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Full Paper

Application of Surfactant Micellar Solutions as Extractants and Mobile Phases for TLC-Determination of Purine Bases and Doping Agents in Biological Liquids

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Abstract: Separation of caffeine and its metabolites (theophylline and theobromine) and doping agents (spironolactone, propranolol, and ephedrine) and determination of caffeine in serum sample and propranolol and ephedrine in urine were studied on normal-phase thin layers ("Sorbfil-UV-254"). Aqueous organic solvents and aqueous micellar surfactant solutions were compared as the mobile phases for separation. The acceptable separation of purine bases and doping agents was achieved by micellar Thin Layer Chromatography and normal-phase Thin Layer Chromatography. Anionic surfactant solution with added 1-propanol was the best eluent as for caffeine, theophylline, and theobromine separation, as for doping agents. The best characteristics of caffeine extraction from serum, and propranolol and ephedrine from urine were achieved when micellar eluent based on non-ionic Tween-80 surfactant was used.

Keywords: micellar TLC; surfactant; serum; urine; doping agents; purine bases

1. INTRODUCTION

The determination of the biologically active substances (drugs, dopes, toxic materials *etc*.) is relevant for pharmacology, forensic medical examination, and clinical researches. The features of such determinations are the low concentration of analytes in biological liquids and the change of chemical form of analytes in the presence of biocomponents, for example, due to binding with the proteins. Extremely low concentrations of drugs and their metabolites must be determined by pharmacokinetic researches when the composition of biological liquids is monitored over a long period after the drug injection in the organism [1-6].

The quantitative determination of drugs in biological liquids is usually realized by high performance liquid chromatography (HPLC) [7, 8]. Thin layer chromatography (TLC) technique is often applied for preliminary estimation of the sample composition and for screening of numerous samples before HPLC determination. The principle of TLC technique was formulated by Izmaylov and Shrayber in 1938, but this technique has been developed up to now [9]. Organic solvents or solvent mixtures are used in separation on normal-phase plates in TLC. Most of these solvents are flammable, many are toxic or carcinogenic. The use of micellar solutions as eluents in TLC was suggested by Armstrong in 1979 and micellar thin layer chromatography (MTLC) was initiated as a new variant of TLC [10]. The analytical capabilities of MTLC were considered in some reviews [11, 12] and articles [13-19]. Micellar eluents are biodegradable, nonflammable, and they have very low toxicity, and moreover, they are cheap. One of the main advantages of micellar eluents in HPLC is the possibility of drugs' determination in bioliquids without prior separation of proteins presented in the samples, simplifying sample preparation of the bioobjects [5, 19]. These advantages are the reason of development the chromatography techniques with micellar eluents [12, 14-17, 20].

The purpose of this work is to study the possibility of application of various chary type solutions in separation and identification of purine bases and dopes by TLC on a polar sorbent, and surfactant application in biological samples preparation.

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2. MATERIAL AND METHODS

Reagents

Acetone, ammonia, diethyl ether, 1-butanol, 1-propanol (Makrokhim, Ukraine), chloroform (Ukrkhimexpo), methanol, and ethanol. Samples of caffeine, theophylline, theobromine, spironolactone, propranolol, and ephedrine were supplied by Sigma (96 %). The anionic surfactant sodium dodecylsulfate (SDS, 96 %, Merck); cationic surfactant cetylpyridinium chloride (CPC, 99-101 %, Merck); nonionic surfactant Tween-80 (density 1.074 g/cm^3, Appli Chem GmbH) were used for eluent preparation.

Equipment

Glass chambers for TLC, plates for TLC "Sorbfil-UV-254", the graduated capillaries with a scaling factor of 1 μL, UV-lamp (UV-254, UPM-FIMET) with wavelength 254 nm, pH-meter (673 M) equipped with glass electrode ESL 43-07 and Ag/AgCl reference electrode EVL-1M3.

Detection of the purine bases and spironolactone was performed under UV exposure at 254 nm, in this case, dark-violet spots on a green fluor background of plates were observed. Detection of propranolol and ephedrine was performed after spraying the acidified solution of potassium permanganate, after that spots of analytes became yellow on a crimson background of a plate [21]. The R_f value, shape, and color intensity of spots were considered as a characteristic of chromatographic spots.

3. RESULTS AND DISCUSSION

Caffeine is one of extensively used bioactive substances with a well-known therapeutic effect [22]. Theophylline and theobromine are the metabolites of caffeine and also could be used as independent pharmaceutical substances. Caffeine, theophylline, and theobromine are derivatives of purine and belong to a group of alkaloids. Structural formulas are presented in Figure 1.

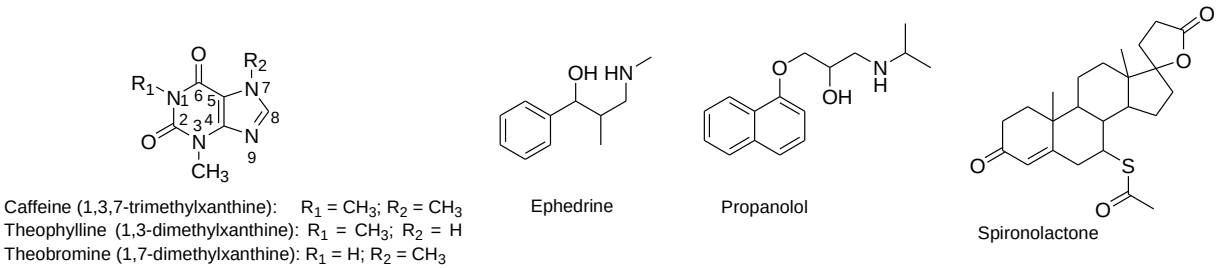


Figure 1. Structure formulas of purine bases and doping agents.

Theophylline and theobromine are ampholytic substances. Caffeine is a weak base and does not reveal the acidic properties [22, 23]. The quantity of caffeine and its metabolites in serum were determined in forensic medical investigation, a dope-control, in pharmacokinetics investigation [24]. Spironolactone, propranolol, and ephedrine were the concern the doping preparations, which are brought in Medical board of the International committee of a doping test [25, 26].

3.1 Identification and separation of purine bases

3.1.1 Application of surfactant solutions as eluents for separation of caffeine, theophylline, and theobromine

Dependences of chromatographic characteristics on the surfactant type, surfactant concentration in eluent, acidity of eluent, and additives of organic modifiers were studied. The surfactant concentration was varied in wide range, including concentration values below and above critical micelle concentration (cmc).

Application of micellar solutions of the anionic surfactant SDS as eluent gives the possibility to obtain theophylline separately; however, spots of theobromine and caffeine have interfered with each other. In this case, using the individual component solutions had the maximal difference of R_f values which were observed for SDS concentration 0.02 mol L^{-1} (Figure 2). SDS solution with this concentration was used in further investigations.

The R_f values of components separated are practically independent on pH values in the range from 2 to 9, however, the color intensity of spots is the best at pH 7**.**

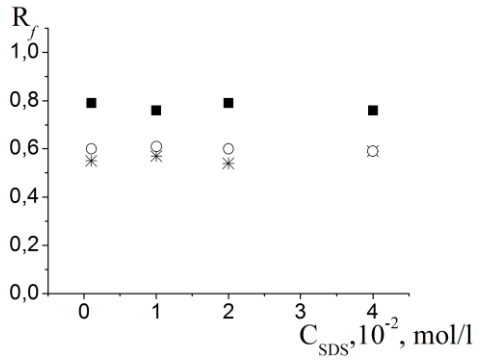


Figure 2. Dependence of R_f of caffeine **(*)**, theophylline **(■),** and theobromine **(○)** on SDS concentration.

The separation selectivity of micellar eluent can also be controlled by addition of small amounts of organic solvents [8].

The influence of additives of aliphatic alcohols ethanol, 1-propanol, and 1-butanol to the micellar solutions on separation was studied [10, 27]. The general form of chromatogram did not change in the presence of ethanol. When 1-propanol and 1-butanol were added into the micellar eluent, the separation characteristics were improved. The best result was obtained by addition 1-propanol (volume fraction 6%) and 1-butanol (volume fraction 0.1%) (Figure 3 A, B).

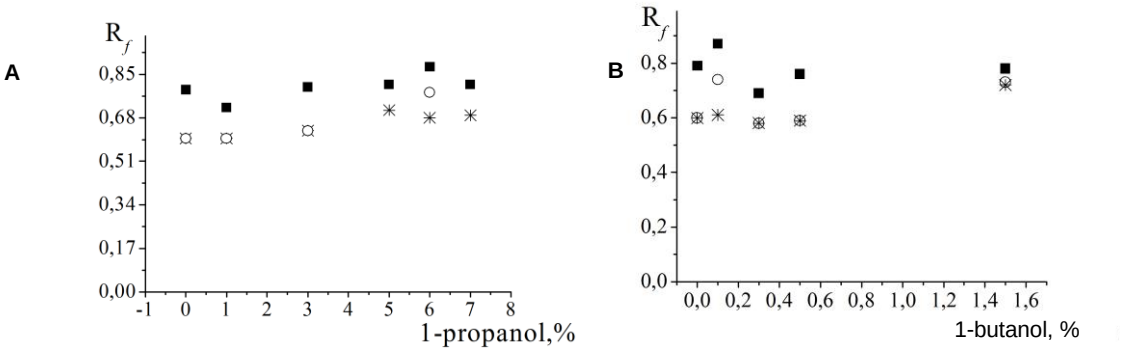


Figure 3. Dependence of R_f of caffeine **(*)**, theophylline **(■)** and theobromine **(○)** on the volume fraction of modifier *A* —1-PrOH (v/v) and *B* —1-BuOH (v/v) in $2 \cdot 10^{-2}$ mol L^{-1} SDS solution.

Application of micellar solutions of the non-ionic surfactant Tween-80 as eluent was studied. The R_f values of purine bases were practically independent on surfactant concentration (Figure 4), however, the intensity of chromatographic spots was the best of all solutions of Tween-80 with a concentration of $5 \cdot 10^{-4}$ mol L^{-1}.

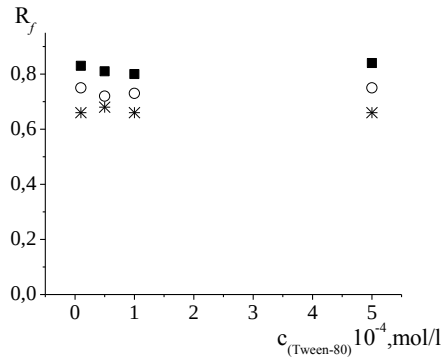


Figure 4. Dependence of R_f of caffeine **(*)**, theophylline **(■),** and theobromine **(○)** on Tween-80 concentration in the eluent.

It should be noted that retardation of purine bases is independent on concentration of SDS and Tween-80 in eluent: the R_f value being practically unchanged by using submicellar and micellar surfactant solution. Obviously, the surfactant effect consists of decreasing of surface tension of mobile phase. Thus, superficial velocity and value of HETP (height-equivalent theoretical plate) decrease; therefore, separation efficiency increases.

Solutions of the cationic surfactant CPC at concentration below the cmc did not provide a separation of purine bases; analytes were transferred together with eluent front. Applications of micellar solutions of CPC as eluent provide poor separation. In the best case (c(CPC)=$2 \cdot 10^{-2}$ mol L^{-1}) R_f values of adjacent spots were different only in 0.03 (Figure 5). This difference had not changed by addition of alcoholic modifiers. Cationic CPC was adsorbed of on the silica surface more than anionic and nonionic surfactants, especially from micellar solutions [28]. As a result, silica surface of TLC-plate takes the properties of reversed phase.

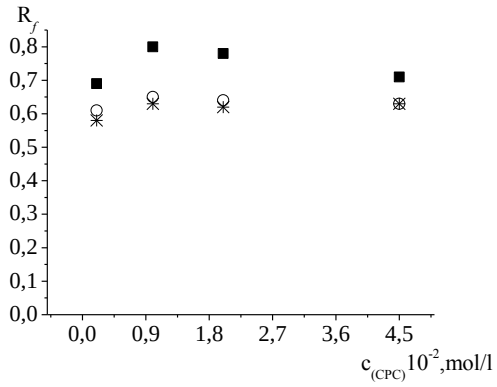


Figure 5. Dependence of R_f of caffeine **(*)**, theophylline **(■),** and theobromine **(○)** on CPC concentration in micellar eluent.

Really, the obtained consequence of spots (figures 2, 4, 5) is reversed in comparison with the results obtained for aqueous-organic eluents (Figure 1). This fact agrees with literature data of peculiarities of surfactant solutions as eluents and their modifying action on the surface of silica stationary phase [10, 11].

The following peculiarity was observed by application surfactant solutions as eluents for purine bases separation: the chromatographic chamber did not require long-time pretreatment for saturation by solvent vapor. This peculiarity is caused by low volatility of aqueous surfactant solutions, which contains a small amount of organic solvent-modifier. Water has lower volatility in comparison with organic solvents, which are usually applied as eluents in TLC. Furthermore, solvent-modifier is solubilized by surfactant micelles that decrease its volatility.

3.1.2 Separation of normal-phases plates with aqueous-organic eluents

TLC with aqueous-organic eluents was used as a reference technique for testing the MTLC one. The eluent, which contained diethyl ether, acetone, and liquid ammonia, was chosen for the separation of purine bases according to literature data [29]. The other eluents described in literature, contained more toxic components, such as chloroform. The composition of the eluent was selected for the self-made plates in authors' laboratory [30]; therefore, quantitative composition of the eluent was optimized additionally with respect to plates "Sorbfil", which were used in this study.

The best separation and maximal difference of R_f value was observed on the plates "Sorbfil" by using the eluent which contained diethyl ether: acetone: concentrated ammonia in volume ratio 20:40:2 (Figure 6).

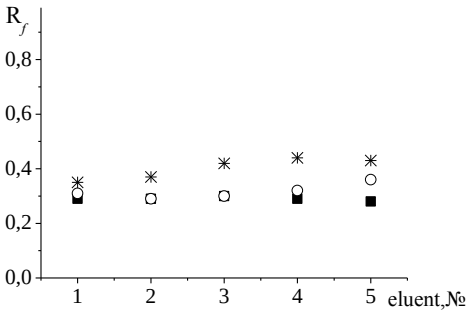


Figure 6. Dependence of R_f of caffeine (*), theophylline (■), and theobromine (○) on eluent composition: diethyl ether: acetone: liquid ammonia in relationship: No. 1—(30:30:1); No. 2— (25:30:1); No. 3— (20:40:1); No.—(15:45:1); No. 5 — (20:40:2).

The basic characteristics of TLC-separation techniques are compared with use eluent on the basis of surfactants and a mix of organic solvents: analysis time, a difference of R_f values of the next spots, toxicity are presented on the Table 1.

According to Table 1, the micellar eluent containing $5 \cdot 10^{-4}$ mol L^{-1} Tween-80 at pH 7, gives the chance to receive the same distance between the adjacent spots, and having advantages in comparison with mixture diethyl ether: acetone:liquid ammonia; analysis time is reduced by 10 times, use of toxic reagents is excluded.

3.1.3 Recovery, identification and determination of caffeine in biological liquids by MLTC technique

Micellar eluents were tested as extracting agents and were used for identification of caffeine from serum with using spiked samples. The spiked sample was prepared by addition of a known amount of caffeine into caffeine-free serum. The spiked sample was shaken up and kept for 24 hours at -4 ºC.

Caffeine forms a complex with proteins in biological fluids [31]; therefore, sample preparation should involve the procedure of caffeine release by the destruction of the complex. Application of micellar eluents eliminates this necessity because of the solubilization of proteins by the surfactant.

Table 1. Comparison of techniques of purine bases separation with use aqueous-organic and micellar eluents.

Compounds	**Composition of eluent**	**Characteristic**		
		Analysis time, min	**ΔR_f**	**toxicity**
1.caffeine 2. theobromine 3. theophylline	$5 \cdot 10^{-4}$ mol L^{-1} Tween-80 pH 7	10	1-2:0.09 2-3:0.09	Tween-80 – it is not toxic, doesn't possess resorptive action, doesn't cause irritation
	diethyl ether: acetone: liquid ammonia (20:40:2)	100*	1-2:0.09 2-3:0.1	Moderately to highly hazardous, dangerous

*time of chromatogram development – 40 minutes, time of chamber saturation of the steams of eluent – 90 minutes

The same surfactant solutions, which were used as eluents for TLC-separation, were studied as extracting agents for caffeine from serum.

Micellar solutions of CPC didn't extract caffeine from serum, because the solubilization of serum proteins by cationic surfactant had not been effective [3]. Protein fraction remains close to start line on the TLC-plate, separating of caffeine had not been observed. The precipitation of protein by addition of cationic surfactant into biological liquids was obtained during sample preparation. Then caffeine was determined by micellar liquid chromatography. The same effect prevents direct serum injection in the column of MLC [4, 5].

When micellar solutions of anionic surfactants (SDS) were applied the protein fraction transferred together with the solvent front thus separation of caffeine spot from the serum was observed. However, chromatographic process developed quite slowly, the separation continued over two hours. Caffeine spot and a front line had irregular shapes (Figure 7).

These difficulties were not removed by additions of 1-propanol to micellar solutions of SDS (Table 2).

The addition of a known amount of the eluent to the serum is used for preliminary binding of protein at the sample preparation. The results at different volumetric ratios of serum to the surfactant solution were obtained. Volumetric ratio of probe and micellar eluent 1:10 was chosen as optimal.

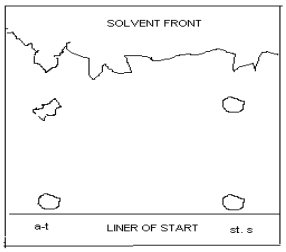


Figure 7. Chromatogram of serum (left) and standard of caffeine (right); eluent – $2 \cdot 10^{-2}$ mol L^{-1} SDS and 6 % 1-PrOH (v/v).

The best recovery and separation of caffeine from the serum was achieved with micellar solution of non-ionic surfactant Tween-80. In this case, protein fraction transferred together with the solvent front, and chromatographic spot of caffeine had regular shape without tails. The spot of recovered caffeine had the same size and intensity as the spot of standard solution with conformable concentration of caffeine (Figure 8).

Table 2. Dependence of R_f and time of analyses on volume fraction 1-PrOH in micellar eluent (c(SDS)=$2 \cdot 10^{-2}$ mol L^{-1}).

Volume fraction of 1-PrOH	4%	6%	8%	10%	12%	15%
R_f or characteristics of spot	spot moves with the front line	0.68	0.69	0.69	0.71	spot moves with the front line
			The time of analysis was too extended			

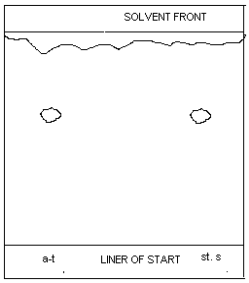


Figure 8. Thin-layer chromatogram of serum (left) and standard solution (right) of caffeine; eluent – $1 \cdot 10^{-4}$ mol/L Tween-80.

Visual semi-quantitative determination of caffeine was performed using standard scale with several known concentration of caffeine. The 10 μL of spiked samples and 10 μL of standard solutions with different caffeine concentrations were applied on the TLC plate. The intensities of caffeine spots in spiked samples and in standard solutions were compared. Working caffeine concentration was 15 mg L^{-1} (caffeine quantity in the blood is controlled in range 5-225 mg L^{-1} [32, 33].

The schematic example of caffeine determination is presented in Figure 9. The intensity of caffeine spot in serum sample was the same as the intensity of spot contained 150 ng of caffeine, agreeing with the additive injected into the serum.

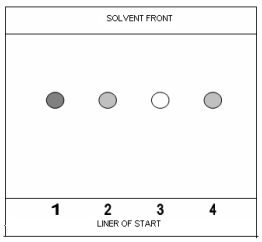


Figure 9. The scheme of determination of caffeine quantity in serum by comparing with it quantity in standard solution. Standard solutions of caffeine: 1 – 225 ng, 2 – 150 ng, 3 – 75 ng; 4– probe.

The technique of micellar TLC with Tween-80 solution as eluent was tested by identification of caffeine in real samples of rat's serum. Three rats were taken for analyses: 1 - without the caffeine injection, 2 - with intraperitoneal introduction of 1 mL of 2.3 mol L^{-1} caffeine solution; 3 - with intraperitoneal introduction of 2 mL of 2.3 mol L^{-1} caffeine solution. The dose of caffeine was chosen according to requirement: no more than 3 mg of caffeine on 1 kg of bodily weight of live rat [23]. The decapitation of rat under ethereal narcosis was performed after caffeine injection. The serum was obtained after blood sampling, cooling during 15 min at -4 ºC and centrifugation of packed red blood cells.

Sample preparation of the serum was performed as addition of 9 g of Tween-80 to 2.5 mL of serum to avoid dilution of sample. The prepared sample was shaken and kept during 24 hours at -4 ºC.

The spot of caffeine was not found on the thin-layer chromatogram of serum of rat No. 1 (blank sample). After the separation of serum samples No. 2 and No. 3 the spots with R_f value 0.7 were obtained. That spots correspond to R_f value of caffeine in standard solution. The spot of rat serum No. 2 sample was less intensive than the spot of rat serum sample No. 3.

Thus, micellar eluent of Tween-80 allows the determination and identification of caffeine in serum that simplifies TLC-analyses of biological fluids.

3.2 Identification and separation of spironolactone, propranolol, and ephedrine

3.2.1 Application of surfactant solutions as eluents for separation of spironolactone, propranolol, and ephedrine

Selection of suitable surfactants for identification and separation of the chosen dopes by TLC method carried out by the same scheme as for purine bases.

Solutions of cationic surfactants do not elute spironolactone, propranolol, and ephedrine. Individual zones of dopes were observed at elution of each component by solution with SDS concentration $1.0 \cdot 10^{-3}$ mol L^{-1}, φ (1-butanol) =3 %, pH 2 (Figure 10). The sport of spironolactone had the biggest size. Separation of analytes mixture by this eluent was not possible.

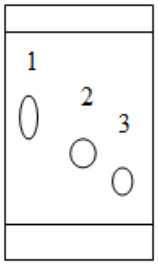
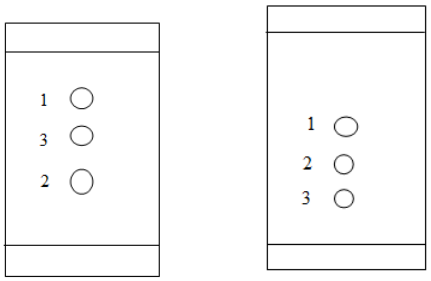


Figure 10. The scheme of the chromatogram of spironolactone (1), ephedrine (2), and propranolol (3), received with eluent based on anionic surfactants (c (SDS) $=1.0 \cdot 10^{-3}$ mol L^{-1}, φ (1-butanol) =3 %, pH 2).

The micellar solution with $5 \cdot 10^{-4}$ mol L^{-1} Tween-80 and an addition of 1 % 1-propanol at pH 2 provides satisfactory separation of three dopes (Figure 11,a). On chromatograms received, earlier noted feature of micellar eluent — presence of the second front of a micellar *pseudo*-phase-type was observed [12, 13]. All components of the mixture settle down in the first part of eluent's front.



a) b)

Figure 11. The scheme of the chromatogram of spironolactone (1), ephedrine (2), and propranolol (3), received with two eluents: a) c (Tween-80)= $5 \cdot 10^{-4}$ mol L^{-1}, φ (1-propanol)= 1 % at pH2; b) methanol: liquid ammonia in the volume ratio (49.5: 0.5).

The technique of separation and identification of dopes with micellar eluent has been compared with the TLC technique in which as a mobile phase a mixture methanol: liquid ammonia in the volume ratio (49.5: 0.5) have been used [34]. In comparison with surfactants eluent solutions here chromatographic zones of propranolol and ephedrine are located upside-down, in comparison with normal-phase TLC (Figure 11,b).

The basic characteristics of types of TLC-separation techniques were compared: analysis time, difference of R_f values of the neighboring spot and toxicity are presented on the Table 3.

Apparently from Table 1, micellar eluent, containing $5 \cdot 10^{-4}$ mol L^{-1} Tween-80, 1% 1-propanol at pH 2, gives the chance to reach the same distance between the neighboring spots, and has advantages in comparison with mixture methanol : liquid ammonia: analysis time is reduced in 4 times, use of toxic reagents is excluded.

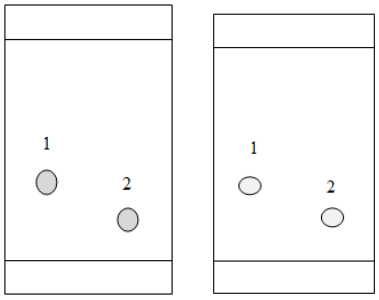
Table 3. Comparison of dopes' separation techniques with use of aqueous-organic and micellar eluents.

Compounds	**Composition of eluent**	**Characteristic**		
		Analysis time, min	ΔR_f	toxicity
1.spironolactone **2.ephedrine** **3.propranolol**	$5 \cdot 10^{-4}$ mol L^{-1} Tween-80, 1% 1-propanol pH 2	35	1-2:0.13 2-3:0.07	Tween-80 is not toxic, doesn't possess resorptive action, doesn't cause irritation
	methanol: liquid ammonia (49.5:0.5)	150*	1-3:0.13 3-2:0.18	highly hazardous dangerous

*time of chromatogram development – 40 minutes, time of chamber saturation of the steams of eluent – 90 minutes

3.2.2 Unmasking of dopes from urine by micellar solutions of surfactant

Unmasking of dopes from urine by surfactant micellar solutions was carried out on a modeling sample of urine. The completeness of extraction was supervised, by in parallel taking dopes from the same tests by a standard technique [1]. For this purpose, various quantities of a mobile phase added to a modeling mix (urine (10 mL) +dopes (0.06 g). It has been established that ephedrine and propranolol was effectively extraction by Tween-80 micellar solution, also it has been established that increasing of nonionic surfactant concentration fall in a taking solution gives the chance to define smaller quantities of analytes (Figure 12).



a) b)

Figure 12. The scheme of chromatogram, received with eluent: c (Tween-80)=5.10^{-4} mol L^{-1}, φ(1-propanol)=1 % at pH 2 (ephedrine (1), propranolol (2)); a) unmasking of ephedrine, propranolol from urine by surfactant micellar solutions; b) unmasking of ephedrine, propranolol from urine by a standard technique.

4. CONCLUSIONS

Micellar solutions of anionic surfactant sodium dodecylsulfate, and non-ionic one, namely Tween-80, can be used as eluents for separation of mixture of caffeine and its metabolites theophylline and theobromine by MTLC technique. Identification of spironolactone, ephedrine, propranolol are feasible by use non-ionic Tween-80.

The application of MTLC method for the analysis of biological liquids (serum, urine) was investigated. Better results were obtained when micellar eluent based on Tween–80 was used for serum and urine analyses.

The use of micellar eluents for TLC separation, identification, and semi-quantitative caffeine and dope determination in serum has the following advantages:

1. Sample preparation is simplified because there is no necessity of protein matrix separation; solubilized protein moves together with eluent front;

2. Total analysis time is reduced due to two factors: there is no necessity of chamber saturation, duration of chromatographic process is reduced;

3. Application of micellar eluent makes analysis ecologically safe, inexpensive, and quick.

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