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SPONTANEOUS REVESICULATION - NEW METHOD FOR THE PREPARATION OF LIPOSOMES

Rapid communication

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Abstract

In this paper we present a utilization of the novel process for preparation of liposomes from soya bean phospholipids, which is based on spontaneous revesiculation. We study this process by two analytical methods - measurement of conductivity and quazielastic light scattering. On the basis of results from these measurements we can confirm that spontaneous revesiculation is new, easy and quick method for preparation of liposomes.

1. Introduction

Liposomes are polymolecular aggregates formed from closed phospholipid bilayer, which are stabilized via hydrophobic interactions. Liposomes can be formed with varying size (generally 50 nm to 5 µm in diameter), and lamellarity (one or more phospholipid bilayers formed uni- or multilamellar liposomes), depending on the used preparation method. The amphiphilic character of phospholipids and the organization of liposomes in closed structure give the possibility to encapsulate hydrophobic compound in bilayer membrane or hydrophilic molecules in the internal cavity [1-3]. These features enable liposomes to be used as models for biological membranes in medical science [4], molecular biology [5], food [6] and cosmetic industry [7] and also in analytical chemistry [8-15].

2. Problem

Common method for preparation of liposomes is based on reconstruction of the dry lipid film prepared from phospholipids dissolved in organic solvent [16-21]. Unfortunately even minute amount of organic solvent presented in prepared liposomes is highly undesirable for its utilization in analytical chemistry. For this reason we developed the new progressive method for preparation liposomes - so called "spontaneous revesiculation". This method is based on subsequent titrimetric addition of concentrate phospholipid solution to the aqueous solution. The process of spontaneous revesiculation and liposomes created in this way were intensively investigated via conductometric titration and quazielastic light scattering.

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3. Results and discussion

Conductometric titration curve (Fig. 1) include well visible turning points, which correspond with formations and revesiculation of liposomes. Liposomes contained in stock solution of asolectin (8 g.l^{-1}) disintegrate as the result of the sudden dilution. Conductivity rise with raising concentration of asolectin in solution. The growth of conductivity is slowed down in the interval from 1 to 1.5 g.l^{-1} . Formation of more stable liposomes, which are resistant to next addition of asolectin is the most probable explanation. The process of spontaneous revesiculation was monitored also in dependence on temperature in interval from 20 to 60°C (results not shown).

Conductometric measurements were supported with assistance of quazielastic light scattering (Fig. 2). Results from these measurements confirm relation between turning points on conductometric titration curve and formation of phases containig various sized liposomes. As we mentioned above spontaneous revesiculation of liposomes from stock solution into smaller and more stable liposomes occurs in solution with concentration of asolectin about 1.5 g.l^{-1} .

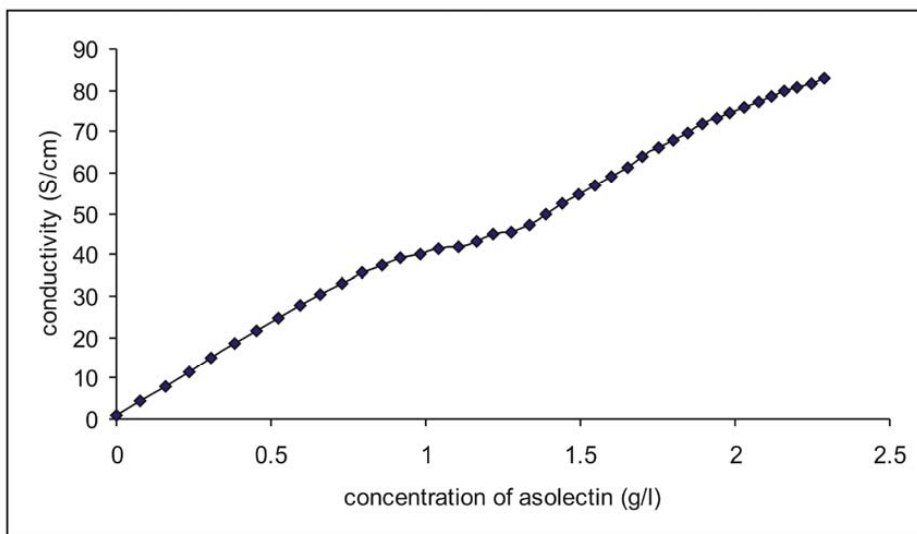


Fig. 1: Conductometric titration curve: dependence of conductivity on concentration of asolectin (stepwise addition of 8 g.l^{-1} stock solution in 5 seconds interval at 20°C).

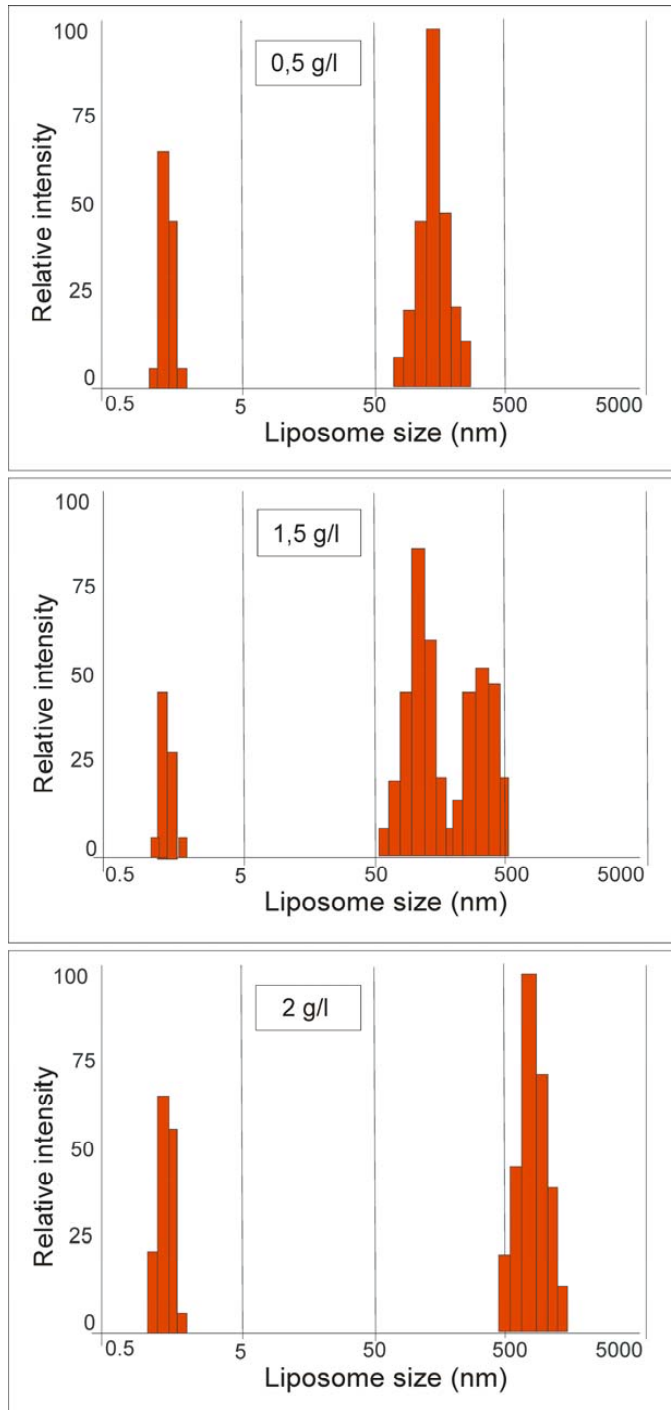


Fig. 2: Size distribution of liposomes in samples with concentration of asolectin 0.5 - 2 g.l⁻¹ at 20°C.

4. Conclusion

Spontaneous revesiculation is valid method for preparation of (phospholipid) liposomes, which size is dependent on total concentration of phospholipid in solution. The formation of liposomes was confirmed by two orthogonal methods (conductivity measurement and quasielastic light scattering).

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**STUDY OF CYPERMETHRIN HYDROLYSIS BY MICELLAR
ELECTROKINETIC CHROMATOGRAPHY**

Short Communication

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Abstract

A study of alkaline hydrolysis of cypermethrin in methanol or tetr. butanol is covered in this article. Cypermethrin and its residues were quantified by micellar electrokinetic chromatography using 100 mmol.L⁻¹ sodium phosphate pH 2.5, 50 mmol.L⁻¹ SDS and 10 mmol.L⁻¹ γ -cyclodextrin as the background electrolyte. Oxidation by potassium permanganate following after hydrolysis enabled quantification of pyrethroids containing 3-phenoxybenzoic moiety.

Key words: cypermethrin, micellar electrokinetic chromatography, hydrolysis, γ -cyclodextrin, oxidation

1. Introduction

Pyrethroid insecticides widely used in many states all over the world are nowadays in high research interests because of its environmental and ecotoxicological risk relevance. Cypermethrin, (R,S)- α -cyano-3-phenoxybenzyl-(1R,S)-cis-trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate, is a synthetic insecticide easily hydrolysable in the environment by carboxyesterases (see the scheme in Fig.1) [1-4] or it can be oxidased by cytochrome P450s [5] or by alcohol dehydrogenases [6]. The hydrolytic products can be found in mammalian cells as well as in food [7,8]; therefore many analytical methods allowing identification and quantification of these products were established, e.g. gas chromatography [7 - 10] and high performance liquid chromatography [11-13]. Interesting possibilities give electromigration techniques, in the beginning mainly isotachopheresis [14, 15], later capillary zone electrophoresis [16,17] and nowadays capillary electrochromatography [18]. They allowed fast and high efficient separation of the pesticide residues with low consumption of electrolytes as well as samples. The aim of our work is to contribute to the hydrolysis studies using micellar electrokinetic chromatography as an analytical technique for separation of cypermethrin residues.

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2. Experimental

2.1 Chemicals

Phosphoric acid, boric acid, sodium hydroxide, α -cyclodextrin, hydroxypropyl- β -cyclodextrin, γ -cyclodextrin, sodium dodecylsulfate (SDS), methanol and tert. butanol were obtained from Sigma (St. Louis, USA), potassium permanganate was obtained from Lachema (Czech Republic). All of chemicals were analytical grade quality. Cypermethrin and its main metabolites were prepared as described elsewhere [14]. Soil used in hydrolysis experiment was fortified by cypermethrin. Deionised water (Elga, Bucks, England, $18 \text{ M}\Omega \cdot \text{cm}^{-1}$) was used for preparation of all solutions.

2.2 Apparatus and conditions

All experiments were performed on a modular system Spectra PHORESIS 100 (Thermo Separation Products, Fremont, CA, USA) with UV-Vis detector Spectra Focus (Thermo Separation Products). Uncoated fused-silica capillaries (CACO-Silica Tubing and Optical Fibers, Slovakia) $75 \text{ cm} \times 45 \text{ cm} \times 0.75 \text{ }\mu\text{m}$ were used. All experiments were performed at the ambient temperature. The background electrolyte was prepared by diluting of appropriate amount of phosphoric acid in deionised water and then the solution was titrated with NaOH to pH 2.5; then SDS and α -cyclodextrin were added to the background electrolyte.

Standards were dissolved in the ten times diluted background electrolyte. Samples were injected for 0.5 s by vacuum. The capillary was washed daily before the first experiment with $0.1 \text{ mol}\cdot\text{L}^{-1}$ NaOH solution for 10 min, then with water (10 min); $0.1 \text{ mol}\cdot\text{L}^{-1}$ HCl (10 min); again with water (10 min); and finally with the working electrolyte for 15 min. The capillary was washed with BGE between the individual analysing runs. All measurements were done five times.

2.3 Hydrolysis and oxidation

Hydrolysis of cypermethrin standard was carried out as follows: 2.5 mL of $20 \text{ mol}\cdot\text{L}^{-1}$ cypermethrin solution in methanol or tetr. butanol was added to 2.5 mL of sodium borate buffer at pH 12.0. The mixture was then boiled under reflux for the certain time; the solution was then cooled to ambient temperature and diluted to 25 mL by deionized water. In the case of cypermethrin hydrolysis in a soil, 1.000 g of the soil was extracted by methanol in Soxhlet extractor for 24 hours; then the methanol was evaporated to the volume of 10 mL. The hydrolysis setup was the same as for standard. Oxidation was executed by warming up 1.0 mL of the hydrolytic solution with 100 μL of $0.1 \text{ mol}\cdot\text{L}^{-1}$ potassium permanganate solution to 60°C for 30 min.

3. Results and discussions

3.1 Micellar electrokinetic chromatography

From the structure (see Fig. 1), cypermethrin and its residues can be assumed as neutral compounds that can be favorably separated by micellar electrokinetic chromatography (MEKC). Because cypermethrin is unstable in an alkaline environment a separation has to be lead under acidic conditions. $100 \text{ mmol}\cdot\text{L}^{-1}$ sodium phosphate pH 2.5 with SDS was chosen as a starting electrolyte; voltage -18 kV was used. SDS concentration

range was studied using 25 mmol.L⁻¹, 50 mmol.L⁻¹, 75 mmol.L⁻¹, 100 mmol.L⁻¹, and 150 mmol.L⁻¹ SDS. Concentration 50 mmol.L⁻¹ SDS was the best choice counting both separation time and resolution. But separation only of four compounds of our interest was obtained; 3-phenoxybenzoic acid was not resolved. Cyclodextrins were tested as additives influencing the selectivity [19]. Only addition of γ -cyclodextrin provides good resolution of all the studied compounds. Influence of γ -cyclodextrin concentration on migration times is displayed in Fig. 2; concentration 10 mmol.L⁻¹ gives the highest resolution and therefore it was chosen for the next study. The residues and cypermethrin were identified by UV spectra and migration times. Calibration curves were linear from 2.5×10^{-5} mol.L⁻¹ to 3×10^{-3} mmol.L⁻¹ with correlation coefficients ranging from 0.9854 to 0.9999. Detection limits were: 1.3×10^{-5} mol.L⁻¹ for cypermethrin, 0.9×10^{-5} mol.L⁻¹ for 3-phenoxybenzaldehyde, 1.1×10^{-5} mol.L⁻¹ for 3-phenoxybenzoic acid and 0.7×10^{-5} mol.L⁻¹ for 3-phenoxybenzylalcohol. The precision of peak areas represented by its R.S.D. did not exceeded 3 % for interday assays and 20 % for interday assays.

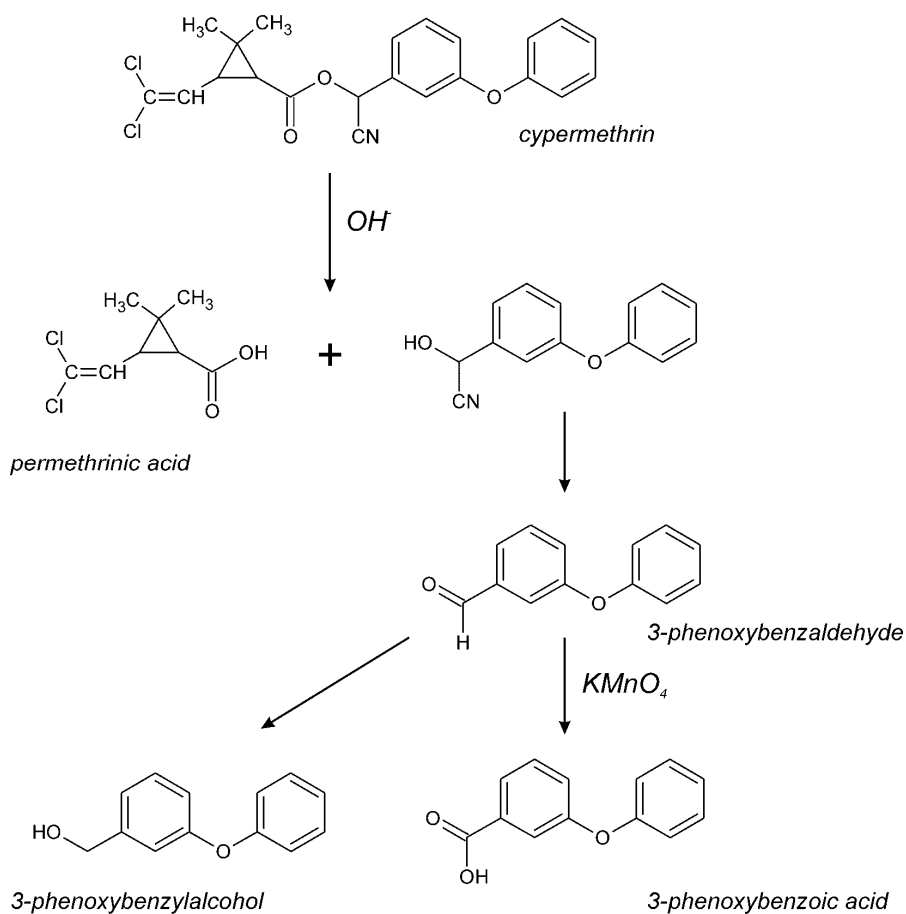


Fig. 1: Scheme of conversion of cypermethrin.

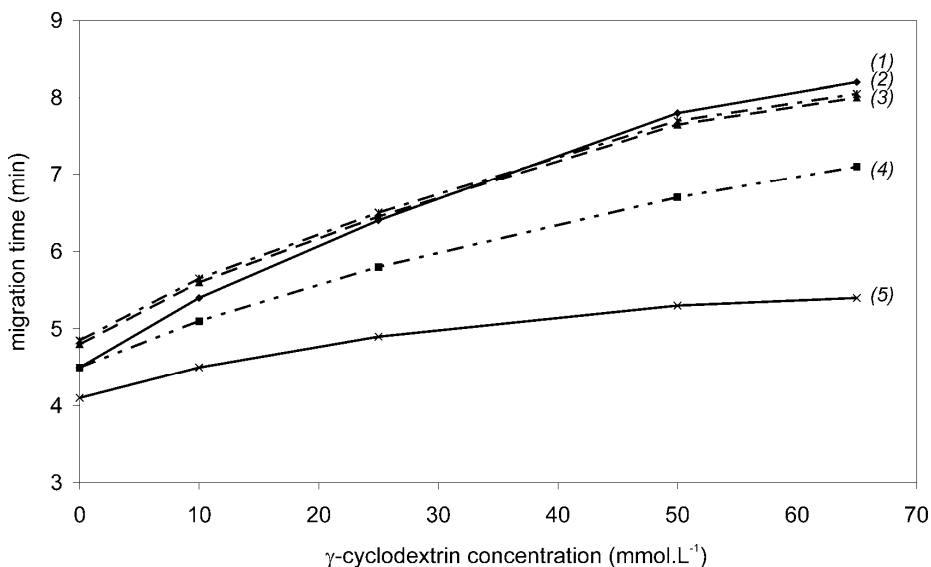


Fig. 2: Influence of γ -cyclodextrin concentration on migration times of analytes
 (1) 3-phenoxybenzoic acid, (2) permethrinic acid, (3) 3-phenoxybenzylalcohol,
 (4) 3-phenoxybenzaldehyde, (5) cypermethrin

2. Hydrolysis and oxidation

Kinetics of cypermethrin alkaline hydrolysis was studied in two solvents: methanol and tert. butanol. Profiles of the hydrolysis are displayed in Figure 3. Hydrolysis belongs to the reactions with first order kinetic equation [20]:

$$-\frac{dc_A}{dt} = kc_A$$

where k is the rate constant. Calculated rate constants from plotting data according to the equation (1) are $(310 \pm 60) \times 10^{-5} \text{ L.mol}^{-1}.\text{s}^{-1}$ for methanol and $(19.8 \pm 0.8) \times 10^{-5} \text{ L.mol}^{-1}.\text{s}^{-1}$ for tert. butanol.

A primary hydrolytic product 3-phenoxybenzaldehyde (Fig. 1) can be oxidized by the strong oxidizing agent, e.g. potassium permanganate, forming 3-phenoxybenzoic acid. This reaction is quantitative and it can be used for determination of total content of pyrethroids containing 3-phenoxybenzoic moiety. In the case of hydrolysis study, the reaction serves as a confirmation that the hydrolysis process is not affected by similar pyrethroids.

Examples of electrophoretic analyses of cypermethrin methanolic soil extracts after 60 min alkaline hydrolysis and after oxidation are shown in Figure 4. Decrease of cypermethrin peak and increase of 3-phenoxybenzaldehyde peak after hydrolysis is visible; in the electrophoregram from oxidation only peak of 3-phenoxybenzoic acid is detectable that is in correspondence with the presumptions.

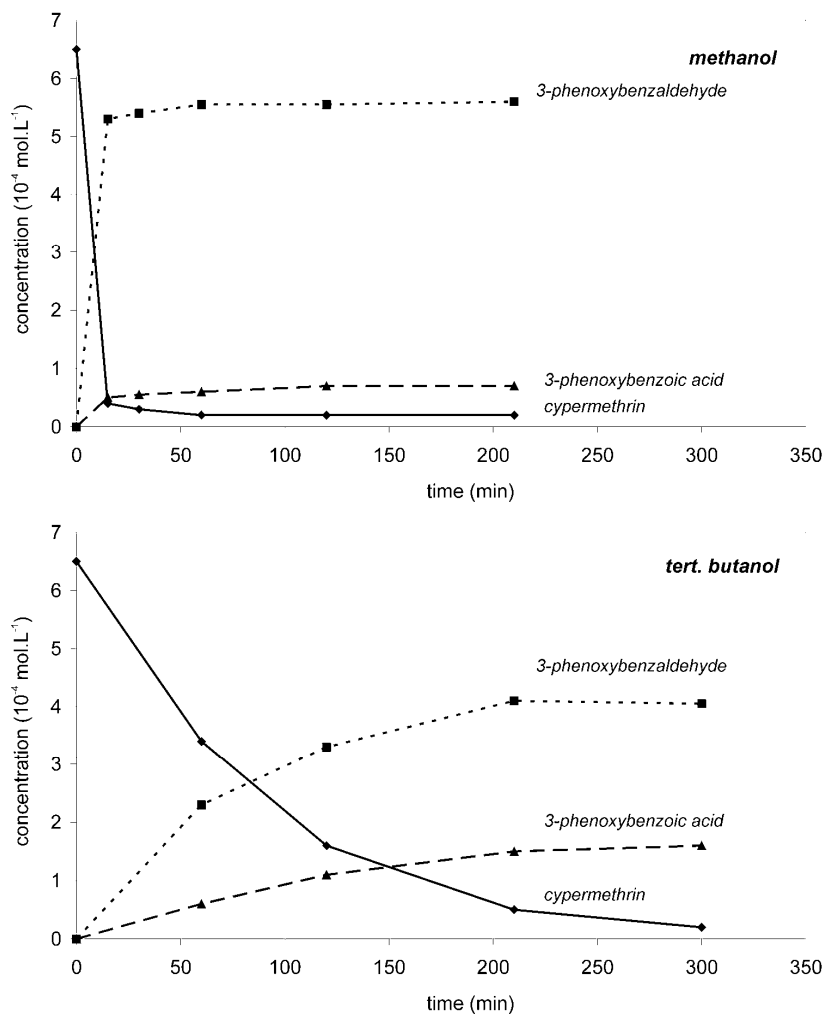


Fig. 3: Profiles of cypermethrin hydrolysis

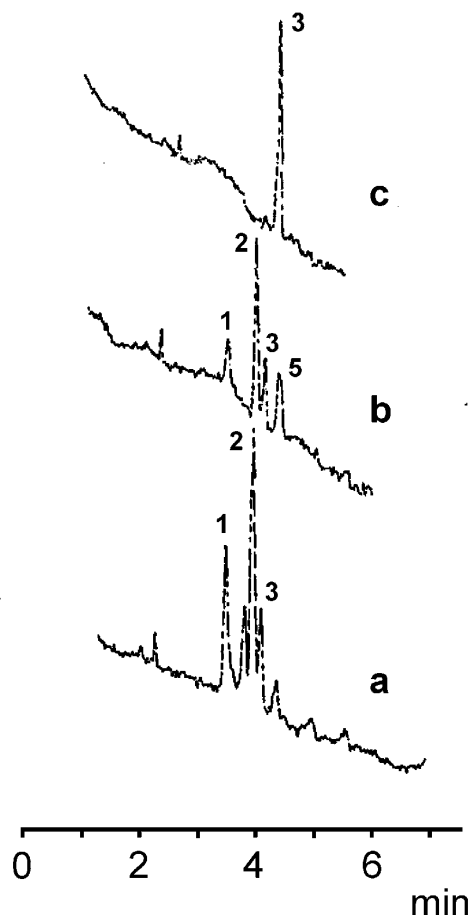


Fig . 4: Analyses of cypermethrin extract from soil
 (a) methanolic extract, (b) after alkaline hydrolysis of the extract, (c) after oxidation of hydrolytic products; (1) 3-phenoxybenzoic acid, (2) permethrinic acid, (3) 3-phenoxybenzylalcohol, (4) 3-phenoxybenzaldehyde, (5) cypermethrin; BGE: 100 mmol.L⁻¹ sodium phosphate pH 2.5, 50 mmol.L⁻¹ SDS and 10 mmol.L⁻¹ γ -cyclodextrin.

4. Conclusions

Micellar electrokinetic chromatography was successfully used for separation of cypermethrin and its hydrolytical products. Presented method was employed for study of hydrolytic process in methanol and tert. butanol. The hydrolysis in methanol was 15times faster than that in tert. butanol; moreover during hydrolysis in tert. butanol higher amount of 3-phenoxybenzoic acid is produced. Oxidation of one hydrolytic product, 3-phenoxybenzaldehyde, was then used for confirmation of observed results and quantification of similar pyrethroids.

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MEMORIES ON DETERMINATION OF PHENOLS IN SOILS

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Abstract

Head-space solid phase microextraction was suggested as a promising technique for isolation of phenolic compounds from contaminated soil. Proposed method was compared with simultaneous distillation-extraction and liquid extraction in Soxhlet extractor. Some fundamental aspects of rather problematic analysis complicated mainly by inhomogeneity of the matrix, mutual sorption affinity between analytes and matrix and troublesome availability of the reference material were discussed. Special attention was paid to the efficiency and selectivity of the extraction process. Detection limits below the ppm level were achieved by extraction techniques under investigation using gas chromatographic analysis with flame ionization detector. In excess of order of magnitude better detection limits were obtained for halogenated phenols with electron capture detector.

Keywords: phenols, soils, solid phase microextraction, simultaneous distillation-extraction

1. Introduction

Relevance of analytical monitoring of phenol and its derivatives in environment is generally well known, because of their widespread use in industrial processes [1-3] including the manufacture of plastics, dyes, drugs, antioxidants, pesticides, paper, petrochemical products and so on [4-8]. Isolation of such polar analytes from solid samples, especially soils, may be rather difficult with regards to low volatility, strong adsorption of phenols on solid particles and common problems with inhomogeneous samples [9]. The paper tries to overcome these problems by combination of three independent preconcentration techniques.

The attention is naturally concentrated on utilization of solid phase microextraction (SPME). The presentation of the SPME in capacity of powerful tool for analysis of soils is one of the main objectives of this paper. SPME is fast, simple and inexpensive preconcentration technique, which doesn't require any organic solvent [10-20]. Headspace modification [21-29] is suitable for extremely acid or basic samples, for samples containing high concentration of salts, proteins and other interfering components and for samples with

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colloid or even solid particles. This important feature inherent to headspace techniques should be accentuated in this paper and practically demonstrated on the example of so many problematic analysis of rather polar and handily adsorbed compounds in soils [30-37].

Simultaneous (or Steam) Distillation-Extraction (SDE), originally developed for analysis of food and agricultural products [38-50], found lastly a wide utilization in analysis of environmental samples and other matrices [51-60]. So far, wide pallet of organic compounds has been extracted from different matrices including low molecular weight alcohols and esters [61], nitrosoamines [62], pesticides [63], polychlorinated biphenyls [64], polychlorinated dibenzodioxins and polychlorinated dibenzofurans [65], metabolites [66], terpenes [67-69] and of course last but not least phenols [70-75].

Soxhlet extraction (SE) [76-85] is generally considered as an efficient and well established extraction method [86-91] serving often for direct valuation of other extraction techniques [92-103]. It has been used as referential method in this study in accordance with recommendation of US - EPA Method 8040 and 3540 [104,105].

2. Experimental

Gas chromatograph Hewlett Packard 5890 (Agilent, Palo Alto, CA, USA) with DB-5 column (30 m x 0.53 mm x 1.5 μm) was used for all experiments. Flame ionization detector (FID) (250°C, helium as a carrier gas, 7 ml/min) and electron capture detector (ECD) (nitrogen as a carrier gas 7 ml/min) were used. Carrier helium and nitrogen and other auxiliary gases (hydrogen, air, nitrogen as a makeup gas) were purchased from Linde, Technoplyn, Czech Republic.

SPME fiber holder with 85 μm polyacrylate fiber (Supelco, Bellefonte, PA, USA) were used. Sample of soil (4 g) was added directly to the 20 ml of 0.1 mol.l⁻¹ HCl saturated by NaCl in the standard 30 ml EPA vial. Mixture was sampled for 60 minutes in the haedspace at ambient temperature. Analytes were desorbed at 250°C for 2 minutes and analyzed using temperature program: 80°C - 2 min - 6°C/min - 250°C - 5 min.

For the SDE experiment soil sample (10 g) was extracted two times by 75 ml of 0.1 mol.l⁻¹ sodium hydroxide for 30 minutes in ultrasonic bath. Mixture was centrifuged; supernatant with two boiling chips was placed in the extraction flask, acidified by hydrochloric acid to pH~1, saturated by sodium chloride (60 g) and extracted by diethylether (10 ml) for 90 minutes. Extract was evaporated to 1 ml and analyzed by GC (1 μl injected) using temperature program: 100°C - 2 min - 4°C/min - 250°C - 5 min.

Soxhlet extraction was used as a standard referential method (US - EPA Method 8040, 3540). 10 g of soil and 10 g of dry Na₂SO₄ was placed in extraction cartridge and extracted by 300 ml of dichloromethane for 24 hours by 4 - 5 cycles per hour. Extract was evaporated in Kuderna Danish evaporator to 5 ml and analyzed by GC (1 μl injected) using temperature program: 100°C - 2 min - 4°C/min - 250°C - 5 min.

3. Results and discussion

3.1 Solid phase microextraction

The possibility of extraction of samples containing solid particles was declared as one of the most important advantages of the headspace modification of the Solid Phase MicroExtraction (HS-SPME). The fact predestinates HS-SPME for analysis of soil samples too. The sample of soil was added to the 20 ml of water, treated by hydrochloric acid and so-

dium chloride, and extracted by headspace technique. The simple model of headspace extraction of aqueous sample, including two partition equilibria: 1. water - headspace. 2. headspace - fiber coating, was extended by third equilibrium: 3. soil – water. So, the overall partition coefficient soil – fiber is given as a product of partition coefficients of all particular equilibria. In order to be comparable with previous data, measured with liquid samples, discussed values of partition coefficients were calculated by using of phase ratio given by volumes of water (20 ml) and fiber coating (~0.5 μ l). Resulting values of partition coefficients and corresponding recovery values are summarized in Table I together with recoveries of simultaneous distillation-extraction and Soxhlet extraction.

Although SPME gives the lowest extraction yields of all techniques under investigation, limits of detection are comparable to other discussed techniques, because whole extracted amount gets into gas chromatograph, while only about 0.1 % of the total extract volume is used in the case of SDE and SE. That's why in the case of SPME, partition coefficient seems to be much better expression of extraction efficiency than recovery value.

With regards to inhomogeneous matrix and low extraction recoveries some difficulties with calibration should be expected. As calibration curve method is unsuitable for such complex samples, the standard addition method could be recommended. The kinetics of the equilibrium between soil and added phenols should be accentuated in this case in order to achieve equilibrium distribution between soil and aqueous phase [106-109].

Typical chromatogram obtained by GC- FID at concentration level 10 mg.kg⁻¹ is shown on Fig. 1A. Limit of detection of HS-SPME depends on the quantity of soil, which can be agitated sufficiently. The limit of detection was estimated to 0.1 – 0.01 mg.kg⁻¹ under described conditions.

Electron capture detector was used for further improvement of sensitivity and selectivity for halogenated derivatives, which have the main toxicological and hygienic impact. For SPME - GC - ECD about order of magnitude higher responses were observed for halogenated derivatives of phenol in comparison to flame ionization detection. Typical chromatogram on the concentration level 1 mg.kg⁻¹ is shown on Fig. 1B.

3.2 Simultaneous distillation-extraction

Simultaneous distillation–extraction has been presented in one of our previous works as a substantial instrument for determination of phenols in soils as well as for investigation of distribution of phenols in environment¹⁰. For the reason, optimized SDE process was suggested as one of the comparative methods. The procedure consists in extraction of phenols (weak acids) with 0.1 M NaOH in the form of respective phenolates. Alkaline solution serves as an extraordinary efficient extraction agent, which is over and above able to release adsorbed phenols in virtue of partial hydrolysis and corrosion of the surface of lignin particles and some other solid surfaces responsible for the sorption capacity of soils. Remaining solid particles are then removed by centrifugation, supernatant is acidified by hydrochloric acid to convert phenolates to neutral phenols, sample is transferred to the SDE apparatus and free phenols are extracted by diethylether.

Recovery values obtained by SDE are given in Table I. Regarding the high extraction power of the complex extraction procedure (involving almost dissolution of the solid soil particles and subsequent reconstitution of the analytes), the recovery value are considerably high, mostly even higher than reference values obtained by Soxhlet extraction. Moreover, the distillation step brings so important selectivity for polar and hydrogen bond forming com-

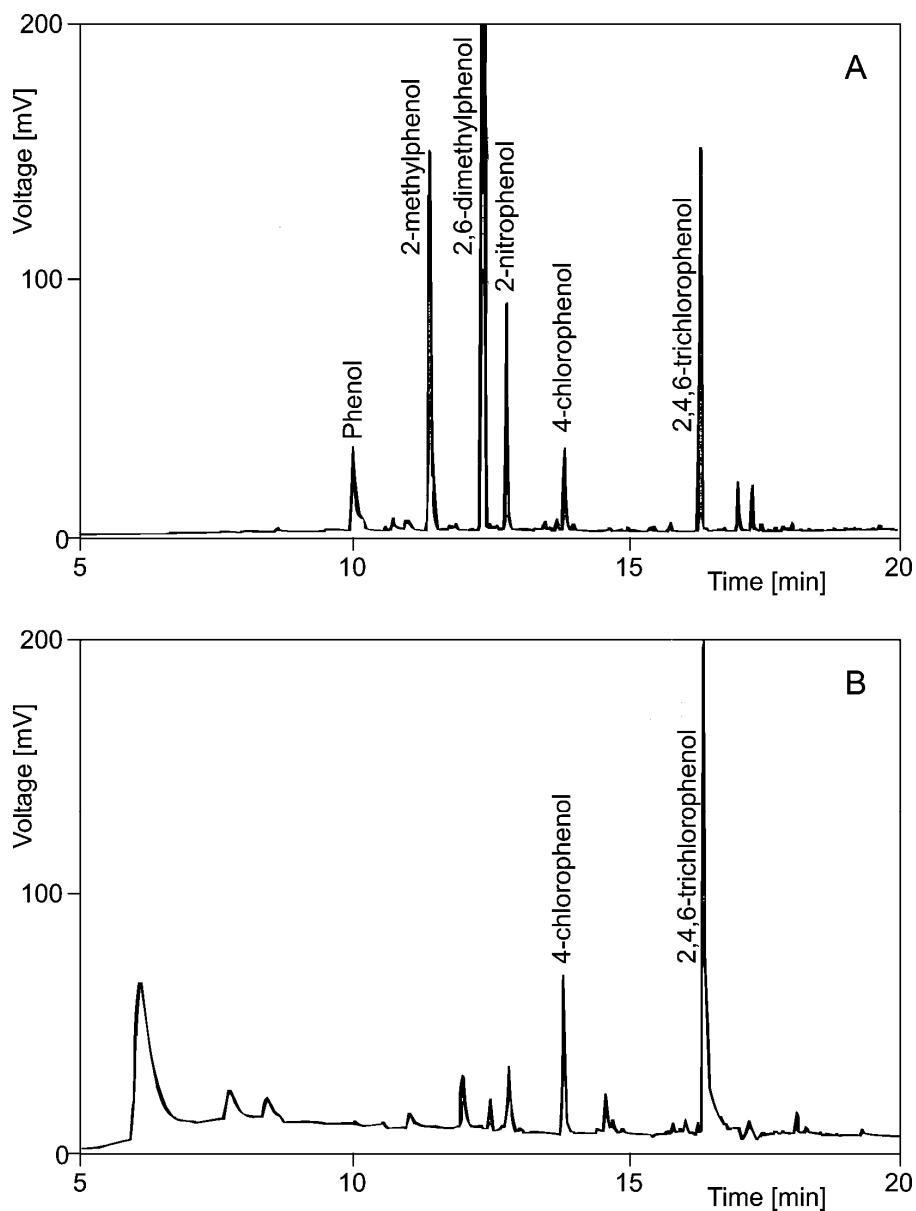


Fig. 1: (A) SPME – GC – FID chromatogram of soil with 10 mg.kg⁻¹ of each phenol; (B) SPME – GC – ECD chromatogram of soil with 1 mg.kg⁻¹ of each phenol.

Table I: Comparison of extraction efficiencies obtained by discussed methods (for conditions see Experimental; SPME – Solid Phase Microextraction (Head – Space modification), SDE – Simultaneous distillation-extraction, SE – Soxhlet extraction).

	SPME		SDE	SE
	Partition coefficient	Recovery (%)	Recovery (%)	Recovery (%)
Phenol	8.54	0.021	73.27	50.19
2-methylphenol	8.66	0.022	76.96	44.19
2,6-dimethylphenol	13.28	0.033	58.26	15.31
2-nitrophenol	9.56	0.024	57.50	37.64
4-chlorophenol	1.77	0.004	80.44	80.44
2,4,6-trichlorophenol	5.51	0.014	81.94	88.67

pounds, which are susceptible to vapor distillation (Fig. 2). Selectivity of SDE procedure represents great advantage of SDE prior to Soxhlet extraction, involving wide range of different chemical individuals from strictly nonpolar, low molecular weight compounds to strongly polar and even macromolecular and polyfunctional chemicals. After all, complicated cleanup should be included after Soxhlet extraction, while SDE procedure doesn't require any more sample treatment. Shorter extraction time and lower solvent consumption are additional advantages of SDE prior to Soxhlet extraction.

3.3 Soxhlet extraction

Soxhlet extraction was named after Franz Ritter von Soxhlet, who introduced this extraction method in 1879. It had been the most widely used extraction method for more than one hundred year. Since it is efficient and well-established technique, it is often used as the benchmark for comparing other methods. Moreover, number of standard protocols prescribes Soxhlet extraction for determination of a wide range of organic compounds. For instance, standard EPA method for determination of phenols in soils prescribes Soxhlet extraction in 300 ml of dichloromethane for 24 hours.

Recovery values obtained by Soxhlet extraction are given in Table I. Mostly, the values are somewhat lower than those obtained from SDE procedure. This fact slightly decreases the credibility of the Soxhlet extraction as a referential extraction method. The low extraction efficiency can be ascribed to relatively low desorption and solvation capability of cooled, condensed solvents. The same reason is usually mentioned as a main cause of the slowness and time consumption (Soxhlet extraction can usually take between 6 to 48 hours).

Some other drawbacks of Soxhlet extraction consist in relatively large volume of the solvent required for extraction and low selectivity of the extraction. Usually a solvent evaporation step and suitable cleanup procedure have to be implemented into the analytical protocol based on Soxhlet extraction. The necessity of proper cleanup may be clearly evidenced in Fig. 2. On the other hand, absence of any kind of selectivity is one of the fundamental qualifications for the technique aspiring for the reputation of the universal extraction method.

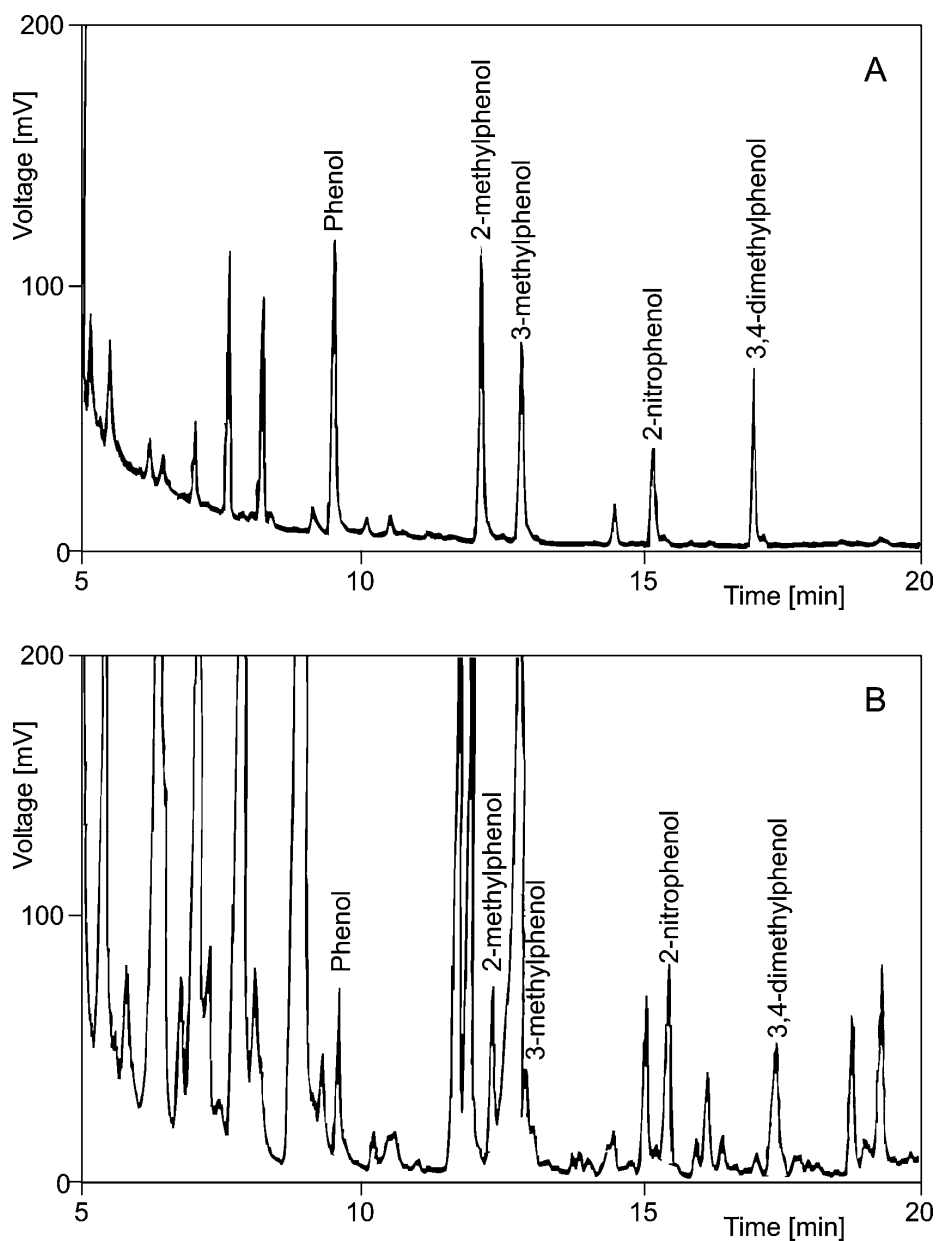


Fig. 2: (A) Simultaneous distillation-extraction: GC – FID chromatogram of extract at concentration level 1 mg.kg^{-1} ;
 (B) Soxhlet extraction: GC – FID chromatogram of extract at concentration level 1 mg.kg^{-1} .

4. Conclusion

Headspace Solid Phase Microextraction (HS-SPME) is suitable technique for extraction of low molecular weight phenols from soils at ppm concentration level. Since the whole extracted amount passes into the gas chromatograph, the technique offers similar detection limit in comparison with SDE and SE, although the recovery values are mostly below 0.1 %. Both, SPME and SDE methods, offer higher selectivity for phenols derivatives in comparison to standard Soxhlet procedure. In addition, SDE gives higher recoveries than Soxhlet extraction. Shorter extraction time and lower solvent consumption are additional advantages of SDE.

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**ELECTROCHEMISTRY ON-LINE WITH MASS SPECTROMETRY:
NOTES ON CURRENT STATE OF THE TECHNIQUE**

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Abstract

Electrochemistry combined on-line with mass spectrometry is a relatively new analytical technique which has spread over the last decade. This trend has been supported by the increased commercial availability of instruments as well as by the fact that ESI became a common ionization technique in mass spectrometry, well compatible with electroanalytical flow-measurements. This connection has proven to be a powerful analytical tool in various fields of application which include on-line electrochemical derivatization for enhanced MS selectivity, studies of reaction mechanisms, in-vitro mimicking of metabolic processes and protein or peptide specific cleavage.

Keywords: electrochemistry, mass spectrometry, on-line

Current state of the on-line electrochemistry with mass spectrometry

The first serious attempts to combine electrochemistry (EC) with mass spectrometry (MS) are dated in the early 70's. Nevertheless, this pioneering research stayed rather unnoticed by the main analytical stream over several next decades. The interests in this hyphenated technique, however, have been rapidly growing in recent years and it will become even more evident from the continuously increasing number of scientific papers dealing with this topic [1].

Although the main scope of this article is to discuss some interesting features of "flow" electrochemical techniques (working electrode placed in the stream of transport electrolyte), some alternative approaches will be pointed out briefly as well.

Among the most important studies are those concerning direct coupling of a conventional electrochemical cell with a mass spectrometer: the analyte is introduced into the MS ion source via polymeric (Teflon or silicon) membrane incorporated in the specially modified electrochemical cell. This method is also known as Membrane Introduction Mass Spectrometry (MIMS). The main disadvantage of this approach seems to be molecular trans-

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port rate of the non-polar species from the water through the membrane and the resulting response delay. The MIMS method has been developed further in the last two decades: the incorporation of the two-stage vacuum system and most frequently, a cyclic voltammetry (CV) measurements, enabled simultaneous and fast on-line MS detection of volatile electrode reaction products. First reviews covering this new technique – “differential electrochemical mass spectrometry” (DEMS) - appeared at the beginning of the 90’s, but DEMS has never been widespread or extensively analytically used and has remained meanwhile purely academic method, even if future development can certainly be expected.

One of the most important analytical aspects of the electrochemistry on-line coupled with mass spectrometry (EC-MS) is its capability to be combined further with high-performance liquid chromatography (HPLC). The expansion of the electrospray ionization (ESI) technique in the recent years plays also the major role in the current EC-MS development [2]. Doubtlessly, ESI is the best suitable ionization technique well compatible with electrochemistry.

This can be explained by the fact that in ESI the ions are transferred from the electrolyte into the gas phase with only weak and gentle fragmentation as a result of the process known as “coulombic explosion”, unlike the electron impact (EI) or other “hard” ionization techniques. Since the reactions involved in ESI are connected with charge transfer processes, the mobile phase should preferably contain some volatile buffer components, similar to those used commonly in EC-LC which is also the reason why ESI suits the studies of mechanisms of electrochemical processes very well.

However, there are also some inherent limitations using the ESI technique: the efficiency of the ion emission decreases as the flow rate of the mobile phase increases – at the rates within 10-100 nl/min, the efficiency of 100% is reached. It is known that electrolytes present in the mobile phase suppress the overall ESI-MS sensitivity whereas volatile organic modifiers such as methanol or acetonitrile enhance the selectivity. Low electrolyte concentration and thus low mobile phase conductivity results in electrode response decrease.

In ESI, high voltages (in the kV range) are applied at the nozzle tip. As a result, faradaic currents are generated which are responsible for the potentially-driven electrochemical side reactions. These reactions can cause numerous interfering electrochemical side-processes in the mobile phase, and therefore they should be eliminated or at least minimized. In such case, the MS ion source basically mimics an electrochemical cell in a potentiostatic mode [3.4]. It is obvious that MS provides more complex information about the target molecule than the electrochemical detection itself: the quantification is then performed on the basis of MS data and not by measuring electrochemical currents. Using this approach, the majority of commonly known EC drawbacks – such as baseline instability or signal irreproducibility – can be effectively eliminated. EC-MS then becomes a valuable final analytical step after previous LC separation.

Importantly, LC-EC-MS is characterized by nearly unlimited compatibility with LC gradient-elution techniques and the separation process can be easily and efficiently optimized [5].

Combined EC-MS technique can be particularly useful in case that either

1. separation is required for the study of electrochemical properties of an individual compound in the mixture, or
2. separation is required for electrochemical conversion product(s)

In the first case, ECD follows the classical reversed-phase LC and the effluent then enters the MS ion source. In such situation, only one reaction product can be detected. In the second case the whole mixture is first converted (most often oxidized), followed by the liquid-chromatographic separation and final MS detection. In both cases it is possible to incorporate an optional UV-VIS spectrophotometer prior to MS to maximize the information yield of the each single analysis.

Even if diverse electrochemical cells of various construction types can be generally used for EC-MS, the large-surface 'flow-through' type electrodes are most often utilized. The main reasons are long maintenance intervals and its low tendency to the surface passivation/deactivation. Large sample batches can be analyzed without tedious interruptions.

Last but not least, the instruments utilizing this cell type have become largely commercially available in recent years. Porous graphite coulometric working electrodes, combined with the maintenance-free Pd/H₂ reference electrode, have promoted significantly the current development of the EC-MS hyphenated technique. With large electrode surface, high conversion degrees at moderate flow-rates of the mobile phase can be achieved (<100 µl/min). The current papers dealing with EC-MS technique can be divided into four fields of application [6]:

On-line electrochemical derivatization for the purposes of MS-detection sensitivity enhancement

The electrochemical cell performs as an electrochemical on-line reactor. A large number of compounds which are normally inactive in MS (no or very low ion current is obtained) can be detected sensitively if previous electrochemical on-line oxidation has been applied.

Electrochemical reaction mechanisms studies

Another possibility is to monitor electrode reaction products of the molecules of which the proper mechanism is unknown [7,8]. The on-line arrangement is particularly advantageous if labile products or short-lived intermediates have to be identified [9].

Biological processes in-vitro mimicking

Presumably one of the most attractive features of the combined EC-MS technique is the ability to mimic some of the oxidative metabolic pathways taking place in living organisms. The resulting products can be then characterized by MS [10,11].

Based upon experimental results when electrochemically simulating some oxidative reactions catalyzed by the P450 cytochrome complex it has been concluded that it is possible to mimic in-vitro one-electron oxidations, such as N-dealkylations, S-oxidations, P-oxidations, alcohol oxidations and dehydrogenations. Reactions catalyzed by P450 cytochrome are initiated directly via hydrogen atom, whereas O-dealkylations and hydroxylations occur on the non-substituted aromatic ring. Electrochemical oxidation, on the contrary, will not mimic such type of catalyzed reactions. Valuable information concerning sensitivity of various substrates towards oxidation has been obtained in that manner, as well as additional data concerning the involved reaction mechanism.

EC-LC-MS was also used for the *in-vitro* simulation of acetaminophen detoxification in human liver. It was found out that this process could be very well mimicked by the on-line electrochemical oxidation at a porous glassy carbon working electrode [12]. Such EC-MS

usage is a very promising starting point for further metabolic studies and drug metabolism mimicking in living organisms [9,13,14].

Specific protein/peptide cleavage

Other application field of EC-MS technique represents fast analysis of peptide oxidation products. Tyrosine contained in peptides is readily oxidized while yielding various peptide fragments as a result of hydrolysis on C-terminating tyrosine bond. The limiting factor is whether tyrosine is phosphorylated or not. By using EC-MS the peptides can be cleaved and then mapped [15,16].

Metallic complexes can also be analyzed by EC-MS since different oxidation states depending upon applied working electrode potential can be identified. If a high-resolution mass spectrometer is used, the data of redox states of metalloproteins can be determined.

The immense publication growth in the EC-MS area in the past years indicates that this technique will soon find a large usage in miscellaneous scientific fields among which the metabolomic and peptide structural studies play a prime role. Nevertheless, the full potential of the EC-MS technique is probably still waiting for its complete discovery and numerous important applications in this area are yet to be expected.

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ELECTROCHEMICAL BEHAVIOUR OF NORFLURAZON AT MERCURY ELECTRODES, AND ITS ANALYTICAL APPLICATION

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Abstract

Norflurazon (systemic herbicide, Fig. 1) is reducible at mercury electrodes to the different extent depending on media acidity, area of mercury cathode and its contact time with an electrode surface. The electrode reaction, investigated using direct current and pulse voltammetry, controlled-potential coulometry, and HPLC-MS, is a combination of the electro-reduction and a kinetic process. Four-electron (by voltammetry) or six-electron (by coulometry) irreversible reduction of a norflurazon protonized form was observed in strongly acid media. Simple compounds (HCl, NH₃) are released and five-membered pyrrole cycle is formed at electroconversion in these types of media. Only two-electron reduction proceeds in weakly acid solutions and no signal is observed in neutral pH values. A new diffusion wave strongly affected by kinetics is formed in alkaline media. Norflurazon was determined in 0.01 M HCl using differential pulse voltammetry and adsorptive stripping voltammetry with limits of detection 43 nmol.L⁻¹ (13 ppb) and 18 nmol.L⁻¹ (5.8 ppb), respectively.

Keywords: herbicide, norflurazon, mercury electrodes, coulometry, voltammetry

1. Introduction

Norflurazon **I** (4-chloro-5-methylamino-2-(α,α,α -trifluoro-*m*-tolyl)pyridazin-3(2*H*)-one) belongs to the group of diazine compounds with herbicide activity [1].

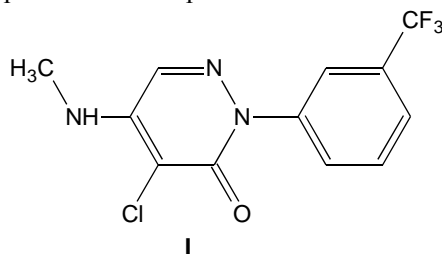


Fig. 1: Structure of norflurazon

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It is an effective component of commercial herbicide preparations, e.g. Sandoz-9789, Zorial and Solicam [2-4], used for the pre-emergence control of annual grasses and broadleaved weeds and some perennial grasses and sedges in cotton, nuts, soybeans, citrus, vines, pome and stone-fruit, cranberries, hops, etc. Norflurazon is adsorbed by the roots and reduces carotenoid biosynthesis, which causes the chlorophyll depletion with the subsequent inhibition of photosynthesis [5].

Norflurazon is adsorbed in the surface layer of soil depending on the organic matter content [6]. Photodegradation and microbial action are involved in the dissipation process, effecting demethylation to less toxic and herbicide inactive product [5]. The half-life for norflurazon in soil varied from 22 to 180 days [4, 5]. Considering high persistence, the control of norflurazon in soil, crops or fruits is necessary in term of ecology as well as human health. Liquid chromatography is the method most frequently employed for the analysis of norflurazon and its metabolites in different matrices [7-9]. Redox properties of this herbicide, that play an important part in its biological activity, can be used for analytical purposes. Favourable structure of pyridazine ring polarized by the electrophilic atom of chlorine and by the trifluoromethyl group at the side ring enables to observe the redox behaviour using voltammetric methods.

Very little of attention has been paid to study of electrochemical properties of norflurazon and related compounds. The mechanism of the electrochemical reduction of chloridazon, a diazine herbicide with the structure very similar to norflurazon, was studied only in past years [10-13]. Therefore the aim of our work was to contribute to the elucidation of the electrochemical behaviour of norflurazon at the mercury electrode in aqueous solutions, and to develop a voltammetric procedure for the determination of, especially, low levels of this herbicide as a low-cost and fast alternative to standard HPLC method.

2. Experimental

2.1 Apparatus

An OH-102 (Radelkis, Budapest, Hungary) polarographic analyzer with a three-electrode cell containing a dropping mercury electrode (DME) ($t = 3.6$ s, $m = 1.98$ mg.s⁻¹), a saturated calomel electrode (SCE), and an auxiliary Pt electrode were used for DC polarography. The fast polarographic and pulse voltammetric measurements were performed with two types of instrument: first a PA-4 polarographic analyzer with a static mercury drop electrode (SMDE) and 4105 X-Y recorder (all Laboratorní přístroje, Prague, Czech Republic) and second a computer controlled Eco-Tribo polarograph (Polaro-Sensors, Prague, Czech Republic), equipped by Polar.Pro software v. 4.0. DME ($t = 2$ s) served as a working electrode at fast and differential pulse polarography (DPP) and a working pencil type hanging mercury drop electrode (HMDE) was employed for voltammetric measurements. Saturated calomel or silver-silver chloride served as the reference electrodes and a small platinum sheet was used as the auxiliary electrode. Voltammograms were recorded at a scan rate of 20 mV.s⁻¹ and pulse amplitude -50 mV (FSDPV). The Eco-Tribo polarograph with a mercury pencil electrode was used also for cyclic voltammetry.

The controlled-potential coulometry measurements were performed with the OH 404 (Radelkis) coulometric analyzer with a mercury-pool cathode (26.4 cm²). The SCE served as a reference electrode; an auxiliary platinum electrode in the anode space was separated by sintered glass. The electrolysis of norflurazon solution ($c = 0.1$ mmol.L⁻¹, total volume 50 ml)

was performed in 10 mmol.L⁻¹ HCl in acetate buffer pH 4.7, and in 1 mmol.L⁻¹ NaOH at constant potentials -0.90 V, -1.20 V and -1.80 V, respectively, relative to SCE. The sample was electrolysed at least for a period of 60 min until the measured current dropped to the residual current value of the supporting electrolyte. Samples were taken from the electrolytic cell in the sequence of 0, 30 and 60 minutes after a start of the electrolysis.

The products of the coulometric reduction of norflurazon were identified by HPLC/MS system. The analyses were performed with a system Gold liquid chromatograph (pump 125 S and diode-array detector 168, scan 190-390 nm, all Beckman, Fullerton, CA, USA). The separation was carried on LiChroCart column (250 mm×4 mm i.d.) packed with LiChrospher 60 RP Select B, 5 μm (Merck, Darmstadt, Germany). Twenty microliters of the sample were injected for analysis. A binary linear mobile phase gradient (water–methanol: 0 min 95/5, 5 min 95/5, 35 min 0/100) was used at a flow-rate of 0.75 mL.min⁻¹. The flow was split between a diode-array detector and a mass spectrometer in a ratio of ~85:15. Mass spectrometric detection was performed using the LCQ mass spectrometer (Finnigan Mat, San Jose, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) source. The response of the mass spectrometer was tuned to the norflurazon standard. Optimised parameters were: source current 5.0 μA, APCI vaporizer temperature 450 °C, sheath gas flow 40 arbitrary units, capillary temperature 150 °C, capillary voltage 4.0 V.

pH was measured using an inoLab 720 pH meter with a combined pH electrode SenTix 41 (WTW, Weilheim, Germany). The pH meter was calibrated by the use of aqueous standard buffers WTW.

2.2 Chemicals

The norflurazon standard (98.6 %) was purchased from Supelco (Bellefonte, USA). The stock solution of norflurazon ($c = 1 \text{ mmol.L}^{-1}$) was prepared by dissolving the standard in methanol (p.a., Lach-Ner, Neratovice, Czech Republic) and stored in the dark. Methanol was also used for the preparation of more diluted solutions. HPLC-grade methanol (Merck) was used to prepare the mobile phases. All other chemicals and solvents used for the preparation of supporting electrolytes and buffer solutions were of analytical reagent grade (Lachema, Brno, Czech Republic). Elgastat ultra high purity water (18.2 MΩ.cm⁻¹, Elga, Bucks, U.K.) was used to prepare all solutions. Air oxygen was removed by passage of pure nitrogen (99.999 %).

3. Results and discussion

3.1 Mechanism of electrode reaction

The electrochemical reduction of norflurazon at a mercury electrode proceeds in whole pH range. One DC wave/DPP-peak has been observed in acidic media ($E_{1/2} = -0.785 \text{ V vs. SCE}$ in 0.12 M HCl and $E_{1/2} = -1.015 \text{ V}$ at pH 4.41) corresponding to a diffusion-controlled process. A voltammetric peak splits in two peaks at the higher scan rate (20 mV.s⁻¹, FSDPV) in mineral acids (Fig. 2). The splitting has not been observed at slow scan rate (2 mV.s⁻¹, DPP) indicating that the electrode process proceeds in one step. It results from comparison of the DPP and FSDPV peak areas that the electrode process is relatively slow and involves a kinetic reaction.

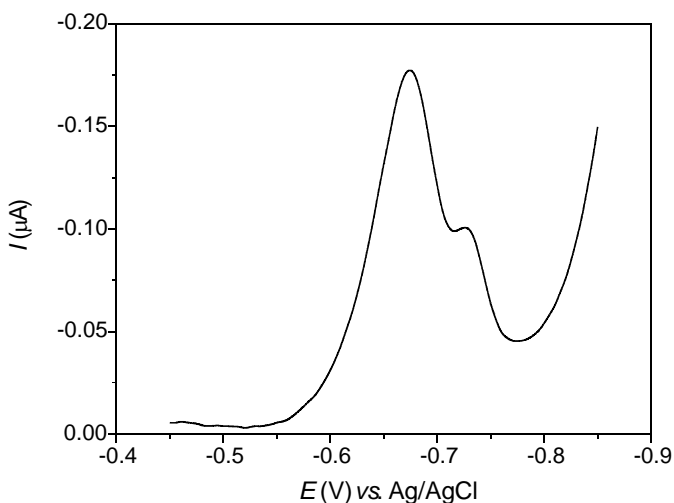


Fig. 2: FSDPV-peak of norflurazon ($c = 0.01 \text{ mmol.L}^{-1}$) in 1M HCl, at scan rate 20 mV.s^{-1} .

The polarographic current is markedly dependent on the acidity of measured solution. The limiting current value decreases with increasing pH in strongly acidic media, remains constant at pH between 2 and 5, decreases at $\text{pH} > 5$ and drops to zero at pH about 9 (Fig. 3). It is evident from a course of the dependence $I = f(\text{pH})$ (Fig. 3) that the acidity and the related protonation of the analyzed molecule play an important part in the reduction process. At least two dissociation equilibria of redox couple of analyte can be taken into consideration.

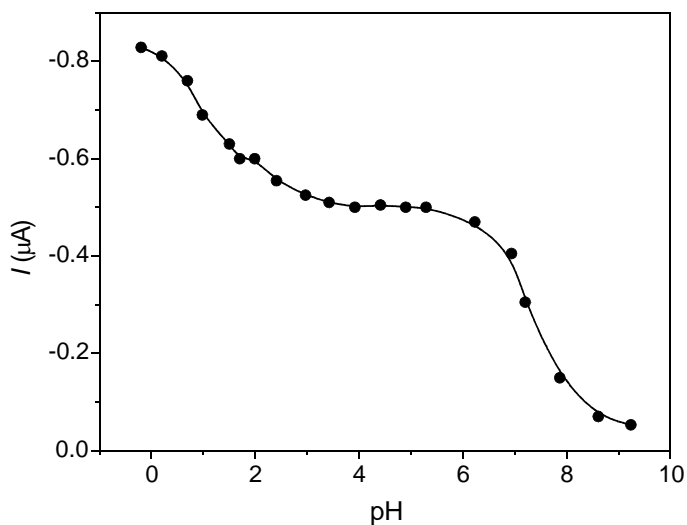


Fig. 3: Dependence of the DC-wave current value of norflurazon ($c = 0.1 \text{ mmol.L}^{-1}$) on pH in HCl and Britton-Robinson buffers.

An influence of the medium acidity on the electrode process is obvious also from a dependence of the half-wave potentials on pH (Fig. 4) consisting of three straight lines with two intersect which correspond to the dissociation constants of the oxidised ($pK_{ox} = 1.75$) and reduced ($pK_r = 6.90$) form of norflurazon.

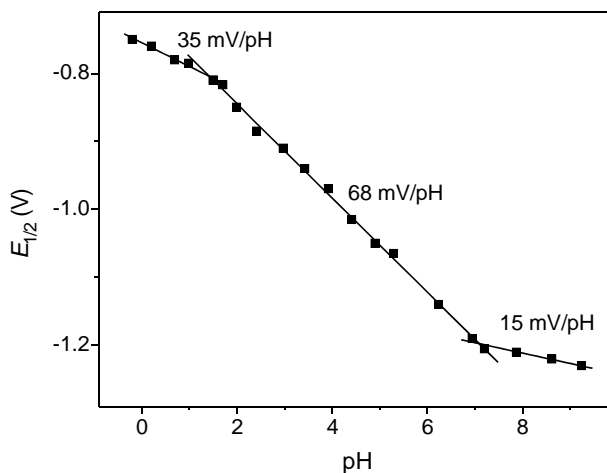


Fig. 4: Dependence of $E_{1/2}$ (vs. SCE) of norflurazon ($c = 0.1 \text{ mmol.L}^{-1}$) on pH in HCl and Britton-Robinson buffers.

The influence of acidity on the reduction of norflurazon is closely associated with its protolytic reaction. It can be assumed on the basis of structural analogy between norflurazon and chloridazon [13] that only the protonated form of norflurazon is reducible. This form is stabilized by mesomeric and tautomeric effects (Fig. 5).

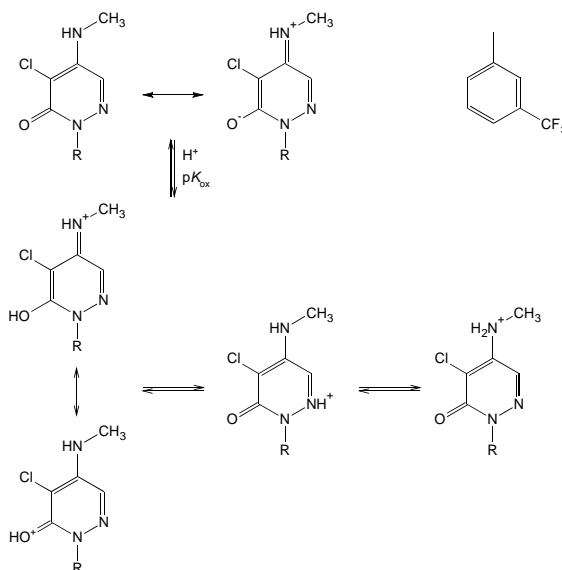


Fig. 5: Mesomeric and tautomeric stabilization of the protonated form of norflurazon.

The height of the DC wave of norflurazon in strong acidic media is comparable to the height of the four-electron wave of chloridazon and approximately twofold in comparison with two-electron wave of benzil (Fig. 6) under the same experimental conditions. That means the electrode reduction of norflurazon is four-electron. The same number of electron was determined by controlled-potential coulometry at the mercury-pool electrode in 0.01M HCl.

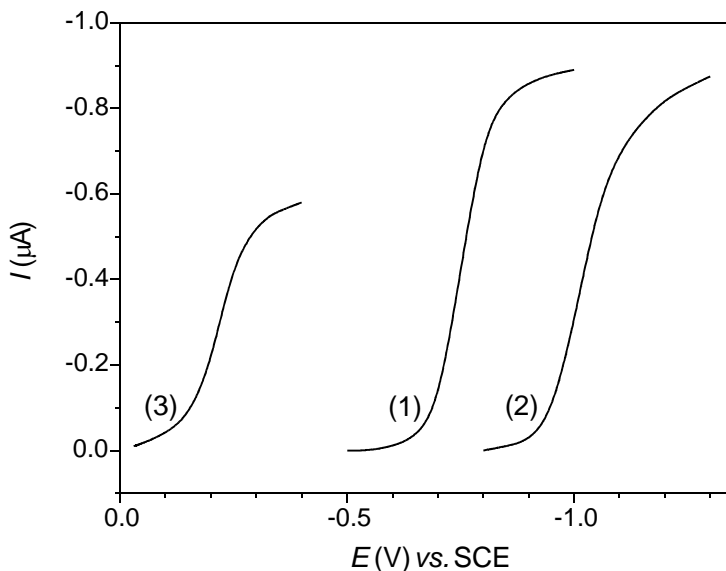


Fig. 6: DC curves of norflurazon (1) at pH 1.0, chloridazon (2) at pH 2.6 and benzil (3) at pH 1.2; concentration of depolarizers 0.1 mmol.L⁻¹.

The products of the coulometric reduction in 10 mmol.L⁻¹ HCl have been analyzed by HPLC/MS (Fig. 7). The electrolysed solution contained, besides norflurazon itself (quasi-molecular ion $[M+H]^+ = 304$), also another three products characterized by the quasi-molecular ions at m/z 306, 291 and 257, respectively. The ion $[M+H]^+ = 306$ corresponds to 1,6-dihydronorflurazon, $[M+H]^+ = 291$ and 257 correspond to 3-chloro-4-(methylamino)-1-[3-(trifluoromethyl)phenyl]-1,5-dihydro-2H-pyrrol-2-one and 4-(methylamino)-1-[3-(trifluoromethyl)phenyl]-1,5-dihydro-2H-pyrrol-2-one, respectively, that are formed by reduction and protogenolysis of 1,6-dihydronorflurazon.

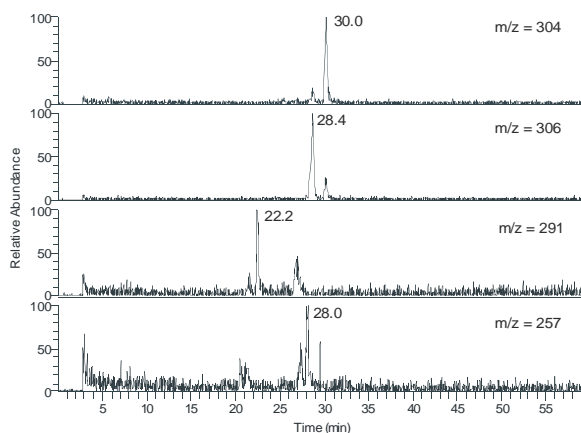


Fig. 7: Reconstructed chromatograms for the selected m/z values. The data were obtained by HPLC/MS analysis of norflurazon solution ($c = 0.1 \text{ mmol.L}^{-1}$) reduced for 60 min in acidic solution (0.01M HCl in water with 10 % (v/v) of methanol).

On the basis of the above-mentioned facts the scheme of electrochemical reduction of norflurazon in strong acidic media ($\text{pH} < \text{p}K_{\text{ox}} = 1.75$) can be proposed (Fig. 8).

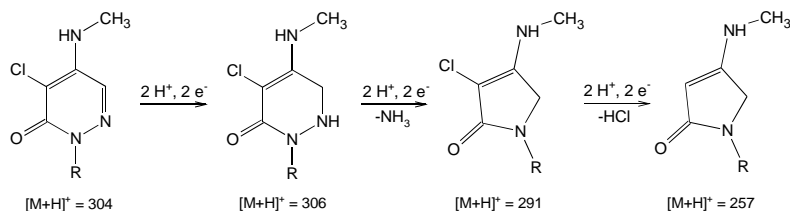


Fig. 8: Electrochemical reduction of norflurazon at the mercury electrodes in strongly acidic media.

According to the HPLC/MS analysis of the reduction products of norflurazon in strongly acidic media ($\text{pH} < \text{p}K_{\text{ox}}$) up to six electrons can be involved in the electrode process. On the other hand only four-electron process was observed using polarographic and voltammetric techniques. It can be supposed that long-term contact (60 min) of the species with the electrode surface under conditions of electrolysis on the mercury-pool cathode leads to the further transformation of the original molecule and its reduction products. Furthermore the third reduction step is apparently kinetically controlled, so that it need not develop in the time scale of voltammetric experiments.

Electro-reduction of norflurazon is partially attended by adsorption. It follows from the cyclic voltammetric data recorded at scan rates $10 - 200 \text{ mV.s}^{-1}$ in solution of 0.1 mmol.L^{-1} norflurazon in 10 mmol.L^{-1} HCl. The plot of $\log I = f(\log v)$ is linear ($y = 0.61x + 1.69$). The slope closed to the value of 0.5 (a theoretical value for purely diffusion-controlled process) means that the adsorption is not very significant (about 20 %).

The mechanism of electrochemical reduction of norflurazon is changed in slightly acid solutions ($\text{pH} > \text{p}K_{\text{ox}}$). Most of the species is not protonized (94.7 % at pH 3). A part of energy

of electrode reaction is consumed on protonation which is the primary process. The reduction proceeds at more negative potentials and only two electrons are exchanged. The current value at pH 6.2 is comparable to the limiting current of two-electron wave of benzil (Fig. 9). The reduction gradually ceases with increasing pH and the cathodic wave disappears (Fig. 3).

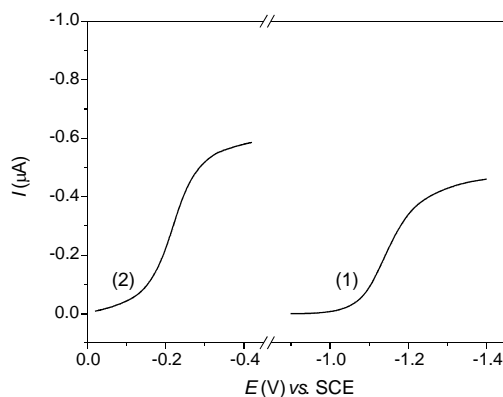


Fig. 9: DC curves of norflurazon (1) at pH 6.2 and benzil (2) at pH 1.2; concentration of depolarizers 0.1 mmol.L⁻¹.

Two-electron process has again been confirmed by controlled-potential coulometry in acetate buffer (pH 6.0) and HPLC/MS analysis of the reduction products. Peaks corresponding to quasi-molecular ions [M+H]⁺ at *m/z* = 304 (norflurazon) and 306 (a product of the two-electron reduction) were found in the mass spectrum after HPLC separation. No further products were found in the reduced solution. The mechanism of the reduction process is described on Fig. 10.

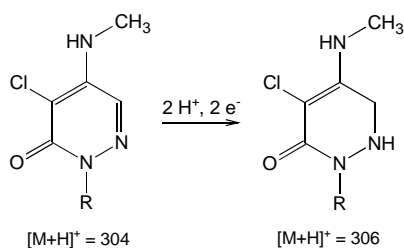


Fig. 10: Electrochemical reduction of norflurazon in slightly acidic media.

The mechanism of the irreversible electrode reduction of norflurazon at different acidity (Figures 8 and 10) is in agreement with the course of the dependence of $E_{1/2}$ on pH (Fig. 4). The dependence is defined by Eq. (1)

$$E_{1/2} = E^f - p \frac{0.0592}{\alpha z} \text{pH} \quad (1)$$

where p is the number of protons. The value of E^f (-0.775 V relative to the SCE) was found from the plot (Fig. 4) as the intercept (pH = 0). The product z was calculated from DC curves (from the difference $E_{1/4} - E_{3/4}$ and also from the ratio of the current at a given potential and the limiting current [14], Table 1). Values of p were calculated for particular linear parts of the dependence of $E_{1/2}$ on pH.

Table 1: Values of αz and p (number of protons) calculated from DC curves in acidic, neutral and slightly alkaline media.

pH	αz ^{a)}	αz ^{b)}	Mean value		p	Slope calculated (V.pH ⁻¹)	Slope found (V.pH ⁻¹)
			αz	α			
< 1.7	1.064	0.954	1.009	0.252	0.574	-0.034	-0.035
1.7 – 6.9	0.831	0.747	0.789	0.395	0.934	-0.068	-0.068
6.9 – 8.0	0.441	0.367	0.404	0.202	0.085	-0.013	-0.014

^{a)} Calculated according to: $E_{1/4} - E_{3/4} = \frac{0.0517}{\alpha z}$

^{b)} Calculated according to [14]: $E_{1/2} - E = \frac{0.0592}{2\alpha z} \log \frac{2x(3x-1)}{5(1-x)}$, where $x = \frac{I_E}{I_{im}}$

Slopes of the straight lines of the dependence $E_{1/2} = f(\text{pH})$ (Fig. 4) correspond to the slopes calculated for particular intercepts confirming the proposed mechanism of the electrode reaction of norflurazon at a mercury drop in acidic, neutral and slightly alkaline media.

A new four-electron DC-wave appears in neutral and alkaline media (pH > pK_r = 6.9). It consists of two waves with closed half potentials (Fig. 11a), characterizing two consecutive two-electron processes. The half potentials of both waves are practically independent on pH of the solution. Both electrode processes are markedly separated on the DPP voltammogram (Fig. 11b). Linear dependences of the overall limiting current on concentration of the depolarizer and on the square root of the mercury reservoir height suggest diffusion-controlled character of the electrode process. However more complex character of the reduction affected by kinetics and/or adsorption as in the case of chloridazon reduction [13] ought to be taken into consideration.

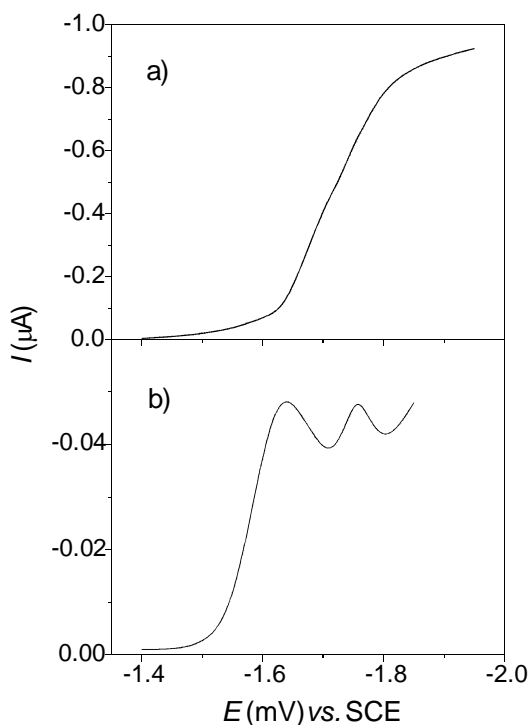


Fig. 11: *I-E* curves of norflurazon: a) DC-tast, $c = 0.1 \text{ mmol.L}^{-1}$, $\text{pH} = 9.45$; b) DPP, $c = 0.01 \text{ mmol.L}^{-1}$, $\text{pH} = 9.75$.

The HPLC/MS analysis after coulometric reduction was used to elucidate the mechanism of the electrochemical transformation of norflurazon in alkaline media. Three products with quasi-molecular ions at $m/z = 306$ (dihydronorflurazon), 270 and 272, which correspond to a dechlorinated product and its dihydro-form, were identified in the electrolysed solution. It can be assumed on the basis of all experiments that two-electron reduction proceeds in the first step followed by splitting-off the hydrogen-chloride and restoring of the reducible double-bond, which is reduced in the second step. The mechanism of electrochemical reduction of norflurazon in alkaline media described on Fig. 12 can be designate as ECE.

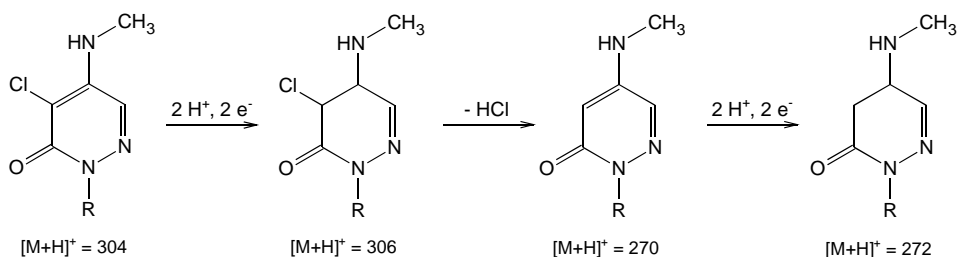


Fig. 12: Reduction of norflurazon in alkaline media.

Electrochemical behaviour of the structurally relative compounds, norflurazon and chloridazon, is considerable similar. However some differences are evident. It is connected to different distribution of electron density owing to trifluoromethyl group of norflurazon and positive induction effect of methyl group bound to the amino group in the position 5. Decreased acidity of the oxidized form and increased basicity of the reduced form of norflurazon occur in consequence of the structural differences. Changes of electron density lead to a shift of reduction potential of norflurazon to the more positive values in comparison with chloridazon.

3.1 Determination of norflurazon

The most suitable electrolytes for calibration and determination of norflurazon by FSDPV at a static electrode (SMDE) are solutions of dilute mineral acids (e.g. HCl). Acidic Britton-Robinson and McIlvaine buffers can also be used but they reduce the sensitivity of the method, owing to decreasing current intensity at increasing pH. Generally, adsorptive methods (e.g. AdSV) make it possible to reduce limits of detection. The adsorption of norflurazon evaluated using cyclic voltammetry at different scan rates (see above) is not significant (about 20 % in 10 mmol.L⁻¹ HCl) and it cannot lead to effective increase of sensitivity of AdSV in comparison with FSDPV. AdSV is suitable only for low concentration of norflurazon ($c = 5 \cdot 10^{-7}$ mol.L⁻¹ at $t_{acc} = 25$ s, Fig. 12). Characteristics of the calibration regression straight lines and limits of detection in HCl are summarized in Table 2.

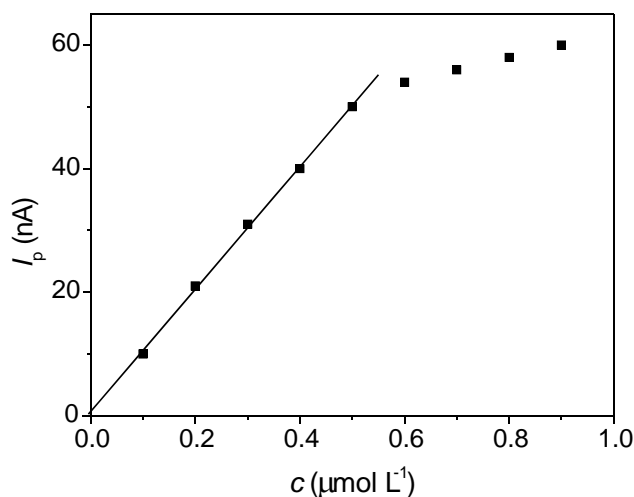


Fig. 13: Calibration dependence of norflurazon at AdSV in 0.01M HCl and $t_{acc} = 25$ s.

Table 2: Characteristics of the calibration regression straight lines ($y = ax+b$), and limits of detection ($LD = 3 s_b/a$) in 10 mmol.L⁻¹ HCl.

c (nmol.mL ⁻¹)	Method	n	$a \pm s_a$ (nA)	$b \pm s_b$ (nA.mL.nmol ⁻¹)	Corr. coeff.	LD (nmol.mL ⁻¹)
1.0 – 10.0	FSDPV	9	9.975±0.048	0.547±0.269	0.9999	0.081
0.2 – 2.0	FSDPV	9	11.917±0.151	0.550±0.170	0.9994	0.043
0.1 – 0.5	AdSV	5	99.012±1.852	0.701±0.583	0.9994	0.018

The determination of small amounts of norflurazon in model samples was realized in 10 mmol.L⁻¹ HCl as supporting electrolyte using the method of standard additions. Following results were obtained using FSDPV technique: mean = 310 ng.mL⁻¹ (number of measurements $n = 5$), relative standard deviation $s_r = 1.58$ %, relative error of the mean $\varepsilon = 1.47$ % and = 60.9 ng.mL⁻¹ ($n = 4$), $s_r = 3.75$ %, $\varepsilon = -0.21$ %. The AdSV technique allowed a determination of norflurazon with following results: = 29.4 ng.mL⁻¹ ($n = 4$), $s_r = 4.52$ %, $\varepsilon = -3.49$ %. Calculated t-tests of the Student distribution are less than critical values (for significance level of 0.05) confirming accuracy of used methods of determination.

4. Conclusion

The reduction pathway of norflurazon at the mercury electrodes depends on pH of the supporting electrolyte. The electrode reaction is a mainly diffusion controlled process combined with a kinetic reaction and also affected by slight adsorption in acidic media.

A multistep reduction of the protonated form of norflurazon accompanied by release of NH₃ and HCl was confirmed by HPLC–MS analysis of the product of coulometric reduction at the mercury pool cathode. In strongly acidic media the original cyclic skeleton is cleaved and a five-membered pyrrole ring is formed after release of ammonium. The product of electrolysis can be subsequently reduced. The voltammetric reduction in this media is a four-electron process. In slightly acid media ($\text{pH} > \text{p}K_{\text{ox}} = 1.75$) only a two-electron response was obtained corresponding to the reduction of the 1,6-azomethine bond of the pyridazine ring. The speed of protonation is the rate determining step so that no current response can be observed in neutral media. The four-electron reduction proceeds in alkaline media involving a kinetic reaction with elimination of HCl.

Diluted mineral acids (e.g. 0.01M HCl) are the most suitable electrolytes for the FSDPV determination of norflurazon. Low concentration of norflurazon under $5 \cdot 10^{-7}$ mol.L⁻¹ can be determined using the AdSV with the limit of detection 18 nmol.L⁻¹.

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FORMATION AND VARIOUS MUTUAL TRANSFORMATIONS OF MOXONIDINE

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Abstract

Drugs supplied on regulated markets must be analyzed by validated method mostly described in Pharmacopoea. For this purpose must be used impurities again described at Pharmacopoea. Synthesis of various moxonidine impurities, the conditions of their formation and mutual transformations are described.

Keywords: synthesis, impurities, moxonidine

1. Introduction

4-Chloro-N-(4,5-dihydro-1H-imidazol-2-yl)-6-methoxy-2-methylpyrimidin-5-amine (2), known as moxonidine (Fig. 1) is a presynaptic 2-adrenoreceptor agonist [1,2]. This compound affects on the cardio-circulatory system and is known as an antihypertensive agent [3]. The described preparative methods are based on a reaction of N-(1-acetyl-4,5-dihydro-1H-imidazol-2-yl)-4,6-dichloro-2-methylpyrimidin-5-amine (1) (scheme 1) with nucleophilic agents. The procedures mostly differ in bases used 4-6. The products formed are accompanied with various side-products that are known from Pharmacopoea as impurities A, B, C, D (Fig 1). Some of them are of simple structure but their synthesis has not been described. The impurities type and their ratio differ depending on the reaction conditions.

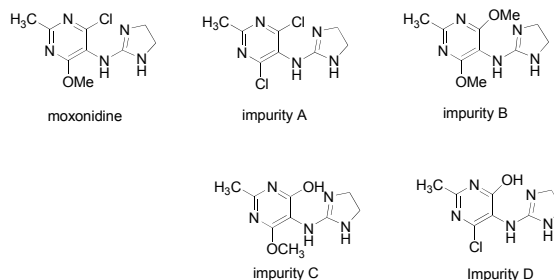
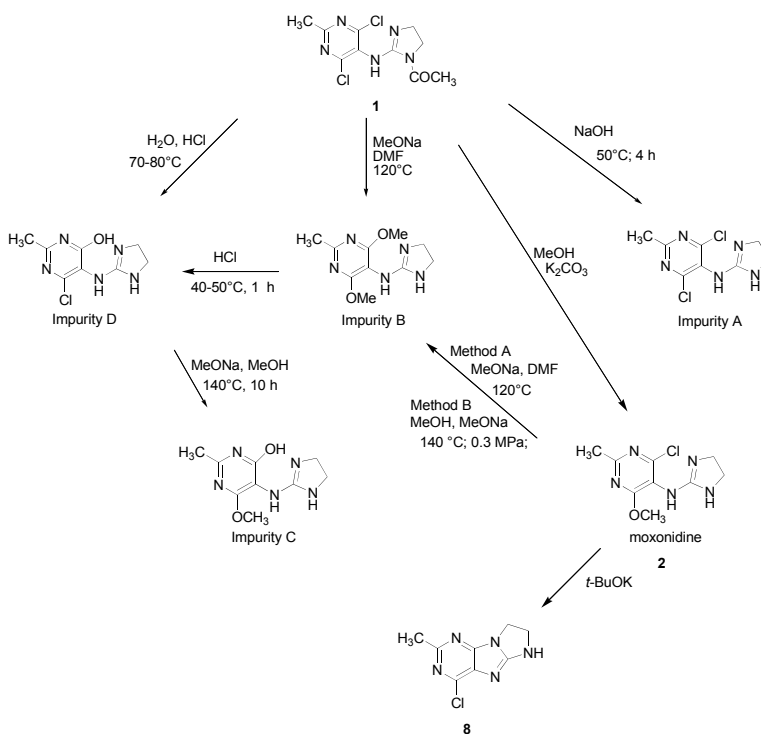


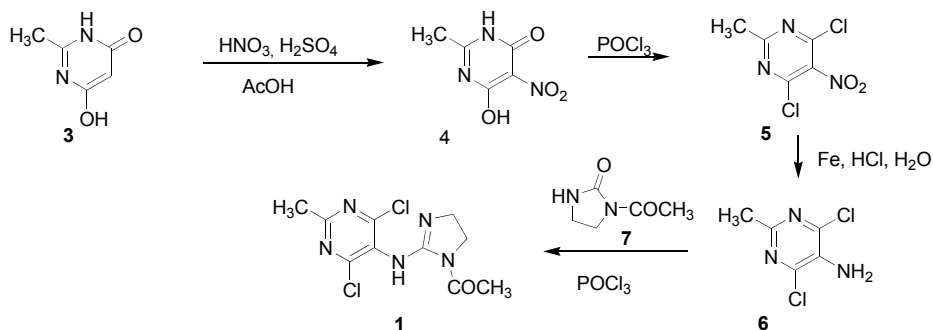
Fig. 1: Structures of moxonidine and related impurities

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Scheme 1

The key intermediate of moxonidine synthesis is aminopyrimidine **6**, which was prepared in several steps from dihydroxypyrimidine **3**. After the nitration, the hydroxy group was substituted by chlorine with use of POCl_3 and finally the nitro group was reduced. The aminopyrimidine **6** was coupled with acetylimidazolidinone **7** and the moxonidine precursor **1** was obtained (Scheme 2).



Scheme 2

In the final step, one of the chlorine atoms of the precursor **1** was substituted by the methoxy group. If the sodium methoxide was used in various solvents (like MeOH, DMF and others [5,6]), the reaction was fast but the product was considerably contaminated by the impurity B. At lower temperature only deacetylation took place and the impurity A was formed in a high yield, whereas the longer reaction time lead to the higher amount of dimethoxy derivative (impurity B). If the reaction was carried out in MeOH in presence of milder bases⁴ (like carbonates), the substitution was sufficiently fast, but only small amount of dimethoxy derivative (impurity B) was formed. Impurities C, D and also compound **8** were not formed using this procedure. However, all impurities described at Pharmacopoea were synthesized in the independent way (Scheme 3).

Impurity A was prepared by the reaction of acetyl derivative **1** with sodium hydroxide at 50 °C for 4 hours.

Impurity B was obtained by the reaction of starting compound **1** with sodium methoxide in DMF at 120°C for 4 hours. This compound was also synthesized by the heating of moxonidine with sodium methoxide under similar conditions.

Impurity C was prepared from impurity D by the heating of the mixture of sodium methoxide in methanol in autoclave at 140°C for 10 hours. As in the case of the impurity B, impurity C can be synthesized from moxonidine, but harsher conditions are required (autoclave).

The most difficult was the preparation of impurity D, which was finally prepared by heating of the starting material **1** in diluted hydrochloric acid at elevated temperature for 4 hours. This compound was also prepared by the cleavage of ether bond in impurity B. In the meantime we tried to prepare t-butyl ether from moxonidine, as we supposed to be simpler way for the removing of this group. Surprisingly, different reaction was observed and polycondensated product **8** was isolated (Scheme 1).

2. Experimental

Melting points were measured in the Kofler apparatus and are uncorrected. HPLC was performed on Agilent 1100 with UV light detection 225nm and mobile phase acetonitrile/water/phosphoric acid using gradient method.

MS characterisation was carried out using the DEP-CI-MS-MS (direct exposure probe-chemical ionisation-tandem mass spectrometry) technique with quadrupole ion trap mass analyzer and methane as a CI reagent gas.

NMR spectra were measured with a Bruker Avance 300 spectrometer operating at 300 MHz (¹H) and 75 MHz (¹³C). The compounds were dissolved in DMSO-d₆ and measured at 300 K.

2.1 Moxonidin (**2**)

Dichloropyrimidine **1** (16.0 g, 55.5 mmol) was suspended in methanol (128 ml), potassium carbonate (15.4 g, 111.4 mmol) was added and reaction mixture was refluxed for 20 minutes. Then the reaction mixture was cooled to ambient temperature and pH was adjusted by acetic acid (12.8 ml) to 7, water (110 ml) was added and the solution was filtrated with charcoal (3.0 g) and pH was adjusted to 8 – 9 by water solution of ammonia (10.4 ml). Precipitated solid material was cooled to 0 °C and filtered off, washed with water and dried in vacuum at 70 °C. The yield of substance **2** was 11.2 g, 85 % theory with purity above 99.5 % HPLC. This crude substance **2** was stirred in methanol at 40 °C for 1 hour. After cooling

the suspension was filtered off and washed with cooled methanol, yield 12.8 g, 95 % theory, mp 217 – 219 °C (lit⁵ mp 217-219 °C).

¹H NMR (DMSO-d₆, 300 MHz) δ 6.19 (2H, s, NH), 3.84 (3H, s, OCH₃), 3.31 (4H, s, CH₂), 2.4 (3H, s, CH₃).

¹³C NMR (DMSO-d₆, 75 MHz) δ 163.23, 158.00, 156.64, 149.99, 127.35, 53.83, 41.72, 24.47.

Full MS, *m/z* (relative intensity) 242,244 (100,33) [M(³⁵Cl)+H]⁺, [M(³⁷Cl)+H]⁺, 270,272 [M(³⁵Cl)+C₂H₅]⁺, [M(³⁷Cl)+C₂H₅]⁺, 282,284 [M(³⁵Cl)+C₃H₅]⁺, [M(³⁷Cl)+C₃H₅]⁺; MS²(243,w5,EV1.5), *m/z* 227, 212, 210, 206(100) [M+H-HCl]⁺, 201, 199, 191, 176, 165, 151, 149, 137.

2.2 Synthesis of 4,6-Dichloro-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-2-methylpyrimidin-5-amine (Impurity A)

Dichloropyrimidine **1** (20.0 g, 69.4 mmol) was suspended in solution of sodium hydroxide (13.9 g, 34.75 mmol) in water (1400 ml). Suspension was stirred at 50-55 °C for 4 hours, the solid was filtered off, washed with water and dried in vacuum at 80 °C. The yield of white crystals was 15.6 g, 91 % theory, mp 213 – 215 °C

¹H NMR (DMSO-d₆, 300 MHz) δ 6.53 (2H, s, NH), 3.36 (4H, s, CH₂), 2.48 (3H, s, CH₃).

¹³C NMR (DMSO-d₆, 75 MHz) δ 157.96, 157.42, 153.76, 138.38, 41.66, 23.91.

Full MS, *m/z* (relative intensity) 246,248,250 (100,65,12) 246 [M(³⁵Cl)+H]⁺, 274 [M(³⁵Cl)+C₂H₅]⁺, 286 [M(³⁵Cl)+C₃H₅]⁺; MS²(248,w7,EV1.5), *m/z* 212, 210 [M(³⁵Cl)+H-HCl]⁺, 174, 133.

2.3 Synthesis *N*-(4,5-Dihydro-1*H*-imidazol-2-yl)-4,6-dimethoxy-2-methylpyrimidin-5-amine (Impurity B)

Procedure A

Sodium methoxide (5.63 g, 104.1 mmol) was dissolved in DMF (30 ml), the solution was heated to 65-70 °C and dichloropyrimidine **1** (5.0 g, 17.4 mmol) was added. Then, the reaction mixture was heated to 115-120 °C and stirred for 11 hours. After cooling the water (50 ml) was added, the solid product was filtered off and washed with water. Crude Impurity B was crystallized from methanol (50 ml) and dried at vacuum dryer at 80 °C. The yield of Impurity B was 3.93 g, 99 % theory, mp 239-246 °C (lit⁵ mp 230 °C).

Procedure B

Moxonidine (**2**) (5.45 g, 22.6 mmol) was suspended in methanol (100 ml) and sodium methoxide (1.65 g, 71.8 mmol) was added. The suspension was heated in autoclave under pressure of nitrogen to 135-140 °C for 11 hour. Methanol was evaporated *in vacuo*, the solid residuum was suspended in water and acetic acid (7 ml) was added. The solution was filtered with charcoal, ammonium hydroxide (8 ml) was added and pH was adjusted to 9 - 10. The precipitated product was filtered off, washed with water and dried in vacuum at 80 °C. The yield of the impurity B was 3.95 g, 74 % theory, mp 239-245 °C.

Procedure C

Moxonidine (**2**) (2.0 g, 8.28 mmol) was dissolved in DMF (20 ml) and sodium methoxide (2.68 g, 49.7 mmol) was added. The reaction mixture was heated to 120 °C for 24 hours. Then water (130.0 ml) was added, precipitated product was filtered off, washed with water and dried at 80 °C in vacuum. The yield of the impurity B was 1.07 g, 54 % theory, mp 239- 245 °C.

¹H NMR (DMSO-d₆, 300 MHz) δ 5.85 (2H, s, NH), 3.78 (6H, s, OCH₃), 3.26 (4H, s, CH₂), 2.37 (3H, s, CH₃).

¹³C NMR (DMSO-d₆, 75 MHz) δ 161.96, 157.86, 109.23, 88.34, 53.17, 41.79, 25.01.

Full MS, *m/z* (relative intensity) 238 (100) [M+H]⁺, 266 [M+C₂H₅]⁺, 278 [M+C₃H₅]⁺; MS²(238,w3,EV1), *m/z* 223(100), 222, 208, 206 [M+H-CH₃OH]⁺, 204, 195, 194, 183, 176, 165, 151.

2.3 Synthesis 5-(4,5-Dihydro-1H-imidazol-2-ylamino)-6-methoxy-2-methylpyrimidine-4-ol (Impurity C)

Impurity D (10 g, 43.9 mmol) was dissolved in methanol (120 ml) and sodium methoxide (7.11 g, 131.7 mmol) were added. The reaction mixture was heated in the autoclave under nitrogen to 140 °C for 10 hour. The reaction mixture was acidified with hydrochloride acid (51 ml) to pH 6. Precipitated product was filtered off, washed with water and methanol. After drying at 80 °C in vacuum the yield was 7.53 g, 77 % of the theory, mp 173-176 °C.

¹H NMR (DMSO-d₆, 300 MHz) δ 12.87 (1H, s, OH), 9.45 (1H, s, OH), 8.22 (2H, s, NH), 3.87 (3H, s, OCH₃), 3.6 (4H, s, CH₂), 2.35 (3H, s, CH₃).

¹³C NMR (DMSO-d₆, 75 MHz) δ 164.47, 160.50, 159.06, 158.91, 99.55, 54.45, 42.48, 21.16.

Full MS, *m/z* (relative intensity) 224 (100) [M+H]⁺, 252 [M+C₂H₅]⁺, 264 [M+C₃H₅]⁺; MS²(224,w3,EV1), *m/z* 209, 207, 194, 192, 181, 166, 151(100), 126, 124.

2.4 Synthesis of 6-Chloro-5-(4,5-dihydro-1H-imidazol-2-ylamino)-2-methylpyrimidine-4-ol (Impurity D)

Procedure A

Starting dichloropyrimidine **1** (20.0 g, 69.4 mmol) was dissolved in a mixture of water (50 ml) and conc. hydrochloride acid (32 ml). The solution was kept at 70-80 °C for 6 hours. The mixture was filtered with charcoal (0.5 g), filtrate was cooled to ambient temperature and pH was adjusted by ammonium hydroxide to 6. The precipitated product was filtered off, washed with water and dried in vacuum. The crude product was crystallized from methanol (60 ml) and dried at vacuum again. Yield 13 g, 83 % theory, mp above 350 °C.

Procedure B

Impurity B (0.2 g, 0.84 mmol) was dissolved in conc. hydrochloride acid (3,0 ml) at ambient temperature. The solution was then stirred at 60-70 °C for 1 hour. The precipitated solid was filtered off and washed with ethylacetate. After drying in vacuum the yield was 0.1 g, 50 % of the theory, mp above 350 °C.

¹H NMR (DMSO-d₆, 300 MHz) δ 10.14 (1H, s, OH), 8.5 (2H, s, NH), 3.65 (4H, s, CH₂), 2.36 (3H, s, CH₃).

¹³C NMR (DMSO-d₆, 75 MHz) δ 159.19, 159.13, 158.33, 155.32, 117.42, 42.56, 20.9.
Full MS, *m/z* (relative intensity) 228,230 (100,33) [M(³⁵Cl)+H]⁺, [M(³⁷Cl)+H]⁺, 256,258 [M(³⁵Cl)+C₂H₅]⁺, [M(³⁷Cl)+C₂H₅]⁺, 268,270 [M(³⁵Cl)+C₃H₅]⁺, [M(³⁷Cl)+C₃H₅]⁺;
MS²(229,w5,EV1.5), *m/z* 192(100) [M+H-HCl]⁺, 174 [M+H-H₂O]⁺, 151, 123.

2.5 Synthesis of 4-Chloro-2-methyl-7,8-dihydro-6H-imidazo[1,2-*e*]purine (8)

Moxonidine (**2**) (10 g, 41.4 mmol) was dissolved in DMF (100 ml), potassium *t*-butanolate (13.9 g, 124.2 mmol) was added and the mixture was stirred at 60 °C for 18 hours. Then the reaction mixture was diluted with water (60 ml) and acidified with hydrochloride acid (10 ml) to pH 6. The water layer was extracted with ethylacetate (3 x 40 ml), organic layer was washed with water, dried with sodium sulfate and evaporated. The solid residuum was crystallized from ethanol. The yield of white crystalline product was 2.7 g, 37 % of the theory, mp 301-304 °C.

¹H NMR (DMSO-d₆, 300 MHz) δ 7.82 (1H, s, NH), 4.25-4.02 (4H, m, CH₂), 2.59 (3H, s, CH₃).

¹³C NMR (DMSO-d₆, 75 MHz) δ 162.99, 157.22, 152.19, 139.97, 134.95, 47.94, 40.85, 25.14.
Full MS, *m/z* (relative intensity) 210,212 (100,33) [M(³⁵Cl)+H]⁺, [M(³⁷Cl)+H]⁺, 238,240 [M(³⁵Cl)+C₂H₅]⁺, [M(³⁷Cl)+C₂H₅]⁺, 250,252 [M(³⁵Cl)+C₃H₅]⁺, [M(³⁷Cl)+C₃H₅]⁺ ;
MS²(211,w5,EV1.5), *m/z* 183, 174(100) [M+H-HCl]⁺, 147, 133, 106.

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