolar and hydrophobic interactions. Gallic acid as the first eluted compound on RP has the longest retention on HILIC but the retention of the other compounds does not correspond to the predicted behavior on HILIC. TSKgel provides sufficient resolution of critical pair catechin-vanillic acid. Bad shape of peaks of quercetin and hippuric acid can be seen. An improvement in sufficiently buffered mobile phase can be expected. Testing of those chromatographic systems for the analysis of urine is now in progress.

Conclusion

We studied conditions of separation using four different types of columns: silica gel either in normal phase system or HILIC one, HILIC containing carbamoyl groups and C18 phases. Almost complete separation was achieved using RP, however the column reequilibration of Kinetex C18 was somewhat longer. Unmodified silica gel provided only a poor separation of hippuric, vanillic, p-hydroxybenzoic and caffeic acids. When a silica gel column is used in HILIC system a better separation is achieved, however, with strong tailing of peaks of catechin and quercetin. TSKgel seems to provide a unique selectivity for the studied mixture. All the studied polar stationary phases provided excellent separation of catechin and vanillic acid, which is the critical pair when a reversed phase is used.

Acknowledgements

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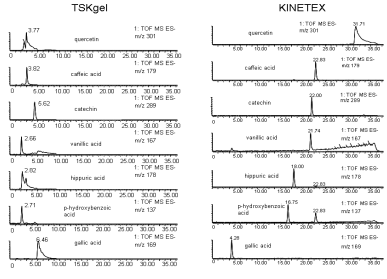

Fig. 1: LC/MS analysis of mixture using TSKgel Amide-80 and Kinetex C-18.

Application of affinity capillary electrophoresis for quantitative characterization of hexaarylbenzene-based receptor binding with alkali metal ions in methanol

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Introduction

Hexaarylbenzene derivatives have received in recent years great attention because of their potential application in the emerging area of molecular electronics and nanotechnology. It has been previously described by employing NMR spectroscopy and X-ray crystallography that hexarylbenzene-based receptor (R) (see Fig. 1) binds a single potassium cation K^+ because it synergistically interacts with the polar fence formed by six ethereal oxygens and with the central benzene ring via cation- interaction [1]. Nevertheless, according to the above study, a binding constant of K-R^+ complex could not be measured by NMR spectroscopy. Recently, capillary electrophoresis (CE) especially in the mode of affinity capillary electrophoresis (ACE), has been recognised as one of the useful analytical methods for studying non-covalent molecular interactions and determining binding constants in aqueous, non-aqueous or mixed hydroorganic media in a relatively simple and accurate way [2-4]. The advantages of employing ACE to study molecular interactions include the requirement of a small amount of the sample with the relatively low analyte concentration, mostly short analysis times and an ability to apply nonpure samples provided that ACE can separate the analyte of interest from the impurities. Due to the potential use of R in the sensing devices, it was our interest to describe quantitatively its interactions with several alkali metal ions, such as Li^+, Na^+, K^+, Rb^+ and Cs^+.

Experimental

The CAE analyses were carried out in a home-made CE apparatus equipped with a UV photometric detector (206 nm), in methanolic background electrolyte (BGE: 0.05 M $ClCH_2COOH$, 0.025 M Tris and alkali metal chlorides, pH_{MeOH} 7.8), in fused silica capillary (total/effective length 306/200 mm, ID/OD 50/375 μm), using hydrodynamic sample injection (10 mbar, 5s). Receptor R (20 M) in Cl_2CH_2/CH_3OH and EOF marker, mesityl oxide (2.5 mM) in CH_3OH, were used as sample solutions. The applied separation voltage was 12 kV, current was in the range of 10-28 A. A chromatography station Clarity (DataApex, Czech Republic) was used for data acquisition and the program Origin 6.1 (OriginLab Corp., USA) for the non-linear regression analysis.

Results

In the Tris-chloroacetate BGE neutral R interacts not only with alkali metal ions but also with $Tris^+$ cation. It has been previously reported that R forms 1:1 complex with potassium cation [1]. Therefore, it is assumed that alkali metal and $Tris^+$ cations react with R competitively as R can bind only one of them at a time. The effective electrophoretic mobilities of $M\text{-}R^+$ and $Tris\text{-}R^+$ complexes were determined from the measured migration times of the analyte and of the EOF marker. The migration behaviour of R, in the presence of M^+ and $Tris^+$ ions can be described by the following equation:

$$m_{R,eff} = \frac{m_R^* + K_{MR}^*[M^+]m_{MR}}{1 + K_{MR}^*[M^+]} \qquad (1)$$

where m_{MR} and m_R^* are the effective mobilities of $M\text{-}R^+$ and $Tris\text{-}R^+$ complex, respectively, K_{MR}^* is the apparent binding constant of the $M\text{-}R^+$ complex in the presence of $Tris^+$ cation and $[M^+]$ is concentration of the cation. The apparent binding (stability) constants (K_b) of the $M\text{-}R^+$ complexes in methanol were obtained from the dependence of the effective electrophoretic mobilities of the receptor R, corrected to reference temperature, 25 °C, and to constant ionic strength, 25 mM, on the concentration of alkali metal ions in BGE using a non-linear regression analysis according to Eq. (1).

Conclusion

The applied ACE method was found to be suitable and effective tool for the quantitative evaluation of strong as well as weak interactions between receptor R and various alkali metal ions in methanol. The selectivity of R expressed by binding constants K_b towards alkali metal ions was found to increase in the order of Na^+ (K_b 0.7) << Li^+ (1.15 ± 0.09) < K^+ (3.20 ± 0.22) < Cs^+ (3.72 ± 0.22) < Rb^+ (4.01 ± 0.21).

Acknowledgements

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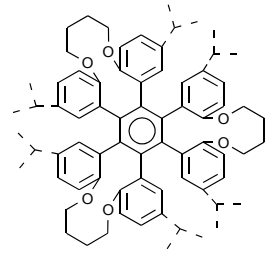

Fig. 1: The structure of the hexaarylbenzene-based receptor (R).

Comparison of two different IMAC techniques used for the enrichment of phosphorylated peptides

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Introduction

Protein phosphorylation is one of the most common of all protein modifications and has been found in nearly all cellular processes. Anomalous protein phosphorylation and disregulation of protein kinases or phosphatases can result in oncogenesis. MS analysis of proteolytic protein digests represents one of the most powerful tools for the study of protein phosphorylation. It has been shown that the enrichment of phosphopeptides from peptide mixtures significantly improved phosphoprotein characterization. Immobilized metal affinity chromatography (IMAC) is one of the methods used for this purpose.

Experimental

Two types of iminodiacetic acid (IDA)-modified sorbents were used: magnetic non-porous poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) microspheres with hydrophilic properties containing coupled IDA (prepared as described previously [1]), and the commercial non-magnetic sorbent TopTip IMAC also containing IDA (Glygen, USA). Both IMAC sorbents loaded either with Fe(III) or Ga(III) ions were used for the phosphopeptide enrichment from the -chymotryptic digest of porcine pepsin A. The fraction of peptides adsorbed to immobilized metal ions was analyzed using matrix-assisted laser desorption/ ionization – time-of-flight mass spectrometry (MALDI-TOF MS).

Results

In our study, model phosphoprotein porcine pepsin A, containing one phosphate group, was used to compare two IMAC sorbents, differing only by a nature of inert support, in terms of their ability to enrich phosphopeptides. Fe(III) or Ga(III) ions were immobilized via IDA either to magnetic non-porous poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) microspheres or to the commercial non-magnetic sorbent (TopTip IMAC). The optimum conditions for the phosphopeptide separation were investigated. MALDI-TOF MS analysis of porcine pepsin peptides adsorbed to IMAC magnetic microspheres containing Fe(III) or Ga(III) ions showed the presence of only two peptide ions. The predominant peptide ion (*m*/*z* 1321) was previously identified as peptide EATphosSQELSITY containing one phosphorylated serine residue [2]. The second peptide ion (*m/z* 1343) represents adduct of the predominant peptide with sodium ion. In the case of the second IMAC sorbent (TopTip with Fe(III) and esp. TopTip with Ga(III) ions), besides the phosphopeptide also non phosphorylated peptides were found in the adsorbed fraction.

Conclusion

Our results have shown that a nature of the sorbent used for the phosphopeptide enrichment by IMAC separation with immobilized Fe(III) and Ga(III) ions affects the phosphopeptide separation process, esp. in the case of acidic protein containing a high number acidic amino acid residues.

Acknowledgements

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The impact of extraction method, procedure, conditions and hydrolysis digestion for the content of phenolic acids and quercetin derivatives in Centella asiatica

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Introduction

Centella asiatica L. (syn. *Hydrocotyle asiatica* L.) belongs to the family Apiaceae (Umbelliferae). In India, the plant is commonly known as "Mandukaparni", in Srilanka and Indonesia as "Thankuni Sak". Centella asiatica is used in the China herbal medicine for thousands of years to treat various diseases. In classical Indian Ayurveda literature, it is considered to be one of 'Rasayana' (rejuvenator) drugs and it is said to improve the texture of skin and posses anti-inflammatory, anticancer and collagen synthesizing activities [1].

It is well known that medicinal plants collected at different times and from different localities may considerably differ in their types and quantities of chemical components, therefore, resulting in different therapeutic efficacy. Hence the quality control of herbal medicine is an important concern for both the health authorities and the public.

Experimental

The HPLC system equipped with auto sampler SIL-20AC HT and photodiode multi-wavelenght detector (SPD-M20A Prominence Diode Array Detector), SHIMADZU (Kyoto, Japan) was applied. UV-VIS spectra were recorded on a Spectrophotometer UV Unicam HELIOS α, Spectro-Lab (Warsaw, Poland). 1 cm quartz cuvette was using.

Results

In the present work, a method involving ethanolic extraction, HPLC – RP- C18 column chromatography with photodiode array detection is developed for determining the level of quercetin derivatives (e.g., quercitrin, hyperoside, rutin, rhamnetin, keampherol and miricetine) and derivatives of benzoic and cinamic acids in Centella Asiatica. The impact of extraction method (water-bath extraction and ultrasonic extraction), procedure and various extraction conditions (time and temperature) and acidic and basic hydrolysis was tested. The total amount of studied flavonols and phenolic acids was compared with total content of polyphenols using Folin-Ciocalteu's method. The obtained results were compared with those achieved by pharmacopoeian method. The spectrophotometric method for qualitative and quantitative analysis of polyphenols in dry plant extracts was applied.

Conclusion

The developed method was validated for specificity, repeatability, recovery and accuracy. The results demonstrate that HPLC-PDA method can be suitable for routine analysis of quality control and quantity evaluation of botanical products and extracts from plants containing flavonols and phenolic acids.

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Determination of selected food additives in various salad by HPLC

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Introduction

Chemical preservatives have played an increasingly important role in the food industry since they are used to prevent the growth of bacteria, yeasts and moulds in food [1]. The most popular preservatives are sorbic and benzoic acids their salts. However, excessive addition of these preservatives may be harmful to consumers, because of the tendency to induce allergic contact dermatitis, convulsion and hives etc [2]. The use of sorbic and benzoic acids, as well as their salts (sodium; potassium; calcium sorbates and sodium; potassium; calcium benzoate) is allowed by European legislation and its presence must be declared on the label.

From both technological and health standpoints, it is essential to develop analytical methods for determination of these compounds in food, in order to ensure sufficiently accurate results. Most of the methods reported so far for the determination of these additives in food have been based chromatographic methods such as high-performance liquid chromatography HPLC [2,3,4].

In this study HPLC with photodiode array detection was applied for the determination of the sodium benzoate, potassium sorbate, citric and ascorbic acid in food samples.

10 salads (marked as 1-10) were purchased from local markets. According to declaration of producer all salads do not acquired preservatives in composition.

Experimental

The HPLC system, equipped with an autosampler SIL-20AC HT and a photodiode multi-wavelength detector (SPD-M20A Prominence Diode Array Detector), SHIMADZU (Kyoto, Japan) was applied. Analyses were carried out on Discovery RP-C_{18} column (5 μm particle size, 150×4,6 mm, SUPELCO), maintained at 30°C. Mobile phase was a mixture of 0.005M ammonium acetate, 15% acetonitrile and acetic acid to pH =4 (90:5:5).

The detection was performed at the ë max: 263 nm (for ascorbic acid and potassium sorbate); 254 nm (for sodium benzoate) and 212 nm (for citric acid). The chromatographic data were recorded and processed by the *LCsolution version 1.23 SP*.

Calibration curves for all compounds were constructed using five calibration solutions in the range: 20-100 mgL^{-1}.

All samples of salad were obtained from different supermarkets. Prior to analyses, samples were minced and homogenized. The food products samples (10 ± 0.0001 g) were extracted with 30 mL of redistilled water using an orbital shaker for 60 min. The extracts were separated using centrifuge at 9000 rpm for 30 min, followed by double filtration. All extracts were transferred into a 50 mL volumetric flask, made up to the mark with mobile phase and analysed with HPLC method.

Results

Peaks on the chromatograms were identified by comparison of the retention times with reference standards and by addition of the individual reference standard to extracts. Precision was evaluated as the within-day and between-days coefficient of variation (CV) [5]. Within-day analyses were determined by injection of the standard solutions five times per day. Intralaboratory reproducibility was determined by analysis of the standard solutions during 5 consecutive days. The regression parameters of calibration curves are listed in Table 1.

The obtained results indicated that all samples contained predominantly ascorbic acid. The obtained level of this compounds ranged form 45 8 to 1664 38 mg100g^{-1} what resulted from technological process. The obtained citrate acid amount pointed at variable contents of this ion in analyzed salad, while the average concentrations varied from 22±4 to 113±12 mg.100 g^{-1}. It should be noted that these food additive (citric and ascorbic acids) are not allowed by Polish legislation and can be used in accordance with good manufacturing practice.

According to declaration of producer all salads do not acquired preservatives in composition. However, sodium benzoate was detected in six samples whereas potassium sorbate in eight salads. The mean amounts of benzoates varied between 0.95-70 mg.100g^{-1},whereas sorbates between 78-358 mg.100g^{-1}. The CV values for all compounds determination varied from 1.72% to 7.99%, what indicated satisfactory precision of proposed method.

Conclusion

In conclusion the presented results provide evidence that the developed HPLC method for simultaneous determination of potassium sorbate, sodium benzoate, citric acid and ascorbic acid in food samples and apple is reliable and reproducible. A clear separation of all tested additives from other components of salads is achieved within 15 minutes.

Presented method may be applied for routine analysis and quality control of food samples, due to simplicity, speed of sample preparing and short analysis time.

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Table 1: Linear regression calibration parameters of tested compounds determination by HPLC method (n=5).

analyte	potassium sorbate	sodium benzoate	citric acid	ascorbic acid
b	48168	1404	803	257
a	198771	8339	14931	9199
R^2	0.9990	0.9988	0.9990	0.9905

Magnetic inhibitor reactor as a tool for the separation of aspartic proteinases

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Introduction

Affinity based separation techniques are widely used in proteomic studies not only for isolation of specific proteins, but they are also applied e.g. in a removal of high abundant proteins from a sample, or in peptide separation for the study of protein posttranslational modification. Besides that these techniques provides a tool for the study of protein - protein interactions, protein-binding properties or to distinguish individual forms or isoforms of one protein. Multiple forms of enzymes can differ in their ability to interact only slightly with suitable ligands and this phenomenon can be utilized for their separation.

The aim of the present study is to elaborate a simple affinity-based method for the separation of two aspartate proteinases occurring in human gastric mucosa.

Content and changes in the ratios between individual human aspartic proteinases and their zymogens were found to be important from the diagnostic point of view.

In our study, we have used two synthetic peptide inhibitors to study their interaction with pepsin A and pepsin C and after the immobilization of peptides to apply them for distinguishing pepsin A and pepsin C.

Experimental

Two synthetic heptapeptides (Val-D-Leu-Pro-Phe-Phe-Val-D-Leu and Val-D-Leu-Pro-Phe-Tyr-Val-D-Leu) obtained from Vidia (Jesenice u Prahy, Czech Republic) were used in our study. The inhibition effect of the heptapeptides on the enzyme activity of porcine pepsin A and rat pepsin C was investigated using hemoglobin as a substrate. Proteolytic activity of the enzyme was determined by the method of Anson and Mirsky [1] with TCA-soluble peptides. The amount of formed peptides was determined using BCA method [2]. Proteinase peptide inhibitors were further immobilized to magnetic particles (Glyoxal agarose beads (20–75 μm), BioScience Bead Division of CSS., West Warwick, RI, USA) by coupling the free amino group of the peptide to active –CHO substituents of the magnetic carrier as described previously [3].

Results

The ability of prepared synthetic heptapeptide inhibitors containing D-amino acid residues (Val-D-Leu-Pro-Phe-Phe-Val-D-Leu and Val-D-Leu-Pro-Phe-Tyr-Val-D-Leu) to interact with porcine pepsin A and rat pepsin C was investigated by two different approaches: a) using kinetic measurements to determine inhibition constants and to evaluate a type inhibition of the enzyme reaction; b) by a study of interaction of both aspartate proteinases with the hepta-

peptides immobilized to magnetic particles to find conditions for their separation.

Conclusions

The investigated differences in the interaction of two synthetic heptapeptide inhibitors of porcine pepsin A and rat pepsin C contribute not only for better understanding their preferences in a substrate hydrolysis but also they contribute to an elaboration of simple method for the separation of pepsins and in combination with MS, for the enzyme detection and determination.

Acknowledgements

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Analytical method development for investigation of the role of hydrazone metabolites in isoniazid intoxication

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Introduction

Isoniazid ranks among the most important therapeutically used antituberculotic agents. During a long time of its preclinical and clinical investigations a number of information about the fate of the drug in organism as well as its toxicity profile have been obtained. However, in the case of neurotoxicity, many aspects are still elusive. It's clear that neurotoxicity is associated with the depletion of active form of vitamine B6 (pyridoxal 5-phosphate, PLP) [1]. The presence of condensation products of isoniazid and PLP was repeatedly mentioned as a potential explanation for this event [2]. However, a strong analytical evidence to support this hypothesis is still missing. This lack of data might be caused by absence of appropriate analytical methods for this study. The aim of this work was **1/** to develop pilot analytical method suitable to analyze the samples from preliminary *in vivo* experiment **2/** optimize the analytical conditions to be MS compatible and suitable for the analysis of samples from the *in vivo* experiment focused on investigation of the role of hydrazone metabolites in isoniazid intoxication.

Experimental

1/ Following experimental conditions (method 1) were used to analyse samples from the preliminary *in vivo* experiments. LiChroCART 250 × 4 mm, Lichrospher 100 RP-18 packing, 5 µm (Merck) and mobile phase composed of part A (water solution of 0.01 mol/L NaH_2PO_4 with addition of 1 mM EDTA and 5 mM heptansulfonic acid, pH 3 adjusted with H_3PO_4) and part B (methanol). Following gradient profile was developed: 0. - 22. min. 10 – 51 % B, 22. - 26. min. 51 % B, 26. - 27. min. 51 – 10 % B, 27. - 40. min. 10 % B. Mobile phase flow was 1 mL/min, UV detection was performed at 260 and 297 nm. Analytes were extracted from plasma using precipitation. **2/** For further method optimization different columns (LiChroCART 100 RP-18, Lichrospher 250 × 4 mm, 5 µm – Merck; Discovery HS F5 150 × 3 mm, 5 µm - Supelco; Zorbax SB-Aq 150 × 4.6 mm, 3.5 µm - Agilent; Ascentis Express HILIC 150 × 2.1 mm, 2.7 µm - Supelco; HILIC Atlantis 150 × 2.1 mm, 3 µm - Waters and ZIC-HILIC 150 × 2.1 mm, 3.5 µm - Sequant) were tested for separation of isoniazid, acetylisoniazid, pyridoxal, pyridoxol and pyridoxal isonicotinoyl hydrazone under the MS-compatible conditions.

Results

In the preliminary study, the appropriate separation of IZN, its active metabolite – acetylisoniazid (AcIZN), whole group of B6 vitamins – pyridoxal (PL), pyridoxol (POL), pyridoxamine (PAM) and potential hydrazone metabolites – pyridoxal isonicotinoylhydrazone (PIH), pyridoxal isonicotinoyl hydrazone-5-phosphate (PIH-5-phosphate) was achieved on C18 analytical column using the gradient profile of the mobile

phase (method 1). Thereafter, this method was applied to the analysis of samples taken after i.v. administration of toxic dose of isoniazid to rabbits followed by i.v. application of pyridoxal. This preliminary experiment was designed to get information about the presence of particular analytes in the real samples and obtain data about their concentration range. This analysis revealed the presence of IZ, AcIZN, PL, POL and PIH in the samples, while PAM and PIH-5-phosphate were not detected. Unfortunately, the active form B6 vitamins – PLP, which is supposed to play an important role in isoniazid intoxication, could not be detected due to the coelution of the compound with plasma matrix and low response to UV detection. These results stimulated further development of a LC-MS method capable to analyze IZN, AcIZN, PL, POL, PIH and PLP in biological samples.

As the previously used chromatographic conditions weren't MS compatible, further efforts were focused on finding novel stationary phase and analytical conditions capable to separate IZN, AcIZN, PL, POL, PLP and PIH using LC-MS. Among all HPLC columns tested, Zorbax SB-Aq and ZIC-HILIC stationary phase gave the most promising results. The best separation on Zorbax SB-Aq column was achieved employing a mixture of 2 mM ammonium formate (pH 4.5) and methanol (90:10) at a flow rate of 0.35 mL/min. However, there was still partial coelution of POL and PLP. The best separation on ZIC-HILIC column was achieved with mobile phase containing a mixure of 2 mM ammonium formate (pH 3.5) and acetonitrile (20:80) with a flow rate of 0.2 mL/min. However, in this case the partial coelution of isoniazid and acetylisoniazid was observed. Considering MS detection, the lack of full separation could not be a substantial issue for further method development. However, potential ion suppression effect needs to be carefully evaluated.

Conclusion

In this study, a HPLC-UV method capable to separate all analytes under the study was developed and applied to preliminary *in-vivo* experiment simulating the treatment of isoniazid intoxication. Thereafter, Zorbax SB-Aq and ZIC-HILIC columns were selected as proper stationary phases for further LC-MS method development for the investigation of the role of hydrazone metabolites in izoniazid intoxication.

Acknowledgements

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Development and validation of a liquid chromatographic method for the determination of trandolapril and verapamil in tablets

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Introduction

The angiotensin-converting enzyme inhibitors (ACE inhibitors) are widely used for the treatment of mild to moderate hypertension and heart failure, either alone or in conjunction with other drugs [1]. Trandolapril, the esterified prodrug of the active metabolite trandolaprilat, is a nonsulfhydryl ACE inhibitor used in hypertension and congestive heart failure therapy. Verapamil, a slow calcium channel antagonist, was introduced as an antianginal agent but now it is widely used in cardiac dysrhythmias.

In the literature there are limited reported methods referring to the determination of trandolapril and verapamil in pharmaceutical preparation [2]. The focus of the present study was to develop and validate a rapid, sensitive and selective liquid chromatographic procedure coupled with diode array detector for the simultaneous determination of trandolapril and verapamil in pharmaceutical formulations.

Experimental

Trandolapril, verapamil and ramipril standards were obtained from pharmaceutical companies. Ramipril was used as IS. Phosphoric acid, hydrochloric acid, sodium hydroxide and methanol were obtained from Merck (Darmstadt, Germany). Trandolapril and verapamil based commercial tablet formulations were obtained from the national market.

The HPLC analysis was carried out on a Shimadzu HPLC system with a pump (LC-20 AD), a DAD detector system (SPD-M 20A) and column oven (CTO 20 AC). This equipment has a degasser system (DGU 20 A). A X Terra RP-18 column (250 x 4.60 mm ID x 5m) was used at 40 ^{o}C. Mettler Toledo MA 235 pH/ion analyzer with combined glass electrode was used for pH measurements. The mobile phase composition was methanol–water at 55% (v/v), containing 15 mM phosphoric acid. The pH of the mobile phases was adjusted to 2.7 by the addition of sodium hydroxide. Flow rate was 1.2 ml/min. Standard mixture of verapamil (5 µg/ml) (a) - trandolapril (25 µg/ml) (b) and ramiprilin (20 µg/ml) (IS) was given in Fig. 1.

Results

Chromatographic determination of trandolapril and verapamil in commercial tablet formulations was achieved by means of reversed-phase liquid chromatographic procedure. Under the current chromatographic conditions, trandolapril and verapamil were eluted at 3.0 and 5.1 min, respectively. The calibration curves and equations for studied compounds were calculated by plotting the peak area ratios of I.S. versus concentration of the compounds in the range of 0.5–18 µg/mL for verapamil and 0.05–1.00 µg/mL for trandolapril (Table 1). These results showed highly reproducible calibration curves with correlation coefficients of 0.9999. When working on standard solutions and according to the obtained validation

parameters, results encourage the use of the proposed method described for the assay of trandolapril and verapamil in its pharmaceutical dosage form. The results obtained from the analysis of tablet dosage forms are summarized in Table 2. The quantities found were in conformity with the values claimed by the manufacturers.

Conclusion

The proposed RP-LC method enables a rapid and accurate assay with a run time lower than 6.0 min. This analytical procedure provides a useful insight into the quantitation of trandolapril and verapamil in tablets and could be further applied as a routine method to support a wide range of pharmacokinetic studies.

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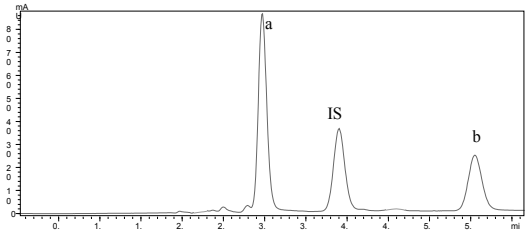

Fig. 1: Chromatogram of standard mixture of verapamil (5 µg/ml) (a) - trandolapril (25 µg/ml) (b) and ramiprilin (20 µg/ml) (IS).

Table 1. Statistical evaluation of the calibration data of verapamil and trandolapril by RP–HPLC.

HPLC

Compounds	Linearity Range (µg/mL)	Slope	Intercept	SE of slope	SE of intercept	Correl coeff.	LOD (µg/mL)	LOQ (µg/mL)
Verapamil	0.50-18.00 n=8	2.179	-0.3107	0.0082	0.083	0.9999	0.0082	0.0250
Trandolapril	0.05-1.00 n=7	0.494	0.0016	0.0020	0.0035	0.9999	0.0178	0.0540

Table 2. Results of the assay and the recovery analysis of verapamil and trandolapril in pharmaceutical dosage forms.

	Verapamil	Trandolapril
Labeled claim (mg)	240	4
Amount found (mg)[a]	239.873	3.958
RSD (%)	0.135	1.564
Bias (%)	0.053	1.053
Added (mg)	240	4
Found (mg)[a]	240.0394	4.0046
Recovery (%)	100.0164	100.1157
RSD% of recovery	0.0851	0.3487
Bias (%)	-0.0164	-0.1157

[a)] Each value of the mean five experiments.

An automated capillary zone electrophoresis on-line coupled with capillary isotachophoresis and a hydrodynamically closed separation system

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Introduction

A combination of capillary zone electrophoresis (CZE) with capillary isotachophoresis (ITP), as operating for the column-switching technology, was published for the first time some 20 years ago [1]. Although offering very significant analytical potentialities, this ITP-CZE combination is still employed rather rare in practice. Very likely, automated capillary electrophoresis (CE) equipment, as based on the column-coupling technology, is offering a set of CE techniques and these can be considered to bring efficient analytical tools for the electroseparations. While considering these facts, such a particular concept of automated CE equipment was proposed by D. Kaniansky et al. [2]. At present, this is equipment (ItaChrom II EA 202A) as commercialized by Villa Labeco (Spišská Nová Ves, Slovakia).

Our particular study is focused for the use of ITP-CZE as operating for the automated CE instrument. Importantly, this is a hydrodynamically closed separation system as almost eliminating for the hydrodynamic and electroosmosis transports and operating only for the electrophoresis transport (see, details as shown in a relevant review [3]). Our approach was electrophoretically operating: (a) using the ITP stage as directly transferred the analytes for the CZE stage (ITP-CZE) while running for CZE, (b) using for the discrete spacers (DS) in the ITP stage and, subsequently, transferred for given analytes as present in a boundary layer (between the ITP zones) as loaded the CZE stage while given for a particular part of the analytes. A set of the ITP(DS)-CZE runs with a given analyte as providing for particular parts of the analyte while well-defined for the electrolyte solution. Apparently, this is a practical way to enhance for the CE resolutions of the analytes.

Experimental

Our experiments were carried out with a prototype of automated CE equipment with the column-coupling and operating for the column-switching [3]. At present, it is commercialized ItaChrom II EA 202A by Villa Labeco, Spišská Nová Ves, Slovakia. For the ITP column was employed with an 800μm I.D. capillary tube (a total length was 90 mm) made of FEP (tetrafluoroethylene and hexafluoropropylene, 3M, St. Paul, MN, USA) and an on-column conductivity sensor. The CZE column for a 320 μm I.D. capillary tube (a total length was 180 mm) as made of FEP. Its photometric absorbance detector (a fiber based the detection technology or a capillary insertion to the detection cell) was set at a 254 nm detection wavelength. The sample solutions were loaded for a 30μm loop by the injection valve.

For the electrolyte solutions were chosen, (1) for the ITP stage: the leading electrolyte (LE) as containing for 10mM HCl with 14.5mM beta-alanine and 0.2 % HEC (pH=3.2); for the terminating electrolyte (TE) solution was employed 15mM caproic acid with 60mM

β-alanine and 0.05 % HEC (pH=4.6), (2) for the CZE stage: the background electrolyte (BE) solution was 100 mM MES with 10 mM HIS and 0.1 % HEC, or using also 8 mM β-cyclodextrine (pH=5.1)

Results

Automated ITP-CZE runs, while loading 50 analytes, was shown to resolve for 35-37 analytes in the CZE stage as operating the electrolyte solutions as described in the Experimental section. It should stress as providing 50 analytes, as operating very tightly in ITP, and, subsequently, were transferred to the CZE stage. Apparently, it can be considered to resolve rather restrictive as regarding very differing migration properties of the analytes. Therefore, we were running for smaller numbers of the analytes as showing benefits for better resolutions. Very likely, this can be linked with an elution model as proposed by Giddings with Davis [4].

In general, we were investigated in details to use discrete spacers as operating for ITP [ITP(DS)] and, in addition, providing groups of the analytes (migrating isotachophoretically in boundary layers between the ITP zones of the discrete spacers). Such specific ITP configurations and transferred to the CZE stage were running automatically to provide enhanced resolutions of the analytes (48 resoluted analytes from 50). Reproducibilities of such ITP(DS)-CZE for the analytes were found very good. Apparently, we can state as our operation was based for suppressing the hydrodynamic and electroosmotic transports in ITP(DS)-CZE.

Conclusions

The electroseparation (using a column-coupling technique with a hydrodynamically closed separation system) was studied for both the ITP-CZE and ITP(DS)-CZE. Our investigation was focused, especially, for the ITP(DS)-CZE. Using the SIMUL program (Bob Gaš with co-workers) can be considered to be very essential in understanding for the ITP(DS)-CZE. We feel, such a concept was introduced for the first time in CE. This might be to follow further in this approach (clearly, especially, for the analytes as included for very complex matrices).

Practically, the use of automated ITP(DS)-CZE is analytically very beneficial. Although, running, in total, for a long time seems to offer new approaches in this context.

Acknowledgements

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Fractionation of humic substances by immobilized metal ion affinity chromatography

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Introduction

Immobilized metal affinity chromatography (IMAC) is gaining widespread popularity as an effective tool for the separation and characterization of a variety of biological macromolecules [1]. IMAC is based on the ability of separated ligands in solution to form coordination complexes with metal ions that are fixed to the immobilized multi-dentate ligands attached to the surface of stationary phase within a column. IMAC is most commonly used in biochemistry, molecular biology and related branches of science for isolation of ligands that bind soft metal ions [2], but it has been also employed to isolate natural organic ligands from aquatic environments and to separate humic substances (HS) [3]. HS possess a variety of sub-structures and functional groups, such as carboxylic, aromatic hydroxyl, aliphatic hydroxyl and amine groups, which may provide potential binding sites for metals via their lone electron pairs. Thus, IMAC may be applied to separate different HS fractions based on their affinity with the immobilized metal ions [4]. The aim of this work was to develop analytical procedure for the separation and fractionation of HS using IMAC methodology. For this purpose we have chosen Al(III) ions as an immobilized metal.

Experimental

Chromatographic analyses were carried out by the HPLC system Elite LaChrom (Merck–Hitachi, Darmstadt, Germany) consisting of L-2130 pump provided by quarternary low-pressure gradient with solvent degasser, L-2200 autosampler, L-2400 UV-VIS spectrophotometric detector, L-2300 column oven, organizer and PC data station with software EZChrom. Packed glass chromatographic column CGC (30×3 mm) produced by Tessek (Prague, Czech Republic) with chelate sorbent Iontosorb Salicyl (approximately 250 mg). The column was equilibrated with 5 ml Labconco water, and then 10 ml acetate buffer solution. After elution, the column was regenerated by washing with 10 ml solution of EDTA. The working standard of HS was isolated from peat from Cerová territory, Slovakia [5].

Results

Sorption characteristics of Al(III) for chelate sorbent procedure included four steps: column packing and equilibrium, immobilization of metal ions, fraction collection and column regeneration. In our case, we used chelating sorbent Iontosorb SALICYL, which containing salicylic acid bound via azo group in side chains of modified bead-form cellulose [6]. This chelate sorbent was selected, because of two reasons. At first the salicyl functional group is one of functional groups of HS that are occurring naturally. This approach can represent model of metal immobilization which occurs in HS in natural systems. At second Al(III)-salicyl chelate has high constant of stability (log b = 14.1). Resulting sorption capacity is highest at pH 5.5 giving value 36 mmol of aluminium per 1 g of the chelating sorbent. This pH value we used for further immobilization of metal ions on Iontosorb SALICYL sorbent.

In the following part of our work, we have HS by IMAC technique. As the first step, the effect of pH of acetate buffer solution on the retention of HS was investigated. Volume 10 ml of these samples was injected on IMAC column. Then the sample was eluted with acetate buffer solution only with one pH value (alternatively, pH = 8.9, 6.7 or 3.9) for 5 min at flow-rate 1 ml min and then retained of sample was eluted with pH = 2.0 eluent 5 min at flow-rate 1 ml min. Different ability of HS to be retained in IMAC column filled with Iontosorb SALICYL sorbent at different values of pH of elution environment was utilized for design of IMAC method with buffer-based pH gradient. The buffer-based pH gradient had been realized by elution of solution from the highest value of pH = 8.90 up to elution by solution of HCl with pH =2.00. In the Fig. 1 it is shown that it is possible to separate HS to individual fractions according to their different affinity to immobilized ions of Al(III).

Conclusion

Based on presented results, Al(III)-IMAC method seems to be promising tool for fractionation of humic substances. Obtained fractions can be characterized further by numerous methods. With help of this simple method it is possible to gain additional information about their character, structure, functionality and possibilities for their utilization in different areas of industry, agriculture and medicine. Potential of this approach to model particular soil processes is under investigation.

Acknowledgements

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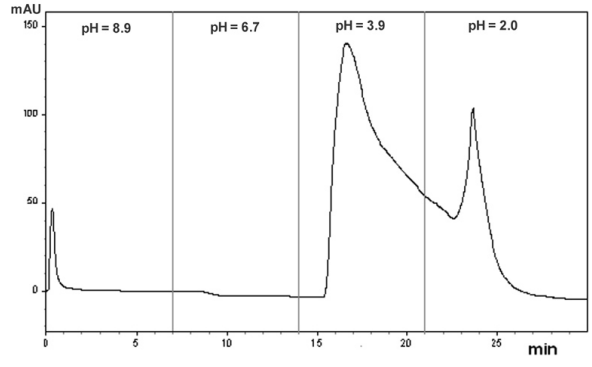

Fig. 1: Chromatograms of humic substances in Al(III)-IMAC experiments with buffer-based pH gradient for gradual elution of analytes.

Diagnosing Inherited Metabolic Disorders by Capillary Electrophoresis

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The work deals with capillary electrophoretic methods with various detection principles suitable and applicable for diagnosing some inherited metabolic disorders (IMDs) at the metabolite level. In practice, it is very important quickly detect and/or determine a number of species allowing timely diagnosis and following possible treatment of IMDs. Therefore, the presentation is strictly focused on rapid and selective capillary electrophoretic methods requiring minimal body fluid samples (urine, blood, cerebrospinal fluid) where metabolites typical for a certain disease or a group of IMDs are commonly elevated. Capillary electrophoretic methods summarized in this review are usable for screening of some IMDs because of their low operating and time-consuming expenses usually limiting routine utilization of other separation techniques with the excessive need of sample volume whose taking from newborn infants has ever been very complicated.

Determination of histamine and histidine in various food samples by capillary isotachophoresis

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Introduction

Histamine (1H-imidazol-4-ethanamine) is a heterocyclic amine and is obtained through decarboxylation of free amino acid histidine. This compound is the most frequently studied biogenic amine in food samples due to its toxicological effect. Histamine is also used as an indicator of food quality and hygiene [1].

Until now, there have been many kinds of methods for determining the presence of histamine in food: enzymatic, immunological and chromatographic. HPLC is one of the most reliable ones commonly applied to fish and related kinds of samples [2].

Capillary isotachophoresis (cITP) has high sensitivity and precision and enables the determination of sample than are problematically analyzed by HPLC or other method [3,4]. For this reason, we applied this method for simultaneous determination of histamine and its precursor (histidine). The results obtained for amine determination were compared with HPLC and spectrofluorimetric method [5].

Experimental

Isotachophoretic separations were performed using a Villa Labeco EA 100/101 isotachophoretic analyzer equipped with a conductometric detector. Samples of 30 l fixed volume were injected via a sample valve by internal sample loop. The isotachopherograms were evaluated with the software supplied with analyser (KasComp Ltd., Slovakia). Analysis of histamine were performed with LE: 10 mM KOH + valine (pH = 9.9) and TE: 20mM TRIS-HCl (pH = 8.3). In the case of histidine: LE 7 mM NH_4OH + 15 mM MES +1% HEC and TE: 15 mM ε-ACA + 5 mM HAC + CH_3OH were applied.

HPLC analyses were performed with a Shimadzu chromatograph UFLC with DAD detector and a column C_{18} (15cm x 0.46cm), Supelco Discovery, Germany. Mobile phase was a mixture of 0.1M acetic acid, acetonitrile and methanol (90:5:5). Detection was carried out in the UV region at 225 nm.

Calibration curve for histamine was constructed using six calibration solutions in the range: 10-100 mgL^{-1}. Results were calculated as an average of five replicates. The calibration curve for histamine, was found resulting in the regression equation of $y = 16179x + 134040$ with the determination coefficient $R^2 = 0.9997$.

Fluorescence measurements were performed using a Fluorescence Spectrophotometer F-7000 HITACHI equipped with a Xenon flash lamp. All analyses were performed in 10 mm quartz cells at 20 ^oC.

All food samples (meat and meat products, fish, cheese) were obtained from different supermarkets.

Results

The histamine and histidine ions were identified using the relative step height (RSH) parameter. Precision was evaluated as the within-day and between-days coefficient of variation (CV) [6]. Within-day analyses were determined by injection of the standard solutions five times per day. Intralaboratory reproducibility was determined by analysis of the standard solutions during 5 consecutive days. The regression parameters of calibration curves for both compounds are listed in Table 1.

The food were minced, homogenized and extracted with 0.1M HCl (for histamine) or methanol (for histidine) using an orbital shaker for 30 min. The extracts were separated using centrifuge at 9000 rpm for 30 min, followed by double filtration. All extracts were transferred into a 50 mL volumetric flask, made up to the mark and analysed with one-dimensional cITP. The typical isotachopherograms of samples are presented on Figure 1. The obtained results were compared with HPLC and spectrofluorimetric methods.

Conclusion

Capillary isotachophoretic (cITP) procedures for the determination of histamine and histidine are presented as an alternative to the HPLC and spectrofluorimetric techniques. cITP is a simple, sensitive and inexpensive method and therefore well suited for routine analysis of food samples.

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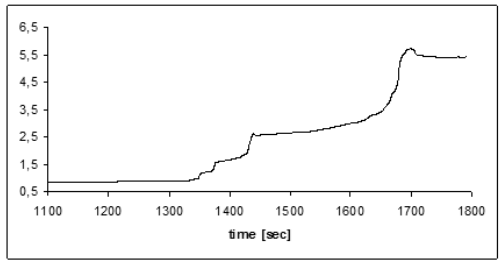

Fig. 1: The isotachopherogram of food samples (chicken).

Table 1: Linear regression calibration parameters of tested compounds determination by cITP method (n=5).

analyte	b	a	R^2	LOQ [mg·L^{-1}]	LOD [mg·L^{-1}]
Histamine	0.6000	0.7422	0.9982	2.24	7.48
Histidine	0.2333	1.3238	0.9977	1.75	5.82

Possibilities of employment of zirconia-based stationary phases in drug analysis

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Introduction

Generally the physico-chemical properties of related substances occurring as impurities in a drug substance are often similar to the drug itself. On that account a targeted development of new separation methods is required to enable the analysis of these complicated samples. Recently, the zirconia-based stationary phases have been extensively studied thanks to their positive chromatographic properties (high thermal and chemical stability, unique type of selectivity combining the reversed phase and ion exchange mechanisms) in many areas of analytical chemistry. Our research group exploits these alternative materials in the field of drug analysis as reported previously [1,2,3].

Experimental

In this work we dealt with the analysis of methylergometrine maleate, a synthetic ergot alkaloid routinely used for its uterotonic effect in the clinical praxis. The analysis of ergot alkaloids often includes a challenging element – the presence of compounds together with their epimers, e.g. active methylergometrine – inactive methylergometrinine (Fig. 1). The structure of analysed compounds arises from a heterocycle with two differently basic nitrogen atoms. Two related compounds possess an additional carboxylic group. According to the European Pharmacopoeia the purity of methylergometrine is assessed by a gradient HPLC assay (principal peak of methylergometrine and nine impurities) based on classic C18 silica sorbents [4]. However, the pH value of the eluent is about 11 which may cause problems with lifetime of columns. Thus an alternative approach utilizing the more stable zirconia stationary phases was investigated. The whole work was performed on the Shimadzu HPLC system equipped with DAD detector. The method development was carried out with the decomposed sample of identifying solution. Besides all specified impurities it was containing some additional degradation products which were also the objects of separation. Moreover we report the analysis of extra one impurity, probably 1'-*epi*-methylergometrinine.

Results

Since the spectrum of analytes counts numerous pairs of geometric isomers the carbon-coated zirconia (Zr-Carb) column was tested firstly. However, similarly as on oktadecyl-modified Zr-Carb sorbent, extremely strong retention was observed without any eluted peaks although 50% acetonitrile and 50% tetrahydrofurane were used as the mobile phase. That is why further work was performed on polymer-modified zirconia columns. The elution of carboxylic acids on polystyrene-coated zirconia (Zr-PS) at acidic conditions (pH 4) was due to extremely slow interaction kinetics very problematic. Moreover Zr-PS column offered slightly worse separation results comparing to that achieved on the polybutadiene-coated zirconia (Zr-PBD). Thus the Zr-PBD stationary phase possessing similar separation

properties as silica based C18 columns was chosen. An interesting trend for both carboxylic acids was observed when acidifying the buffer pH. Except for the increasing retention of them an obvious tendency to coelution of both peaks was observed. Hence a final development of the method at basic conditions was a goal with promising separation at pH 8.5. The subsequent adjustment of gradient profile and small changes in the method settings resulted into a satisfactory separation of all analytes. Optimal chromatographic conditions were Zr-PBD 150 × 4.6 mm (5 μm) as stationary phase, gradient elution with mobile phase consisting of A) 25 mM phosphate buffer (pH 8.7) and B) 50% (v/v) acetonitrile in water, UV detection at 310 nm, flow rate of 1 ml/min and separation temperature 25 °C, respectively. Prior to the validation procedure a stability evaluation was performed. Predominant degradation products originating in the solution after thermal exposition are lysergic and iso-lysergic acid and methylergometrinine. Conversely a number of unknown decomposition products occured after the light exposition. Finally the validation of analytical method for assay of related substances was carried out successfully. The results show the suitability of method for the pharmacopoeial purposes with all quantification limits within the allowed amount of individual impurities. Some small changes in method conditions did not lead to any undesirable coelution.

Conclusion

Polybutadiene coated zirconia column was utilized for the separation of methylergometrine and its pharmacopoeial impurities. Potential new impurity (probably 1'-*epi*-methylergometrinine) was included. The method could serve also for analysis of other unspecified impurities originating in solution. The validation results exhibit a sufficient repeatability, accuracy and sensitivity of the method. By comparison with current pharmacopoeial method there is notable a dramatic reduction of organic solvent consumption what is beneficial from environmental point of view.

Acknowledgements

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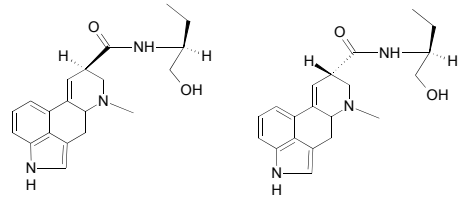

Fig. 1: Methylergometrine (right), methylergometrinine (left).

HPLC or CZE for separation of benzodiazepines?

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Introduction

Benzodiazepines (BZDs) are a class of psychoactive drugs [1]. They are useful in treating anxiety, insomnia, agitation, seizures and muscle spams, as well as alcohol withdrawal [2]. Although BZDs are notably safer in use than their predecessor barbiturates, they can still have severe side effects and, moreover, possess a potential for abuse. They are clinically used as racemates so that enantiomeric resolution is not necessary for drug quality control or screening purposes [3].

Experimental

The separation conditions with C8 and C18 columns and acetonitrile/deionised water adjusted to pH 3.0 with acetic acid as mobile phases were used and optimized in reversed-phase HPLC. In CZE method 6 mM phosphate buffer, pH 2.0 was chosen as background electrolyte.

Results

The aim of this work was to develop fast and simple analytical methods for chromatographic and electrophoretic separations of seven 1,4-BZDs, namely oxazepam, flunitrazepam, nitrazepam, diazepam, chlordiazepoxide, bromazepam and lormetazepam.

HPLC

The first column tested was Zorbax SB-C8. The most suitable mobile phase composition found was ACN/deionised water with acetic acid (HAc), pH 3.0, 30/70 (*v/v*). Baseline resolution of all seven analytes was achieved and the analysis time was 20 minutes. Using the column with more hydrophobic stationary phase – Zorbax SB-C18, the analysis time was substantially reduced. The baseline resolution of all seven analytes was achieved within 7 min with mobile phase composed of higher amount of ACN (40 volume %). Various linear gradient elution profiles were tested to decrease the analysis time. As the peak symmetry was similar under the both isocratic and gradient elution conditions and the analysis time did not differ substantially the isocratic mode was chosen as the optimized separation conditions – see Fig. 1. Under the isocratic conditions there is no need to equilibrate the column before each analysis.

CZE

Under optimized CZE conditions the mixture of six compounds from the 1,4-benzodiazepine group was baseline separated within 17 minutes. Nitrazepam was excluded from the studied set because of its instability in buffer of pH 2.0. This compound gave two peaks in the electropherogram. It was not able to quantify the peak of lormetazepam. Its mobility was very

close to zero and thus the peak was in resonance with the stationary system peak.

Conclusion

The methods for separation of compounds from the 1,4-benzodiazepine group were optimized under RP HPLC and CZE conditions. The LOD and LOQ values were established for all the analytes under optimized separation conditions in HPLC and for five analytes in CZE.

Acknowledgements

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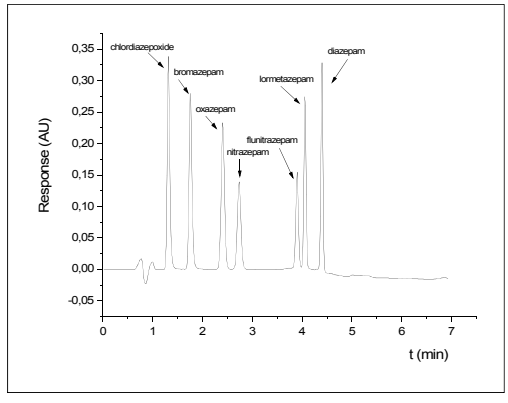

Fig. 1: Chromatogram of separation of seven 1,4-benzodiazepines in gradient elution mode. Column: Zorbax SB-C18, mobile phase: ACN/deionised water with HAc, pH 3.0, 40/60 (*v/v*) with a linear gradient to 90 vol. % of acetonitrile applied from 2nd to 3rd minutes, flow rate: 2 ml/min, column temperature: 25 °C, UV detection: 240 nm.

On-line preconcentration and separation of brominated phenols by isotachophoresis coupled with capillary zone electrophoresis

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Introduction

Brominated phenols (BPs) present a significant group of flame retardant compounds because of their low cost and high efficiency. They are mainly added into polymer matrices for purpose of inhibition or suppression of combustion process. Tetrabromobisphenol A (TBBPA) as one of the most produced compound can be found in electronic equipment, packaging, and construction materials in concentration between 10% and 20% .
Methods for determination of TBBPA and its derivates were recently reviewed by Covaci et al. [1]. For environmental analysis of BPs, GC-MS is the mostly used analytical tool [2,3] followed by HPLC equipped with both UV [4] and MS [5] detectors. Blanco et al. [6,7] used aqueous and non-aqueous capillary electrophoresis with UV detection for separation of BPs. In this work, a method for separation and determination of nine BPs (3,3',5,5'-tetrabromobisphenol A (TBBPA), 2-bromophenol (2-BP), 3-bromophenol (3-BP), 4-bromophenol (4-BP), 2,4-dibromophenol (2,4-DBP), 2,6-dibromophenol (2,6-DBP), 2,4,6-tribromophenol (2,4,6-TBP), pentabromophenol (PBP), 4-bromo-3-methylphenol (4-B-3-Me-P)) was developed utilizing isotachophoresis hyphenated to capillary zone electrophoresis (ITP-CZE) equipped with UV detector. The method was successfully applied to the analyses of BPs's spiked tap and river waters samples requiring only simple filtration.

Experimental

Automated capillary electrophoresis analyzer EA202A (Villa-Labeco, Spišská Nová Ves, Slovak Republic) assembled in the column-coupling configuration of the separation unit, was used in this work for performing the ITP–CZE runs. The samples were injected by a 30 µl internal sample loop of the injection valve of the analyzer. EA202A analyzer was provided with Knauer UV detector K2000 (Knauer, Berlin, Germany) and the detection wavelength was set to 220 nm.

Results

A method for determination of nine brominated was developed by on-line coupled ITP-CZE. For ITP step, 1×10^{-2} mol.L^{-1} hydrochloric acid with 3×10^{-2} mol.L^{-1} ammediol pH 9.1 was used as the leading electrolyte, and 3×10^{-2} mol.L^{-1} β-alanine with 2×10^{-2} mol.L^{-1} sodium hydroxide pH 10.05 was used as the terminating electrolyte. As the background electrolyte for CZE separation, 2.5×10^{-2} mol.L^{-1} β-alanine with 2.5×10^{-2} mol.L^{-1} lysine pH 9.6 was used. All electrolytes contained 0.1% (w/v) hydroxyethylcellulose to suppress electroosmotic flow. Separation of BPs standard mixture at concentration 2×10^{-7} mol.L^{-1} is shown in Figure 1. Detection limits in order of tens of nmol.L^{-1} were achieved. When determining the recoveries

from spiked water samples, unsatisfactory results were obtained. This can be explained by the adsorption of BPs on the hydrophobic surfaces. It presents a significant problem because of FEP material used in the separation system. An addition of 1×10^{-5} mol.L^{-1} naphthalene-1,3,6-trisulfonic acid (NTS) to the sample was performed. NTS adsorbs preferentially onto surfaces during storage, injection, and separation avoiding greater losses of analytes during the separation process and improving their recoveries.

Conclusion

Developed ITP-CZE method was successfully applied to determination of BPs in spiked tap and river water samples, even an adsorption of BPs caused decrease of recoveries. Utilization of naphthalene-1,3,6-trisulfonic added to the sample improved recovery in most cases. Good repeatability of migration times (less than 0.33% RSD) and good repeatability of peak areas (less than 7.19% RSD) at concentration level 5×10^{-8} mol.L^{-1} were observed. Minimum required sample clean-up procedures and simple ITP-CZE automation enables the method to be easily used.

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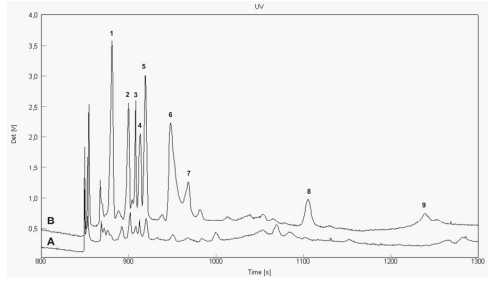

Fig. 1: ITP-CZE separation of standard mixture of BPs (concentration 2×10^{-7} mol.L^{-1}). Electropherogram A presents injection of terminating electrolyte, electropherogram B presents injection of standard mixture. Peak assignment: 1 – TBBPA; 2 – 2,6-DBP; 3 - 2-BP; 4 – 2,4-DBP; 5 – 2,4,6-TBP; 6 – PBP; 7 – 3-BP; 8 – 4-BP, 9 – 4-B-3-MeP.

Separation and identification of monogalactosyldiacylglycerols and digalactosyldiacylglycerols by RP-HPLC/ESI-MS

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Introduction

Monogalactosyldiacylglycerols (MGDGs) and digalactosyldiacylglycerols (DGDGs) are glycolipids forming the major part of biological membranes in photosynthesizing organisms. They are abundant in photosynthetic parts of plants, algae and some bacteria. MGDG and DGDG molecular species differ in fatty acids linked to *sn*-1 and *sn*-2 positions on the glycerol backbone [1]. Reliable methods for analyzing MGDGs and DGDGs are needed to better understand their biological functions and to study their anticancer activities [2, 3].

Until now the HPLC analysis of MGDGs and DGDGs has been performed using isocratic elution, which does not ensure sufficient chromatographic separation. The aim of this work is to develop an RP-HPLC/MS method for efficient chromatographic separation and unambiguous identification of MGDGs and DGDGs in plant samples.

Experimental

MGDGs and DGDGs were isolated from leaves of *Melissa officinalis*, *Eucalyptus globulus* and *Arabidopsis thaliana*. Fresh leaves were extracted by modified method of Folch [4]. The crude extracts were separated on silica TLC plates using chloroform:methanol:water (80:18:2, v/v/v) mobile phase. MGDGs and DGDGs were visualized by spraying TLC plates by primuline solution as describe elsewhere [5]. The TLC zones corresponding to MGDG and DGDG were scraped off the plates and extracted by chloroform:methanol (2:1, v/v). The solvent was finally evaporated under nitrogen stream and the residues were dissolved in chloroform:methanol (2:1, v/v).

The HPLC was performed on a HPLC/MS system with an ion trap spectrometer LCQ FLEET equipped with an electrospray ion source (Thermo Fisher Scientific, USA). A stainless steel column NUCLEOSIL C18, (250 x 2 mm, 5 μm, Macherey-Nagel, Germany) with a C18 precolumn (4 x 2 mm, Phenomenex, USA) was used. The mobile phase for MGDGs was mixed from water (A) and methanol (B). The linear gradient increased from 75% of B to 100% of B in 80 min, followed by 20 min at 100% of B.

The mobile phase for DGDGs was mixed from water (A), acetonitrile (B), and methanol (C). The linear gradient increased from 35% of A and 65% of B to 80% of B and 20% of C in 50 min, followed by a linear segment to 100% of C in 10 min and finally by 20 min at 100% of C. The flow rate of the mobile phase was 200 μl/min and the column temperature was 30 °C.

Results

We examined the retention behavior of MGDGs and DGDGs in the solvent systems with water, acetonitrile and methanol. Binary systems with acetonitrile and water appeared to be

improper for separating molecular species of both lipid classes. The elution strength of acetonitrile is not sufficient enough for complete elution of all molecular species and the peaks tend to be wide and distorted. MGDGs can be separated in the binary mobile phases containing water and methanol. The chromatographic resolution between molecular species is greatly influenced by a gradient steepness and composition of the mobile phase at the beginning of analysis. High initial concentrations of water increase retention of MGDGs and may cause that some MGDGs are not eluted at all. The best separation of MGDG molecular species was observed in a linear gradient 75% - 100% of methanol. Pure methanol has also high elution strength for DGDGs causing quick elution of all molecular species. However, binary gradients with methanol and water did not perform well, as incomplete separation and partial strong retention of the molecular species was observed. Much better results were achieved using ternary solvent system with water, methanol and acetonitrile. When ionized by electrospray, MGDGs and DGDGs provided sodium adducts $M+Na^+$. The main fragmentation pathways led to elimination of fatty acids and dehydrated galactose as neutral molecules.

Conclusion

We developed HPLC/ESI-MS(/MS) methods for analyzing complex mixtures of MGDGs and DGDGs. The methods allow separation and detection of more than 30 molecular species of MGDGs and 40-60 DGDGs in plant samples. These results represent a considerable improvement over the results published so far.

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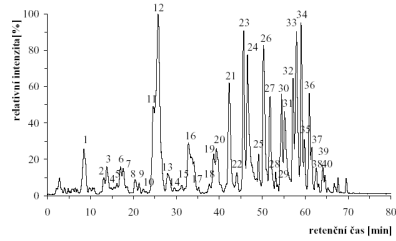

Fig. 1: Basepeak chromatogram of DGDGs from *Eucalyptus globulus* (57 DGDGs identified in 40 chromatographic peaks).

Properties of acid phosphatase from potato tubers immobilized to magnetic particles

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Introduction

A number of applications of enzymes immobilized to various supports in proteomic studies are still increasing. Immobilized proteinases, which are used for the preparation of proteolytic digests for further analysis, predominate. Immobilized enzymes of other type, e.g. protein phosphatases and kinases, are applied only rarely. Generally, an application of immobilized enzymes (enzyme reactors) has several advantages as compared with that of soluble ones, e.g. increased enzyme stability, easy manipulation, possibility of repeated use, and increased thermostability. For immobilization of acid phosphatase in the present study we have chosen magnetic beads.

Experimental

Acid phosphatase from potato tubers (Sigma-Aldrich, Praha, Czech Republic) was coupled to Magnetic Glyoxal 4% Agarose Beads (20–75 μm, BioScience Bead Division of CSS, West Warwick, RI, USA) containing 20 μequiv. of aldehyde groups per 1ml of magnetic beads as described previously [1]. The phosphatase activity was determined using p-nitrophenyl phosphate as a substrate [2].

Results

In the present study, we have compared kinetic properties and stability of immobilized acid phosphatase from potato tubers with those of soluble enzyme. Coupling of acid phosphatase to magnetic beads only slightly affected the pH dependence of the activity: the pH optimum of the soluble form of enzyme was determined at pH 5.5, while that of the immobilized one was characterized by the broader pH optimum curve with maximum at pH 5.0. Contrary to these findings, coupling of acid phosphatase to magnetic beads significantly increased thermostability of the studied enzyme. After incubation of the immobilized enzyme at 50°C for 5 hrs, its activity only slightly decreased (to 92%) as compared with that at 25°C, while the activity of the soluble enzyme reached only 18% of the original activity. Immobilized acid phosphatase fully retained its activity after 3 months of storage at 4°C, while the activity of the soluble enzyme decreased to about 40% of original activity under the same conditions. The immobilized and soluble forms of acid phosphatase did not differ in the inhibition with Zn(II) or Co(II) ions, but the immobilized form of the enzyme was less sensitive to the effect of Fe(III) ions. The K_m value of the soluble enzyme was lower, using p-nitrophenyl phosphate

as a substrate, than that of the immobilized form.

Conclusion

The immobilization of acid phosphatase from potato tubers to magnetic beads did not significantly affect the enzyme activity, but it resulted in the increase of enzyme thermostability and the immobilized enzyme retained its activity after storage. Acid phosphatase coupled to magnetic beads was found to be the advantageous tool for the dephosphorylation of porcine pepsin A.

Acknowledgements

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Immobilized Metal Affinity Electrophoresis as a Tool for Phosphoprotein Enrichment

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Introduction

Post-translational modification of proteins is a fundamental regulatory mechanism, and characterization of protein modifications is primary task for understanding protein function. The reversible process of protein phosphorylation – dephosphorylation is one of the most common of all protein modifications. Disregulation of protein kinases or phosphatases, enzymes involved in these processes, can result in oncogenesis. However, the results of phosphoproteomic studies are largely influenced by low abundancy of phosphorylated proteins, and the reversible nature of the modification. Therefore, pre-analytical separation (or enrichment) of phosphorylated molecules is an advantageous, sometimes necessary, step of the analysis. Besides affinity-based chromatographic techniques that are often used, affinity electrophoretic separation represents an alternative tool for this purpose. In the present study we have modified conditions of immobilized metal affinity electrophoresis (IMAEP) described recently [1,2] for an entrapment of phosphoproteins that could be analyzed y MS.

Experimental

Polyacryalmide electrophoresis was performed both under the denaturing in the presence of SDS [3] and the not-denaturing conditions [4]. The preparation of IMAEP gels containg Fe (III) ions described by Lee et al. [1,2] was modified. $FeCl_3$ solution was added to the solution for the preparation of upper part of the separation gel. The electrophoretic behaviour of phosphoproteins in polyacrylamide gel containing embedded Fe(III) ions was compared with that in gels without metal ions.

Results

Polyacrylamide gels with embedded Fe(IIII) ions were applied to study the electrophoretic behavior of phosphoproteins under both the native and the denaturing conditions. It has been shown that phosphoproteins containing even low content of phosphate groups (1-2 phosphate groups per mol, as e,g, ovalbumin, or pepsin) are entrapped in the upper part of polyacrylamide gel containing embedded Fe (III). Mobility of non-phosphorylated proteins was not affected by the presence of iimmobilized Fe(III) ions. Phosphoproteins entrapped in the metal ion containing part of the polyacrylamide separation gel can be extracted from the and used for the analysis, e.g. using MS. The phosphoprotein capture was observed both in the absence of SDS or the presence of SDS in polyacrylamide gel.

Conclusions

Immobilized metal affinity electrophoresis (IMAEP) represents and an alternative tool that can be used for the separation or enrichment of phosphoproteins prior analysis. This method can be advantageously applied esp. in the cases of complex mixtures of different proteins.

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Determination of selected phenothiazine derivatives by capillary isotachophoresis

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Introduction

Phenothiazines (PH) are widely used in therapy of different nervous disease. Among these drugs there are promethazine hydrochloride (PMT), thioridazine hydrochloride (TD) and chloropromazine hydrochloride(CPM). Methods which are applicated for the determination of this group of pharmaceutical generally based on theirs chemical properties. Among these methods there are titrimery [1,2], spectrophotometry [3-6], fluorimetry [7,8], voltamperometry [9] and chromarography [10-12].

In our paper we described the isotachophoretic method for determination of three phenothiazine derivatives. The elaborated conditions were tested on pharmaceutical preparations. The results are discussed in respect of the accuracy, precision and statistical parameters and compared with these obtained by HPLC and pharmacopoeial methods

Experimental

All used reagents (analytical grade) were purchased from Sigma (Germany) and POCH (Poland), whereas Diphergan, Thioridazin and Fenactil from local pharmacy. Redistilled water (specific conductivity below 2 SmL^{-1}) was used for sample solution preparation.

Isotachophoretic separations were performed using a Villa Labeco EA 100/101 isotachophoretic analyzer equipped with a conductivity detector. The isotachopherograms m were evaluated with the PC software package supplied with analyser (KasComp, Slovakia).

Results

Phenothiazines were analysed applying following electrolytes systems: leading (LE) - 10 mM sodium acetate + 0.08% hydroxethylocelulose (HEC) + acetic acid to pH = 5.5 and terminating (TE) – 10 mM β-alanine (I); LE - 10 mM morpholinoethanosulphonic acid (MES) + 5 mM ammonium and TE 10 mM ε-aminicaproic acid (EACA) + 0.08% HEC (II) and LE - 10 mM sodium acetate + 0.08% HEC + acetic acid to pH = 5.5 and TE -5 mM erythromycin (III). The driving current of the preseparation column and analytical column was 250 A.

Phenothiazines were identified with the relative step height (RSH) which was calculated using the equation: $RSH = h_p/h_t$, where h_p is the height of the zone PH step and h_t is the height of the terminating zone step. To the purpose of calibration curves construction at each concentration levels five 5 repetitions of measurements were made. The statistical parameters of calibration curves are presented in Tab.1. The results of analyzed pharmaceutical preparations are listed in Tab.2.

Table1: The statistical parameters of calibration curves.

	Equation of calibration curve	R^2	LOQ	LOD
System I				
PMT	y=0.1864x+0.385	0.9988	22.7	9.70
TD	y=0.0489x-0.295	0.9964	38,1	16.1
CPM	y=0.1169x-2.516	0.9989	23.7	8.80
System II				
PMT	y=0.1349x-2.80	0.9982	28.90	10.2
TD	y=0.1905x-4.28	0.9985	26.90	9.90
CPM	y=0.1202x+1.66	0.9991	19.80	8.30
System III				
PMT	y=0.1859x-1.610	0.9980	28.90	11.30
TD	y=0.1996x – 5.01	0.9993	20.20	7.40
CPM	y=0.1610x – 1.71	0.9986	25.30	9.80

Table 2: Results of pharmaceutical preparations analysis (n=15).

Pharmaceuticals	Range of analysis, mg/L	Recovery, %	CV, %
System I			
Diphergan	30 - 100	95.96	4.39
Thioridazin	40 - 200	98.68	4.34
Fenactil	30.100	99.14	3.11
System II			
Diphergan	30 - 100	97.23	3.05
Thioridazin	30 - 100	98.13	3.57
Fenactil	30 - 100	98.21	4.16
System III			
Diphergan	30 - 100	95.62	4.11
Thioridazin	30 - 100	96.84	4.32
Fenactil	30 - 100	97.26	4.25

Conclusion

It is shown that the capillary isotachophoresis method is suitable for the quick determination of studied phenothiazines. This method was successfully applied for determination of PMT, TD and CPM in their dosage form. One therefore conclude that cITP can be suitable for routin analysis in quality control laboratory.

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Chiral separation of R,S-tolterodine by capillary electrophoresis employed negatively charged cyclodextrins

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Introduction

Enantiomers of (*R,S*)-2-[3-(diisopropylamino)-1-phenylpropyl]-4-methyl-phenol (tolterodine) is a competitive muscarinic receptor antagonist used for the treatment of urinary incontinence and other overactive bladder symptoms, such as urgency and high-micturition frequency [1]. The enantiomeric purity of tolterodine is very important issue for quantitative estimation of enantiomers of tolterodine. For enantiomeric separation of R,S-tolterodine LC method was published using Chiralcel OD-H chiral column [2] and only one work employed capillary electrophoresis (CE) method [3] using sulfated-γ-cyclodextrin (S-γ-CD). S-γ-CD is relatively expensive chiral selector that must be used in higher concentration (electrokinetic chromatography mechanism at negative separation polarity). From this point of view, the study of separation ability of other negatively charged cyclodextrine for R,S-tolterodine was performed.

This work deals with study of enantiomeric separation of R,S-tolterodine using of three negative charged cyclodextrines sulfated-α-cyclodextrin (S-α-CD), sulphated-β-CD (S-β-CD) and phosphated-γ-cyclodextrin (P-γ-CD) by CE.

Experimental

Capillary electrophoresis HP 3D Agilent (Waldbronn, Germany) equipped with on-column diode array detector was used. The separation was performed in fused silica capillary of 48.5 cm total length and 50 μm I.D. (MicroSolv Technology Eatontown, NJ, USA). The capillary was thermostated to 25 °C. The capillary was rinsed 15 min by 0.1 M NaOH, 15 min by deionized water and then by running electrolyte at the beginning of each working day. Between individual analyses the capillary was washed 1 min by 0.1 M NaOH, 1 min by deionized water and 2 min by running electrolyte. The detection wavelength was set at 200 nm. Injection was performed by pressure of 50 mbar. The applied voltage was +20 kV for experiments with P-γ-CD and -15 kV for experiments with S-α-CD and S-β-CD. All the measurements were performed five times.

Phosphoric acid, sodium hydroxide, TRIS, S-α-CD, S-β-CD and P-γ-CD were obtained from Sigma (St. Louis, MO, USA).

Results

An acidic background electrolyte (BGE) with sulfated-β-cyclodextrin (S-β-CD), sulfated-α-cyclodextrin (S-α-CD) and phosphated-γ-CD (P-γ-CD) were used to create conditions for chiral separation of R,S-tolterodine. Baseline separation of the enantiomers was achieved during 10 min for S-β-CD, 20 min for S-α-CD and during 7 min for P-γ-CD. When the

S-α-CD and S-β-CD were used for chiral separation, the negative voltage was applied and diastereomeric complexes between tolterodine enantiomers and sulphated cyclodextrins migrated as anions. On the other hand when P-γ-CD was used as selector the positive voltage was used, no enantiomeric separation was obtained with high concentration of P-γ-CD and negative applied voltage. The different migration order of enantiomers was obtained for S-α-CD in comparison with S-β-CD. The electropherograms of chiral separation of R,S-tolterodine using the all studied negatively charged cyclodextrines is in Fig. 1.

Conlusion

The methods for enantiomeric separation of R,S-tolterodine using three negative charged cyclodextrins in acidic background electrolyte were developed. All of studied cyclodextrins allow separation of R,S-tolterodine with $R_S > 1.5$. The reversed migration order was achieved when the S-α-CD and S-β-CD used as chiral selectors which is advantageous for determination of minor enantiomers.

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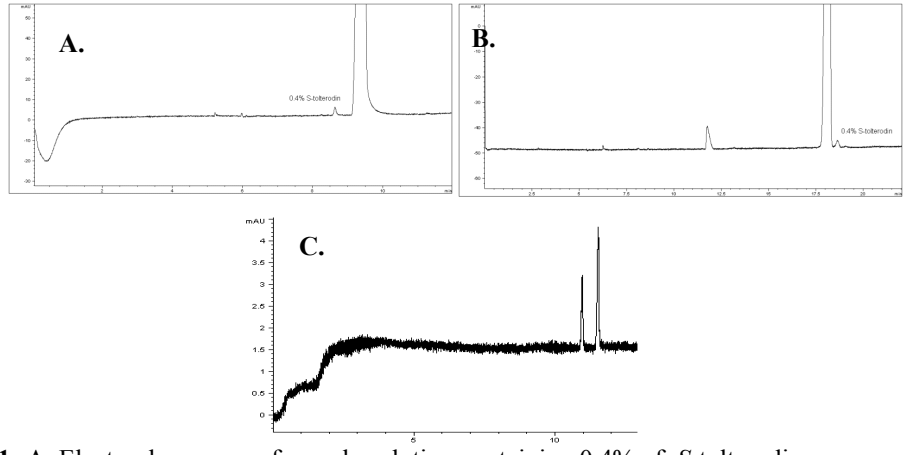

Fig. 1: A. Electropherogram of sample solution containing 0.4% of *S*-tolterodine. Conditions: 30 mM TRIS/phosphate, pH 2.5, with 4% of S-β-CD, -15 kV, applied pressure 5 mbar.
B. Electropherogram of sample solution containing 0.4% of *S*-tolterodine. Conditions: 30 mM TRIS/phosphate, pH 2.5, with 4.5% of S-α-CD, -15 kV, applied pressure 5 mbar. C. Electrophrerogram of sample solution containing 35% of *S*-tolterodine. Conditions: 100 mM phosphate/TRIS pH 2.5 with 1% of P-γ-CD, +20 kV.

Optimalizace separačních podmínek a vývoj metody EMMA pro "in-capillary" studium enzymové aktivity CYP2C9 s diklofenakem

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Úvod

Cytochromy P450 (CYP) jsou skupinou enzymů, kterou spojuje monooxygenasová aktivita a identická prostetická skupina, ale liší se ve struktuře apoproteinu. Funkcí CYP je biotransformace endogenních a exogenních látek na polárnější deriváty. Klíčovou roli hrají mimo jiné v metabolismu léčiv a z farmakokinetického hlediska patří k nejčastěji studovaným enzymům [1].

Jelikož je přímá kvantifikace hmotnosti enzymu nemožná, enzymy se popisují pomocí katalytické aktivity. Tu lze vcelku snadno stanovit měřením rychlosti reakce katalyzované enzymem. K tomu účelu může sloužit celá řada analytických technik, jejichž vhodnost je určována povahou enzymu, jeho čistotou a účelem stanovení. Ideální analytická metoda je specifická, citlivá, jednoduchá a rychlá. A právě kapilární elektroforéza (CE) se svými módy splňuje takřka všechny zmíněné podmínky [2].

CE je separační analytická metoda, kterou při studiu enzymové aktivity lze využít pro separaci reakční směsi a stanovení přítomného produktu. Tento přístup se v souvislosti s CE označuje jako „pre-capillary" stanovení. Nicméně kapilára nemusí sloužit jen pro separaci reakční směsi, ale její vnitřní prostor může být využit také jako miniaturní reakční cela. Substrát a enzym se jednoduše odděleně nadávkují do kapiláry, elektroforeticky se promíchají a následně se stanoví množství vzniklého produktu. Jelikož reakce probíhá přímo v kapiláře, označuje se tento přístup jako „in-capillary" stanovení anebo jako elektroforeticky zprostředkovaná mikroanalýza (EMMA) [3]. Výhodou EMMA je nízká spotřeba substrátu i enzymu a jedná se o jistou formu automatizace [2].

Metoda

CYP2C9 se podílí na metabolismu přibližně 10% terapeuticky významných léčiv. V lidských játrech je zastoupen z 20% z celkového množství CYP a je jednou z nejvýznamnějších isoforem CYP. Pro studium aktivity CYP2C9 byl jako modelový substrát použit diklofenak, který podléhá 4'- a 5-hydroxylaci. 4'-hydroxydiklofenak je hlavní metabolit, zatímco 5-hydroxydiklofenak je minoritní produkt [1].

Reakční podmínky a výchozí separační podmínky byly převzaty z práce Konečného, *et al.* [1], která byla zaměřena na „pre-capillary" studium enzymové aktivity CYP2C9 s diklofenakem. Reakční pufr byl 50mM fosfátový pufr (pH 7,4) s obsahem 5,1 mM $MgCl_2$ a 0,1 M KCl. NADPH nezbytný pro reakci CYP byl kontinuálně regenerován glukosa-6-fosfát dehydrogenasovou reakcí. Teplota reakční směsi byla po celou dobu reakce udržována na 37°C.

Výchozí separační podmínky se sestávaly z křemenné kapiláry s vnitřním průměrem 50 μm o délce 64,5 cm (56,0 cm efektivní délka). Základní elektrolyt (BGE) byl tvořen 20mM fosfátovým-tetraborátovým pufrem (pH 8,6) s 50 mM SDS. Reakční směs byla dávkována tlakem 50 mbar po dobu 4 sekund. Separační napětí 24 kV, teplota kapiláry 25°C a UV.

detekce o vlnové délce 200 nm byly parametry zajišťující ideální separaci a citlivou detekci.

Výsledky

Separační podmínky z práce Konečného, *et al.* [1] byly optimalizovány pro „pre-capillary" stanovení enzymové aktivity CYP2C9 s diklofenakem. Předběžné analýzy zaměřené na otestování vhodnosti daných podmínek i pro „in-capillary" studium ukázaly na nedostatky především v promývání kapiláry. Docházelo k častému ucpávání a relativní směrodatná odchylka (RSD) pro migrační časy diklofenaku a 4'-hydroxydiklofenaku byly vyšší jak 6,1% (n=5). Z toho důvodu byla realizována obsáhlá optimalizace promývání kapiláry, kdy různé kombinace 0,1M NaOH, 1M NaOH, 0,1M HCl, 1M HNO_3 a acetonitrilu byly testovány s cílem minimalizovat RSD pro migrační časy. Kombinace 0,1M HCl a 0,1M NaOH v zmíněném pořadí se ukázala za jednoznačně nejvhodnější postup promývání kapiláry. RSD pro migrační časy obou analytů nepřesáhly 0,2% (n=5).

S ohledem na rychlost analýzy a citlivost stanovení byly dále optimalizovány koncentrace a složení BGE, separační napětí a délka kapiláry, která byla zkrácena na 48,5 cm (40,0 cm efektivní délka). Dále byla zvýšena teplota kapiláry na 37°C, a to ze zjevných důvodů, kdy v případě EMMA enzymová reakce probíhá přímo v kapiláře.

Vzhledem ke skutečnosti, že reakční pufr a BGE se liší svým složením a pH, byla zvolena za jednoznačně nejvhodnější variantu zonálního módu EMMA technika částečného plnění. Její charakteristikou je, že odděleně nadávkované zóny substrátu a enzymu jsou ohraničeny zónami samotného reakčního pufru. Zóny reakčního pufru zajišťují optimální podmínky pro enzymovou reakci a brání jinak nevyhnutelné denaturaci enzymů na rozhraní zón enzym/BGE.

Závěr

Byly optimalizovány separační podmínky a vyvinuta metoda EMMA pro „in-capillary" studium enzymové aktivity CYP2C9 s diklofenakem.

Poděkování

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Optimization of capillary electrophoretic method for determination of physiologically relevant nucleotides and coenzymes in cell extract

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Introduction

Nucleotides are essential in many biochemical pathways. They represent the universal currency of energy in biological systems, play important roles in the regulation of the metabolic states of living cells, and represent the precursors for DNA and RNA synthesis [1]. Due to their importance for metabolism, the main aim of this work was to optimize capillary electrophoretic method for the selective and rapid determination of 17 energetically important metabolites (purine and pyrimidine nucleotides, adenine coenzymes and Acetyl CoA). For improvement of concentration sensitivity, the capillary zone electrophoresis (CZE) was combined with the on-line preconcentration technique -field enhanced sample stacking.

Experimental

All the CZE experiments were performed using an Agilent ^{3D}Capillary Electrophoresis System equipped with a diode array UV-VIS detector (DAD). Experimental data were collected and evaluated by ^{3D}CE ChemStation Software. Metabolite samples were dissolved in deionised water and injected by pressure of 50 mbar for 14 s into the fused-silica capillary (effective length: 56 cm; internal diameter: 75 m). Data were collected at a detection wavelength of 260, 280 and 340 nm. Peaks were identified by spiking the sample solution with a standard of the respective metabolite and by comparing the UV spectra of peaks obtained from DAD.

Results

The optimal separation was reached by using voltage 23 kV (positive polarity), temperature of capillary 21 °C and direct detection at 260, 280 and 340 nm. 50 mM concentration of phosphate buffer (pH 5.8) provided the best resolution of peaks. Resulting method showed RSD in the range from 0.65 to 1.40 % for migration time and from 2.37 to 5.99 % for relative peak area of metabolites (n=10). Optimized method was used for analyzing of cell-extract of bacterium *Paracoccus denitrificans.*

Conclusion

The optimal CZE conditions for separation of 17 energetically important metabolites were found and afterwards the optimized methodology was applied on the cell-extract of *Paracoccus denitrificans.*

Acknowledgements

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Ion pairing of selected cations with dodecylsulphate micelles in electrophoretic systems

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Introduction

As interactions in micellar electrophoretic systems are not fully understood yet predictions and simulations of such systems are rather complicated. The simulation program PeakMaster [1, 2] can predict correctly behavior and properties of the electrophoretic systems where no aggregation occurs. If background electrolyte contains a surfactant in concentration above its critical micelle concentration (CMC) predictions of the PeakMaster fail and the simulations are not in agreement with experiments. We suppose that this disagreement is caused by ion pairing of cations present in the system with the micelles. This interaction leads to decreasing of effective mobilities of these cations. In case of alkaline cations the interaction should differ according to the atomic number and diameter of the cation. The smaller the cation, the bigger solvation shell. As a consequence, smaller cations exhibit weaker interactions with the micelle and thus their effective mobility should be less influenced by the presence of micelles.

Experimental

We measured dependencies of effective mobilities of selected cations (Na^+, K^+, Cs^+, $Tris^+$) on concentration of dodecylsulphate in background electrolyte above its critical micelle concentration. CMC was determined using the method proposed by Lokajová et al. [3]. The method uses a ”jump” in the dependence of slow system eigenmobility on concentration of an surfactant. The steep change in this dependence indicates formation of micelles and so determines the value of CMC.

Results

The effective mobilities of selected cations were decreasing with increasing concentrations of the surfactant above its CMC. These dependencies seemed to be linear with different slopes for different cations. To compare interactions of these cations with the micelle we plotted relative effective mobilities against the surfactant concentration (Fig. 1). Steeper decrease of the relative effective mobility (i.e. higher absolute value of the slope) reflects stronger interaction. In addition to the alkaline cations, organic cation $Tris^+$ was also studied and its interaction with the micelle appeared to be slightly stronger than the interaction of Cs^+.

Conclusion

The order of the higher absolute value of the slope of the dependencies of the alkaline cations can be related to their interaction force with micelles. It was found that the higher absolute value of the slope increase with increasing value on atomic number and decreasing hydrodynamic radius. This was in agreement with our presumptions as well as with the works of other authors [4,5,6], in which the authors used other methods to investigate such interactions.

Acknowledgements
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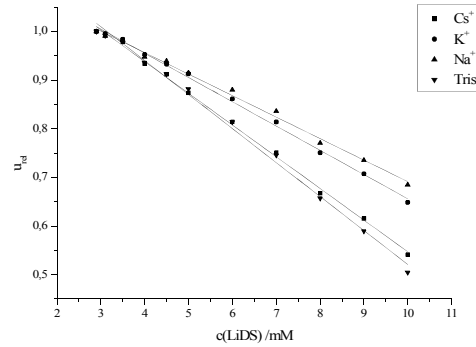

Fig. 1: Dependencies of the relative mobilities $u_{eff}(c)/u_{eff}(CMC)$ of various cations on concentration of lithium dodecylsulphate.

Analysis of poly(5-(4'-aminophenylamino)-10,20-diphenylporphyrin by MALDI-TOF

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Introduction

Porphyrins are among the most-studied organic chromophores. These macrocylic compounds have a great potential in catalysis, sensors, biomedicine and photosensitizer applications due to their rich electron-transfer chemistry [1]. Among these materials, metalloporphyrins have been used as attractive agents for electrode modification and they can be useful in photoelectrochemical process due to their strong light absorption in the visible-near-ultraviolet region [2,3]. A next step for porphyrines is to use their characteristics to produce electricity or fuels. Matrix assisted laser desorption (MALDI) coupled to QqTOF analyzer has become very useful mass spectrometric method for analysis of synthetic polymers [4,5]. Based on exact mass measurement and study of MS/MS spectra this analytical technique provides information about molecular weight distribution, repeated units as well as end groups of polymer [6,7]. In this work MALDI-QqTOF was used to characterize new promising conductive polymer; poly(5-(4'-aminophenylamino)-10,20-diphenylporphyrin (PAPADPP).

Experimental

Mass spectrometer equipped with a hybrid analyzer QqTOF and vacuum MALDI source (Waters, Milford, USA) was used. The studied material was dissolved in tetrahydrofuran (10 mg of PAPADPP in 1 mL). Dithranol was used as matrix (20 mg/mL solution in tetrahydrofuran). Both solutions were properly mixed and deposited on the sample plate. After evaporation the plate was loaded into the ion source and mass spectra were recorded. The setup of mass spectrometer was as follows: positive ion mode, laser parameters: wavelength: 337.1 nm; pulse energy 300mJ (10 Hz), flow rate of cooling gas: 10 mL/h, collision energy 10 V and collision gas flow 0.32 mL/min. A mixture of polyethylene glycols (PEG) 600, 1000, 2000 and 4000 was used for TOF calibration. PEG 10000 was measured as a reference compound.

Results

Figure 1 shows the spectrum of PAPADPP. Major peaks at m/z 568.6869, 1133.4474, 1699.6552, 2265.8647, 2831.1118, 3397.3125, 3963.6026 and 4529.8223 correspond with

m/z of expected oligomers (precision of measurement is 10.5, 3.7, 8.3, 13.5, 4.2, 5.6, 12.4 and 10.4 ppm, respectively). Oligomers containing 6-8 units can be seen in the inserted spectrum focused on higher masses. It can be seen that the highest signals provide quasimolecular ions of dimer and trimers. The highest yield of those oligomers was proven at each setup of quadrupole scan range profile. Moreover, fragments of particular oligomers can be observed in the spectra (i.e. 1043.4121, 1609.6391, 2175.8562, 2741.1372, 3307.2983, 3873.5731 and 4439.8130). These peaks belong to the loss of aminophenyl unit (Dm/z=90.0344). This fragmentation pattern was unambiguously proven by MS/MS spectra. To conclude, the MS experiments show–that the PAPADPP is a mixture of oligomers containing 1–8 units of (5-(4'-aminophenylamino)-10,20-diphenylporphyrin). Dimer and trimer are evaluated as the most abundant components.

Conclusion

In this work, novel porphyrin polymer (5-(4'-aminophenylamino)-10,20-diphenylporphyrin) was characterized by MALDI-QqTOF mass spectrometry. The highest found oligomer contains 8 units. Dimer and trimer are the most abundant components of the studied material.

Acknowledgements

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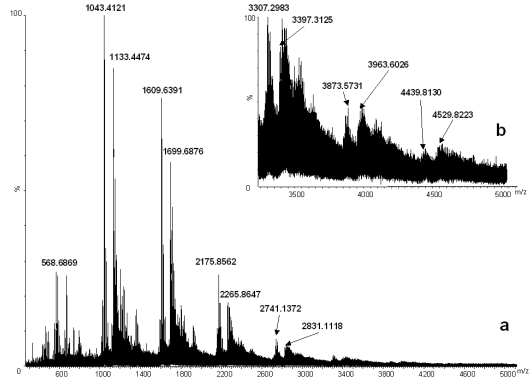

Fig. 1. Maldi MS Spectrum of PAPADPP (a); inserted spectrum focused on the higher masses with 6-8 units (b).

An impact of zwitterionic micelles to the separation of humic acids by ITP-CZE

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Introduction

Humic substances (HSs), ubiquitous natural materials, occur in huge amounts in soils, sediments and waters as products of the chemical and biological transformations of animal and plant residues. HSs are chemically complex and polyelectrolyte-like materials that range in molecular mass from a few hundred to several thousand. In fact, these are attributable for main fractions: (1) humic acids (HAs), (2) fulvic acid (FAs), (3) humin.

A series of capillary zone electrophoresis (CZE) papers was published for the humic and fulvic acids and these were operating for a hydrodynamically opened separation system. A resolving power for these acids was found rather low for this particular CZE approach. On the other hand, our study was employed for the HA and FA constituents and, especially, as using the ITP-CZE combination with a hydrodynamically closed separation system. In addition, such a specific CE approach was taken to introduce for a humic sample and eliminating for the aggregations of these constituents as used the electrolyte system solutions at pH=10 or so. Further, relatively close effective mobilities of the HAs and FAs were investigated to enhance for differentiating power of these constituents as reflecting for suitable separation mechanisms while linked with zwitterionic detergents (adding the detergents to the background electrolyte in the CZE stage).

Experimental

Capillary electrophoresis equipment with the column-coupling and operating for the column-switching, a prototype equipment as introduced by D. Kaniansky et al. [1], was used in our experiments (currently, ItaChrom II EA 202M is commercialized by Villa Labeco, Spišská Nová Ves, Slovakia). For the ITP column was employed with an 800mm I.D. capillary tube (a total length was 90 mm) made of FEP (tetrafluoethylene and hexafluoropropylene, 3M, St. Paul, MN, USA) and an on-column conductivity sensor. The CZE column for a 320 μm I.D. capillary tube (a total length was 180 mm) as made of FEP. Its photometric absorbance detector (a fiber based the detection technology or a capillary insertion to the detection cell) was set at a 254 nm detection wavelength and followed with the on-column conductivity sensor. The sample solutions were loaded for a 30mm loop for the injection valve. In addition, it was included for the samples with the aid of a 701 N microsyringe (Hamilton, Bonaduz, Switzerland).

For the electrolyte solutions were chosen, (1) for the ITP stage: the leading electrolyte (LE) as containing for 10mM HCl, 39mM etanolamine and 0.2% HEC (pH=10); for the terminating electrolyte (TE) solution was employed for the electrolyzed deionized water and minimizing carbonate and electrochemically formed hydroxide; (2) for the CZE stage: the background electrolyte (BE) solution was containing 20mM beta-alanine, 30mM etanolamine, 0-50mM DDAPS, 0.05% HEC (pH=10).

Results

In dependence on pK values of FAs and HAs and pH values of the electrolyte systems tend to aggregate in aqueous solutions. Apparently, the separations are performed at a high pH (at pH=10 or so) while eliminating or, at least, minimizing humic aggregate formations (dissociations of phenolic groups).

Dodecyldimethylammoniopropanesulfonic acid (DDAPS), a zwitterionic molecule, indicates a zero charge as covered for zwitterionic micelles in the background electrolyte solution. A critical micelle concentration (CMC) of DDAPS is ranging from 2 to 4 mmol/l. The usage of DDAPS in CE for HSs was not published yet. In the present work, HA constituents were isotachophoretically separated into 5 fractions (HAs were migrating in the interzonal boundary layers as formed by the ITP zones). A resolution for HAs in the CZE stage (including ITP-CZE), using the BE without DDAPS or at concentration under CMC, was very similar to the ITP resolution. Considerable changes in the zone electrophoresis profile, in the presence of a higher concentration of DDAPS (above CMC) in the BE, were significantly observed. These electroneutral DDAPS micelles are attributable for complexation with HAs and differentiating the effective mobilities of HAs. Reproducibilities for the CZE profiles (fingerprints) of the studied humic constituents were found common.

Hydroxyethylcellulose (HEC) at a low concentration (e.g., at a 0.1%) in the electrolyte solutions as an electroosmotic flow suppressor was used. An influence of a high viscosity BE, achieved by very high concentration (1.8 %) of HEC, on the separation of HAs were also studied.

Conclusions

The ITP-CZE separation (using a column-coupling technique with a hydrodynamically closed separation system in an anionic mode) was studied for the first time with humic acids (HAs).

In this context, was introduced for the use of DDAPS in the BE solutions (applied over the CMC while acting like a pseudo-stationary phase) to optimize the CZE separation of humic constituents. In fact, the analytes are distributing between the pseudostationary phase and the plain buffer solution during of the CZE separation. Due to these micelles can be employed to modulate the selectivity in the separation of closely related charged compounds (HAs) by introducing lipophilicity as an additional differentiating parameter.

HEC while present in the BE solution at a very high concentration, has evident but not significant impact on the effective mobilities of HAs in the CZE stage of the ITP-CZE separation.

Acknowledgements

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Effects of organic solvents on on-line electrokinetic preconcentration in capillary electrophoresis

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Introduction

Capillary electrophoresis (CE) is well known separation technique that with common UV detection suffers from a lack of detection sensitivity. However, the CE enables on-line sample preconcentration diminishing this disadvantage. Four main preconcentration techniques were established up to now: stacking, sweeping, isotachophoresis, and dynamic pH junction. Indeed, the combination of presented techniques is possible and could provide high preconcentration factors, e.g. over 500,000 [1, 2]. Interesting on-line preconcentration method was presented by Horáková et al. [3] from our group. It is based on electrokinetic injection of weak acid diluted in electrolyte at pH 9.5 to the pH boundary formed by electrolyte at pH 2.5. After this preconcentration, analytes are mobilized and separated due to partitioning between SDS micelles and the free electrolyte.

In this contribution, we studied the effect of organic solvents on a performance of the electrokinetic on-line preconcentration of model compounds benzoic acid, ferulic acid, and cinnamic acid. In general, organic solvents are well known for their general effects on separation performance in CE. They change among others electrolytes' electrical permittivity, viscosity, and conductance [4]. In this view, the use of organic solvents could be profitable in electrokinetic preconcentration because all the variables mentioned have the influence on the mechanisms of the preconcentration.

Experimental

Separations were performed on the capillary electrophoresis system HP ^{3D}CE (Agilent Technologies, Waldbronn, Germany) with diode array detection; the detection wavelength was 200 nm and 280 nm. Uncoated fused silica capillaries capillaries (Polymicro Technologies, Phoenix, USA) with 50 μm i.d., total capillary length 48.5 cm, effective length 40.0 cm, were used in these experiments. The capillary cassette was thermostated at 25 °C. The capillary was rinsed every day before the initial experiment with 0.1 M NaOH (10 min), deionised water (20 min), and then with the analysis buffer (10 min). The capillary was further rinsed with 0.1 M NaOH (5 min), deionized water (10 min), and then with the buffer (5 min) before each analysis. Electrolytes were prepared by dissolving appropriate amounts of the acid in deionized water and pH was adjusted by 50 % (w/w) sodium hydroxide to the desired value. SDS was added to the final electrolyte and the solution was properly mixed.

Results

A study of addition of two common organic solvents methanol (MeOH) and acetonitrile (ACN) to the injection electrolyte was performed. They were added to the injection

electrolyte in concentrations of 0 – 80 % (v/v) and 10 min injection of 10^{-6} mM benzoic acid was studied with SDS-mobilization (50 mM SDS). The increase of organic solvent in injection electrolyte led to the non-linear increase of peak area of benzoic acid. However, the injection reproducibility in the terms of current stability was lower with higher concentration of the organic solvent.

The use of 80 % (v/v) MeOH seemed to be the most interesting regarding the effectiveness of the on-line preconcentration. Therefore, a mixture of benzoic acid, ferulic acid, and cinnamic acid was analyzed with 30 min of electrokinetic injection (see Fig. 1). Detection limits, 0.3 nM for benzoic acid, 0.7 nM for ferulic acid, and 1.0 nM for cinnamic acid were much better in comparison with previously published electrokinetic accumulation methods without any addition of organic solvent. Moreover, in comparison with a classical CZE method, the electrokinetic injection from mostly methanolic electrolyte provided ca 70,000 fold lower LODs. Indeed, the limitation of this method is still in the analysis of real samples in which also other ions could be presented in a sample and therefore a sample clean up step

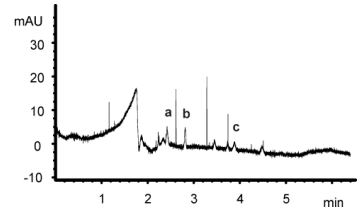

Fig. 1: An example of preconcentration from the mostly methanolic injection electrolyte a) cinnamic acid, b) ferulic acid, c) benzoic acid.

Conclusion

In this contribution, we showed that the addition of organic solvent to the injection electrolyte could enhance the preconcentration performance. Subnanomolar detection limits of hydroxybenzoic acids were achieved with 80 % methanol in the injection electrolyte within 30 min injection what represents more than 70,000 fold improvement of LODs in comparison with common CZE method.

Acknowledgements

The financial support by the Research project MSM 6198959216 is gratefully acknowledged.

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Fractionation and characterization of humic substances by RP-HPLC method using stepwise gradient

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Introduction

Humic substances (HSs) are ubiquitous natural materials occurring in huge amounts in soils, sediments and waters as a product of the chemical and biological transformation of animal and plant residues [1]. HSs are also complex mixture of amorphous, yellow to black colored, hydrophilic, polyelectrolyte-like, polydisperse bio-macromolecules and probably no their two molecules are identical. With respect to their supramolecular structure, there is still a lot of controversis about their nature.. HS are divided to humic, fulvic acids and humin. In this work, reversed phase high performance liquid chromatography (RP-HPLC) using stepwise gradient of dimethylformamide (DMF) in buffered aqueous mobile phase and a wide-pore (30nm) octadecylsilica column was evaluated for purpose of fractionation and characterization of humic acids by tandem flourometric and diode-array detection.

Experimental

Fractionation and characterization of HSs was carried out by the HPLC system LaChrom (Merck-Hitachi, Darmstadt, Germany) consisting of pump L-7100 provided by a quarternary low-pressure gradient, autosampler L-7200, LiChrospher ODS WP 300 RP-18 (250 x 4) mm, (30nm wide-pore) column and guard column, column oven L-7300, diode-array detector L-7450A, fluorescence detector L-7480, interface D-7000, PC data station with software HSM version 4 and on-line four channel solvent degasser L-7612.

Stepwise gradient program was set from 0.0 to 3.6 min isocratic 0% (v/v) B in A, and from 3.7 min, every 4 min there was isocratic step added increasing content of B in A by 10% (v/v) up to the last step increased by 9% (v/v) ending in 99% (v/v) B in A, maintained till 55.0 min isocratic 99% (v/v) B in A, from 55.1 min to 60.0 min linear decrease from 99% (v/v) B in A to 0% (v/v) B in A and between runs 10 min reequilibration was maintained [2,3].

Solution A: 1% DMF / 99% phosphate buffer (c = 5mM, pH = 3)

Solution B: 100% DMF

Two types of HAs was analysed in this work, the first one was commercially developed standard of HSs from Sigma - Aldrich company (Aldrich) and the second one was soil from Dunajská Streda (DS J) isolated by combinated IHSS/Nagoya fractionation scheme [4,5].

Results

In the first step the RP-HPLC method was used to obtain elution profiles of all samples and for fractionation (collecting all 11 fractions). In the second step the RP-HPLC method was applied for characterization of each fraction, which means re-injection all collected fractions. The results showed that ten-step gradient can induce distinct features of HAs. Combination of very good DMF solvating and disaggregating properties for HSs together with wide pore RP sorbent improves surface interactions of the analytes and suppresses influence of size exclusion effects. Thus it provides reproducibility of characterization profiles and robustness of the methods.

Conclusion

Obtained data indicate, that this mode of application of RP-HPLC system could be employed as a separation system for more detailed characterization of such complicated natural biopolymers such as analysed HAs and obtain so more information about their attributes.

Acknowledgments

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Extraction of water-soluble proteins from Bt-transgenic and non-transgenic maize species and tryptic digests peptide profiling by capillary electrophoresis

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Introduction

The progress in gene engineering has led to introduction of genetically modified organisms (GMOs) into production of foods. Transgenic *Bacillus thuringiensis* (Bt) maize belongs to insect-resistant GMOs, which are expanding rapidly worldwide. Bt hybrids with the event MON 810 were constructed by introducing the *cry1Ab* gene from *Bacillus thuringiensis,* in order to produce its own insecticide,ä-endotoxin, for European corn borer (*Ostrinia nubialis*). Due to the public concern on GMOs role in the environment and food and feed safety, the development, growth and use of GMOs are under regulation in many countries, especially in the EU. In order to fulfill these regulations, reliable and powerful analytical methods to detect and quantify GMOs and/or their products in foods are needed. Besides the detection of transgenic DNA, protein analysis represents another way to detect GMOs. Protein fractions from transgenic Bt and non-transgenic maize varieties were analyzed by perfusion and monolithic RP-HPLC [1]. Capillary zone electrophoresis (CZE) is being increasingly used in protein analysis [2] and peptide mapping [3], but up to now, rather limited research has been focused on the development of CZE methods for separation of the water-soluble (WS) proteins from crops [4, 5]. The aim of this study was to develop a new method for extraction of water-soluble proteins (albumin fraction) from the maize flours of transgenic Aristis-Bt and non- transgenic Aristis and Coventry maize varieties and to test separation conditions for analysis of tryptic digests of water-soluble proteins (albumin fraction) from the maize flours of transgenic Aristis-Bt and non- transgenic Aristis and Coventry maize varieties by CZE.

Experimental

Maize WS proteins were extracted following the first step of the Osborne extraction procedure [6] with some modifications. Water-soluble extracts were obtained by two ways. Flour doses (maize Coventry) of 40, 100 or 250 mg were extracted with 1 mL of deionized water either with continuous vortexing or sonicated in the bath sonicator for 5, 10, and 15 min. Filtered supernatants were evaluated by Lowry assay [7] for protein contents. WS proteins were digested by TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone) treated trypsin immobilized on agarose, following procedure of the producer (Pierce). Filtered digests were analyzed by CZE in three types of BGEs: i) classical acid/salt-based BGE1, 100 mM H_3PO_4, 50 mM Tris, pH 2.25 (Tris-phosphate) ii) highly concentrated weak acids-based BGE2 and BGE3, 200 mM formic acid, 200 mM acetic acid, pH 2.05, and 500 mM acetic acid, pH 2.54, respectively, and iii) isoelectric BGE4, 200 mM iminodiacetic acid, pH 2.26, using home-made CZE analyzer [8] equipped with bare fused silica capillary (total/effective length 31/20 cm, ID/OD 50/375 μm) and UV-absorption detector operating at 206 nm.

Results

From the dependence of UV-absorbance of the extracts at 280 nm and determined concentration of extracted proteins on the flour dose (concentration) and the extraction time for both vortexing and sonication procedures, the final extraction method was developed: 300 mg of the flours of the maize varieties were extracted with 1.5 mL of deionized water at room temperature with continuous vortexing at 800 rpm for 8 min. The mixture was centrifuged for 5 min at 18890 g and the supernatant was filtered through 0.45 μm polyvinylidendifluoride filter. Among tested BGEs, the best resolution of tryptic digests of WS extracted proteins was achieved in isoelectric buffer, 200 mM iminodiacetic acid, pH 2.26 (see Figure 1). The well resolved components of trypsin digests profile in BGE1 and BGE 4 were characterized by effective electrophoretic mobilities and corrected peak areas (peak areas divided by their migration times). Significant differences were found in the corrected peak areas among transgenic Aristis-Bt and non- transgenic Aristis and Coventry maize varieties.

Conclusion

The found significant qualitative and quantitative differences in CZE-UV profiling of tryptic digests of WS extracted proteins of Bt-transgenic and non-transgenic maize species could be used to distinguish the Bt variety from the wild maize. This approach could be potentially employed to detect and/or differentiate presence of these maize varieties in foods.

Acknowledgements

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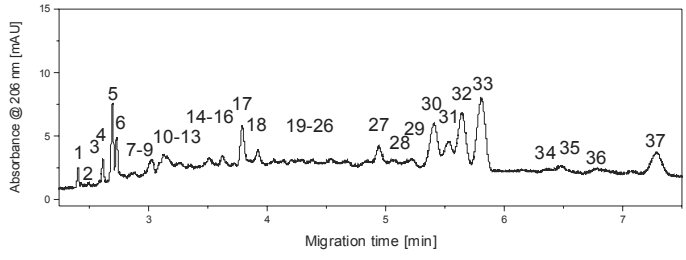

Fig. 1: CZE-UV profile of tryptic digest of water soluble proteins extracted from Coventry maize species in 200 mM iminodiacetic acid, pH 2.26.

Development and validation of RP-LC method for determination of Ceftazidime and ceftizoxime in pharmaceutical formulations

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Introduction

Cephalosporins are structurally and pharmacologically related to the penicillins. Like the penicillins, cephalosporins have a β-lactam ring structure that interferes with the synthesis of the bacterial cell wall. They are used for the treatment of infections caused by Gram (+) and Gram (–) bacteria. They are among the safest and the most effective broad-spectrum bactericidal antimicrobial agents and therefore, they are the most frequently prescribed class of antibiotics [1,2].

Ceftazidime is a third generation cephalosporin often used in Pseudomonas aeruginosa infections [3]. The antimicrobial activity of ceftazidime is mainly due to inhibition of the biosynthesis of peptidoglycan which is a component of the bacterial cell wall. Ceftizoxime is a new broad-spectrum semisynthetic cephalosporin antibiotic with β-lactamase stability.

Numerous assays have been developed for ceftazidime and ceftizoxime; most of these use HPLC methods [4], capillary electrophoretic [5], spectroscopic [6] and electrochemical methods [7].

In this study, the adequate condition for the chromatographic determination of these compounds in pharmaceutical dosage forms was established. The method developed was successfully applied to the determination of these drug compounds in their commercial pharmaceutical dosage forms. Each analysis requires no longer than 12 min.

Experimental

Ceftazidime, ceftizoxime and cefixime standards were obtained from pharmaceutical companies. Cefixime was used as IS. Phosphoric acid, hydrochloric acid, sodium hydroxide and methanol were obtained from Merck (Darmstadt, Germany). Cephalosporin based commercial tablet or capsule formulations were obtained from the national market.

The HPLC analysis was carried out on a Shimadzu class HPLC system with a pump (LC-20 AD), a DAD detector system (SPD-M 20A) and column oven (CTO 20 AC). This equipment has a degasser system (DGU 20 A). A X Terra RP-18 column (250 x 4.60 mm ID x 5m) was used at 30 ^{o}C. Mettler Toledo MA 235 pH/ion analyzer with combined glass electrode was used for pH measurements. The mobile phase composition was methanol–water at 20% (v/v), containing 25 mM phosphoric acid. The pH of the mobile phases was adjusted 3.2 by the addition of sodium hydroxide. The monitoring wavelength was 260-290 nm. Standard mixture of cephalosporin was given in Fig. 1.

Results

The most widely used criterion for the optimization of reversed-phase chromatography is the resolution between peak pairs. As known, achieving a good resolution between all of the working compounds is the main goal of chromatographic separation.

In this study three conditions (20%, 25%, 30% (v/v)) was selected and separation factors were calculated. The calibration curve and equation for ceftazidime and ceftizoxime were calculated by plotting the peak area ratios of cefixime to I.S. versus concentration of the

compounds in the range of 1–16 μg.mL^{-1} for ceftazidime and 1–20 μg.mL^{-1} for ceftizoxime. These results showed highly reproducible calibration curve with correlation coefficient of >0.999. The low values of standard error of slope and intercept and greater than 0.999 correlation coefficient for ceftazidime and ceftizoxime established the precision of the proposed method. The developed method was validated according to the standard procedures.

Conclusion

The reliability of the LC methodology was investigated statistically. The method was applied pharmaceutical formulations containing a single active ingredient was shown to be linear, sensible, accurate, and reproducible. The method developed would serve as a versatile analytical tool suitable for the simultaneous analysis of these drugs and would be of interest for quality control and clinical monitoring laboratories.

Acknowledgements

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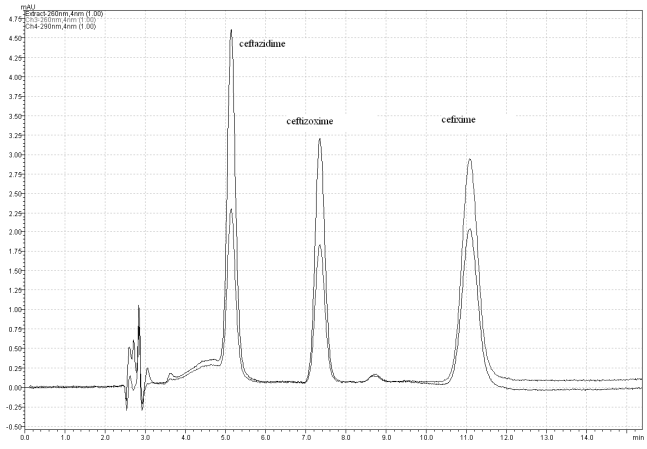

Fig. 1: Chromatogram of real sample.

Optimization of LC method for separation of Cefdinir and Cefixime and their analysis in pharmaceutical dosage forms

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Introduction

Cephalosporins are antibiotics from the β-lactam family which inhibit bacterial cell wall synthesis, commonly used for the treatment of infections caused by Gram-positive and Gram-negative bacteria. Cefixime (CEF) and cefdinir (CF) are semi synthetic and generally classified as a third-generation cephalosporin antibiotic for oral administration.

A literature survey about chromatographic determination of cephalosporin reveals several LC methods [1–4]. Among those proposed for pharmaceutical analysis, the chromatographic conditions are generally applicable to a particular cephalosporin or to a limited number of these drugs.

The purpose of the proposed work was to develop a fast and reliable isocratic liquid chromatographic method for vast scale screening of CEF and CF commercialized in oral dosage forms aimed to the detection of counterfeit and substandard drugs.

Experimental

CEF, CF and Ceftazidime (CFZ) were kindly donated by Bilim Pharm. Company (İstanbul, Turkey). Ceftazidime (CFZ) was used as IS. Cephalosporin based commercial tablet or capsule formulations were obtained from the national market.

Phosphoric acid, hydrochloric acid, sodium hydroxide and methanol were obtained from Merck (Darmstadt, Germany). Deionized, double distilled water was used for the mobile phase preparation. The chromatographic apparatus consisted of a Shimadzu class LC HPLC system with a pump (LC-20 AD), a DAD detector system (SPD-M 20A) and column oven (CTO 20 AC). This equipment has a degasser system (DGU 20 A).

The chromatographic stationary phase was X Terra RP-18 column (250 x 4.60 mm ID x 5) (Waters) was used at 30 ^{0}C. Mettler Toledo MA 235 pH/ion analyzer with combined glass electrode was used for pH measurements.

The optimized mobile phase was 20% (v/v) methanol–water binary mixtures containing 25 mM phosphoric acid delivered at a constant flow rate of 1.0 mL min^{-1}. The pH of the mobile phases was adjusted to 3.2 by the addition of sodium hydroxide. The monitoring wavelength was 260-290 nm. Standard mixtures of cephalosporin was given in Figure 1.

Results

In the present work, the retention behavior of three cephalosporin drugs CEF, CF and CFZ (I.S) in RP-LC, was investigated using different elution conditions on C-18 column, in order to determine separation conditions. Their structural formulas are shown in Fig. 1.

Reversed phase LC is the separation method of choice for the analysis of this class of compounds because of the presence of an aromatic moiety, a cyclic structure and one or more ionizable functional groups.

From the literature [5-7] it was clear that elution on a C18 column with an acidic mobile phase of low ionic strength could be a good starting point for method development. By using

phosphate buffers it was experimentally observed that narrow and well-shaped peaks were obtained at pH values around 3.0. Different concentrations and type of organic modifiers (methanol and acetonitrile) were then tried and methanol was chosen as organic modifier. A representative chromatogram of the analytes is shown in Fig. 2.

Conclusion

The proposed reversed-phase procedure showed the successful baseline separation of studied cephalosporin compounds in a twelve-minute chromatographic run using isocratic elution. It provides a fast, accurate and reliable method for screening of cephalosporin drugs aimed to the detection of counterfeit and substandard drugs and can be easily reproduced by a variety of laboratories.

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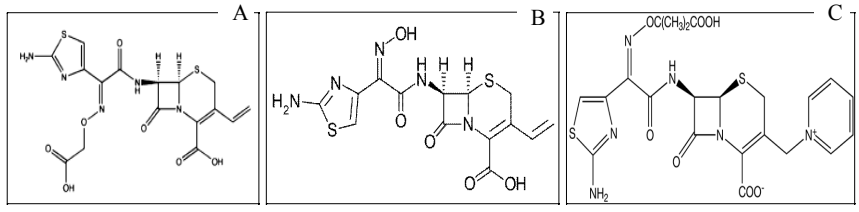

Fig. 1: Chemical structures of studied cephalosporins; A: Cefixime, B: Cefdinir, C: Ceftazidime (IS).

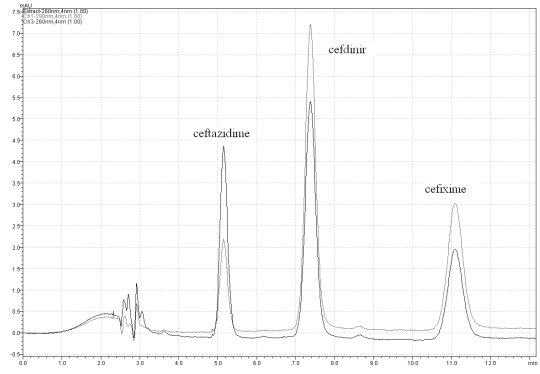

Fig. 2: Chromatogram of standard mixture.

CZE/LIF - A Powerful Tool for the Analysis of N-linked Glycans Released from Human Glycoproteins

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Introduction

Glycosylations are the most abundant post-translational modifications observed in human cells, where more than 50% of all proteins are believed to be glycosylated [1]. These glycosylated proteins play important roles in cell-to-cell interactions, immunosurveillance and a variety of receptor-mediated and specific protein functions through a highly complex repertoire of glycan structures. Accordingly, metabolic disfunctions and disease states may be reflected in the appearance of abnormal glycans or altered quantitative proportions within the glycome. Differential glycomic measurements between healthy and disease states may even have a significant clinical diagnostic potential [2].

Mass spectrometry (MS) is currently recognized as the ultimate technique for characterization of biomolecules, including glycans released from human glycoproteins [3-5]. Despite the wealth of information provided by the most powerful MS and tandem MS techniques, isomeric structures are not readily identified. Therefore, analytical separation techniques provide alternatives for the analysis of glycan pools containing isomeric structures. In present work, capillary zone electrophoresis (CZE) coupled with LIF detector as a powerful tool for identification of N-linked glycan structures derived from glycoproteins is demonstrated. Complex mixtures of derivatized N-glycans were resolved on a polyacrylamide coated capillary with reduced electroosmotic flow. To detect N-glycans using high sensitive LIF detection, a highly fluorescent dye 1-aminopyrene-3,6,8-trisulfonate (APTS) as a derivatization reagent was attached to the reducing end of oligosaccharide molecule through reductive amination [6]. Glycan structure identification was elucidated using a combination of strategies, including comparison with standard glycan libraries and the enzymatic digestions of N-linked glycan pools using specific and non-specific exoglycosidases.

Experimental

The experiments were carried out with the P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter, Fullerton, CA, USA) coupled with LIF detector using a 488 nm argon-ion laser. Fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 60 cm total length, 50 cm effective length and 25 μm I.D., coated with a linear polyacrylamide according to a modified Hjerten's procedure [7,8], and Tris-HCl buffer (40 mM, pH 6.5) as a working electrolyte were used for all separations.

Results

Purified N-glycan pools released from standard glycoproteins (fetuin, asialofetuin, α_1-acid glycoprotein, human immunoglobulin, ribonuclease B and ovalbumin) using PNGase F were analyzed in order to generate a migration time database used for an identification of N-glycans released from more complicated samples. In addition, N-glycan pools released from glycoprotein standards were enzymatically digested using specific and non-specific exoglycosidases (sialidase, α-fucosidase, β-galactosidase and β-N-acetylglucosaminidase) to positively identify isomeric structures. Female serum samples, collected from healthy disease-free individuals and the patients diagnosed with different breast cancer stages, were distributed into 4 subgroups (I - IV) according to the severity of cancer development and enzymatically digested using PNGase F. Purified released N-glycans were analyzed under same separation conditions as standard glycans and their identification was provided under strategies mentioned above. By doing so, 47 glycan structures, many being structural isomers, were identified.

Conclusion

CZE-LIF is a suitable method for the rapid, highly efficient and reproducible profiling of N-linked glycans derived from purified human glycoproteins. CZE significantly complements other means for glycan mapping, such as MS, due to its ability to resolve structural isomers of N-glycans. Comprehensive glycan structure identification is based on comparison with standard glycan libraries and selective enzymatic digestions of N-linked glycan pools using specific and non-specific exoglycosidases. CZE-LIF may be used as a clinical diagnostic and prognostic tool to identify low abundance glycan biomarkers that may indicate a presence of cancer disorder and its progression. Based on the analysis of N-glycans released from female blood serum samples from healthy individuals and breast cancer patients with different stages of progression, we were able to identify 47 glycan structures.

Acknowledgements

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Analysis of ibuprofen and its metabolites in human urine using LC IT TOF MS technique

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Introduction

Therapeutic drugs and their metabolites are usually present in biological samples (urine, serum, etc.) at trace concentration levels. Of these are attributable for samples as included for several hundreds of constituents and, in addition, representing different chemical/biological properties and still covering for very significant concentrations of the analytes. These two categories of the analytes indicate a combination of powerful separation techniques with a sensitive and/or selective detection technique. Liquid chromatography/mass spectrometry (LC/MS) based techniques provide unique capabilities for pharmaceutical analysis. LC/MS methods are applicable to a broad range of compounds of pharmaceutical interest, and they feature powerful analytical figures of merit (sensitivity, selectivity, speed of analysis, and cost effectiveness).

LC-IT-TOF-MS is proved to be a powerful analytical technique, for example, as separating and identifying biologically important compounds such as therapeutic drugs and their metabolites as joined with analytical constituents of different complex biological mixtures. Main advantages of this coupled system are in its high separation efficiencies, short analysis times, high detection selectivities and sensitivities and still consuming low sample amounts. LC-IT-TOF-MS allows obtaining both chemical and structural information by utilizing both the fragmentation power of the ion trap, and the high resolution and mass accuracy of the time-of-flight mass spectrometer.

This work deals with the qualitative and quantitative analysis possibilities of ibuprofen and its metabolites present in a complex biological matrix (human urine) by using a LC-IT–TOF-MS technique.

Experimental

LC-MS analyses of our samples were performed by using Shimadzu LCMS-IT-TOF ™ (Shimadzu, Kyoto, Japan). This MS analyzer was combining an electrospray ionization (ESI), a 3D quadrupol ion trap (IT) and an orthogonally accelerated time-of-flight analyzer (TOF) as providing both the high sensitivity and high resolution of ions. LC experiments were performed on Ascentis C18 column (100/2.1 mm; 5μm) as using a gradient elution (water – acetonitrile) with a 0.2 ml/min flow rate. The MS – MS^n experiments acquired for the data within 50-1000 m/z values in both the positive and negative modes.

All chemicals as used in this work were obtained from Merck (Merck, Darmstadt, Germany), Sigma-Aldrich (Steinheim, Germany) and Fluka (Busch, Switzerland). Formic acid, acetonitrile, and water for LC-MS were purchased from Merck (Merck, Darmstadt, Germany). Nurofen StopGrip® samples (Reckitt Benckiser Healthcare Ltd., Slough, United Kingdom) were obtained from a local pharmacy.

Results

After oral administrations of Nurofen StopGrip, urine was collected in 0, 1, 2, 3, 4, 5 and 6 hours (urine samples for 4 healthy volunteers). Each of the volunteers was prescribed one table (the table containing 200 mg of ibuprofen and 30 mg of pseudoephedrin hydrochloride). A particular urine sample was diluted with acetonitrile (1:1) and, subsequently, centrifuged at 5000 RPM (5 minutes for a 10 μl volume) and injected into the LC-IT-TOF-MS analyzer. The samples were separated chromatographically on an Ascentis C18 column (a gradient elution for a 0.2 ml/min flow rate) and the effluent as transferred through the ESI interface. MS-MS^3 data acquisition was performed, within 50-1000 m/z values, in both positive and negative ionization modes. A total LC-MS time was 12 minutes.

MS spectra of the urine samples were compared for MS-MS^3 spectra of ibuprofen standard (Sigma-Aldrich). The concentration level of ibuprofen in urine samples was determined from the parameters of calibration line. Pharmacokinetic curves were constructed for ibuprofen and its main metabolites. Metabolites were positively identified in the samples of the volunteers as using MetIDsolution software (Shimadzu).

Conclusion

Rapid LC-MS analytical method with simple sample pretreatment for analysis of ibuprofen and its metabolites in urine samples was developed in this work.

Acknowledgements

This work was supported by grant of Slovak Research and Development Agency No. VVCE-0070-07 and grant of Slovak Grant Agency No. 1/0882/09.

APCI-LC-MS/MS method for the analysis of the novel anticancer agent-Bp4eT in aqueous media

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Introduction

Development of novel anti-cancer drugs ranks among the most progressive fields in current drug discovery. Special attention has been drawn to new anti-cancer strategies which have potential to overcome resistance to standard chemotherapy. Selective targeting of iron (Fe), an essential micronutrient, has been identified as a novel strategy for anti-cancer drug development. One of the new Fe chelator, with marked anti-tumor activity and selectivity against neoplastic relative to normal cells, is 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT).

Apart from the high anti-cancer activity of Bp4eT, this compound might be viewed as a challenge from the analytical point of view. Bp4eT exists in two isomeric forms (Z/E). While the solid substance has been shown to consist mainly as the Z isomer, in aqueous media equilibrium exists between *Z* and *E* isomers. Quantitative analysis of Bp4eT is somewhat difficult since analytical standards of both isomers are not available and isolation or synthesis of pure isomers would be hampered by rapid interconversion of one isomer to another. In addition, the shift in the absorption spectra of both forms hindered the UV-based quantification. On the other hand, theoretical analysis pointed to the possibility of MS/MS detection as an appropriate method that would overcome this analytical issue, providing additional benefits such as sensitivity and selectivity.

The aim of the study was to develop and validate sensitive LC-MS/MS method for determination of Bp4eT in aqueous media and use the method in the solubility study to suggest a formulation suitable for *i.v.* administration of this agent in advanced preclinical experiments.

Experimental

Chromatographic separation was performed using a Shimadzu Prominence System coupled on-line with a Thermo Finnigan LCQ Advantage Max ion-trap mass spectrometer with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources. Final HPLC analysis was achieved on Polar RP column (150 × 3 mm, 4 µm, Phenomenex) with a mobile phase consisting of 2 mM ammonium formate, acetonitrile and methanol (30:63:7; v/v/v). A flow rate of 0.25 mL/min, column temperature of 25°C and 4-methyl-3-thiosemicarbazone (N4mT) as an I.S. were used. Quantification was made employing +APCI in SRM mode, using the main fragment ions of $[M+H]^+$ (240 m/z for Bp4eT and 229 m/z for N4mT-I.S.). Method was validated over the range 20-600 ng/mL. The solubility of Bp4eT in

aqueous solutions of aqueous media was studied as a function of pH (3.0 -7.0) and in the selected pharmaceutical co-solvents (PEG 300%, PG, ethanol).

Results

At the beginning, it was necessary to develop analytical conditions providing the same ionization intensity for both Bp4eT isomers. Under such conditions, Bp4eT can be properly quantified via simple summation of the peak areas of both isomers. Main MS characteristics of both Bp4eT and I.S. were obtained using direct infusion of the standard solutions into the ion source (ESI and APCI). Both compounds (Bp4eT and I.S.) gave signal only in positive mode. The analogical main fragment ions of $[M+H]^+$ were obtained in MS^2.

Further experiments were focused on the development of chromatographic conditions suitable for LC-MS/MS analysis, with particular attention being paid to properly separate both forms, which is essential for proving the same ionization intensity for both isomers. Adequate separation was achieved on Polar RP column with a mobile phase consisting of 2 mM ammonium formate, acetonitrile and methanol in the ratio of 30:63:7 (v/v/v). It was found that due to the different susceptibility of both isomers to form the $[M+Na]^+$ adduct, which has an impact on the exact quantification based on $[M+H]^+$, ESI could not be used. On the other hand, employing APCI, the same detector response for both isomers was confirmed. Method was subsequently validated. Linearity was tested within the range of 20-600 ng/mL (r2>0.997), the intra/inter-day accuracy and precision (RSD) were 89-105% and 4-11%, respectively. In addition, evaluation of the selectivity and ion suppression did not reveal any significant impact of sample matrix on the MS detection.

It was found that Bp4eT is poorly soluble in aqueous media (from 50 µg/mL at pH 3 to 4 µg/mL at neutral pH). With regard to solubility and irritation property of each buffer, the most appropriate buffer for further optimization was chosen ammonium acetate of pH 4. Further increase in Bp4eT solubility was observed using mixtures of pharmaceutical cosolvents (propylene glycol, polyethylene glycol and ethanol) with saline and acetate buffer of pH 4. Considering the compound solubility in particular co-solvent mixture, the co-solvent toxicity (LD_{50}) and the risk of local irritation based from the low pH, 50% PEG 300 in saline was chosen as the most convenient vehicle for i.v. application.

Conclusion

The APCI-LC/MS/MS method developed in this study enables to exactly quantify the total content of Bp4eT in aqueous media via summation of the peak areas of both isomers. This method was properly validated over the range 20-600 ng/mL and applied to the solubility study. The formulation containing PEG 300 and saline (50:50, v/v) was suggested as a suitable vehicle for intravenous administration of Bp4eT in advanced preclinical experiments.

Acknowledgements

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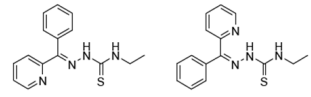
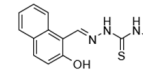


Bp4eT (MW 284)



N4mT (MW 259)

Enantioseparation Mechanisms and Determination of Optimal Chiral Selector Concentration in Single and Multi Chiral Selector Systems

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Introduction

Commercial mixtures of chiral selectors, namely highly sulfated cyclodextrins, are well known for their good separation abilities. It was shown by several authors that multi chiral selector (multi-CS) systems enable to achieve better enantioseparation than single chiral selector ones (single-CS systems) [1,2]. Single-CS separation is primarily controlled by thermodynamic mechanism, it means that enantiomer - CS complexes differ by their distribution constants, while their mobilities are almost the same. In multi-CS systems, the situation is more complex. Overall distribution constants and overall mobilities can be defined for hypothetical complexes between two enantiomers and a mixture of CSs. Further it can be shown that not only overall distribution constants but also overall mobilities considerably differ and thus contribute to the enantioseparation.

Optimal concentration of CS (to achieve maximal difference between effective mobilities of enantiomer – CS complexes) could be determined by equation introduced by Wren and Rowe [3] in 1992. Although this equation is widely used, its application is very limited. The equation is based on the approximation of the same mobilities for both enantiomer – CS complexes. This requirement is valid only for single-CS but malfunctions for multi-CS systems.

Experimental

The enantioseparation mechanisms of single and multi CS systems were studied by means of capillary electrophoresis. Distribution constants and mobilities were determined for lorazepam – CS complexes in the environment of single isomer chiral selector heptakis(6-O-sulfo)-β-CD (DS 7) and commercial available β-cyclodextrin, sulfated sodium salt (DS 7-11).

Results

It was shown in our previous publications [4,5] that multi-CS systems can be simplified and treated like single-CS system. We proposed the equation to calculate the effective mobility of analytes in interaction with a mixture of CSs

$$\mu_i^{eff} = \frac{\mu_i^0 + c_{CS}^{tot} K_i^{over} \mu_i^{over}}{1 + c_{cs}^{tot} K_i^{over}} \quad ;$$

where is mobility of an analyte without presence of any chiral selector, is total concentration of chiral selector, is overall distribution constant and is overall-mobility.

Although this equation is formally the same as for a single CS system, the overall parameters are weighted sums of the individual contributions of all chiral selector isomers. It means that the overall mobility strongly depends on the distribution of the respective enantiomer between all individual CS isomers, which makes the system more complex and complicated. Distribution constants and mobilities were determined for model analyte, lorazepam, using

Distribution constants and mobilities were determined for model analyte, lorazepam, using two different chiral selector systems by ACE like experiments. Heptakis(6-O-sulfo)-β-CD was used as well defined single isomer chiral selector, while commercially available β-cyclodextrin, sulfated sodium salt represented multi-CS system. Excellent fits were obtained for both systems showing predicted behaviour. The single isomer system showed different distribution constants and statistically the same mobilities for enantiomer – CS complexes. In multi CS system both distribution constants and limit mobilities differ significantly.
The thermodynamic selectivity (ratio of distribution constants) is almost the same for the both systems. The additional difference in limiting mobilities ensures better enantioseparation in the multi CS system.
In the next step an optimal concentration of the chiral selector was calculated according to Wren and Rowe simplified equation. It was shown that the theoretical value for single-CS system is in a good agreement with the value determined experimentally. In the case of multi-CS system, no optimal CS concentration exists, the higher the CS concentration, the higher the effective mobility difference. Such behaviour cannot be predicted by simplified Wren and Rowe equation but results as two negative roots of an original quadratic equation when the restriction of the same complex mobilities is released.

Conclusion
The model of enantioseparation for multi chiral selector systems was proposed. This model shows that both electrophoretic and thermodynamic separation mechanisms are combined in multi-CS systems. Different overall distribution constants, significantly different limit mobilities result in multi-CS systems in better separation than can be achieved in single-CS ones. It was also shown that a simplified Wren and Rowe equation to calculate optimal CS concentration can easily be inappropriate in such systems.

Acknowledgements
We gratefully acknowledge funding from the Grant Agency of Charles University, grant No. GAUK 101309, and the Grant Agency of the Czech Republic, grant No. 203/08/1428.

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Reaction of carbon dioxide on zeolite ZSM-5 doped with Fe^{3+} and thiophene

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Introduction

Due to increase of the environmental contamination by greenhouse gases (namely CO_2) seems to be necessary to search for and develop new ways of removal of these pollutants. CO_2 fixation by photocatalytic reduction of carbon dioxide is an attractive process as it can activate chemically inert carbon dioxide by irradiation. Many kinds of heterogeneous catalysts have been tested for the photoreduction of carbon dioxide [1-3]. Earlier [4] we have found that polythiophene, incorporated in the channels of zeolite ZSM-5 produced molecular hydrogen upon irradiation in aqueous media by visible ($\lambda > 400$ nm) light. Now we wish to ascertain possible utilization of such pre-treated zeolite for the reduction of CO_2 and to identify its principal products.

Experimental

Oxidation polymerization of thiophene with Fe^{3+} was performed within the channels of the Na-ZSM-5 zeolite (VURUP Co., Slovakia) according to ref. [4]. Irradiation of the sample (water suspension of the modified zeolite (FeZSM-PT) – 100 mg in 140 cm^3 glass flask) was accomplished by a metal-halogen lamp (250 W, Tesla, ~ 60 W m^{-2}) from the distance of 20 cm through a 5cm water filter at 25 ± 1°C (pH of water suspension was 5.63). During the irradiation the sample was slightly bubbled by CO_2. Absorption spectra were recorded by spetrophotometer Genesys 6 (Thermo-Scientific, USA).

Results

The incorporation of thiophene in Fe^{3+} doped ZSM-5 was accompanied by a color change from the original orange to red-brown. This color change was caused by formation of thiophene oligomers in the channels of the zeolite [4].

Fig. 1 shows absorption spectra of the above described water suspension of FeZSM-5PT after irradiation with visible light. In this picture two new absorption bands are evident at <200 nm and 264 nm respectively. We suppose that these absorption bands belong to acetone and ethyl alcohol or to oxalic acid. In Fig. 1 are depicted absorption spectra of ethyl alcohol, oxalic acid and acetone in distilled water. By comparing these spectra one can see their marked similarity. In our earlier work [4] we found that the irradiation of ZSM-5-PT by visible light in aqueous media gives rise to hydrogen, which under the given conditions undergoes further reaction with oxalic acid and finely can be transformed into ethyl alcohol [5].

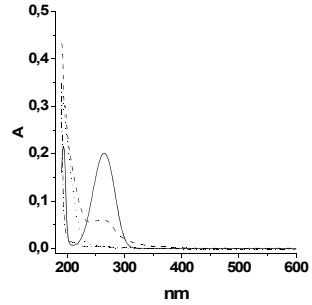

Fig.1: Absorption spectra of water solutios of acetone (1.36 10^{-5} mol dm^{-3}, solid line); oxalic acid (10^{-4} mol dm^{-3}, dotted line); ethanol (1.7 10^{-5} mol dm^{-3}, dash-doted line) and product arising after 7 hours irradiation of water suspemsion FeZSM-5PT (dashed line).

The tentative reaction mechanism can accordingly be expressed by the following reaction scheme:

$$PT + h? \rightarrow [PT]^* \quad (1)$$

$$[PT]^* + CO_2 \rightarrow PT^{+\bullet} + [CO_2^-]^\bullet \quad (2)$$

Since part of water molecules is dissociated, the following reaction can be expected:

$$[CO_2^-]^\bullet + H^+ \rightarrow {}^\bullet COOH \quad (3)$$

$$2\ {}^\bullet COOH \rightarrow HOOC\text{-}COOH \quad (4)$$

After absorption of single energy quantum thiophene oligomers (PT) are raised to an excited state (eq. 1). During interaction between [PT] and CO_2 an anion of CO_2 is formed by charge transfer mechanism. We assumed that the next step involves capture of H^+, abundantly present at pH = 5.63 (eq. 3). The next possible step may be production of oxalic acid (eq. 4), the formation of which has already been assumed by Jitara [6].

Conclusion

In this work was demonstrated that CO_2 conversion into low molecular organic compounds (oxalic acids, ethyl alcohol or acetone) is possible realize by a suitable photocatalyzer (zeolite ZSM-5 doped with Fe^{3+} and thiophene) irradiated with the visible ligh.

Acknowledgements

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Glassy micro-fiber filters as an efficient surface for desorption nano-electrospray ionization mass spectrometry

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Introduction

Desorption electrospray [1] is a powerful tool for analysis of small and large molecules, it is useful for direct analysis of surfaces and for imaging mass spectrometry [2]. Its modification, desorption nano-electrospray (nano-DESI) [3,4], offers longer lasting signal for the same amount of sample, consumption of chemicals as well as contamination of mass spectrometer caused by sample matrix is smaller in comparison to DESI. Nevertheless, character of surface plays an important role in DESI as well as nano-DESI experiments. Different surfaces were tested to enhance signal intensity including nanostructured ones [5].

Glassy micro-fiber filters were used as a target plate for sample deposition and compared with rough glass in this work. Different drugs (caffeine, nicotine, amphetamines and selected benzodiazepines) were employed as testing analytes in whole blood as matrix.

Experimental

Experiments were carried out using home-made nano-DESI ion source and an ion trap mass spectrometer (LCQ, Thermo Finnigan, San Jose, USA) [4]. The capillary tip (PicoTip emitter GlassTip, ID = 2±1 μm; New Objectiv, Woburn, USA) was filled by spraying solution using a micro-syringe. After filling, the spray voltage was applied to the metal coating of the glass capillary tip and set up in the range between +1.5 kV to +3.5 kV.

The standard solutions or spiked whole human blood were spotted on a target plate, either rough glass or glassy micro-fiber filter, type Z7, pores average size 2,5 μm, 30 mm in diameter; Merci, Prague, Czech Republic. Samples were let to dry and analyzed without any other pretreatment.

Ephedrine, methamphetamine, caffeine, nicotine, diazepam, alprazolam, all analytical grade, were kindly provided by Institute of Forensic Medicine and Medical Law, Medical Faculty, Palacký University, acetonitrile, methanol and water (HPLC, gradient grade) were purchased from Sigma-Aldrich (Prague, Czech Republic).

Different DESI spraying solutions were tested and finally a methanol/water mixture (1:1, v/v), acetonitrile/water mixture (9:1, v/v) and acetonitrile, all modified with 1,5% of formic acid (30 μl to 2 mL of spraying solution) were applied.

Results and discussion

The ionization efficiency has depended on the structure of analytes and composition of spraying solution as expected. Above all, the analysis of series of standards showed higher signal intensities for glassy filter in comparison to rough glass. In some cases, A the increase

of intensity was higher than one order. Signal improvement was also observed for spiked blood samples using glassy filter. Except higher response, lesser fluctuation of the signal was observed. Finally, a real blood sample was analysed using spray mixture acetonitrile/water (9:1, v/v) acidified by formic acid. The presence of nicotine was proved by MS/MS spectrum (Fig. 1).

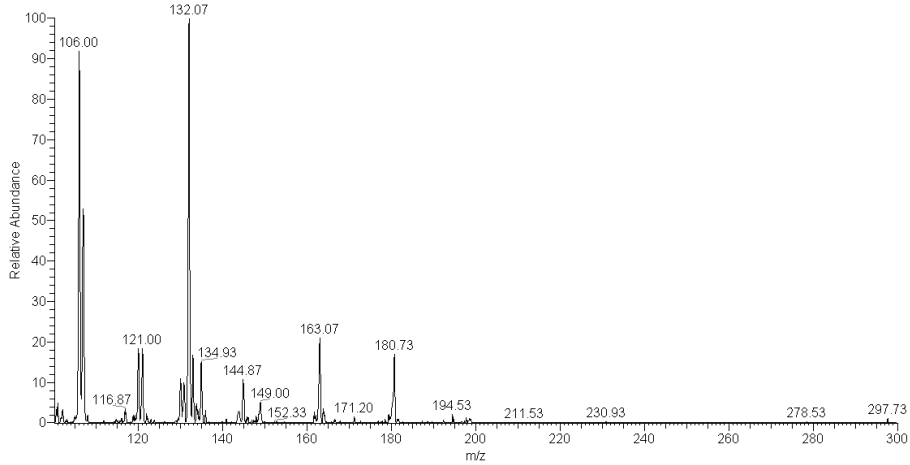

Fig. 1: MS/MS spectrum of real sample of human blood spotted on a glassy micro-fiber filter. The spectrum is in agreement with spectrum of standard of nicotine.

Conclusions

It is evident that character of surface plays important role in nano-DESI (as well DESI) mass spectromeric analysis. Glassy micro-fiber filters offer signal improvement and can be used as good target plate for biological samples (e.g. deposition of blood is easy and spot is concentrated on small area). The detail mechanism of desorption and ionization using this material will be studied in the future.

Acknowledgement

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Comparison of hepcidin adsorption to different types of magnetic particles prior MALDI-TOF MS analysis

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Introduction

Hepcidin is small, cysteine-rich cationic peptide produced by hepatocytes, secreted into plasma, and excreted in urine. Hepcidin is proposed to be the key regulator of iron metabolism and changes in the hepcidin level are associated with several diseases [1]. Determination of the hepcidin content could therefore be useful in diagnosis of iron disorders and would provide further insight into hepcidin regulation in vivo. However, each of methods used for the hepcidin detection and determination in urine and serum has certain limitations [2,3]. At present time an ELISA for human hepcidin and SELDI-TOF-MS based approaches have been often applied to monitor urinary and/or serum hepcidin levels [4]. Prior to the SELDI-TOF-MS analysis, the hepcidin samples are adsorbed to diffrent protein chips: e.g. Normal Phase 20, Immobilized Metal Affinity Capture 30 or CM10 ProteinChip arrays. Another approach of the hepcidin analysis is based on magnetic separation using ion exchange principle.
Aim of the present study was to compare several magnetic sorbents with different coating matrix and/or with different terminal functionalized groups in their ability to adsorb hepcidin prior MS analysis.

Experimental

The following magnetic sorbents were used in the study: commercial Carboxyl or Glyoxal agarose beads (20–75 mm) BioScience Bead Division of CSS; weak cation exchange beads MB-WCX (1 mm) Bruker Daltonics; fluidMAG-CMX nanoparticles (200 nm) with carboxymethyldextran polymer matrix, magnetic silica beads SiMAG-Silanol and SiMAG-Carboxyl (1 mm) Chemicell GmbH or prepared magnetic nonporous hydrophilic microspheres poly(2-hydroxyethyl methacrylate-co-glycidyl methacrylate) containing coupled iminodiacetic acid (IDA), poly(2-hydroxyethyl methacrylate-co-ethylene dimethacrylate) , or poly(glycidyl methacrylate) (1).
Testing samples were prepared by spiking of 25 mM ammonium acetate solution or 50 mM actetic acid solution with stock solution of synthetic standard of hepcidin. Hepcidin samples were adsorbed to magnetic particles at pH 3.0 or pH 6.8 at room temperature.
Adsorbed hepcidin was eluted with 50% ACN/0.1% TFA solution and analyzed using autoflex II MALDI-TOF/TOF instrument from Bruker-Daltonics.
In the case of poly(glycidyl methacrylate) sorbent or Glyoxal Agarose sorbent, active groups were deactivated using ethylamine.

Results

For the determination of hepcidin in biological fluids (urine, blood, serum) fractionation or partial separation seems to be advantageous prior the final analysis, e.g. MS. In our study we

have chosen for this purpose magnetic sorbents differing in composition and modifying groups. Results of our study have shown that hepcidin adsorption to functionalized magnetic particles is related either to a principle of cation exchange or to IMAC separation (in the presence of immobilized metal ions). Inert components of coating layers of magnetic particles were shown to affect the peptide sorption less significantly.
Hepcidin was adsorbed to magnetic sorbents containing linked carboxyl groups (i.e. to weak cation exchange magnetic particles) at pH 6.8 independently on a nature of magnetic particle coating layer. Much less, hepcidin was adsorbed to these sorbents at pH 3.0. Carboxyl Agarose sorbent represents an exception; nearly no hepcidin was adsorbed to these particles. While hepcidin was not adsorbed to magnetic poly(2-hydroxyethyl methacrylate-co-glycidyl methacrylate) containing coupled iminodiacetic acid (IDA) particles, after loading with Cu(II) ions the peptide was on the magnetic sorbent retained and it can be used for its separation from urine samples.

Conclusion
Magnetic sorbents of different composition containing linked carboxyl group or immobilized Cu (II) ions via iminodiacetic acid were shown to be suitable for the hepcidin separation from urine samples prior MS analysis.

Acknowledgements
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Comprehensive GCxGC separation of 209 PCBs with ionic liquid as 2nd column

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Introduction

Separation of complex samples, e.g. 209 PCB's, is in one dimensional (1D) chromatography unsolvable problem especially when we need relative fast analysis. Problem with insufficiently resolution more of peaks is general argument for using of compherensive GCxGC. Selection of column set for 1st and 2nd dimension is very difficult. In general, for 1st dimenesion (1D) we always choose non-polar capillary column and for 2nd dimension we choose short polar column[1]. Ionic liquids are highly polar stationary phases and have wide reange of applycations in gas chromatography. Special character of retention mechanism of ionic liquids, often call as "dual nature" represente their ability to separete both polar and non-polat compounds[2]. This effort is one way to sufficient resolution of all 209 PCB's using of comhrensive GCxGC. In this study was used non-modulated transfer (NMT) compherensive GCxGC [3].

Experimental

A schematic of the instrument used is depicted in Fig. 1. In this study we used HP 5890 gas chromatograph with electronic pressure control, two split/splitless injectors and two flame ionization detectors (FID). Carrier gas was hydrogen 4.0, inlet pressure p_i = 660 kPa, intermediate pressure p_m = 200 kPa. The mean gas velocity was 31 cm/s in 1D column and 294 cm/s in 2D column.Two sets of column were used for the GCxGC separation of 209 PCB's: DB-5×BPX-70 and DB-5×IL-36. Column proportion: DB-5 (40m x 0.1 mm I.D. x 0,1 m); BPX-70 and IL-36 (3m x 0.1 mm I.D. x 0.24 m). All PCB samples (1 l) were injected manually into the first column through the first splitless injector heated at 300 °C and used with a 1 min purge off. FIDs were thermostated at 330 °C. The temperature of columns was programmed from 80°C (for 2 min), increasing at a rate of 3°C/min to 250°C (30 min).

Results

All 209 PCB's were separete using NMT GCxGC with cyanopropyl and ionic liquid statonary phase as 2nd column. Comparison between columns ware demonstrate on the basis of column orthogonality. The degree of orthogonality of the two columns was assessed by Pearson's correlation coefficient (Table 1). This was done for PCB separations on both the DB-5×IL-36 and DB-5×BPX-70 column series. The correlation coefficients show a lower

correlation of the PCB retention data on the DB-5×IL-36 than on DB-5×BPX-70 column series. The degrese of correlation decreases substantially with increasing numbers of PCB's for the DB-5×IL-36 column series but not for the DB-5×BPX-70 series. This data indicates that the DB-5 and IL-36 columns coupled in series are, in contrast to BPX-70 column, almost orthogonal for the separation of 36 and 209 PCB's ($r < 0.014$).

Conclusion

The DB-5×IL-36 column series is almost orthogonal for the comprehensive GC×GC separation of 209 PCB's. Since DB-5 column is non-polar and IL-36 column is very polar, this high polarity difference is largely responsible for the orthogonal separation of PCB's. The IL-36 column may therefore be preferable as the 2D column for any sample separation where such a high polarity span is needed. It was demonstrated that the Pearson's correlation coefficient is a valuable tool to assess the orthogonality of 1D and 2D column series in comprehensive GCxGC, since it evaluates the separation of sample components of any multicomponental sample.

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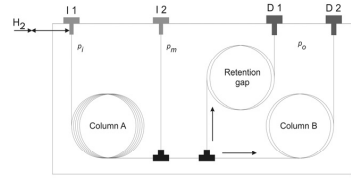

Fig. 1: Schematic of NMT GCxGC instrument.

Table 1: Dependence of Pearson's correlation coefficients on the number of PCBs.

Standard set of PCB's	Number of PCBs	DB-5×BPX-70	DB-5×IL-36
		Corelation coefficient, r	
C-CS-01	39	0.395	-0.0141
C-CS-02	36	0.352	0.0041
C-CS-03	27	0.611	0.3120
C-CS-04	22	-0.353	-0.4590
C-CS-05	20	0.373	0.0001
C-CS-06	18	0.334	0.0039
C-CS-07	14	-0.396	-0.4650
C-CS-08	12	0.562	0.1400
C-CS-09	21	-0.123	-0.1970
	209	0.299	0.0097

Validation of capillary electrophoretic method for in-capillary metabolism of dextromethorphan

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Introduction

Metabolism pathway of dextromethorphan (DEX) was chosen as the example of drug transformation under the action of cytochrome P450 2D6. It principally documents the process of metabolite generation of a tested compound by drug metabolizing enzyme. Cytochrome P450 2D6 belongs to the important enzymes of all P450 isoforms as it is involved in metabolism of about 25% of all commonly prescribed drugs. Recombinant human P450 enzymes have an important role in elucidating the biotransformation role of new drug candidates.

Capillary electrophoresis (CE) has become a common analytical tool in drug analysis for qualitative and quantitative determinations. Moreover, CE enables to conduct in-capillary homogenous enzymatic reactions [1]. We intended to develop in-capillary CYP450 2D6 enzymatic assay.

Experimental

The CE system consisted of an Agilent ^{3D}CE System equipped with a diode array detector. Analyses were conducted in 50 μm I.D. fused silica capillary 50 cm of total length. The capillary temperature was maintained at 37°C. Standard sample solution contained DEX and its metabolites prepared in the incubation buffer (20 mM phosphate buffer, pH 7.4).

Results

The optimal separation of standards was reached in tetraborate buffer (80 mM, pH 9.8) with addition of 2-propanol (8 %, v/v). The UV detection of analytes was performed at 200 nm. The partial filling method enabled to combine separation and incubation buffers within one capillary and thus assure suitable conditions for in-capillary enzymatic reaction.

Preliminary results showed good repeatability of migration times and peak area for all examined analytes (R.S.D. was < 1 % and <10 % respectively). The linear relationship between the concentrations of compounds and the corresponding peak area were obtained. The linear response ranges were tested from 0.1 μM to 50 μM for three metabolites. Correlation coefficients were >0.99 in the tested range of concentrations and limits of detection were 2 μM for 3-hydroxymorphinan and dextromethorphan and 5 μM for 3-methoxymorphinan. Overall validation data will be shown in the presentation.

Conclusion

The described method is suitable for in-capillary metabolism assay of dextromethorphan under the action of CYP 2D6. The validated range is acceptable for examination of parameters of stated enzymatic reaction.

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