Acta Chromatographica 25(2013)1, 97-110
DOI: 10.1556/AChrom.25.2013.1.5

Application of Micelle-Mediated Extraction in Preparation of Body Fluids for HPLC-DAD Screening of Acidic and Neutral Drugs

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Summary. Experimental conditions of cloud-point extraction (CPE) for the selected five acidic and neutral medicaments (salicylic acid, opipramol, carbamazepine, lorazepam, and alprazolam) in human plasma were studied and optimized. Separation and detection of the tested drugs were performed by the high-performance liquid chromatography with diode array detection (HPLC-DAD) method in an appropriate gradient mode using a column Nucleosil C8. Under the optimized conditions, main validation parameters were determined for all the compounds. The extraction yields (%) ranged from 54.12 to 82.17 with intra- and interday repeatability (RSD, %) 5.70–9.92 and 5.79–10.19, respectively. The detection limit was 0.5 μg mL^{-1} for all the tested drugs with exception of salicylic acid (LOD = 2.5 μg mL^{-1}). The linearity of the proposed method was examined for the four drugs: opipramol, carbamazepine, lorazepam, and alprazolam in the concentration range of 0.5–2.0 μg mL^{-1} (correlation coefficient r^2 = 0.995–0.999) and for salicylic acid in the concentration range of 2.5–10.0 μg mL^{-1} (correlation coefficient r^2 = 0.993). The analytical parameters for the medicaments tested in whole blood were unsatisfactory, especially in terms of extraction recovery and repeatability, and application of the developed procedure for this biological matrix requires further study.

Key Words: micelle-mediated extraction, cloud-point extraction, screening drug analysis, acidic/neutral medicaments, body fluids

Introduction

Analysis of body fluids (urine, plasma, or blood) for determination of medicaments is commonly performed in clinical and forensic laboratories. In unknown cases, when there is no information about an overdosed drug, appropriate screening drug analysis is required [1]. For this purpose, usually, immunoassay (for group identification) or chromatographic methods are preferred. High-performance liquid chromatography with diode array detection (HPLC-DAD) and gas chromatography with mass detection (GC-MS) belong to the most commonly employed chromatographic methods in these cases [1]. In application of immunoassay methods, only the dilution of

urine or plasma is often required, however, preparation of body fluids for HPLC analysis is usually more complicated. In this area of analysis, two sample preparation methodologies are routinely exploited: traditional liquid–liquid extraction (LLE) and solid phase extraction (SPE) [1]. Despite some obvious advantages of both these extraction techniques, they also possess some shortcomings, such as time consuming, use of big amounts of toxic solvents or requirement of relatively expensive extraction columns. Therefore, a quick, cheap, and effective micelle-mediated extraction, especially cloud-point extraction (CPE) technique seems to be a good alternative.

In the CPE technique, a micellar (micelle-rich) phase is formed from a homogenous surfactant solution that is added to the sample. Surfactant aggregates (micelles) orientate their hydrocarbon tails towards the center to create a non-polar core. Isolated hydrophobic compounds (most medicaments) present in the aqueous solution are favorable partitioned in the hydrophobic core of micelles. Depending on the nature of the surfactant (ionic, non-ionic or zwitterionic) separation of two phases (i.e. the micelle-rich phase and the aqueous phase concentrating the surfactant close to the critical micelle concentration – cmc) requires appropriate experimental conditions. In the case of non-ionic and zwitterionic surfactant solutions (CPE technique), temperature change results in two-phase separation while other parameters (e.g. pH, addition of ionic salt or organic solvent) are involved in two-phase separation process of ionic surfactants [2].

Generally, the cloud-point extraction technique is not extensively employed in drug analysis, however, a number of papers reporting its applications for determination, mainly, of a single drug in body fluids, such as human urine [3–7], human serum [8, 9], human plasma [10] and rat plasma [11, 12], were presented.

The objective of this work was to present the possibility of application of CPE for HPLC-DAD screening analysis of human body fluids (plasma and blood) examined for acid and neutral medicaments. For this purpose, study of optimal CPE conditions for the selected medicaments in human plasma was carried out, and then, under the assumed conditions, the developed CPE-HPLC-DAD method was validated for both kinds of fluids containing the examined drugs. The selected compounds belonging to the commonly used acidic and neutral drugs, covering a relatively wide range of hydrophobicity (log P ranged from 2.12 to 3.4), were used as model drugs.

Experimental

Apparatus and Chromatographic Conditions

The chromatographic system, Merck-Hitachi LaChrom, consisting of an L-7100 pump and an L-7455 programmable diode array detector DAD (Darmastadt, Germany), was used.

The examinations of separation conditions for the tested drugs were performed on column Nucleosil C8 (125 mm × 4.6mm i.d., 5 μm), supplied by Merck (Germany), which was thermostatted to 25 °C. Ultrasonic bath Vibra Cell and lyophilizator LABCONCO FreeZone 11 were purchased from Sonics&Materials INC. (USA) and Labconco Corporation (USA), respectively. The samples were centrifuged using ultracentrifuge MPW-6 (Mechanika Precyzyjna, Poland).

Chromatographic analyses were carried out using gradient conditions with the mobile phase consisting of phase A: 0.002M aqueous orthophosphoric acid and phase B: acetonitrile. The gradient profile was as follows: 0min – phase A (100%), 0–30min – phase A (30%) and phase B (70%), 30–33min – phase A (100%), and 33–43min – phase A (100%). The flow rate of the mobile phase was 1 mL/min. The drugs were detected by UV-light absorption at 254 nm.

Reagents

Acetonitrile and methanol, both of HPLC-gradient grade, were supplied by Merck (Germany). Non-ionic surfactant Triton X-114 and anionic surfactant sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (Germany). The cationic surfactant dodecyltriethylammonium bromide ($C_{12}NE$) was synthesized at the Faculty of Chemistry in the Jagiellonian University in Krakow. The reagents: 85% orthophosphoric acid, 30% sodium hydroxide, 25% ammonia and isoamyl alcohol, all of analytical grade, were purchased from POCH (Poland). Doubly deionized water (< 1.0 μS/cm) was used throughout.

Examined Drugs and Materials

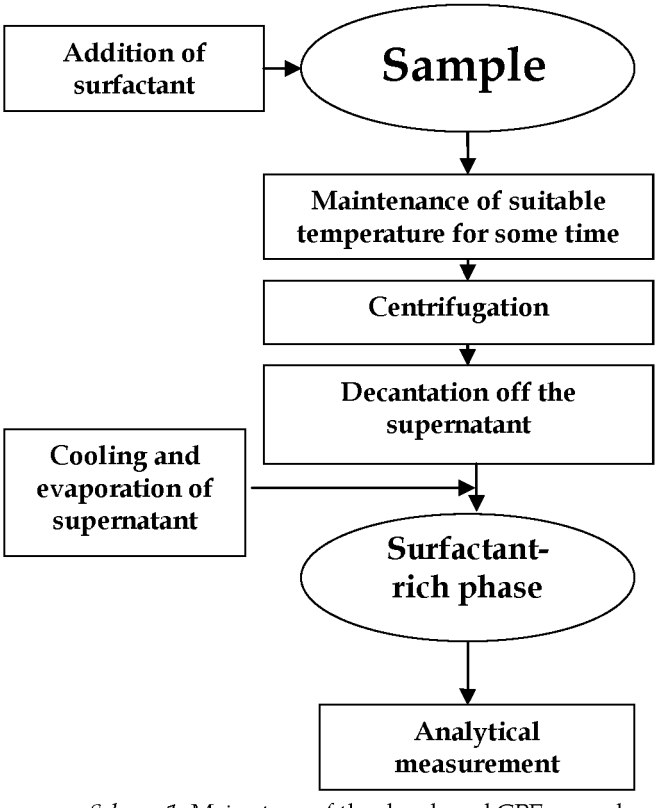
Standard substances of alprazolam, carbamazepine, lorazepam and opipramol were purchased from Sigma-Aldrich (Germany), and standard substance of salicylic acid was obtained from the pharmaceutical factory Polpharma SA (Poland). A stock solution of each drug (10 mg/mL) was prepared in methanol and stored in a refrigerator (4 °C).

Control body fluids, human plasma and whole blood, were obtained from the local blood bank (Krakow, Poland).

Working drug solutions (concentration of each drug usually was 1.0 or 2.0 μg mL^{-1}) were prepared by appropriate dilution of the stock drug solutions with the mobile phase. In order to obtain the samples, the control body fluids were spiked with water diluted standard drugs.

General Extraction Procedure by CPE Technique

The steps of CPE procedure (modified during the study) were shown in *Scheme 1*.



Scheme 1. Main steps of the developed CPE procedure

Results and Discussion

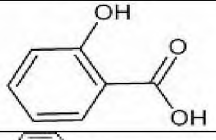
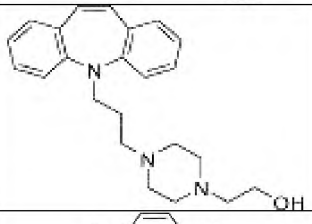
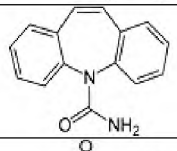
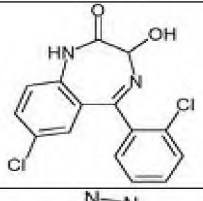
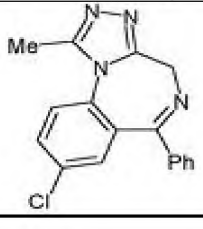
In the study of optimal CPE conditions, the following experimental factors were taken into account: 1) type of surfactant, 2) choice of dye for visualization of the border between the two separated phases, 3) sample pH, 4) selec-

tion of optimal surfactant concentration and solvent volume for micellar phase with the isolated medicaments, and 5) two methods for quantitative evaporation of the micellar phase.

Effect of Surfactant Type

Analyte partitioning in surfactant micelles strongly depends on the octanol–water partition coefficient (log P, see *Table I*). Theoretically, extremely hydrophobic analytes demonstrate very favorable distribution constants between the micelle-rich and the aqueous phases. It has been also recognized

Table I. Structure, dissociation constant (pK_a), and participation coefficient (logP) of the examined medicaments [1]

Drug	Structure	pK_a	log*P*
Salicylic acid		3.0 3.14	2.3[a]
Opipramol		3.8	3.4[a]
Carbamazepine		7.0	2.45[a]
Lorazepam		1.3 11.5	2.4[b]
Alprazolam		2.4	2.12[b]

[a]) Octanol/water).
[b]) Octanol/buffer 7.4.

that solubilization properties of surfactant aggregates (micelles) for hydrophobic organic compounds increase with the increase of the hydrophobic tail length and decrease with the size of polar head [13].

The performed study was started with the selection of an appropriate surfactant as extraction medium for the examined drugs. There were nonionic 7.5% Triton X-114 and aqueous surfactant two-phase systems consisting of two ionic surfactants: 0.05 M cationic C_{12}NE and 0.05 M anionic SDS, mixed in the molar ratio 1.62:1 [14]. Using surfactant, the Triton X-114 extraction procedure was conducted according to the steps shown in *Scheme 1*. The extraction process with the system of two surfactants was performed in a similar way with exception of the fact that the incubation step was omitted and the removed aqueous phase was below the micelle-rich phase (in opposition to the CPE with Triton X-114). In both cases of CPE procedures, the micelle-rich phase with the isolated drugs was dissolved in 150 μL of acetonitrile and then injected onto the chromatographic column.

The obtained results were better for Triton X-114 (extraction yields for the tested drugs was above 60%) and the appropriate extraction yields with the second surfactant system were below 50%. Therefore, the surfactant Triton X-114 was selected for further examinations.

Choice of Dye

Considering that the border between the two separated phases was not clearly visible, addition of an appropriate hydrophobic dye (demonstrating better affinity to the micelle-rich phase than to the aqueous phase) to a plasma sample was used. In order to make this phase border more visible, four different dyes (characterized by relatively low UV absorption) were used: Victoria Blue R, Victoria Blue B, Orange II and Rhodamine B. Each dye (in the amount of 60 μL of 3 mg mL^{-1} solution) was added to two plasma samples with 1 mL Triton X-114 containing the drugs and then subjected to incubation at temperature of 40 °C for 25 min. After the incubation step, the two phases were separated, and a dye was combined mainly with the micelle-rich phase. The micelle-rich phase was colored into orange-red by Rhodamine B and into blue-green by Victoria Blue B. Finally, Rhodamine B was selected as the dye with the best properties concerning visual distinguishing of the two separated phases, and its addition to a plasma sample enabled omitting the centrifugation step.

Effect of pH

For a major part of medicaments (week organic bases or acids), pH belongs to the critical factors regulating the partitioning of the target analyte in micellar phase. For ionizable molecules, maximum extraction efficiency should be achieved at pH values where the uncharged form of the extracted analyte prevails [13].

The influence of sample pH value on extraction yield of the medicaments was examined adjusting plasma samples to three pH: 4.0; 7.0 and 10.0. The obtained extraction recoveries (RV%) for each drug at three sample pHs were shown in *Fig. 1*. For salicylic acid, carbamazepine, and alprazolam, the best pH was 7.0 but for opipramol and lorazepam it was 4.0. However, considering the majority of the tested drugs (with the exception of opipramol), pH = 7.0 appeared to be optimal, and therefore it was chosen for further examinations.

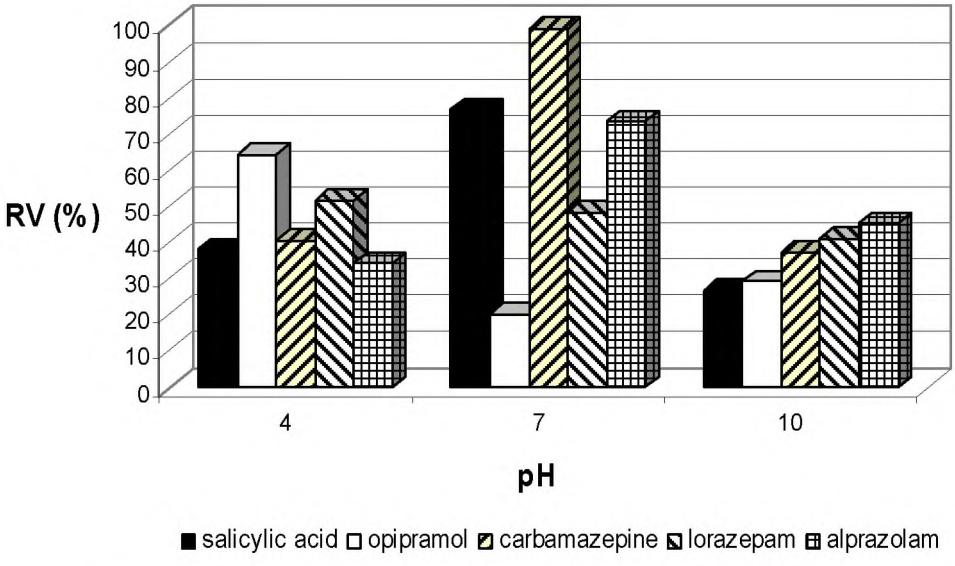


Fig. 1. Effect of sample pH on CPE results of the tested drugs in plasma

Effect of Surfactant Concentration and Solvent Volume (Acetonitrile)

When selecting surfactant concentration, one should consider the compromise between the achievement of appropriately high preconcentration factors and the resultant surfactant-rich phase volume that should be sufficient to make reproducible extractions [13]. The amount of solvent (e.g., acetoni-

trile) also should be optimalized for quantitatively dissolving of evaporated (or dried) micellar phase and achieving appropriate preconcentration factors.

Regarding that surfactant concentration and solvent amount for micellar phase are dependent on one another, the study of these optimal parameters was carried out according to an experimental design 3^2. The effects of surfactant concentration and acetonitrile volume were examined at three value levels: 3.75%; 5.63%; 7.5% and 100; 150; 200 μL, respectively. The obtained results were presented in *Fig. 2*. The best surfactant concentration and acetonitrile volume were as follows: 7.5% and 150 μL for alprazolam, 7.5% and 150 μL for lorazepam, 7.5% and 150 μL for opipramol, 3.75% and 200 μL for carbamazepine, and 3.75% and 150 μL for salicylic acid. Using more concentrated surfactant solutions than 7.5% (e.g. 10.0%) drug recoveries were considerably lower. Finally, concentration of Triton X-114 = 7.5% and acetonitrile volume = 150 μL were selected as the optimal parameters.

Two Methods for Quantitative Evaporation of Micelle-Rich Phase

Handling the micellar phase with the isolate drugs is a critical step for a CPE procedure, especially in terms of reproducibility and credibility of the obtained results. Complete removal of water traces from the micellar phase is usually attained by its evaporation under neutral gas (e.g. nitrogen) [13].

In the study the following two methods for quantitative removal of water from the micelle-rich phase were used:

1) decantation of the upper aqueous phase and evaporation of the micelle-rich phase using hot block (40 °C) under nitrogen stream for 25 min,
2) decantation of the upper aqueous phase and draining the micelle-rich by lyophilization process for cca. 24 h.

The extraction recoveries and repeatabilities for the tested drugs using both treatment methods were placed in *Table II*. For both methods, the drug extraction recoveries were comparable but repeatability of the results was better (with the exception for carbamzepine) for drying of the micellar phase by lyophilization. Considering the long time needed for the lyophilization process, the method using heating in hot block (40 °C) under nitrogen stream for 25 min was chosen.

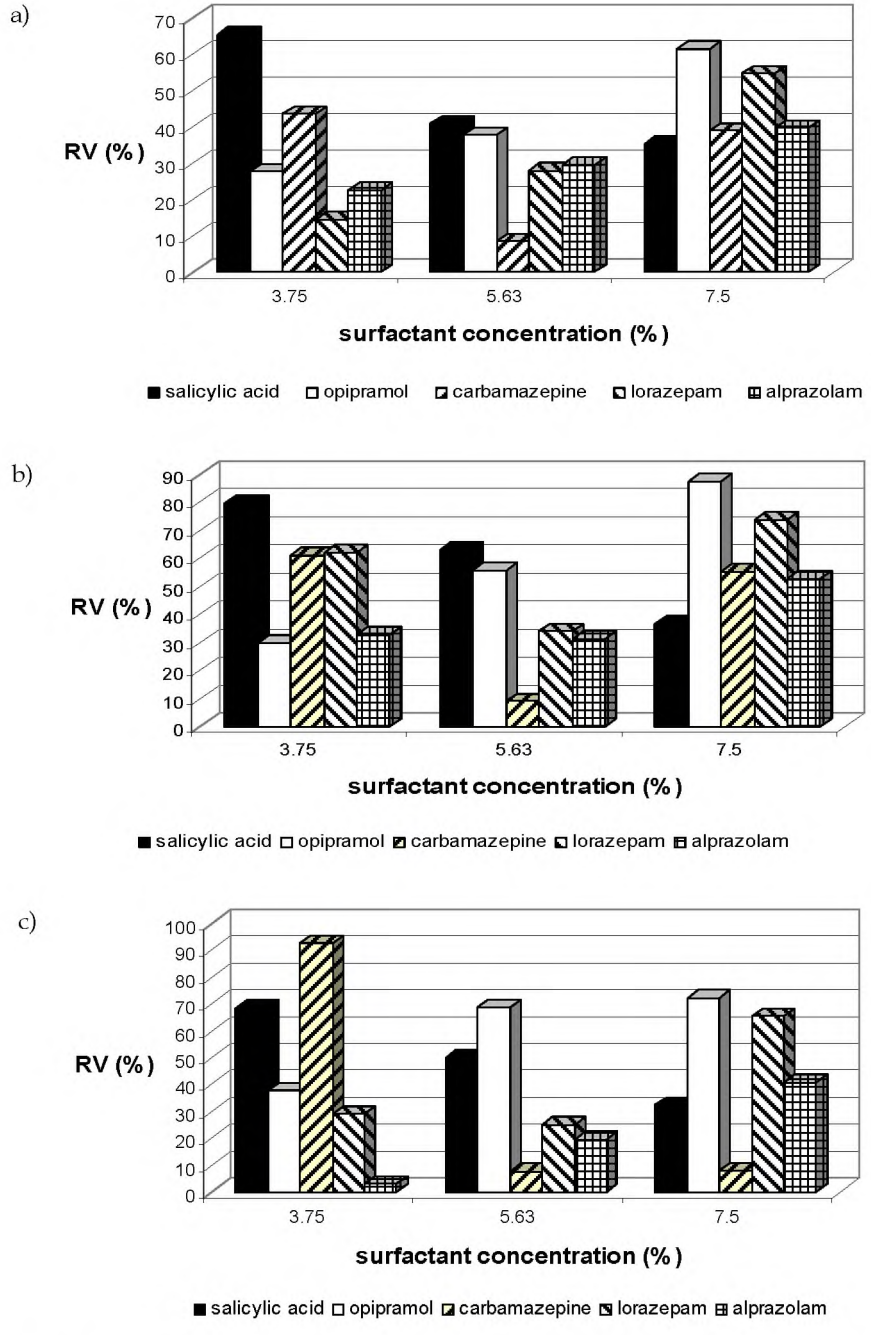


Fig. 2. Effect of surfactant concentration on CPE results of the tested drugs in plasma at three volumes of acetonitrile (the solvent for the micelle-rich phase): a) 100 μL, b) 150 μL, and c) 200 μL

Table II. Comparison of CPE results using two treatment methods of the micelle-rich phase with the isolated drugs from plasma samples

Drug	Treatment of micelle-rich phase			
	Heating in hot block (40°)		Lyophilization	
	Extraction recovery RV (%)	Repeatability (%)	Extraction recovery RV (%)	Repeatability (%)
Salicylic acid	82.17	5.70	82.90	2.54
Opipramol	62.06	7.02	59.08	0.74
Carbamazepine	54.12	8.94	56.46	12.38
Lorazepam	54.50	5.82	52.73	3.07
Alprazolam	64.15	7.74	66.99	2.20

Validation of the CPE-HPLC-DAD Method

Under the CPE conditions, assumed as optimal for all the tested compounds, the main validation parameters such as extraction recovery, intra- and interday repeatability, detection limit, and linearity range were determined. These parameters for the tested drugs present in plasma and whole blood samples were given in *Table II* and *Table IV*, respectively.

Table III. Validation parameters for the drugs determined in plasma and the drug concentration range in plasma corresponding to the evaluated detection limit

Plasma						
Drug	Extraction recovery (%) $n = 4$	Repeatability intra-day (RSD%) $n = 4$	Repeatability inter-day (RSD%) $N = 2$; $n = 4$	Detection limit (μg mL^{-1})	Linearity range, r^2	Concentration range (μg mL^{-1}) in plasma [15]
Salicylic acid	82.17	5.70	5.79	2.50	0.9929	subtherapeutic
Opipramol	62.06	7.02	7.31	0.50	0.9992	therapeutic
Carbamazepine	54.12	8.94	10.19	0.50	0.9977	subtherapeutic
Lorazepam	54.50	5.82	6.11	0.50	0.9954	toxic
Alprazolam	64.15	7.74	8.82	0.50	0.9992	toxic

Table IV. Main validation parameters for the drugs determined in blood and the drug concentration range in blood corresponding to the evaluated detection limit

Blood					
Drug	Extraction recovery (%) $n = 4$	Repeatability intra-day (RSD%) $n = 4$	Detection limit (μg mL^{-1})	Linearity range, r^2	Concentration range (μg mL^{-1}) in blood [16]
Salicylic acid	8.73	20.00	1.25	0.9949	therapeutic
Opipramol	33.97	21.06	0.50	0.9995	–[a]
Carbamazepine	12.03	32.18	0.50	0.9974	therapeutic
Lorazepam	14.39	25.46	0.50	0.9978	toxic
Alprazolam	26.01	29.50	0.50	0.9973	fatal

[a]The data were not found.

Extraction recovery (RV, %) was calculated as the ratio of analyte peak area in body fluid (plasma/blood) extract and analyte peak area in standard drug mixture. Intra- and interday repeatabilities (RSD, %) were determined as the relative standard deviation from four repeatable measurements per day, within one day and two days, respectively. Limit of detection (LOD, μg mL^{-1}) for each drug was determined by estimating the minimum concentration equivalent to three times standard deviation of the mean background noise of signal.

To our knowledge, only salicylic acid from among the tested drugs was reported to be determined spectrofluorimetricly followed by its extraction from human urine by CPE technique [7]. The drug recovery (85–94%) was comparable with that obtained by us, but the repeatability of the results (RSD%, 1.85) was a little better and the detection limit was lower (0.011 μg mL^{-1}) than that obtained in our investigations. However, it should be stressed that concentration levels of salicylic acid in plasma/blood are relatively high (therapeutic concentrations correspond to tens of μg per mL), as well as the proposed HPLC-DAD method is more universally used than the spectrofluorimetric method, especially for screening drug purposes.

Generally, it may be stated that the obtained CPE results in our study are comparable with those reported for neutral and weak acidic drugs achieved by the use of conventional LLE technique. Moreover, in the case of the three tested drugs (carbamazepine, lorazepam, and alprazolam), it is possible to lower (at least twice) the LOD of the proposed method using other light wave for detection of the drugs — e.g. λ = 210 nm (*Fig. 3b*) instead of the most commonly used for medicaments — λ = 254 nm (*Fig. 3a*).

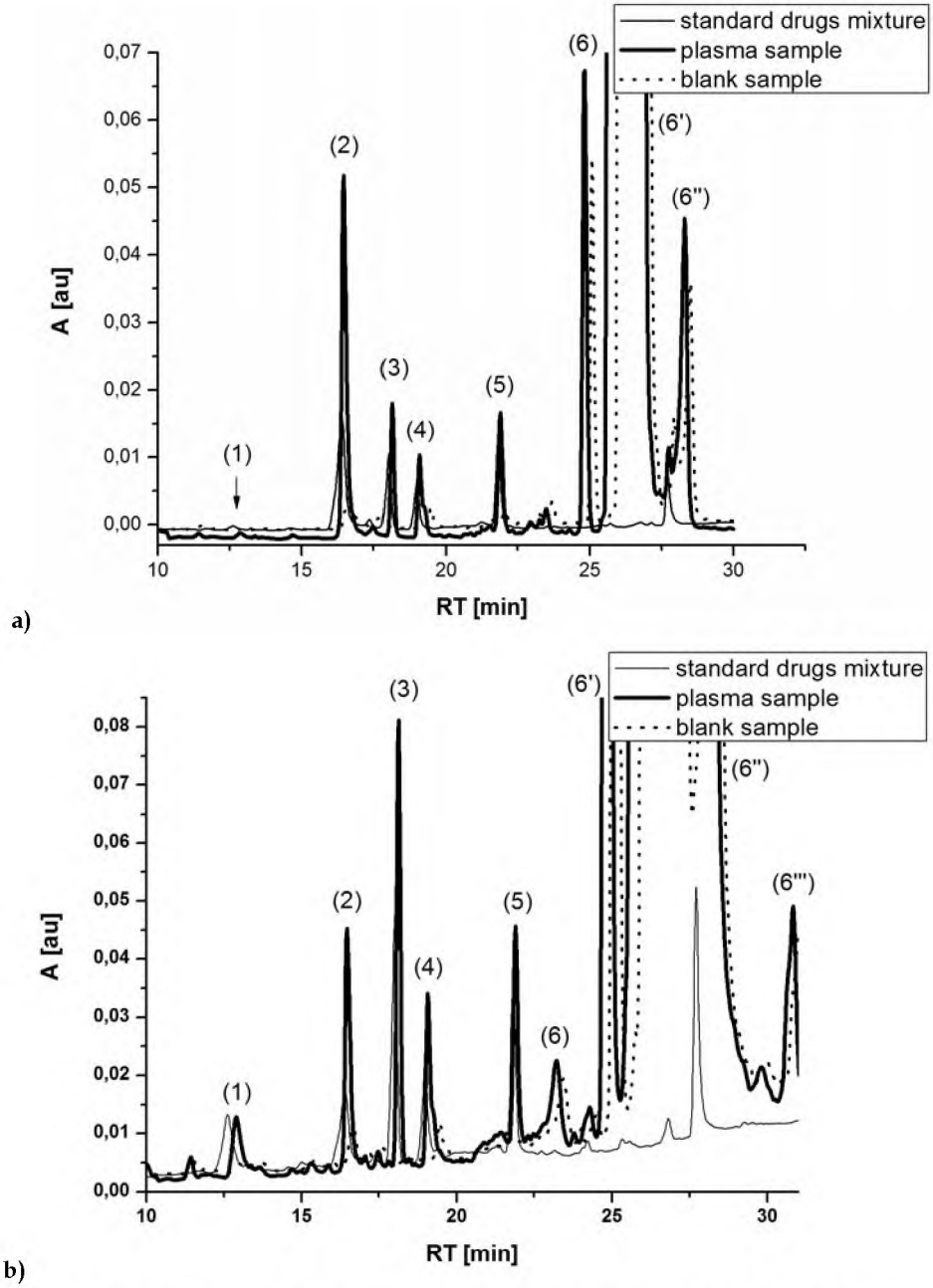


Fig. 3. Chromatograms of the plasma extract after CPE, measured at two UV waves: a) λ = 254 nm and b) λ = 210 nm. 1 — salicylic acid, 2 — opipramol, 3 — carbamazepine, 4 — lorazepam, 5 — alprazolam, 6-6" — peaks corresponding to the surfactant

Conclusions

The possibility of application of the cloud-point extraction (CPE) technique for the preparation of two kinds body fluids: plasma and whole blood screened for acid and neutral medicaments by HPLC-DAD method was examined.

The optimal CPE conditions for the selected five drugs were studied. The experimental parameters such as surfactant type, sample pH, concentration surfactant, solvent (acetonitrile) volume for the micelle-rich phase and two treatment methods of the micellar phase were taken into account. The appropriate dye for improving visualization of the border between two (micelle-rich and aqueous) phases was also proposed.

The results obtained for plasma samples are satisfactory; however, application of the CPE technique for the medicaments tested in whole blood samples requires further study.

Based on the results obtained for the medicaments, especially tested in plasma, it may be concluded that CPE appeared to be an effective, quick, cheap and environmentally friendly technique with extraction yields and repeatability comparable with those obtained by conventional extraction techniques like LLE and SPE.

Finally, it may be stated that CPE technique in combination with HPLC-DAD method may be a good alternative for traditional sample preparation techniques used in toxicological screening drug analysis of human plasma, as well as in some cases of the examined drugs, for therapeutic drug monitoring purposes.

Acknowledgements

The authors are grateful to Dr. Katarzyna Ostrowska for synthesis of the surfactant C_{12}NE at the Faculty of Chemistry in the Jagiellonian University.

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Accepted by DA