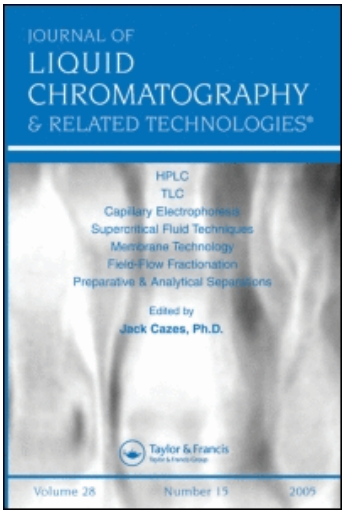

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Determination of Acyclovir in Blood Serum and Plasma by Micellar Liquid Chromatography with Fluorimetric Detection

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DETERMINATION OF ACYCLOVIR IN BLOOD SERUM AND PLASMA BY MICELLAR LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

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ABSTRACT

A method is described for determination of acyclovir (ACV) in blood serum and plasma by micellar liquid chromatography (MLC) on silica. A novel micellar mobile phase containing mixed micelles of sodium dodecylsulfate (SDS) and hexadecyltrimethylammonium bromide (HTMABr) was introduced to MLC and enhanced fluorimetric detection of ACV in acidic media was applied. The method was optimized with respect to detection sensitivity and separation. Addition of SDS to mobile phase caused an increase in the sensitivity of the fluorimetric detection by a factor of five. Additions of isopropanol, tetraethylammonium chloride, triethylammine, Brij 35, and HTMABr to the mobile phase containing SDS were studied. All of them diminished the

positive effect of SDS on the detection sensitivity but the mixture of SDS and HTMABr still yielded in the increase of detection sensitivity by a factor of three compared with the mobile phase without surfactants. Octadecylsilica, nitril, and silica stationary phases were tried out, the last being optimal. The retention of ACV on silica which was too low in a mobile phase containing SDS was shown to increase with additions of HTMABr to the mobile phase. Influences of SDS and HTMABr concentrations, ionic strength, and pH on the relative intensity of fluorescence and the retention of ACV were studied. Under the final conditions (0.050 mol/l SDS, 0.010 mol/l HTMABr, 0.050 mol/l phosphate, pH=2.05) the recovery from serum samples after protein precipitation with perchloric acid was (97.3±3.1)% , the relative standard deviation 3% and the limit of detection 0.8 ng ACV that corresponds to 0.08 μg ACV in 1 ml sample when 10 μl injected.

INTRODUCTION

Acyclovir (9-(2-hydroxyethoxymethyl)guanin, ACV, Fig.1), an acyclic analogue of guanosine, exhibits considerable activity against viruses of the herpes group [1]. Determination of ACV in biological samples has been accomplished by measurments of radioactivity using [8-^{14}C]acyclovir [2], by radioimmunoassay methods [4-6] and by chromatographic methods : HPLC [7-12] and TLC [13,14] the majority of which is reviewed in [15]. Most of the HPLC methods have applied octadecylsilica [7-10] or polymer-based reversed-phase stationary phases [11,12] and photometric detection at 254 or 250 nm. Native fluorescence in acidic media has also been previously adopted in case of nucleosides and their bases and purines [15] and ACV itself [12]. In the latter case, however, the acidic mobile phase (pH=1.8)

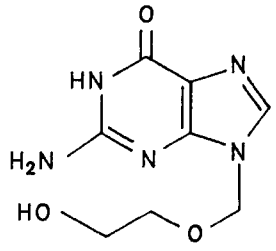

FIGURE 1. Structure of acyclovir.

did not allow the use of octadecylsilica and the use of polyhydroxyethylmethacrylate packing resulted in low retention of ACV.

The fluorescence of acyclovir in acidic media is made possible by the protonation of the molecule [12, 15]. Ionic surfactants are known to shift the protonation equilibria of solutes [16] and, moreover, surfactants are known to enhance the fluorescence of some fluorophores by changing the microenviroment of the solute molecule in the surfactant micelle (higher viscosity, lower relative permitivity) [17]. The increase in fluorescence intensity of some solutes caused by surfactant micelles has been used for determination of critical micelle concentration (CMC) [18-20]. Micelles enhanced fluorimetric detection in HPLC has also been applied [21,22], however, not to the detection of acyclovir. Therefore we have investigated the use of surfactants in order to increase the

fluorescence of ACV so that higher detection sensitivity would be achieved and/or pH of the mobile phase above 2 would be sufficient which would then enable common silica-based stationary phases to be used.

Further, a mobile phase containing both SDS and HTMABr was found to provide a good separation of ACV from serum matrix on silica. Mixtures of anionic and cationic surfactants at comparable ratios are generally considered to precipitate in aqueous solutions due to charge neutralization, however, in excess of one component stable micelles are formed [23]. Thus optimization of the mixed micellar mobile phase composition has been carried out. According to our knowledge, up to now mixed anionic-cationic micellar mobile phases have not been applied in MLC.

MATERIALS AND METHODS

Materials

Sodium dodecylsulfate (SDS), hexadecyltrimethyl-ammonium bromide (HTMABr), Brij 35 (polyoxyethylen(23)-laurylether), tetraethylammonium chloride (TEACl), and perchloric acid were of analytical-reagent grade (E. Merck). Acyclovir (pharmacopeaen quality), triethylammine (TEA) and other chemicals used (RG quality) were obtained from Lachema.

Lyophilized porcine serum Lyonorm U (Lachema) was reconstituted in water for immediate use. Canive plasma

(Beagle dog) was stored at -24 °C and immediately before use was allowed to thaw at ambient temperature and centrifugated at 3000 g (20 min).

Apparatus

The system used was a Hewlett-Packard 1090 chromatograph equipped with a diode-array detector HP 79880A set to monitor the absorbance signal at 256 nm (spectral width 4 nm, reference 596 nm, width 12 nm), a DR 5 binary pumping system, an HP 79846A variable volume injector, an HP 79847A autosampler, and an HP 35900 dual-channel interface. For the system control and data evaluation an HP 79994A workstation based on an HP-310 computer was used. An FS 970 fluorimetric detector (Kratos, Schoeffel Instruments, Trappenkamp, BRD) equipped with a 7-54 prefilter, an excitation monochromator set to 285 nm and an emission cut-off filter 370 nm was used. The photomultiplier gain was set to 6.0 and time constant to 1 s. The fluorimetric detector was connected to the column outlet with the diode-array detector in series.

Determination of Limit of Detection

In a first step, standard deviation of the baseline noise (BN) was determined numerically by a macro program

NOISE.G from chromatograms stored in a digital form. The subjective human factor in the BN determination was limited to the choice of a short (0.5 min typically) part of baseline of blank plasma chromatogram being linear (that means having no other curvature except the noise). This data was then evaluated by the program which uses linear regression and then calculates standard deviation (SD) of residuals ($\bar{y}$-y) along the regression line. The value 5 SD is then calculated which is a good estimation of the noise (mean±2.5 SD is more realistic for short portions of baseline than mean±3 SD) [24].

In a second step, the value 10 SD (equals twice the BN) as the minimal detectable signal in peak height units was multiplied by (peak area/peak height) ratio for the given peak. Thus the minimal detectable signal in area units was obtained which finally after division by the slope of the calibration line (peak area/analyte concentration) yielded the limit of detection in concentration units.

Columns

CGC glass cartriges (3.2x150)mm with Separon SGX (silica), Separon SGX C18 (octadecylsilica) or Separon SGX CN (cyanopropylsilica) of particle size 7 or 5 µm were used (Tessek, Prague, Czechoslovakia).

Void volume was determined by the injection of 10 µl of 1 % fructose solution and using detection at 210 nm.

Mobile Phases

The surfactant was dissolved in phosphoric acid of given concentration and the pH was set by the addition of concentrated sodium hydroxide. When the mixture of SDS and HTMABr was used, a solution of SDS in phosphoric acid was prepared and then at a temperature of 40 °C HTMABr was dissolved using sonication. After cooling down to the ambient temperature of the solution the pH was set to the desired value with concentrated sodium hydroxide.

Sample Preparation

Spiked samples of serum or plasma were prepared by mixing 100 µl of ACV aqueous standard and 900 µl of blank serum or plasma. Then 20 µl of 70 % perchloric acid were added and mixed thoroughly. After cooling in refrigerator at 4 °C for 20 min the samples were centrifugated at 16000 g for 5 min. 10 µl aliquots of the supernatant were injected.

Measurements in Flow Injection Mode

Instead of the column a (0.25x150)mm capillary was installed. The total volume from the injector to the detector was 0.050 ml. 0.1 µl of sample (50 µg/ml) was injected at 30 °C. These measurments were used for relative intensity of fluorescence - mobile phase composition graphs.

Spectrofluorimetry

The measurments were carried out on an Aminco-Bowman spectrofluorimeter (Silver Spring, Maryland) in a quartz cuvette 10x10 mm using the width of the excitation and emission slits of 1 mm. Aqueous ACV solution of 5 µg/ml was used.

The time dependence of the relative intensity of the fluorescence of ACV was tested several times by a quick mixing of a drop of volume 10 µl of ACV solution 500 µg/ml into 990 µl of micellar solution. The excitation monochromator was set to 285 nm and the emission one to 380 nm.

Determination of Dissociation Constant pK_a

The constant pK_a corresponding to the dissociation of proton from the protonated molecule of ACV was determined on a Mettler DL 40 titrator by the titration

of 40 ml of 0.025 mol/l ACV solution (totally $n_{ACV}=10^{-3}$mol) by hydrochloric acid (1.0 mol/l). The determination was carried out in water and in SDS solution (0.050 mol/l and 0.100 mol/l). For each solution the blank values were subtracted in order to obtain the correct volumes of titrant used only for titration of ACV. The moles of titrated (i.e. protonated) ACV n_{ACVH+} were calculated from the volume, concentration and factor of the titrant. The pK_a was calculated according to

$$pK_a = pH - \log\left(\frac{n_{ACV}}{n_{ACVH+}} - 1\right)$$

RESULTS AND DISCUSSION

Fluorescence of ACV in Micellar Media

Emission and excitation spectra of protonized acyclovir were measured in 0.05 mol/l phosphate, pH 2.05, and at the same conditions with SDS and SDS plus HTMABr added (Fig.2.). In the micellar media, the increase of the relative intensity of fluorescence of ACV and shift of the emission maxima by about 15 nm to the short-wavelength region was observed. The latter fact is caused by the effect of the decreased local permitivity inside the micelle upon the fluorescence of ACV [17]. The increase in the relative intensity of

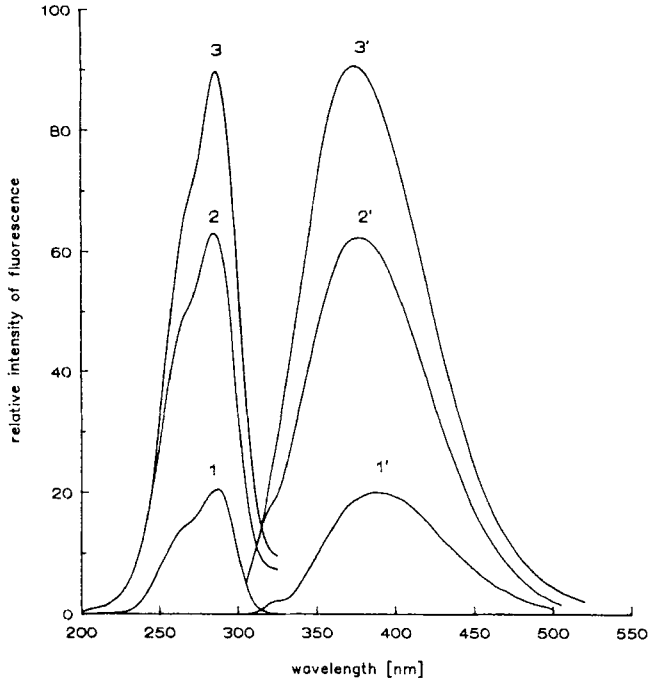

FIGURE 2. Excitation (1-3) and emission (1'-3') spectra of acyclovir in 50 mmol/l phosphate, pH=2.05, without surfactant (1,1'), with SDS addition (2,2'), and with SDS and HTMABr addition (3,3'). For other conditions see Materials and Methods.

fluorescence in micellar media may be also caused by higher viscosity inside the micelles [17] compared to the outside environment, as increased viscosity restricts relaxation motions of the molecules and thus increases the yield of fluorescence. No time dependence or increase of the relative intensity of fluorescence with time after mixing ACV solution and micellar

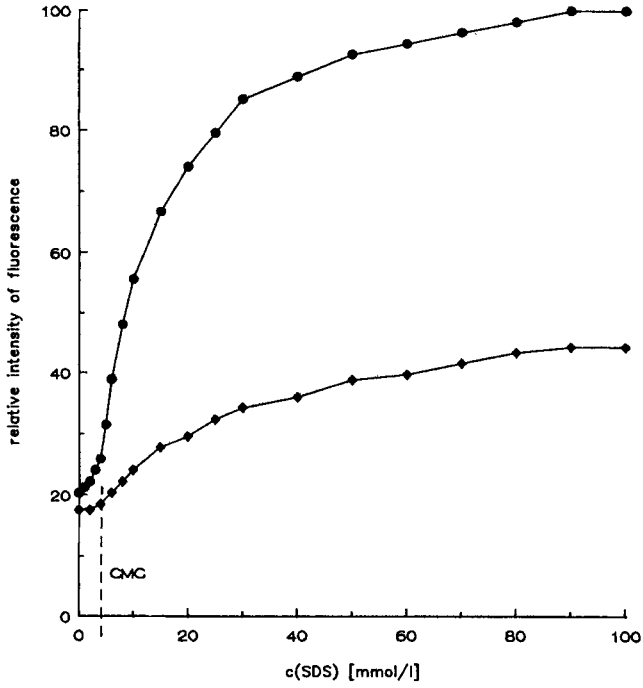

FIGURE 3. Relative intensity of fluorescence of acyalovir as a function of concentration of sodium dodecylsulfate without (●) and with (◆) addition of 5 % isopropanol in a solution of 50 mmol/l phosphate and pH=2.05. For other conditions see Materials and Methods.

solution in the cuvette of the fluorimeter (about 4 s) was observed, as it was in the case of others [19].

A slight increase in the relative intensity of fluorescence with SDS concentration is visible already in the concentration region below the CMC (Fig.3.). This may be caused by the influence of SDS on the dissociation constant pK_a of the protonated ACV [16].

TABLE 1

Dissociation constant pK_a in various media

medium	water	50 mM SDS	100 mM SDS
pK_a ($ACVH^+ = ACV + H^+$)	1.5	1.7	1.9

The constant may increase and so the fraction of protonated ACV increases. This is in agreement with experimentally acquired values of pK_a in water and in presence of SDS .

Generally, the dependence of the relative intensity of fluorescence of ACV on SDS concentration (Fig.3.) has a very similar shape to the curves used for the fluorimetric determination of CMC of surfactants [18-20] and offers a five fold increase in detection sensitivity in the micellar phase. CMC determined from the curve is about 4 mmol/l which corresponds to data from literature with respect to the ionic strength [27].

Because of the need to influence the capacity factor (k') of ACV (on Separon C18 using a mobile phase containing SDS the k' was too high - see Tab.2) and the selectivity of the separation, the influence of additions of other substances and surfactants on the fluorescence of ACV has been studied. In the case of the isopropanol addition, the increase in ACV fluorescence

in micellar media was only two-fold. It can be explained by the changed structure of the micelle [27], and also the influence of the micelles on the pK_a may be less pronounced in the presence of isopropanol. Tetraethylammonium chloride (0.05 mol/l) and triethylammine (0.10 mol/l) have proved to have both a similar effect to that of isopropanol, and could be also explained using the same principles.

The results of additions of Brij 35 or HTMABr to the SDS containing mobile phase are illustrated in Fig.4. Brij 35 clearly interfered and was not studied further. SDS containing micellar mobile phase with HTMABr did not allow as high enhancement of the ACV fluorescence as in the mobile phase without HTMABr. However, there is still a three-fold increase in the relative intensity of fluorescence compared to the solution without surfactants. It may be anticipated that HTMABr can be included with its lipophilic chain into the SDS micelles and does not disturb them as much as the short-chain tetra- or trialkylammonium ions which do not form micelles in aqueous media. The structure of the mixed micelles is known to change with the ratio of the surfactant concentrations [36] that can be the explanation of the shape of the curve in Fig.4. The concentration of HTMABr (0.01 mol/l) used in Fig.4. is above CMC (10^{-4} mol/l in water at ambient temperature)

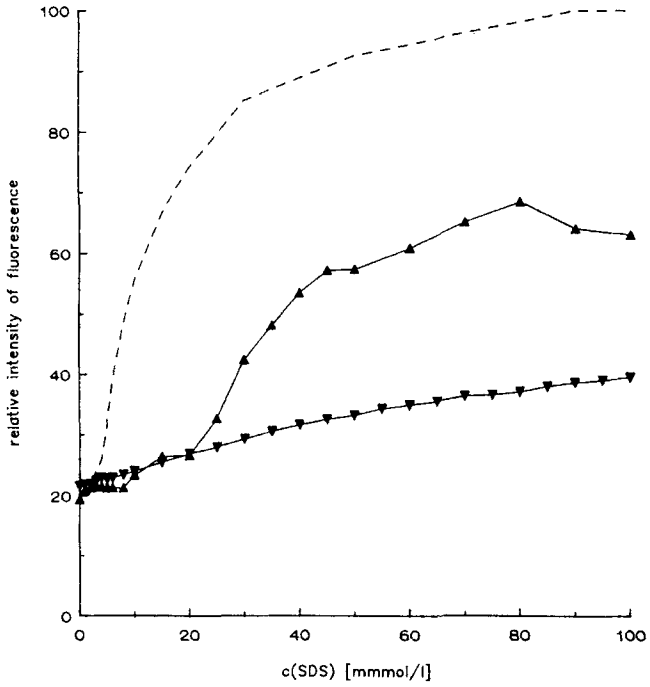

FIGURE 4. Relative intensity of fluorescence of acyclovir as a function of concentration of sodium dodecylsulfate without addition (---) and with addition of Brij 35 (▼) and HTMABr (▲) in a solution of 50 mmol/l phosphate and pH=2.05. For other conditions see Materials and Methods.

[25]. Consequently, mixed SDS-HTMABr micelles carrying mostly a positive charge exist in the solution at a SDS concentration lower than that of HTMABr. At a higher SDS concentrations compared to HTMABr the prevailing charge of the mixed micelles is negative. That may be why the curve raises at SDS concentration higher than 0.1 mol/l, as the protonated ACV molecules can be more easily

included into the micelles becoming mostly negatively charged. As the charge of the micelles is smaller compared to the micelles of SDS, the concentration of the fluorescing protonated ACV is lower and the curve at its optimum (approx. 0.05 mol/l SDS) does not reach the relative intensity of the fluorescence of ACV in SDS solutions without HTMABr. HTMABr alone caused only a slight increase (approx. 1.5x) in the ACV fluorescence compared to the solution without surfactants.

From the experiments carried out it is clear that in the acidic phosphate buffer (0.05 mol/l phosphate, pH 2.05) SDS in concentration at least 0.05 mol/l offers the highest enhancement of ACV fluorescence (5x) and in the presence of additional HTMABr (0.01 mol/l) approx. a 3-fold increase is achieved, whereas the additions of isopropanol, TEACl, TEA, and Brij 35 do not allow a significant increase of ACV fluorescence in micellar media with SDS.

Separations

The key separation experiments are summarized in Tab.2. (conditions in experiment No. 7 were optimized in separate experiments; selectivity was evaluated on the basis of separation of ACV from interfering plasma components).

TABLE 2

Conditions and results of the separation experiments

No.	Stat.Phase	Mob. Phase			Separation	
	Type	Temp. [°C]	Buf.Conc. [mol/l]	Addition [mol/l]	cap.f. k'	selectivity FLUOR.	UV
1	SGX C18	30	0.050	-	17	-	-
2	SGX C18	30	0.100	-	14	-	-
3	SGX C18	30	0.050	TEACl 0.05	10	-	-
4	SGX CN	30	0.050	-	0.8	NO	NO
5	SGX C18	70	0.050	-	6.8	YES	YES
6	SGX	30	0.050	-	0.9	NO	NO
7	SGX	30	0.050	HTMABr 0.01	1.7	YES	NO

Mobile phases all containing 0.050 mol/l SDS at pH=2.05

SDS Containing Mobile Phases

The separation on octadecylsilica in the mobile phase containing SDS resulted in too high retention of ACV (Tab.2. No.1.). The fact that the ACV retention was decreasing with increasing phosphate concentration at a constant pH gives the evidence that it is the result of the interaction of protonated ACV molecules with the anionic SDS sorbed in the stationary phase according to ion-exchange or ion-pair mechanism [25]. To lower the ACV retention it is necessary to increase the ionic strength, or to decrease concentration of SDS in the stationary phase.

In the mobile phase containing 0.10 mol/l phosphate (pH 2.05) the capacity factor of ACV was still too high (k'=14, see Tab.2 No.2.). Further increase of the

phosphate concentration would be harmful for the chromatographic hardware and the stationary phase. That is why additions of TEACl and TEA were tried out as these cations could block the anions SDS better than Na^+ ions due to additional hydrophobic interactions [26]. TAECl (Tab.2 No.3.) was effective in decreasing the ACV retention (k'=10), however, its effect on the relative intensity of fluorescence (see Fluorescence of ACV in Micellar Media) excluded TEACl from further use. TEA had a similar effect.

Decrease of the SDS concentration in the stationary phase can be reached by lowering the hydrophobicity of the stationary phase, i.e. use of shorter alkyl or lower carbon content in the alkylsilica. The nitril stationary phase (cyanopropylsilica) gave too low ACV retention (k'=0.8, see Tab.2, No.4.) and the selectivity of separation was not sufficient.

A further way of decreasing the SDS concentration in the stationary phase is an addition of organic modifier to the micellar mobile phase [27]. However, an addition of 5 % isopropanol had a negative influence on ACV fluorescence (see Fluorescence of ACV in Micellar Media) and could not be used further.

Another way of decreasing the SDS concentration in the stationary phase is the increase of separation temperature [28]. On octadecylsilica at 70 °C the ACV

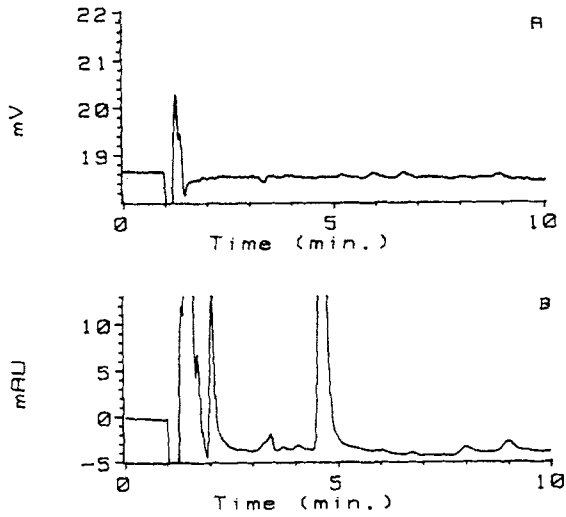

FIGURE 5. Chromatogram of blank plasma registered with fluorimetric (A) and photometric detection (B). Column, Separon SGX C18 (70 °C); eluent, 0.050 mol/l SDS in 0.050 mol/l phosphate at pH=2.05. For other conditions see Materials and Methods.

retention decreased to an acceptable value (k'=6.8, see Tab.2, No.5.). At these conditions the separation efficiency was 4400 theoretical plates. Chromatograms of blank (Fig.5.) and spiked (Fig.6.) serum samples after protein precipitation (see Materials and Methods) show good selectivity with both fluorimetric and UV-photometric detection. Stability of silica based reversed-phase sorbents in acidic aqueous media typically worsen with decreasing pH and increasing temperature. For that reason the separation temperature

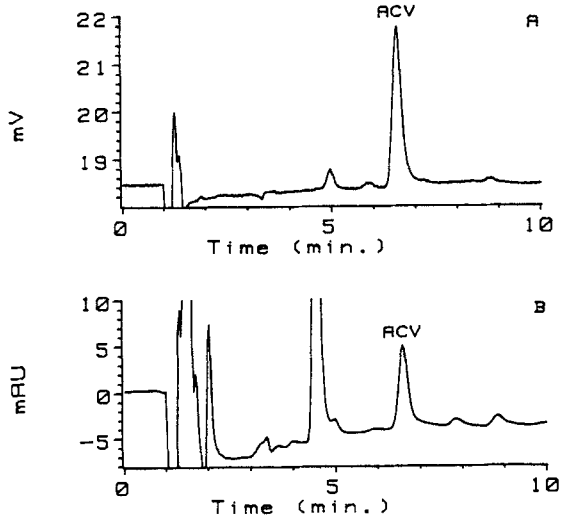

FIGURE 6. Chromatogram of plasma spiked with 5.0 µg/ml ACV registered with fluorimetric (A) and photometric detection (B). Conditions as in Fig.5.

of 70 °C and at the same time mobile phase pH 2.05 is a risk to the long-term stability of the column.

Mixed Micellar Mobile Phases

As the optimized separation on octadecylsilica in SDS containing mobile phase led to high retentions of ACV and the separation on cyanopropylsilica in the same mobile phase gave too low retention of ACV, it was desirable to try out a silica stationary phase. However, the retention of ACV on silica in acidic mobile phase with SDS (see Tab.2, No.6.) is too low to yield the

necessary selectivity of separation of ACV from the interfering plasma components.

Alkylammines (alkylammonium ions) and especially those with a lipophile alkane chain are well known to be sorbed on silica from aqueous solutions under obvious employment of ion interactions with the silanol groups of the silica [29-31]. When a lipophile anion is added, it interacts with the alkylamine (alkylammonium ion) sorbed on the silica. If the lipophile anion concentration compared to the lipophile amine (ammonium ion) is high enough, a dynamically modified silica with prevailing cation-exchange character is obtained [32-34].

This effect causes an increase of ACV retention on silica (Fig.7.-9.). The act of the most influential parameter - pH upon the relative intensity of fluorescence of ACV in 50 and 100 mmol/l SDS solutions is in Fig.7. The shape of the capacity factor dependence can be explained by protonation of ACV which lowers the hydrophobic interaction with the dynamically generated stationary phase, and by the decrease in the sorption of HTMABr with decreasing pH as described by others [35]. The SDS can be expected to act as an eluent and influence the partition of HTMABr between the stationary and the mobile phase in favour of the later one. Consequently, decreased concentration in the stationary

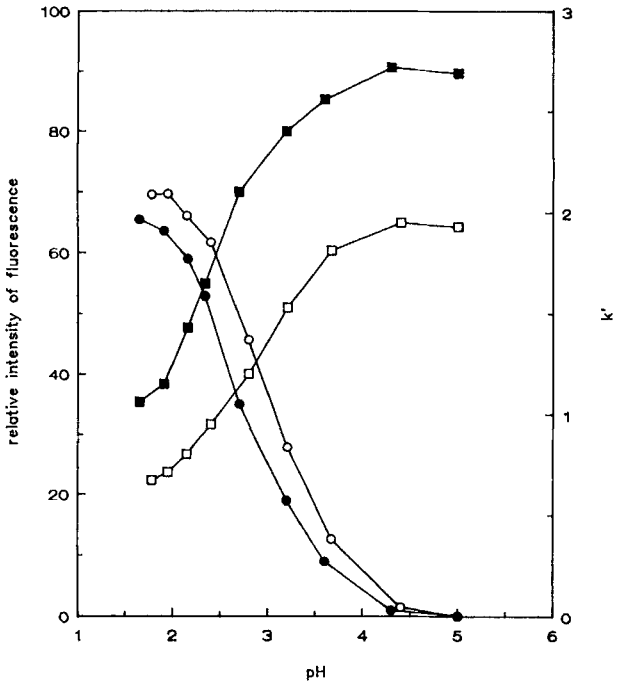

FIGURE 7. Relative intensity of fluorescence (● and ○) and capacity factor (■ and □) of ACV as a function of pH in SDS 50 mmol/l (● and ■) and 100 mmol/l (○ and □), 50 mmol/l phosphate, and 5 mmol/l HTMABr . For other conditions see Materials and Methods.

phase allows less SDS to be sorbed. In this way the negative effect of SDS concentration on ACV retention can be explained. Comparing the two curves in Fig. 7, the higher concentration of SDS (0.100 mol/l) would not allow to reach sufficient ACV retention at low pH so 0.050 mol/l SDS was further used. The pH 2.05 was chosen as optimal because both acceptable ACV retention and detection sensitivity were achieved. The influence of

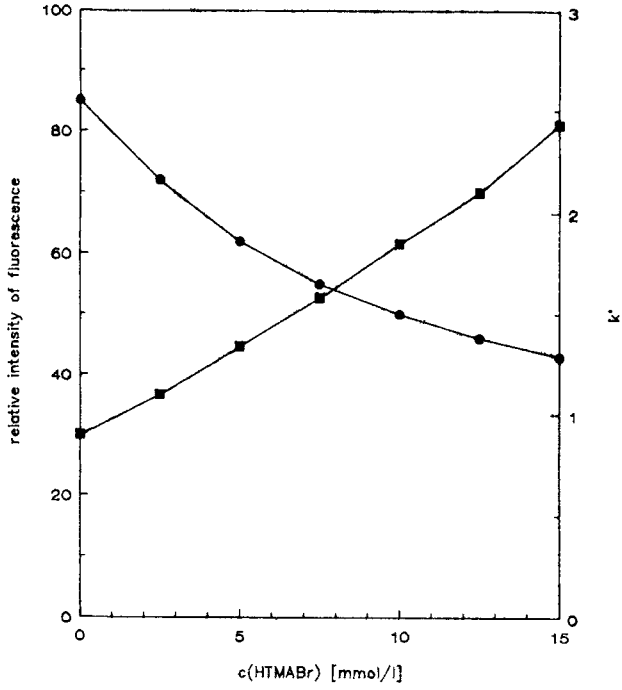

FIGURE 8. Relative intensity of fluorescence (●) and capacity factor (■) of ACV as a function of HTMABr concentration in 50 mmol/l SDS, 50 mmol/l phosphate, and pH=2.05. For other conditions see Materials and Methods.

concentration of HTMABr and phosphate is documented in Fig.8. and 9. In contrast to the octadecylsilica dynamically coated with SDS, here the ACV retention increased with ionic strength (Fig.9.). This may be caused by the more pronounced salting-out effect due to the partial compensation of the opposite charges of SDS and HTMABr which results in an increase of the surfactant concentrations in the stationary phase. Unfortunately, all parameters - SDS concentration, pH,

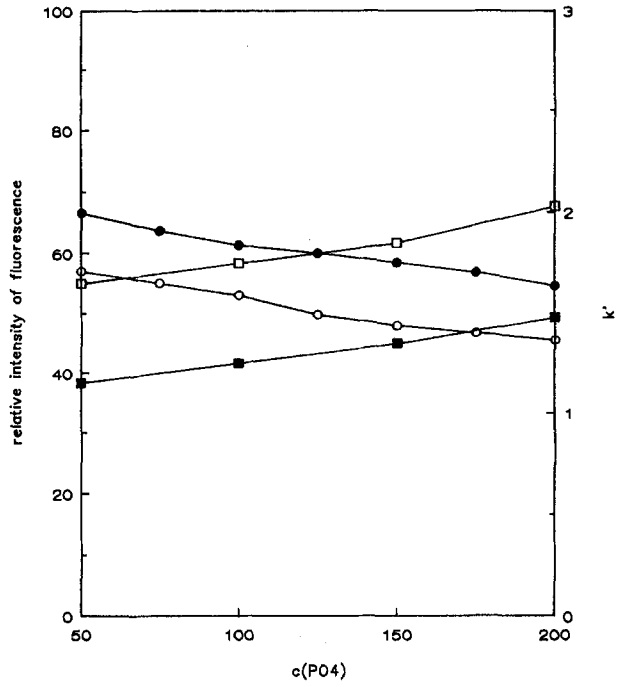

FIGURE 9. Relative intensity of fluorescence (● and ○) and capacity factor (■ and □) of ACV as a function of phosphate concentration in 50 mmol/l SDS, HTMABr 5 mmol/l (● and ■) and 10 mmol/l (○ and □), and pH=2.05. For other conditions see Materials and Methods.

HTMABr concentration, and phosphate concentration are controversial in their effects on ACV retention and detection sensitivity and consequently their optimal choice is not easy.

The increase in the ACV capacity factor when changing the composition from 5 to 10 mmol/l HTMABr is higher than the increase when switching from 50 to 200 mmol/l phosphate. On the contrary, the decrease in the

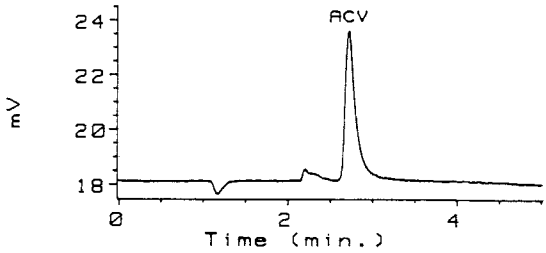

FIGURE 10. Chromatogram of aqueous solution of ACV (5.0 μg/ml). Column, Separon SGX (30 °C); eluent, 0.050 mol/l SDS and 0.010 mol/l HTMABr in 0.050 mol/l phosphate, pH=2.05 .For other conditions see Materials and Methods.

relative intensity of ACV fluorescence is not so rapid with the HTMABr concentration increase as with the phosphate concentration increase. Therefore 10 mmol/l HTMABr and 50 mmol/l phosphate (SDS being 50 mmol/l and pH 2.05) have been chosen as final conditions. Registered at the final conditions, chromatograms of a standard ACV solution, blank serum, and serum spiked with ACV are in Fig. 10 - 12 ; similar chromatograms were also obtained using canive plasma as a matrix instead of porcine serum. Using this separation system, only the fluorimetric detection offered acceptable selectivity. The recovery obtained from serum samples after protein precipitation with perchloric acid was (97.8±3.1)% (c=5 μg/ml, n=6, α=0.05), the relative standard deviation of the determination was 3 % and the limit of detection was determined 0.8 ng of ACV that

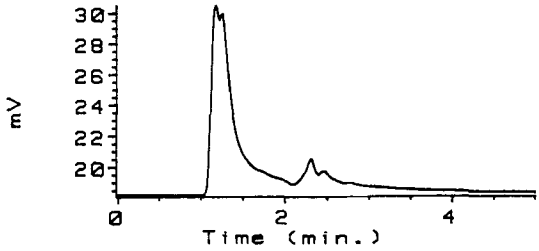

FIGURE 11. Chromatogram of blank serum. Conditions as in Fig.10.

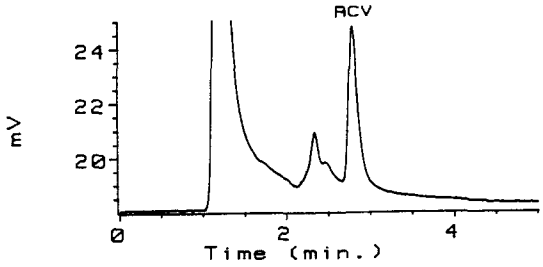

FIGURE 12. Chromatogram of serum spiked with 5.0 µg/ml ACV. Conditions as in Fig.10.

corresponds to 0.08 µg of ACV in 1 ml sample when 10 µl are injected.

CONCLUSIONS

The method of ACV determination in serum and plasma on silica using mixed micellar mobile phase containing SDS and HTMABr has been presented.

A significant increase in the sensitivity of fluorimetric detection can be achieved in micellar media [17-22]. Up to five-fold increase in the sensitivity of

the fluorimetric detection of ACV in an acidic mobile phase results from a SDS addition. In the mobile phase optimized for separation on silica, i.e. containing SDS and HTMABr, a three-fold increase in detection sensitivity is achieved.

The choice of a stationary phase and the optimization of a mobile phase composition with respect to the separation resulted in silica and an acidic mobile phase containing HTMABr and a five-fold excess of SDS (0.010 mol/l HTMABr and 0.050 mol/l SDS in 0.050 mol/l phosphate pH=2.05) as the optimal conditions. The increased retention of ACV on silica after an addition of HTMABr to the mobile phase with SDS is obviously caused by the interaction of the cationic HTMABr with residual silanol groups of silica and secondary increased sorption of SDS [29-34].

Generally, the optimal conditions for the micelles enhanced fluorimetric detection and for the separation may differ considerably, so that optimization with respect to both detection and separation may be impossible.

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