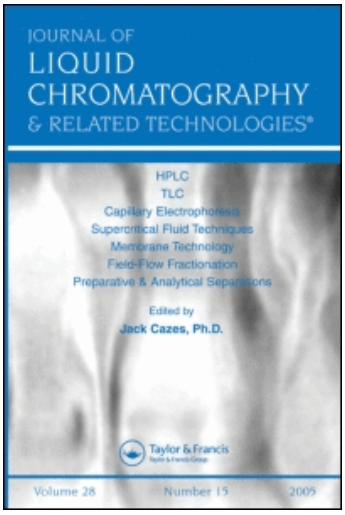

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OIL-IN-WATER MICROEMULSIONS AS MOBILE PHASES FOR RAPID SCREENING OF ILLEGAL DRUGS IN SPORTS

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ABSTRACT

Microemulsions are clear, stable and spontaneously forming liquid disperse systems. They contain an oil, water, a surfactant and a cosurfactant. The system heptane, water, sodium dodecylsulfate (SDS) and n−pentanol was used. The mass diagram was determined. Water rich compositions of the oil−in−water microemulsion type (L1) were investigated as mobile phase in reversed phase liquid chromatography with a 5 μm C18 bonded stationary phase column. The solutes were 11 drugs illegally used in sport. It is shown that the solute retention decreases when the organic content, φ, of the microemulsion (heptane + active blend: SDS and pentanol) increases. A linear relationship between k′ and log φ was found. The selectivity becomes nil, all solutes having the same k′=0.5 value, when the microemulsion organic content was 15.2% (± 0.5% v/v). The active blend (SDS/pentanol) and heptane play a similar role on retention. They have an opposite effect on efficiency. The solute mass transfer increased with the active blend content. It dramatically decreased when the microemulsion heptane content increased. However, heptane

decreased the protein peak width when urine samples were directly injected. The limit of detection of the drug tested was in the nanogram injected range.

Micellar liquid chromatography (MLC) uses aqueous solutions of surfactants at a concentration above the critical micelle concentration. The advantages of MLC are the low cost, low toxicity and non polluting nature of the mobile phases, the enhanced selectivity, a rapid gradient–elution capability, the ability to analyze both hydrophobic and hydrophilic compounds simultaneously. Several reviews on MLC appeared in the literature [1–4].

The drawback of MLC is a poor efficiency due to a low mass transfer between the aqueous mobile phase and the apolar bonded stationary phase. The slow solute mass transfer is due to a surfactant layer formed on the stationary phase [5]. It was shown early that small additions (3–5% v/v) of medium chain alcohol (n–propanol, butanol or pentanol) could enhance the observed efficiency [6–7]. The alcohol desorbs a large part of the surfactant out of the stationary phase surface. This renders the solute mass transfer faster [5, 7].

The addition of medium chain alcohol to micellar solutions is the first step towards microemulsion formation [8]. Microemulsions are liquid disperse systems containing an oil, water, a surfactant, and a medium chain alcohol acting as a cosurfactant. Microemulsions appears as clear liquids with a relatively low viscosity. These liquids present some inhomogeneous character at the microscopic down to the molecular level. The polarity of an oily microdomain is very different of the one of an aqueous microdomain. Microemulsions form spontaneously and are very stable.

It was demonstrated that MLC could analyze directly protein containing samples. Time consuming protein separation or precipitation, or drug extraction steps, or precolumn technology can be avoided [9]. MLC with direct urine or serum injection was used to determine pharmaceutical compounds [10], anticancer drugs [11], or illegal drugs in sport [12]. The aim of this work was to investigate the capability of microemulsion mobile phases in the screening of illegal drugs in sport.

A heptane–water–sodium dodecylsulfate–pentanol system was investigated with an octadecyl (C18) bonded stationary phase. Only the

water–rich compositions (L1 or oil in water microemulsions [8]) were investigated.

EXPERIMENTAL SECTION

Chemicals Heptane was obtained from BDH, Peypin, France. Sodium dodecylsulfate (SDS) was provided by Merck (Darmstadt, Germany) with a 99% purity. The structural formula of SDS is $C_{12}H_{22}$-SO_3^- Na^+, the molecular weight is 288.4 g/mol, the micellar molar volume is 0.246 L/mol. Table I lists some physico-chemical properties of the eleven drugs studied. They were supplied by Sigma Chemical company (St Louis, MO).

Apparatus The chromatographic separations were carried out at room temperature with a high resolution chromatograph consisting of a Spectra Physics Isochrom pump. A Spectra Physics 100 UV detector operated at 254 nm was used with a Spectra Physics Chromjet integrator. The 250x4.6 mm i.d. OD-SA column was filled with an octadecylsilane (C18) monomeric bonded 5 μm Spheri-5 silica (Brownlee Labs). Samples were injected with a Rheodyne valve model 7010 using a 10 μL loop.

Mobile phases A microemulsion forms spontaneously by simple mixing of the right proportion of the constituents. Phase diagrams need to be established to map the microemulsion compositions. Figure 1 shows the mass phase diagram of the heptane-SDS-pentanol-water system used. The SDS/pentanol ratio was kept constant: 1/2 mass ratio which is 1 SDS molecule for 6.5 pentanol molecules. The hatched areas correspond to polyphasic mixtures either two liquid phases (classical emulsions) or a liquid and a solid phase (low water compositions). The dotted area corresponds to a birefringent liquid crystal composition. The open areas map the microemulsion areas, either the L1 oil in water microemulsion area (water rich compositions) or the L2 water in oil and/or bicontinuous microemulsion area (active blend SDS/pentanol rich compositions). The mobile phase viscosity should be low (< *ca* 2 cP) to produce a pressure drop lower than 200 kg/cm^2 (20 MPa or 3000 p.s.i.) at reasonable flow rate (at least 0.5 mL/min). Only the water-rich L1 microemulsions (water content > 87% w/w) could be used. Table II and the Figure 1 inset show the compositions of the microemulsion mobile phases used. Five compositions were selected. Compositions #1, #2 and #3 contain similar amounts of heptane. Compositions #4, #3 and #5 correspond to a dilution line. It means they contain a similar ratio active blend/heptane with increasing amounts of water and decreasing amounts of heptane and active blend (Table II).

Table I: Physicochemical properties and therapeutic effect of the drugs

Drug	formula	m.w.	water solub. mg/L	therapeutic effect
acebutolol	$C_{18}H_{28}N_2O_4$	336.4	i	β adrenergic blocker antihypertensive
chlorthalidone	$C_{14}H_{11}ClN_2O_4S$	338.8	120	diuretic
codeine	$C_{18}H_{21}NO_3$	299.4	8330	narcotic analgesic
hydrochloro-thiazide	$C_7H_8ClN_3O_4S_2$	297.7	l.s.	diuretic
methoxamine hydrochloride	$C_{11}H_{18}ClNO_3$	247.7	$4x10^6$	adrenergic vasopressor
methyltestosterone	$C_{20}H_{30}O_2$	302.4	i	androgen
nadolol	$C_{17}H_{27}NO_4$	309.4	l.s.	β adrenergic blocker
norcodeine	$C_{17}H_{19}NO_3$	285.3	l.s.	analgesic
oxprenolol	$C_{15}H_{23}NO_3$	265.3	i	coronary vasodilatator
phenylephrine hydrochloride	$C_9H_{24}ClNO_2$	203.7	s	α adrenergic antagonist decongestant
probenecid	$C_{13}H_{19}NO_4S$	285.4	l.s.	uricosuric

i=insoluble, s=soluble, l.s.=low solubility; data from The MERCK index, Centennial edition (11th), Merck, Rahway, NJ (1989).

RESULTS and DISCUSSION

As soon as the organic content of a system increased, the viscosity also increased. The pressure drop became high and it was not possible to work at reasonable (~0.5 mL/min) flow rates. The figure captions indicates the pressure drops obtained with microemulsion mobile phases.

Selectivity Table III lists the chromatographic parameters, capacity factor and peak efficiency, of the drugs. Acebutolol was the most retained compound with the five microemulsion mobile phases. Hydrochlorothiazide was the least retained. This shows the unique selectivity of microemulsion mobile phases. With a pure methanol mobile phase, the most retained solutes were oxprenolol and methyltestosterone and the least retained one

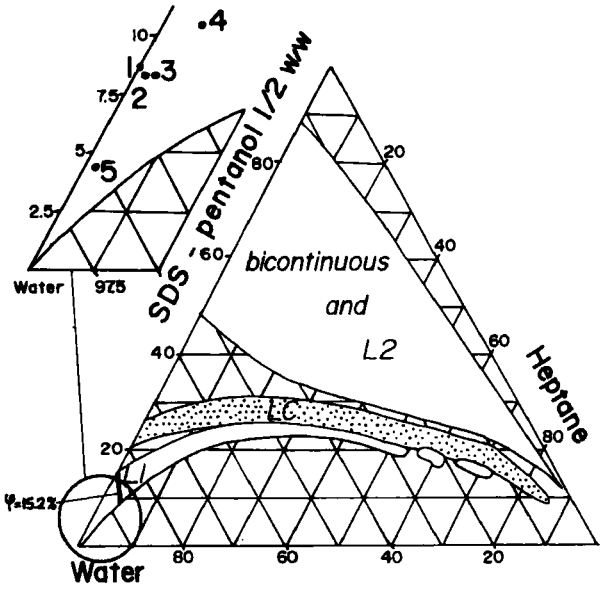

Figure 1 Mass phase diagram of the system water-heptane-SDS-pentanol. The SDS/pentanol mass ratio was 1/2. Hatched areas: biphasic mixtures; dotted area: birefringent liquid crystal; open area: clear microemulsions. The circle at the lower left corner focuses on the compositions used which are located on the inset figure. The numbers refer to Table II. The line corresponding to $\varphi = 15.2\%$ is pointed out (nil selectivity, see text).

Table II: Mobile phase microemulsion compositions

micro-emulsion #	SDS % w/w	pentanol % w/w	heptane % w/w	water % w/w	φ % v/v	protein peak width mL
1	2.88	5.76	0.0	91.36	8.64	2.3
2	2.73	5.51	0.31	91.45	9.21	1.3
3	2.75	5.51	0.59	91.15	9.60	1.3
4	3.52	7.00	1.44	88.04	13.37	1.8
5	1.44	2.87	0.34	95.35	5.09	2.5

Table III Drug capacity factor and peak efficiency

product	microemulsion mobile phase										pure methanol mob.ph.	
	#1		#2		#3		#4		#5			
	k′	N	k′	N	k′	N	k′	N	k′	N	k′	N
acebutolol	16.1	270	14.0	260	12.6	170	5.5	140	29.8	130	13.5	1000
chlorthalidone	1.21	6400	1.14	5500	1.04	4600	0.76	4100	1.83	4000	0.37	4300
codeine	6.54	160	5.50	110	5.23	80	1.07	700	12.3	120	7.26	70
hydr.chl.thiazide	0.68	6500	0.56	5600	0.45	3600	0.19	2600	0.90	3000	0.40	3000
methoxamine	5.64	1600	5.19	1000	4.31	450	1.71	2700	13.3	800	18.1	350
methyltestoster.	5.77	5500	5.42	5000	4.78	3900	2.95	4000	11.5	2500	24	4000
nadolol	3.47	1800	3.07	1100	2.86	900	1.79	1300	5.18	1500	18.2	3300
norcodeine	5.41	400	5.11	350	4.90	300	3.72	400	7.83	300	20.6	400
oxprenolol	15.4	270	11.5	260	10.9	220	2.5	800	27.9	190	23.3	600
phenylephrine	3.41	2800	2.82	2100	2.39	2000	0.98	2600	5.93	2100	17.0	600
probenecid	1.12	1300	1.03	900	0.93	700	0.20	2000	1.55	900	0.09	1200
log φ	0.936		0.964		0.982		1.126		0.707		2	

Table IV: slope and intercept of the k' *vs* log φ lines

compound	slope	intercept	r^2	LOD ng inj.
acebutolol	-58.6	70.9	0.996	2.8
chlorthalidone	-2.59	3.64	0.992	1.2
codeine	-26.6	31.2	0.998	19
hydrochlorothiazide	-1.6	2.13	0.921	1.1
methoxamine	-28.4	32.7	0.976	62
methyltestosterone	-21.0	25.8	0.971	1.6
nadolol	-8.1	10.9	0.995	17
norcodeine	-10.0	14.7	0.995	21
oxprenolol	-61.0	71.1	0.992	31
phenylephrine	-12.0	14.3	0.991	10
probenecid	-3.02	3.84	0.892	0.3

LODs obtained with microemulsion #1 at 0.5 mL/min and UV 254 nm detection.

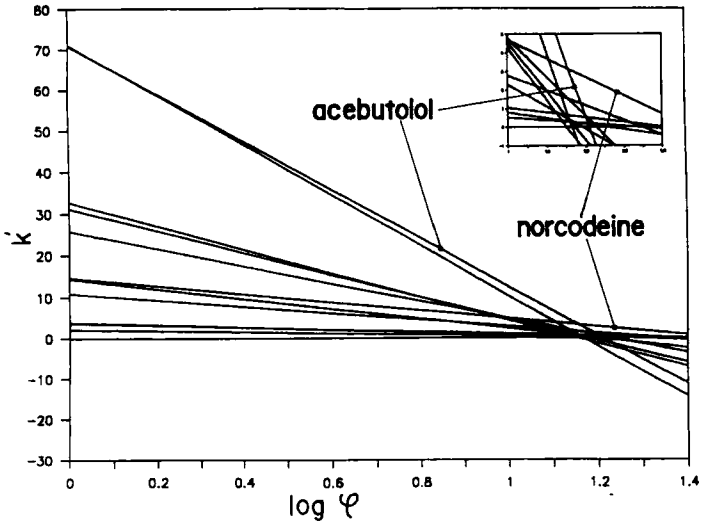

Figure 2 k' *versus* log φ lines showing a common convergence point at log φ close to 1.18. The inset is an enlargement of the convergence point. The norcodeine line is a little bit off. The acebutolol line is not.

was probenecid (Table III). The very hydrophobic solutes, acebutolol, methyltestosterone and oxprenolol, are insoluble in water. All three are highly retained ($k' > 13$) on the C18 stationary phase with a 100% methanol mobile phase. The k' value observed with a microemulsion containing 88% w/w water are four times lower (Table III). The hydrophobic solutes dissolve in the microemulsion oil droplets and are carried in the column by the droplets.

For all drugs, the capacity factors decreased when the organic content (active blend + heptane) of the microemulsion mobile phase increased (Table III). This means that the selectivity between two drugs also decreased. For MLC, it was shown that capacity factor, k', and organic volume fraction, φ, are related by [13, 14]

$$1/k' = A + B\varphi \quad (1)$$

in which A and B are constants connected to the solute partition coefficient with the stationary phase and the micellar phase. The plots of $1/k'$ *vs* φ for

the drugs tested were poorly linear. However the plots of k′ *vs* log φ were linear although there is no theoretical foundation for such a linear relationship. Table IV lists the slopes, intercepts and regression coefficients of the k′ *vs* log φ lines. The lowest regression coefficient was 0.892 obtained for probenecid. Most regression coefficients were higher than 0.99. Figure 2 shows the 11 lines corresponding to Table IV. All lines, but the norcodeine one, cross together in the coordinate range: log φ = 1.18 ± 0.04 and k′ = 0.5 ± 0.5 (inset, Figure 2). log φ = 1.18 corresponds to φ = 15.2% of organic volume percentage. The φ=15.2% line is highlighted in the inset mass diagram (Figure 1). The viscosity of these compositions was high which precluded their experimental use. For these microemulsion compositions, the capacity factor of all solutes should be close to 0.5, with a nil selectivity. The selectivity increases linearly with the log φ decrease.

Efficiency

Methanol mobile phases vs microemulsions. The chromatographic efficiency was measured using the plate number, N,

$$N = 4[W_{0.6H}/t_r]^2 \quad (2)$$

in which $W_{0.6H}$ is the peak width at 60 % of the peak height (expressed in time units) and t_r is the retention time. The low efficiency observed in MLC was extensively studied [5–7, 12]. The solutes should be injected in a mobile phase solution to obtain reproducible efficiencies. Methanol was not suitable as a sample solvent. It produced a significant band broadening at the column head and a huge multiple peak signal at the dead volume. This was due to a perturbation of the adsorbed layer (SDS, pentanol and heptane) by the methanol bolus. It was shown that pentanol and heptane compete with SDS to adsorb on the C18 bonded phase [4, 5, 7]. The observed peak efficiency with microemulsion mobile phases was comparable to the one obtained with pure methanol (Table III). The adsorbed layer should be very reduced compared to the adsorbed SDS layer with pure micellar mobile phases [12]. For the compounds containing an amino group, codeine, methoxamine, norcodeine, oxprenolol and phenylephrine, the efficiency was low. With a pure methanol mobile phase, they are mainly retained by silanophilic interactions. Residual stationary phase silanols interact with the basic amino group producing a high retention and a poor efficiency (Table III). The capacity factors of these solutes were lower and the efficiency higher with microemulsion mobile phases. The pentanol,

heptane and/or SDS adsorption on the C18 bonded surface seems to shield the silanophilic interactions. For some compounds such as acebutolol and nadolol, the efficiency obtained with the microemulsion mobile phases was lower than the one obtained with pure methanol.

Effect of the microemulsion composition Table III shows that the efficiencies obtained with microemulsion #1 and #4 are significantly higher than the ones observed with the three other compositions. There is also a significant efficiency decrease from microemulsion #1 to microemulsion #3. The addition of heptane to microemulsion #1 changes the physico–chemical structure of the medium [8]. Microemulsion #1, without heptane, has a loose structure. The microemulsion droplets only contain pentanol which is also present in the aqueous phase. When heptane is added, it is located in the core of the microemulsion droplets. It changes the droplet core viscosity and polarity. The oil in water structure becomes more rigid. The mass transfer of a solute located inside the oil droplet becomes slow.

At a constant heptane content, the addition of active blend SDS–pentanol increases the observed efficiency. In the selectivity study, it was shown that heptane and the active blend played the same role on solute retention. The organic phase volume, φ, was the important parameter. Heptane and the active blend have an opposite effect on efficiency: additions of heptane to a microemulsion produce a stronger mobile phase, the solute retention times decrease, but the peaks broaden, the efficiency also decreases. Increases of the active blend content decrease the solute retention times without peak broadening.

Analytical capabilities

Limit of detection (LOD) and linear dynamic range The drug limits of detection (LOD) are listed in Table IV. They were measured using microemulsion #1 which produced the highest efficiency with most drugs (Table III). The LODs are in the nanogram injected range. The injection volume was 10 μL. The concentration of the injected samples was in the 0.1 ppm (or mg/L) range. The LODs obtained with microemulsion mobile phase were comparable to the ones obtained with micellar solutions [12]. The linear dynamic range was three orders of magnitude wide or better.

Urine sample analysis Figures 3 and 4 show the chromatograms of the same urine sample injected on the column with different microemulsion

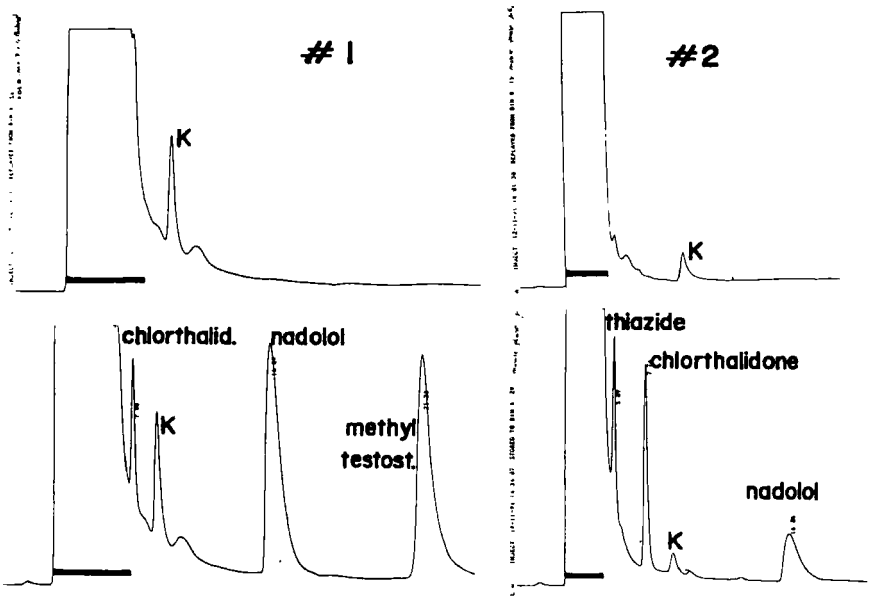

Figure 3 Direct injection of a urine sample after 50% v/v dilution with the mobile phase. Column 25 cm x 4.6 mm id, 5 μm C18 Spheri-5 stationary phase, flow rate 0.5 mL/min, 10 μL injection volume, detection UV 254 nm. Left chromatograms: heptaneless microemulsion #1, pressure drop 184 kg/cm^2, 0.04 a.u.f.s., chlorthalidone 8.5 ppm, nadolol 260 ppm, methyltestosterone 8.5 ppm. Right chromatograms: microemulsion #2, pressure drop 160 kg/cm^2, 0.16 a.u.f.s., thiazide (HCl) 8.5 ppm; chlorthalidone 17 ppm, nadolol 170 ppm. Top: urine blank; bottom: urine spiked with the indicated drugs. The thick lines highlight the protein peak width at base, see text and Table II.

mobile phases. The fresh urine sample was diluted with an equal volume of the microemulsion mobile phase used and directly injected without any filtration or protein precipitation. In all urine samples studied, an important peak was observed close to the dead volume. We suppose it corresponds to UV absorbing proteins or parts of protein material (amino–acids such as histidine, phenylalanine, tryptophan, tyrosine). Peak K, an unidentified compound more retained than proteins, was found in several urine samples. It could be caffeine. A part of the microemulsion diluted urine sample was spiked with known amounts of different drugs, as indicated in the figure captions. The spiked urine samples were also directly injected. No

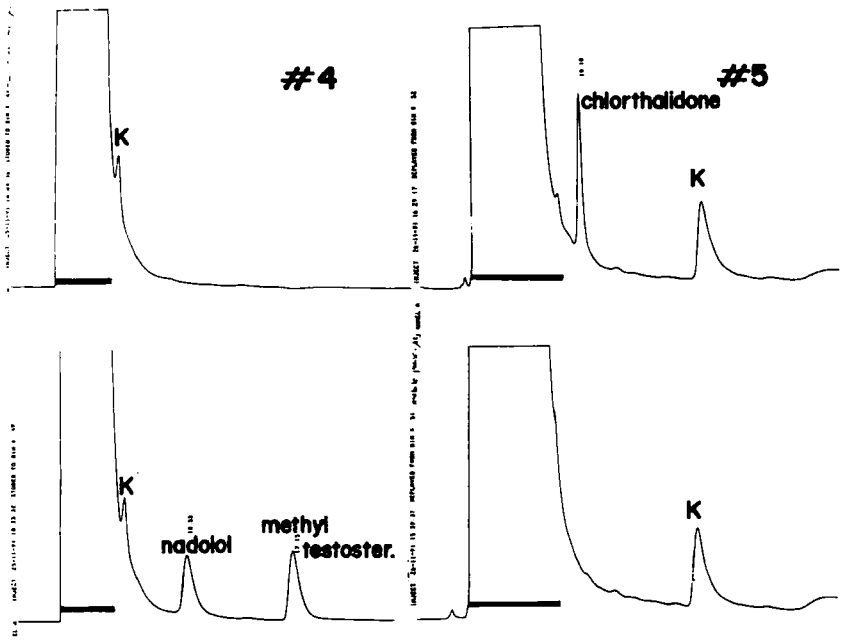

Figure 4 Direct injection of a urine sample. Left chromatograms: microemulsion #4, pressure drop 240 kg/cm^2, 0.16 a.u.f.s., nadolol 250 ppm, methyltestosterone 8.5 ppm. Right chromatograms: microemulsion #5, pressure drop 138 kg/cm^2, 0.04 a.u.f.s., chlorthalidone 24 ppm. All other experimental conditions are listed in Figure 3 caption.

significant change in retention times, peak efficiencies and column pressure drop were observed after 54 direct urine injections.

Figure 3 shows an interesting effect. The small addition of heptane (0.31% w/w) produced a dramatic decrease of protein retention. The protein peak width at base was 4.6 min (2.3 mL) with microemulsion #1 mobile phase (Figure 3, top). It dropped to 2.6 min (1.3 mL) with microemulsion #2 mobile phase (Figure 3, bottom). This was not due to an efficiency change because Peak K efficiency does not change, this is due to a faster elution of proteins. Heptane seems to reduce drastically the protein retention on this C18 bonded phase. The last column of Table II lists the protein peak width at base of the same urine sample eluted on the

same column with the five different mobile phases. It is difficult to predict the protein peak width. Microemulsion #4 has the higher heptane (and active blend) content, the protein peak width was 3.6 min (Figure 4, top). Microemulsion #5, with the lowest organic phase content, but with the same heptane content than Microemulsion #2, produced a broad protein peak (5 min, Figure 4, bottom). A protein peak as sharp as possible is desirable to allow the determination of low k′ drugs (chlorthalidone, Figure 3). The protein peaks obtained with micellar mobile phases (and different urine samples) were much broader, in the 8 mL range, 16 min at 0.5 mL/min [12].

CONCLUSION

MLC cannot pretend to supplant other analytical techniques used for illegal drug screening in sport. However, the use of microemulsion mobile phase can perform some limited drug analysis with acceptable LODs. Direct sample injection is possible. The lowest measurable k′ can be optimized by reducing the protein peak width acting on the microemulsion composition. Only one microemulsion system was studied. The unique selectivity observed with this system may be very different with other systems. The choice of possible microemulsion compositions, with other anionic surfactants, with cationic surfactants or nonionic surfactants, with different oils, hexane, chlorinated solvents, ethers... or different cosurfactants, is so wide that we will not be able to study them all.

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