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Sensitive quantification of chosen drugs by reversed-phase chromatography with electrochemical detection at a glassy carbon electrode☆

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Abstract

Reversed-phase-high-performance liquid chromatographic method with electrochemical detection has proven to be a highly sensitive and selective method for determination of trace components in complex biological samples, and the electrochemical detector becomes an important alternative tool to ultraviolet and fluorescence detectors. A rapid and sensitive method for the accurate determination of metoclopramide, hydrochlorothiazide, imipramine and diclofenac in serum or plasma samples is described. The method is based on liquid–liquid extraction. The compounds were separated on C-18 column as stationary phase with a different binary mixture as mobile phase. Proposed method was validated with respect to specificity, linearity range, limit of detection and quantitation, precision, accuracy and successfully applied in a pharmacokinetic studies.

Keywords: RP-HPLC/ECD; Metoclopramide; Hydrochlorothiazide; Imipramine; Diclofenac; Pharmacokinetics

1. Introduction

Over the recent decades the problem of high sensitivity detection has become a main analytical problem. Analytical method's sensitivity is one of the most important factor is influencing quality of bioavailability as well as therapeutic drug monitoring studies. Among detectors used in HPLC technique electrochemical detector belongs to the most specific and sensitive method. Nevertheless, it is still rarely used. Therefore, development of analytical methods using this kind of detection seems to be very important.

The following categories display compounds that are electrochemically active, on the basis of either their chemical structure or from citations in the scientific literature [1]. Electrochemically active compounds are typically aromatic in nature and contain hydroxyl, methoxyl or amine groups. Metoclopramide as well as imipramine hydrochloride and diclofenac potassium have aromatic and amine groups, which have taken responsibility for electroactive property. In case of imipramine oxidation process did not concern these groups but the ring nitrogen [2]. Although, in hydrochlorothiazide exists chlorinated aromatic ring from the detection point of view important electrochemical reaction is oxidation to chlorothiazide [3]. Aliphatic compounds, such as thiols and amines, are also electrochemically active. Some compounds such as carbohydrates require a special form of detection called pulsed amperometric detection.

In this work the application of glassy carbon electrode for the amperometric detection of electroactive substances such as metoclopramide, hydrochlorothiazide, imipramine and diclofenac, in biological samples was studied in order to demonstrate the usefulness of a electrochemical detection in their assay. Recent advances in electrochemical detectors for HPLC make them a logical choice for determination of easily oxidizable or reductizable substances to achieve significantly lower limit of quantification adequately to drug level in biological material.

Glassy carbon is the electrode material used extensively in electrochemistry for the analysis of electroactive substances and as the basis for surface modified electrodes. The electrochemical properties of this material can be improved by surface treatment [4]. Electrochemical activation of the glassy carbon electrode through oxidation and reduction on the electrode surface is a widely used method to change its characteristics, which can improve the selectivity and sensitivity of the electroanalytical methods [5–8]. Short description of chemical and

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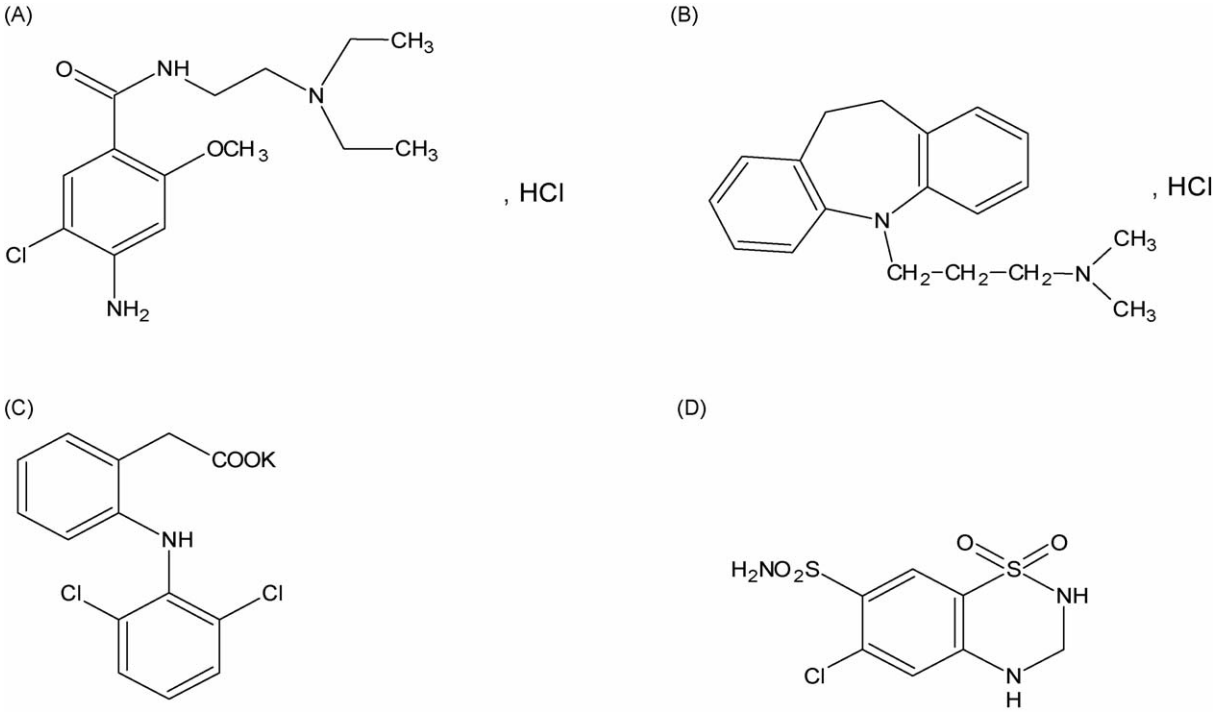

Fig. 1. Structures of analysed substances. A: Metoclopramide hydrochloride; B: imipramine hydrochloride; C: diclofenac potassium; D: hydrochlorothiazide.

pharmacological properties of investigated drug substances is given below; metoclopramide (4-amino-5-chloro-2-methoxy-*N*-2-diethylaminoethyl benzamide) (Fig. 1A) is a drug substance with two basic pharmacological effects; a dopamine receptor blocking effect and an effect on cholinergic mechanisms possibly increasing acetylcholine release. Metoclopramide is an 5-HT3 receptor antagonist. It is frequently used as an antiemetic and for the treatment of gut mobility disorders [9]. Imipramine hydrochloride (10,11-dihydro-*N*,*N*-dimethyl-5H-dibenz[b,f]azepine-5-propanamine) (Fig. 1B) remains one of the most widely used drugs of tricyclic antidepressants class, which has been the mainstay of treatment for major depression for over 30 years [10]. Diclofenac potassium (potassium *o*-(2,6-dichlorophenyl)aminophenylacetate) (Fig. 1C) is non-steroidal anti-inflammatory drug (NSAID) of the phenylacetic acid class. The drug is frequently used as first-line therapy in the major arthritic diseases osteo- and rheumatoid arthritis [11]. Hydrochlorothiazide (6-chloro-3,4-dihydro-2H-1,2,4-benzo-thiadiazine-7-sulfonamide 1,1-dioxide) (Fig. 1D) is used in the treatment of oedema associated with heart failure and with renal and hepatic disorders. It is also used in hypertension, either alone or together with other antihypertensive agents [12].

Different methods have been applied for the determination of metoclopramide, imipramine, diclofenac potassium and hydrochlorothiazide.

Routine analysis of the above mentioned drugs in biological samples can be accomplished by several analytical methods. Samples containing relatively high levels of metoclopramide [13–15], hydrochlorothiazide [16–33], imipramine [34–45] and diclofenac potassium [46–58] can be analysed using UV detection. Although, these methods did not present sufficient accuracy, especially at concentrations below 10 ng/ml. For this reason use of much more sensitive method is necessary. Trace amounts need instrumental techniques, such as derivative gas chromatography [59–68]. Separately described HPLC–MS method reported about significantly lower quantitation limit of hydrochlorothiazide [29]. However, most of these instrumental methodologies require an expensive instrumentation or an extensive sample pre-treatment. Bachman and Stewart [69] and Stewart and Clark [70] made an attempt to apply electrochemical detector to determine hydrochlorothiazide in tablets. Only one method with liquid–liquid extraction has been described for the determination of hydrochlorothiazide from human serum using electrochemical detection [71]. Moreover, a chemical oxidation step of imipramine [2,10,72] as well as diclofenac potassium [73,74] is needed in order to improve the analytical sensitivity, hence, electrochemical methods for the determination of these drugs were utilized. However, it was found during the development of the method that the injections were not reproducible. For this reason a study of a new analytical method from biological material using more sensitive detection was advisable. Kuhlmann et al. [75] reported a very sensitive method by HPLC with electrochemical detection for determination of the other diclofenac salt, diclofenac sodium in human aqueous humor. The low detection limit was achieved using fluorescence detection to determine, imipramine [76–78] and diclofenac potassium [47–54,79,80], however, it was possible after expensive and time-consuming derivatization procedure.

Therefore, the aim of the present work is the development of simple, accurate, reproducible and sensitive method for determination of metoclopramide, imipramine hydrochloride, hydrochlorothiazide and diclofenac potassium in human serum or plasma using rapid and convenient liquid–liquid extraction

and HPLC method based on electrochemical detection. Electrochemical detector is more sensitive than UV-visible detector and much cheaper than laser-inducement-fluorescence detector. In spite of the fact that mass spectrometry is very sensitive and modern for bioanalytical chemistry in particular, it is not freely available. For this reason amperometric detection may be used for analysis of drugs in biological samples. In addition, the present method was applied to the pharmacokinetic studies of healthy volunteers receiving an oral dose of these drugs.

We are also planning comparative studies determination of the electroactive compounds, which were oxidized on glassy carbon electrode, therefore, analytical data concerning imipramine and hydrochlorothiazide were given in an article published earlier for metoclopramide [81] and diclofenac [82] because of varied chemical structure.

2. Experimental

2.1. Reagents and chemicals

Metoclopramide hydrochloride, imipramine hydrochloride, hydrochlorothiazide and diclofenac potassium substance were kindly provided by Pharmaceutical Enterprise Polpharma S.A. (Starogard, Poland).

Cerucal 10 mg tablets were supplied by Asta Medica AWD (Frankfurt, Germany), Tofranil 25 mg dragee was delivered by Novartis Pharma (Norymbergia, Germany), Hydrosaluric 25 mg tablets was the product of Merck Sharp & Dohne Idea Inc. S.A. (United Kingdom) and Cataflam 50 mg was purchased by Novartis Pharma AG (Bazylea, Switzerland).

Methyl parahydroxybenzoate (internal standard) was the product of Fluka (Buchs, Switzerland), chlorpromazine hydrochloride was delivered by Jelfa (Jelenia Góra, Poland), sulphathiazole was supplied by Krka Polska (Warszawa, Poland) and naproxen was from Sigma (St. Louis, MO, USA). Acetonitrile, methanol, dichloromethane were supplied by Merck (Darmstadt, Germany). Phosphoric acid, hydrochloric acid, sulfuric acid, acetic acid, chromic anhydride, diethylether, ethyl acetate, sodium carbonate, sodium hydrocarbonate, sodium acetate, sodium chloride and sodium dihydrogenphosphate were purchased from POCh (Gliwice, Poland). The solvents were used as received (HPLC grade); water was purified by double distillation.

2.2. Instrumentation

The high-performance liquid chromatographic system used was purchased from Knauer (Berlin, Germany) and was equipped with a solvent pump (Mini-Star K-500), column thermostat jet stream 2 plus with injection valve (D-14163), amperometric detector (Merck, L-3500 A) and a computer system for data acquisition (Eurochrom 2000).

A system of three electrodes consisting of an Ag/AgCl as reference electrode, a platinum wire auxiliary and a glassy carbon electrode as working electrode was used. The active surface of the working electrode has to be kept clean, otherwise it loses its sensitivity. It is possible that products of the electrochemical reaction become attached to the electrode surface. These can be removed by periodical clean-ups. We cleaned the glassy carbon electrode with chromic acid (1 g CrO_3 in 10 ml sulfuric acid) to restore its sensitivity using a pipette. Then the working electrode was washed with deionized water and with methanol and dried at room temperature before use.

2.3. Chromatographic conditions

All experiments were performed using Nucleosil 100 C-18, 5 μm 125 mm × 4 mm I.D. column from Dr. Ing. Herbert Knauer GmbH (Berlin, Germany) for metoclopramide hydrochloride, imipramine hydrochloride and hydrochlorothiazide except diclofenac potassium, where LiChrospher column 100 C-18, 5 μm 125 mm × 4 mm from Merck (Darmstadt, Germany) was applied. All columns were placed in column thermostat jet stream with injection valve with a 20-μl loop, which was maintained at a temperature of 25 °C. The Nucleosil 100 C-18 column was chosen because after preliminary experiments symmetrical peaks were obtained for metoclopramide, imipramine and hydrochlorothiazide. Additionally, a LiChrospher 100 C-18 column has been tested, but obtained results were unsuccessful and confirmed that this column is not suitable for the separation of these analysed substances in contrast to diclofenac potassium. In case of diclofenac potassium the best separation was obtained using LiChrospher column.

Chromatographic conditions for independent assays of all analysed drugs were shown in Table 1. As mobile phase mixture of methanol–phosphate buffer or methanol–acetate buffer in suitable ratio was applied, the buffer served as the sup-

Table 1
Chromatographic parameters of analysed substances

Analysed substance	Internal standard	Mobile phase	Detection potential vs. Ag/AgCl (V)
Metoclopramide hydrochloride, t_R = 7.3 min[a]	Methyl parahydroxybenzoate, t_R = 10.3 min	Methanol–phosphate buffer, pH 3 (30:70, v/v), 1 ml/min[b]	1.10
Hydrochlorothiazide, t_R = 4.5 min[a]	Sulfathiazole, t_R = 6.5 min	15% methanol in 0.01 M CH_3COOH, 1 ml/min	1.30
Imipramine hydrochloride, t_R = 3.6 min[a]	Chlorpromazine hydrochloride, t_R = 5.9 min	Methanol– phosphate buffer, pH 3 (60:40, v/v), 1.2 ml/min[b]	1.10
Diclofenac potassium, t_R = 7.8 min[c]	Naproxen, t_R = 3.5 min	Methanol–0.008 M H_3PO_4 (68:32, v/v), 1 ml/min	1.15

[a] Column Nucleosil 100 C-18, 5 μm 125 mm × 4 mm.
[b] Phosphate buffer pH 3 (0.2 M NaH_2PO_4/0.1 M H_3PO_4; 85:15, v/v) containing 0.001 M NaCl.
[c] Column LiChrospher 100 C-18, 5 μm 125 mm × 4 mm.

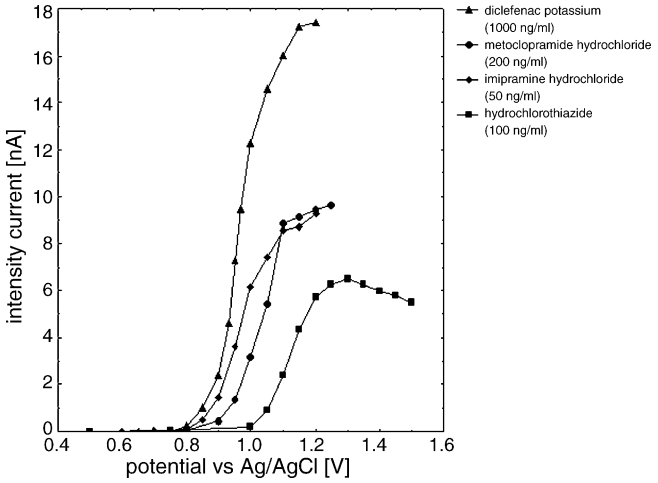

Fig. 2. Hydrodynamic voltammogram of analysed substances.

porting electrolyte. These phases were mixed up, and the air was removed from the phase by degassing for 1 h using ultrasonic apparatus. Measurements were carried out at a flow-rate 1 ml/min except imipramine hydrochloride (1.2 ml/min). Amperometric detector working conditions were as follow: working electrode glassy carbon, reference electrode silver/silver chloride refill and cell volume 1.5 μl. The working potential was practically determined on the basis of hydrodynamic voltammogram for each drug (Fig. 2; Table 1).

2.4. Extraction procedure

Extraction procedure of human plasma or serum samples for all analysed substances was based on one-step liquid–liquid technique (Table 2) with diethylether in presence of 1 M Na_2CO_3 for metoclopramide and imipramine, with ethyl acetate in presence of acetate buffer (pH 3.8) for hydrochlorothiazide, and dichloromethane in presence of 2 M HCl for diclofenac potassium. Diethylether, ethyl acetate and dichloromethane have been chosen for the extraction for drugs, because obtained recoveries of analysed substances were higher than those of after using other organic solvents. The improvement of recoveries of hydrochlorothiazide from human serum as well diclofenac from human plasma was achieved in acid pH. In case of metoclopramide and imipramine good recoveries were obtained with alkaline environment. The proposed sample preparation was selective, efficient, economical and time saving. It was simple and cheaply in comparison with the previous SPE techniques.

To 0.5 ml of human serum (1 ml in the case of metoclopramide) or plasma internal standard solution was added in suitable volume. Finally, 20 μl aliquot was injected into chromatographic column. Standard samples were prepared by spiking blank serum or plasma with known amounts of analysed drug and used for construction of calibration curves.

2.5. Accuracy

The accuracy of the method was determined by assessing the agreement between the measured and theoretical concentration of analysed samples spiked with certified reference substance. The accuracy of the method was measured by replicate analysis of spiked samples containing known amounts (target concentration) of metoclopramide, hydrochlorothiazide, imipramine and diclofenac. In practise the accuracy has been found with recovery studies of spiked samples. The absolute recoveries of certified reference substances were determined at three different concentration levels with nine replicate samples by comparing the height peak response of extracted analyte with that of the certified reference material solution at the same concentration levels and reconstructed into blank serum/plasma extracts. Recovery of certified reference material should be ±2% at concentrations of 80–120% of target concentration [83].

Recovery of internal standard from human serum or plasma was determined at the concentration used in the samples. Good recoveries were observed for all analysed drugs under investigation at all spiking levels and average recoveries were compiled with the requirement over 84% (Table 2).

2.6. Precision

The precision of a method is a statement of the closeness of agreement between mutually independent test results and is usually stated in terms of standard deviation [84]. Precision is usually measured as the standard deviation of a set of data [83]. It is generally dependent on analyte concentration.

The precision was determined by calculating the relative standard deviation (R.S.D.) for the repeated measurements. Inter-assay precision data called repeatability is a type of precision relating to measurements made under repeatable conditions: same method; same material; same operator; same laboratory; narrow time period. Repeatability was obtained by repeatedly analysing, on the same day and in the same laboratory, by the total analysis of six replicate samples, each of which was independently prepared according to the method procedure.

Table 2
Extraction procedures of analysed substances from human serum or plasma

Investigated substance	Biological matrix	Extraction procedure	Recovery (%), ($n=6$)
Metoclopramide hydrochloride[a]	Serum (1 ml)	Extraction with 4 ml diethylether in presence of 0.5 ml 1 M Na_2CO_3	89.2
Hydrochlorothiazide[a]	Serum (0.5 ml)	Extraction with 4 ml ethyl acetate in presence of 0.5 ml 0.2 M acetate buffer at pH 3.8	84.9
Imipramine hydrochloride[a]	Serum (0.5 ml)	Extraction with 4 ml diethylether in presence of 0.5 ml 1 M Na_2CO_3, back-extraction into 0.1 ml 0.1 M H_3PO_4	87.6
Diclofenac potassium	Plasma (0.5 ml)	Extraction with 4 ml dichloromethane in presence of 0.2 ml 2 M HCl	86.2

[a] Initial deproteinization with 4 ml acetonitrile.

Intra-assay or intra laboratory precision measures the effects of different analysts, or repeated sample preparation. It is the standard deviation (usually reported as a relative standard deviation, i.e. the standard deviation divided by the mean, also known as the coefficient of variation) of repeated analyses of aliquots of a single sample on 1 day in the laboratory [83]. Precision data named reproducibility is a concept of precision relating to measurements made under reproducible conditions, i.e. same method; different operator; different laboratories, different equipment; long time period. In our case reproducibility was obtained by repeating the intra-assay experiment on a different day with newly prepared samples.

2.7. Data analysis

All studies were conducted following good analytical practice guidelines. Data concentrations of drugs were received in bioavailability investigations. All concentrations measured chromatographically were established by plotting peak-height ratio (drug/I.S.) versus drug concentration (ng/ml).

These data were sufficient to estimate the pharmacokinetic parameters (AUC, C_{max}, T_{max}) by non-compartmental methods using the pharmacokinetic program WinNonlin, version 4.0.1, provided by Pharsight (USA).

2.8. Pharmacokinetic study

A single 10-mg dose of metoclopramide hydrochloride, 25-mg of hydrochlorothiazide, 25-mg of imipramine hydrochloride and 50-mg of diclofenac potassium were administered orally to healthy volunteers, separately.

The pharmacokinetic of drugs was studied in 18 healthy volunteers (nine males and nine females) for metoclopramide, hydrochlorothiazide and diclofenac potassium, who were taking no concurrent medications or alcohol. The pharmacokinetic studies were carried out in separate investigations at different times. The age of subjects ranged from 23 to 43, from 22 to 28 and from 24 to 42 for metoclopramide, hydrochlorothiazide and diclofenac potassium, respectively. Their body weight was 51–96, 48–97, 50–90 kg and height 163–195, 157–184 and 153–189 cm, respectively. Twenty healthy subjects (10 males and 10 females) participated in the imipramine study, aged 18–48 years. Their body weight was 52–100 kg, and height 153–189 cm.

All of them were informed before study entry and gave prior informed written consent. On the basis of medical history, clinical examination and laboratory investigation, no subject had history or evidence of renal, hepatic or gastrointensinal disease or drug allergy. The study protocol was approved by the Ethical Committee of the Medical University of Gdańsk. After fasting overnight, each volunteer was administered a single oral dose of suitable drug. The participations did not take any other medications for at least two weeks prior and during the day of the study. The volunteers fasted overnight for at least 10 h before the administration of drug and they continued fasting until 3 h post dose when a standard lunch was served. Blood samples (3 ml) were taken into glass test tubes prior to clot and after drug administration at 0, 0.5, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 6.0, 8.0 and 24.0 h for hydrochlorothiazide and at 0, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 8.0, 10.0, 12.0 and 25.0 h for imipramine. Data concerning frequency of administration for metoclopramide (in serum) and diclofenac (in plasma) were published earlier [81,82], respectively.

The serum and plasma (diclofenac) were immediately separated by centrifugation and stored at −20 °C until analysis.

The highest serum or plasma concentration measured for each volunteer was the C a range of C_{max}, and the time at which elimination rate constant (K_e) was estimated using linear regression of ln(concentration)–time curve. The $T_{1/2}$ was calculated by $0.693/K_e$.

3. Results and discussion

Among detectors used in HPLC technique electrochemical detector belongs to most specific and sensitive. Nevertheless, is still rarely used. Therefore, development of analytical methods using this kind of detection seems to be very important, because it leads to lower quantitation limit in compare to alternative chromatographic methods. Many substances do not have chromophores or adsorbed ultraviolet radiation too weakly to achieve limit of quantification adequately to drug level in biological material. For these substances we should choose electrochemical detection for analysis. Proposed method for determination of drug in literature was mainly based on spectrophotometric and fluorimetric detection, which is not sensitive enough to determine all profile of concentration in blood after single oral dose. Preliminary chromatograms received after serum spiked with metoclopramide using amperometric detector (Fig. 3A) and UV detector (Fig. 3B) confirmed significantly difference of sensitivity in both the cases. After comparing both the chromatograms we can see that peak height of metoclopramide received after applied amperometric detection (100 ng/ml) is significantly higher detection than received using spectrophotometric detection (200 ng/ml). On the other hand more convincing example is mean serum concentration–time curve after single oral dose administration of hydrochlorothiazide in the form of Hydrosaluric 25 mg tablet (Fig. 4) and imipramine hydrochloride in the form of Tofranil 25 mg (Fig. 5) for three volunteers, who took part in bioavailability investigations. It is easy to notice maximum of concentration each drug is significantly different in human volunteers, in spite of receiving the same dose. The difference results from large inter-subject variability. The quantitation limit of hydrochlorothiazide using UV-detector in our study was 100 ng/ml, as we can see in Fig. 4, volunteers A and B have a maximum of concentration, which allows to determine, however we cannot determine level of drug in sample for volunteer C because of its too lower value (below quantitation limit). Different situation in comparison with Fig. 4 for hydrochlorothiazide we can see in Fig. 5 for imipramine, where the quantitation limit was 50 ng/ml, only concentrations for subject A may be determined, while volunteer B as well as C achieved too low concentrations to evaluate using UV detector. Thus, in order to achieve these targets, highly sensitive and specific methods of drug analysis are always required. Electrochemical detection is

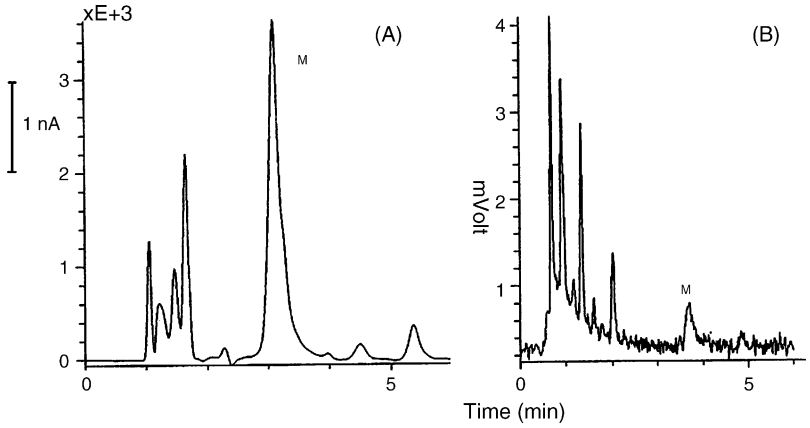

Fig. 3. Chromatogram of human serum extract spiked with metoclopramide hydrochloride (M). A: 100 ng/ml—amperometric detector, potential vs. Ag/AgCl +1.1 V, B: 200 ng/ml—UV detector, λ = 275 nm. Column Nucleosil 100 C-18, 5 μm, 125 mm × 4 mm; mobile phase: acetonitrile–water 40:60 (v/v) adjusted to pH 3 with 100% CH_3COOH; flow-rate 1.2 ml/min; extraction with diethylether in presence of 1 M Na_2CO_3. Scale of the ordinate: 1000 mV = 1 nA (A); 1 mV = 1 mAU (B).

found to fulfill these conditions. The aim of this report is to give justification of the application of amperometric detection for the drugs detectable (and their concentration measurable) in order to reduce quantitation limit.

3.1. HPLC conditions

Optimum conditions, which are necessary for the quantitative analysis of metoclopramide, imipramine hydrochloride, diclofenac potassium and hydrochlorothiazide with maximum sensitivity, were established by varying one parameter at a time by fixing other parameters constant and observing its effect on capacity factor (k') and peak width. Effect of mobile phase composition for metoclopramide and imipramine was studied by varying the ratio of methanol and phosphate buffer pH 3 containing 0.001 M NaCl. The increase of methanol content resulted in the decrease of (k') and peak width. The best separation of metoclopramide and imipramine from serum peaks was obtained when the mobile phase was composed of methanol–phosphate buffer pH 3 in the ratio of 30:70 and 60:40 (v/v), respectively. The results obtained made it possible to choose optimum conditions for the HPLC assay of metoclopramide and imipramine showing peak with retention time 7.3 and 3.6 min, respectively. The typical chromatograms of metoclopramide and imipramine extracted from human serum are presented in Figs. 6 and 7, respectively. In the case of hydrochlorothiazide the best suitable mixture as mobile phase was 15% methanol in 0.01 M acetic acid. A binary mixture of methanol–0.008 M phosphoric acid (68:32, v/v) for diclofenac was applied. Retention times for hydrochlorothiazide and diclofenac were 4.5 and 7.8 min, respectively (Figs. 8 and 9). All analysed substances were found to be electrochemically active, reaching the maximum signal at an oxidation potential versus Ag/AgCl is shown in Table 1. The hydrodynamic voltammograms permitted to select the best potential for determination of each drug (Fig. 2).

3.2. Extraction procedure

Liquid–liquid extraction was used because of the short duration of the clean-up procedure. The proposed sample preparation

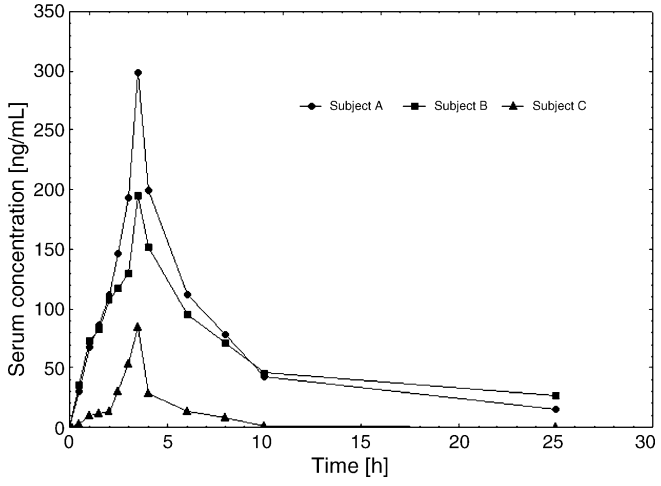

Fig. 4. Serum concentration of hydrochlorothiazide following single oral dose administration of 25 mg tablet Hydrosaluric to three healthy volunteers (A–C).

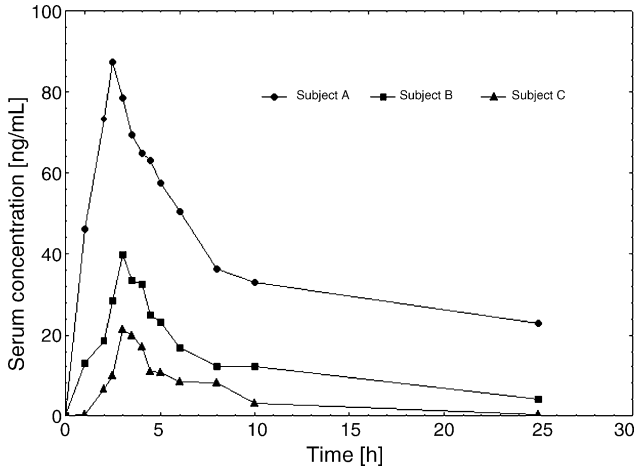

Fig. 5. Serum concentration of imipramine hydrochloride following single oral dose of 25 mg tablet Tofranil to three healthy volunteers (A–C).

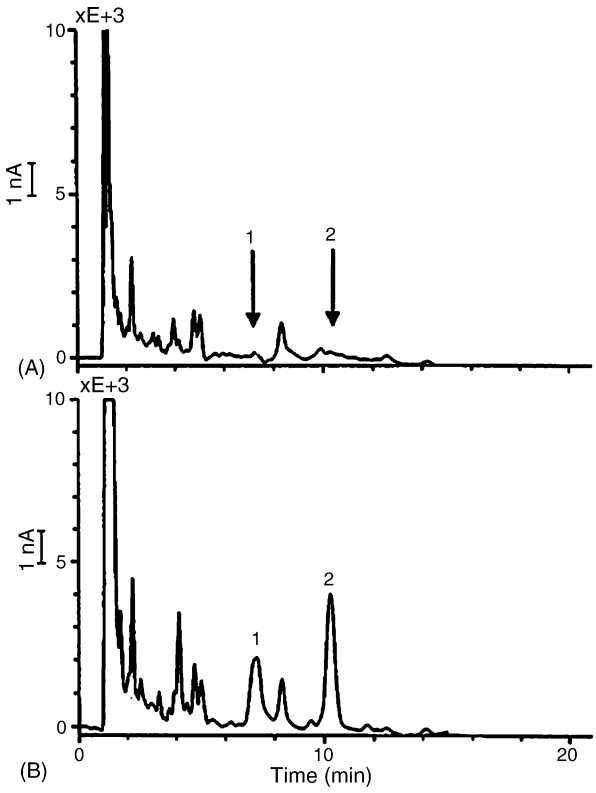

Fig. 6. (A) Chromatogram of blank human serum extract. (B) Chromatogram of human serum extract spiked with metoclopramide hydrochloride concentration 25 ng/ml (1) and 10 ng/ml of methyl parahydroxybenzoate (internal standard) (2). Scale of the ordinate: 1000 mV = 1 nA.

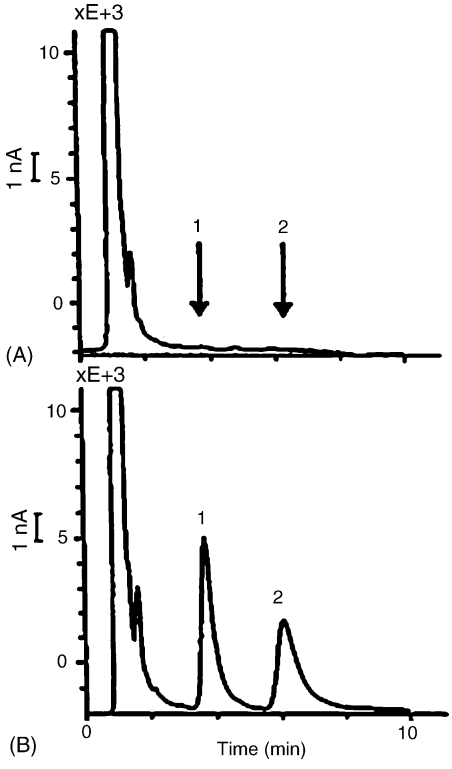

Fig. 7. (A) Chromatogram of blank human serum extract. (B) Chromatogram of human serum extract spiked with imipramine hydrochloride concentration 40 ng/ml (1) and 150 ng/ml of chlorpromazine hydrochloride (internal standard) (2). Scale of the ordinate: 1000 mV = 1 nA.

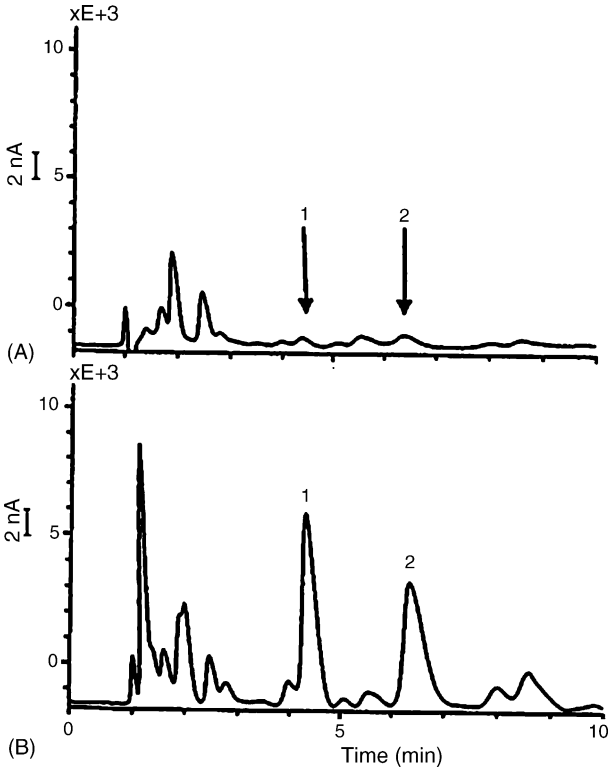

Fig. 8. (A) Chromatogram of blank human serum extract. (B) Chromatogram of human serum extract spiked with hydrochlorothiazide concentration 300 ng/ml (1) and 500 ng/ml of sulfathiazole (internal standard) (2). Scale of the ordinate: 1000 mV = 2 nA.

procedure for HPLC method was optimized to offer the most reliable, effective and rapid approach for routine analysis as well as to permit elimination of time-consuming purification steps, including expensive solid-phase extraction (SPE). Preparation of biological samples for all drugs was simple and cheap in comparison with SPE method, based only on single liquid–liquid extraction.

After serum (for metoclopramide, imipramine and hydrochlorothiazide) and plasma (for diclofenac) extraction, no co-eluting peak was detectable in control samples at the retention time of analysed compounds. Each substance was also completely resolved from the I.S. The run time of one sample has not exceeded 10 min besides metoclopramide (15 min). Typical chromatograms for metoclopramide, imipramine, hydrochlorothiazide and diclofenac are shown in Figs. 6–9, respectively.

3.3. Method validation

The proposed HPLC-ECD assay for the determination of metoclopramide hydrochloride, imipramine hydrochloride, diclofenac potassium and hydrochlorothiazide was validated in accordance with the guidelines for validation of pharmaceutical methods, which have been published by a number of regulations [83–87]. Typical acceptance criteria for validation of an analytical method such as linearity, limit of detection/quantitation, selectivity and specificity, precision and accuracy has given [83], were determined.

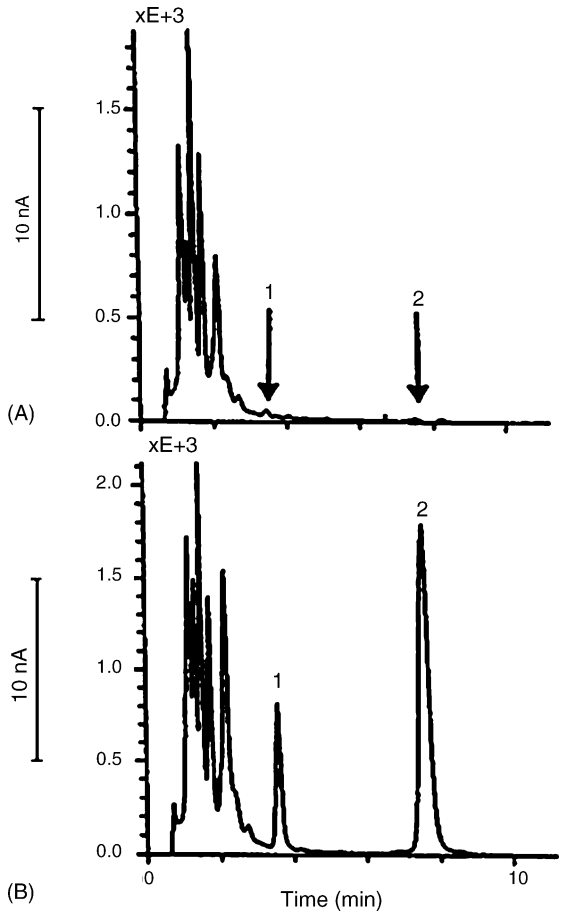

Fig. 9. (A) Chromatogram of blank human plasma extract. (B) Chromatogram of human plasma extract spiked with diclofenac potassium concentration 1500 ng/ml (2) and 3 μg/ml of naproxen (internal standard) (1). Scale of the ordinate: 1000 mV = 10 nA.

3.3.1. Limits of detection and quantitation

The limit of detection of an analyte is often determined by repeated analysis of a blank test portion and is the analyte concentration the response of which is equivalent to the mean blank response plus three standard deviations. Its value is likely to be different for different types of samples [84].

The limit of quantitation is the lowest concentration of analyte that can be determined with an acceptable level of uncertainty. It should be established using an appropriate measurement standard or sample, i.e., it is usually the lowest point on the calibration curve (excluding the blank). The limit should be 10 standard deviations of the blank measurement [84]. The LOD for metoclopramide, imipramine, diclofenac potassium and hydrochlorothiazide assay was found to be 0.8, 1.0, 2.0 and 5.0 ng/ml, respectively (Table 3). The LOQ was calculated to be 2 ng/ml for metoclopramide and imipramine, 5 ng/ml for diclofenac potassium and 10 ng/ml for hydrochlorothiazide. The limit of quantitation of each drug, defined as the lowest sample concentration level can be seen in Table 3.

3.3.2. Recovery from human serum or plasma

Some commercial dosage forms of the studied drugs were successfully analysed by the proposed method. Recovery experiments were performed for each drug in its dosage forms. The results are listed in Table 2. Recovery was determined by comparing values of peak height obtained with that achieved from standard solutions of equivalent concentrations of each drug and internal standard. Recovery studies were performed at eight different concentrations of each drug.

3.3.3. Linearity

Linearity for quantitative methods is determined by the measurement of samples with analyte concentrations spanning the claimed range of the method. The results are used to calculate a regression line against analyte calculation using the least squares method [84]. The linearity of the method was confirmed with precision and inaccuracy below 11% for all analysed drugs excluding diclofenac potassium (below 15%). Range of linearity for these compounds is shown in Table 3. A straight line passing through the origin was obtained. The regression line was calculated using the least square method. The correlation coefficient of the standard curves (peak height–concentration) was greater than 0.999 (Table 3) for all analysed drugs which is a common requirement of validation method [83].

3.3.4. Precision and accuracy

Numerical data for precision and within-day determinations are collected in Table 3. The precision of the assay, calculated as a relative standard deviation for inter-assay variability,

Table 3
Validation of analytical methods

Substance	Range of linearity (ng/ml)	Relative standard deviation (%)	Detection limit (ng/ml)	Quantitation limit (ng/ml)	Regression equation, $H/H_{IS} = b(\pm s)c + a(\pm s)$[a]
Metoclopramide hydrochloride	5–120	10.0–0.3	0.8	2.0	0.006 (±0.00004)c + 0.002 (±0.003), r = 0.9998, n = 8, $s_{y/x}$ = 0.005
Hydrochlorothiazide	10–400	10.6–0.9	5.0	10.0	0.0055 (±0.00005)c + 0.215 (±0.01), r = 0.9998, n = 7, $s_{y/x}$ = 0.02
Imipramine hydrochloride	2–100	11.0–0.6	1.0	2.0	0.05 (±0.0005)c + 0.13 (±0.025), r = 0.9998, n = 7, $s_{y/x}$ = 0.04
Diclofenacpotassium	5–2000	14.0–0.7	2.0	5.0	0.0015 (±0.000009)c + 0.03 (±0.008), r = 0.9998, n = 11, $s_{y/x}$ = 0.02

[a] Where a—intercept; b—slope; s—standard deviations of the slope and intercept; $s_{y/x}$—standard error of the regression; n—number of data points; c—concentration of drug (ng/ml); H/H_{IS}—peak-height ratio (active substance/IS) vs. active substance concentration (ng/ml); r—correlation coefficient.

ranged from 0.8% for 100 ng/ml to 6.0% for 10 ng/ml for metoclopramide, from 1.6% for 50 ng/ml to 6.3% for 10 ng/ml for imipramine, from 1.18% for 200 ng/ml to 10.98% for 50 ng/ml for hydrochlorothiazide and from 0.59% for 1500 ng/ml to 15.78% for 50 ng/ml for diclofenac. On the whole, the precision on the serum or plasma method was acceptable for both intra- and inter-assay.

3.3.5. Specificity

Specifity of the method is that the clean-up procedure allows the final sample to be analysed free of interferences [83]. Selectivity of a method refers to the extent to which it can determine particular analyte(s) in complex mixture without interference from the other components in the mixture. A method which is selective for an analyte or group of analytes is said to be specific [84]. The assay of the specificity of the method has been performed by comparing chromatograms obtained from blank serum or plasma samples analysed using the reported procedure with the chromatograms of serum or plasma samples spiked with certified reference substance such as metoclopramide, imipramine, hydrochlorothiazide, diclofenac and internal standard (Table 1). As can we see in Figs. 6–9 for drugs mentioned above, the results of this assay confirmed that there were no endogenous peaks interfering with peaks of analysed substances and internal standard.

3.4. Application

The concentrations used were based on the range expected during pharmacokinetic investigations.

The validated methods have been successfully applied in bioavailability investigations of metoclopramide [81], imipramine, diclofenac potassium [82] and hydrochlorothiazide in human after oral administration of their standard dose.

Nowadays, electrochemical detection of electroactive compounds at a very low concentration is a recommended method for their determination owing to the excellent sensitivity and relatively fast response.

4. Conclusion

The described method using the amperometric detection with glassy carbon electrode as working electrode proved to be sensitive, accurate and precise than the previously reported methods for the analysis of metoclopramide hydrochloride, imipramine hydrochloride, diclofenac potassium and hydrochlorothiazide. The proposed method may be applied as a routine method in the bioavailability or bioequivalence studies and drug monitoring of these drugs after ingestion of single oral dose. Furthermore, the assay is fast and requires a relatively simple sample preparation. An application of HPLC method with electrochemical detection permits reduction of detection limit and also the blood sample might be lower, 0.5 ml (except metoclopramide), in comparison with the methods previously published. Moreover, determination was possible in serum instead of plasma, which simplified the clinical procedure with the advantage of a minimal use of reagents.

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