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Application of capillaries with minimized electroosmotic flow to the electrokinetic study of acidic drug–β-oleoyl-γ-palmitoyl-L-α-phosphatidyl choline liposome interactions

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Abstract

Interaction of a model set of common drugs varying widely in their polarity as well as in their chemical structure (salicylic acid, acetylsalicylic acid, ketoprofen, phenytoin and propranolol) with β-oleoyl-γ-palmitoyl-L-α-phosphatidyl choline (POPC) liposomes was investigated by means of capillary electrophoresis. Two phosphate buffers differing in their pH (50 m*M*, pH 7.5 and 9.2) were used both for liposome reconstitution and as background electrolytes for capillary electrophoresis using capillaries with minimised electroosmotic flow (EOF). The liposomes showed practically no electrophoretic mobility and formed a stable plug in the capillary. At alkaline pH (9.2), the polyimide coated capillary exhibited residual endoosmotic flow (the EOF marker appeared before the detection window around 40 min as compared to 2.2 min in the untreated capillary; attempts to reveal endoosmotic flow at pH 7.5 were unsuccessful). The concentration of the mixture of the test compounds was 50 μg/ml (except for ketoprofen concentration of which was 5 μg/ml due to the lower solubility of the drug), i.e. large enough to exceed the binding capacity of the injected liposome plug at least at the neutral pH (7.5) which consequently resulted in two regions in the electropherogram, namely that which contained the unbound species and that corresponding to the liposome (lipid)-bound fraction. On the other hand in runs done at high pH of the background electrolyte (9.2) the whole amount injected interacted with the liposomes. Acidic drugs and phenytoin were run with negative polarity at the injection site. It was documented that both at pH 7.5 and 9.2 the investigated solutes interacted with POPC liposomes, though at pH 7.5 the equilibrium between the bound and unbound drugs was in favor of the unbound species. On the contrary, at pH 9.2 binding was considerably stronger and only the liposome bound fraction was seen upon electrophoresis. The well-known instability of phenytoin at room temperature resulted in the formation of an acidic hydrolytic product which was strongly bound to liposomes at the higher pH value. While no binding of phenytoin could be established at pH 7.5, at pH 9.2 this compound was degraded (hydrolyzed) and its degradation product was clearly bound to liposomes. It has to be emphasized that binding experiments must be done separately for acidic/neutral and basic drugs; binding of acidic/neutral drugs must be done at reversed polarity, while in order to reveal binding of basic drugs, positive polarity at the injection site must be used.

Keywords: Oleoylpalmitoylphosphatidyl choline; Liposomes; Salicylic acid; Acetylsalicylic acid; Ketoprofen; Phenytoin; Proprandol

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1. Introduction

Micellar electrokinetic chromatography and later on microemulsion electrokinetic chromatography have been exploited for the separation of a vast number of compounds including drugs ([1–4], for a survey of applications, see Ref. [5]). Sodium dodecyl sulfate appears the most popular pseudophase used, though a number of other surfactants have been tested over the years [6–8]. Besides the synthetic surfactants, it appears quite attractive to investigate the properties of both naturally occurring and synthetic lipid bodies, the liposomes. As these are considered an adequate model of the cell membrane, incorporation of a drug into these particles can offer an information of how easy or how difficult it would be for a compound to penetrate into the cell [9,10]. In other words the idea of the liposome based (micellar) electrokinetic chromatography is not aimed at better separation of e.g. biologically active compounds and their contaminants but preferably towards revealing the chemical/physical affinity to predict drug permeability [9]. The way for calculating, elaborated in Ref. [9], the $\Delta(\Delta G^{\circ})$ values was used throughout this study using the same reference standard (acetylsalicylic acid).

Over the years a number of papers regarding liposomes as carriers in capillary electrokinetic chromatography have appeared [11–14]. The first reports were studying the properties of vesicles formed from an anionic–cationic surfactant mixture (sodium dodecyl sulfate–cetyl trimethylammonium bromide). The liposome-like pseudophases offered better resolution for alkylphenones, for example [15]. Recently particularly phospholipids capable of spontaneous vesicle formation gained interest as carriers of drug molecules [12]. The main mechanism involved in the association of different analytes (drugs, nucleic acids and their fragments) with the phospholipids containing liposomes appears primarily an electrostatic interaction between the negatively charged polar head of the liposome constituting lipid moiety though additional hydrophobic interaction appear to be involved as well [12].

It is quite understandable that the capability of the liposomes to interact with an analyte would depend on the lipids involved in the pseudophase formation. This aspect has been studied in detail in the quoted paper by Wiedmer et al. [12]. A number of negatively charged liposomes composed of zwitterionic and anionic lipids were prepared and the role of the lipid molar ratio, the role of the lipid head group and the background electrolyte composition was investigated. Data regarding the physical state of the liposome (gel vs. fluid) were also available presented. Particular attention has been paid to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC); multilamellar vesicles served as pseudophases. Because their physico-chemical properties depend considerably on their size, the authors of this study [12] investigated both the multi- and small unilamellar vesicles with the result that only the latter are suitable as pseudophase constituents in micellar electrokinetic chromatography. Another report of the same working group [16] brought about additional details about the composition of the phospholipid pseudophase, in particular with respect to the presence of phosphatidyl serine, phosphatidyl glycerol, phosphatidic acid and cardiolipin.

It is, perhaps, worth mentioning that the phospholipids (liposomes) based pseudophases can be studied not only from the viewpoint of their interaction with small molecular mass analytes (typically drugs) but they can serve as a model system for studying phosphatidylinositol anchored proteins or protein adsorption as well [17,18].

2. Materials and methods

A Hewlett-Packard ^{30}CE instrument equipped with HP ChemStation Rev. A.06.03 (509) was used throughout the experimental part of this work. If not specified otherwise the experiments were carried out with negative polarity at the inlet side of the capillary. All buffer components were purchased from Merck (Darmstadt, Germany) and were of the p.a. or highest purity available. Model drugs were obtained from Sigma–Aldrich (Steinheim, Germany). POPC of declared 99% purity was obtained from Sigma–Aldrich (Steinheim, Germany) and used for the preparation of liposome suspension without further treatment; the commercially available POPC was diluted with 50 m*M* phosphate buffer (pH 7.5 or 9.2 as needed) to yield a liposome concentration of

3.25 m*M* and sonicated in a water bath at 4 W for 20 min. The suspension was filtered (0.1 μm inorganic membrane filter, Anotop 10, Whatman, Maidstone, UK) and the suspension thus obtained was directly used for injection into the capillary. Sonication was repeated before every filling of the suspension, however, at a reduced time (10 min). Liposome dispersion was introduced into the capillary by using 50 mbar overpressure for 90 s.

Polyimide modified capillaries [30 cm (19.5 cm to the detector, 50 μm I.D.)] with minimized endoosmotic flow (EOF) were purchased from MicroSolv Technology (Eatontown, NJ, USA). Before use they were rinsed with water (5 min) and run buffer (15 min). In-between the runs the capillary was washed 4 min with the run buffer only. Comparative experiments were done with uncoated capillaries of the same dimensions purchased from Composite Metal Services (Hallow, UK).

All experiments were run at 10 kV, negative polarity at the inlet and except for comparative runs in which positive polarity was used.

Individual peaks in the electropherograms were identified by running spiked samples of the parent mixture. A picture of the liposome suspension used for the plug injection was obtained by the environmental scanning electron microscope XL30ESEM (Philips) in low vacuum mode.

3. Results and discussion

The comparative separations of the test set of drugs were done using standard capillary electrophoresis at pH 7.5 with 50 m*M* phosphate buffer as specified in Section 2. The elution sequence was propranolol, EOF, phenytoin, ketoprofen, acetylsalicylic acid and salicylic acid (which was present as degradation product of acetylsalicylic acid) (Fig. 1). In these experiments standard polarity orientation with the injection site at the anode was used.

In the next stage a polyimide-coated capillary was used into which (after the preliminary tests) a plug of liposome suspension was introduced. These experi-

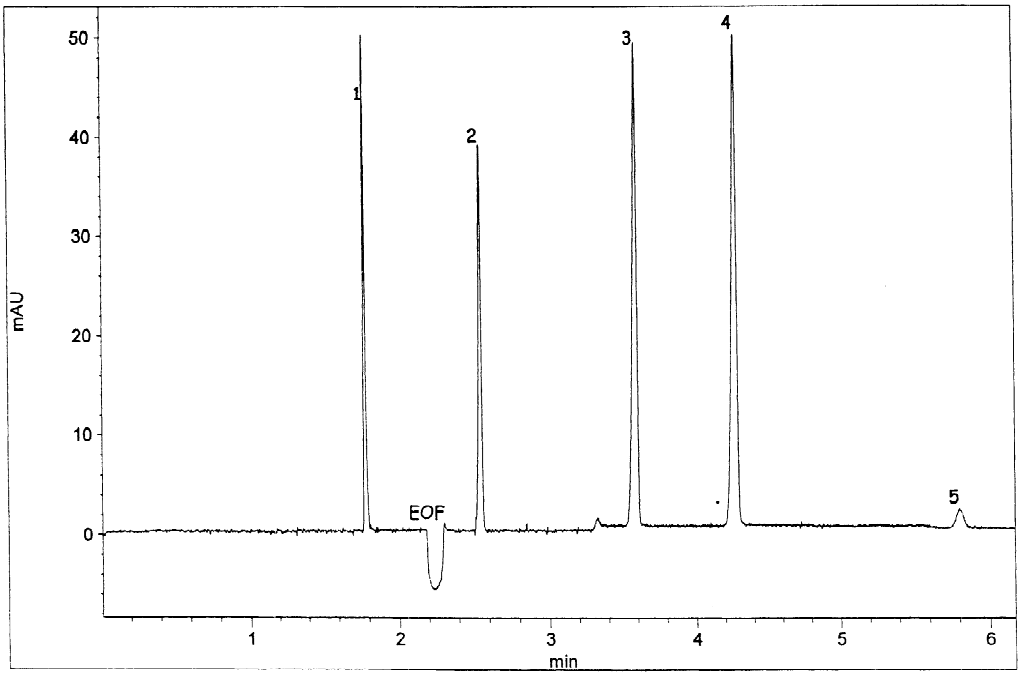

Fig. 1. Capillary electrophoresis of the test solutes in an unmodified fused-silica capillary: 30 cm (19.5 cm to the detector×50 μm I.D. UV detection at 200 nm. Peak identification: 1=propranolol, 2=phenytoin, 3=ketoprofen, 4=acetylsalicylic acid, 5=salicylic acid. Positive polarity (10 kV) at the injection site. Background electrolyte: 50 mol/l phosphate, pH 7.5.

ments have to be done separately for (i) the acidic drugs and phenytoin and (ii) propranolol. Propranolol as a basic drug moved ahead of the endoosmotic flow and, therefore, could not be assayed, while all the other drugs moved anodically and, consequently, had to be studied at the reversed polarity (cathode at injection site). There were two facts that had to be respected at this preliminary stage. First, the polyimide-coated capillary still possessed a residual endoosmotic flow, which at pH 9.2 brought the EOF marker before the detector's window after about 40 min running time (as compared to 2.2–2.3 min in the untreated capillary). Second, phenytoin was found to be subjected at room temperature to hydrolysis yielding (after 1 day storage) a fast anodically moving product which moved closely to the ketoprofen peak. The elution order of test drugs in polyimide coated capillary (no liposomes involved) is shown in Fig. 2. The separation resulting after the introduction of the liposome suspension is demonstrated in Fig. 3. The amount of the injected drugs was large enough to be divided in two categories: (i) the portion which interacted with the liposomes containing plug and (ii) the portion of the unbound drug. The sequence (salicylic acid, acetylsalicylic acid, ketoprofen and phenytoin) as well as the respective run times of the unattached portion of the drugs tested remained unaffected by the presence of the liposome plug (compare Figs. 2 and 3). The interaction of propranolol could not be assessed in this set of experiments as this one being a basic drug moved in the opposite direction (data not shown). The two peaks at 26.0 and 28.4 min running time refer to bound salicylic and acetylsalicylic acid, respectively. The bound portion of ketoprofen could not be distinguished from unbound ketoprofen. The bound portion of ketoprofen appears to be hidden in the tailing part of the ketoprofen peak. Binding of phenytoin to the liposomes could not be assessed as both in the presence and or absence of liposomes only the peak of the unbound drug was observed. However, the product of phenytoin hydrolysis was bound and emerged at 14.8 min run time. (The results are documented in Fig. 4a–c).

The suspension of liposomes used for the experiments was quite homogenous when freshly prepared

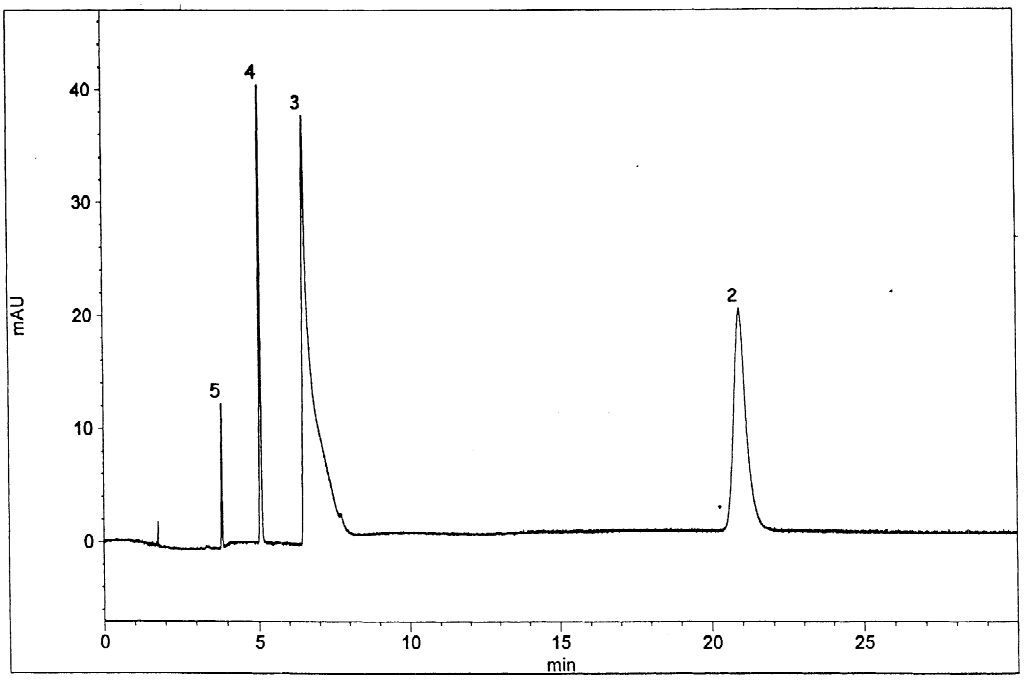

Fig. 2. Capillary electrophoresis of the test mixture in a polyimide coated capillary: 30 cm (19.5 cm to the detector×50 μm I.D. UV detection at 200 nm. Peak identification as in Fig. 1. Negative polarity (10 kV) at the injection site. See also Fig. 3A and B in Ref. [9].

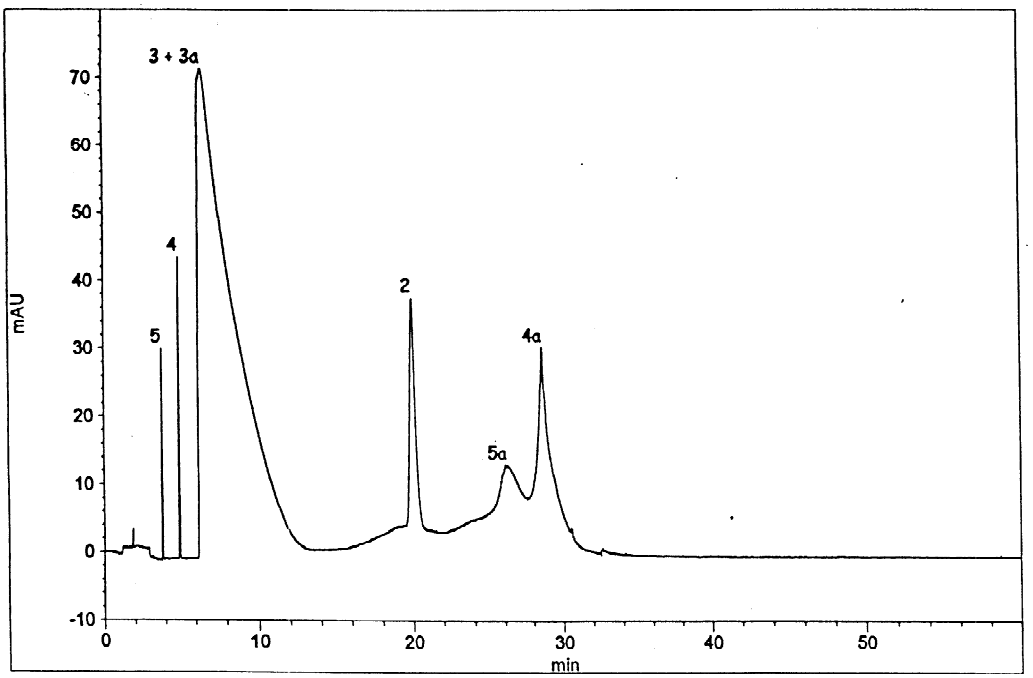

Fig. 3. Capillary electrophoresis as specified in Fig. 2 except that 90-s injection plug of liposome suspension (50 mbar overpressure) was introduced in the capillary. Peak identification as in Fig. 1; 5a=liposome bound fraction of salicylic acid, 4a=liposome bound fraction of acetylsalicylic acid, 3a=liposome bound fraction of ketoprofen. Negative polarity (10 kV) at the injection site. The presented electropherograms have their counterparts in Fig. 3A and B published in Ref. [9].

as seen on the electromicrograph in Fig. 5. However, if the suspension was not freshly prepared daily, the liposomes showed an aging phenomenon resulting in a decrease of their binding ability (data not shown). Upon storage partial precipitation which was difficult to abolish by sonication was apparent. It is feasible to assume that after passing the aged suspension through the 0.1-μm filter less of the liposomes passed through and the amount (concentration) of the liposome particles in the liposome plug inserted in the capillary was decreased. Consequently, also the binding of the liposome plug for individual drugs tested was lowered.

If the pH of the background electrolyte is increased to 9.2 the order of elution of the test mixture in polyimine treated capillary (no liposomes present) was acetylsalicylic acid, salicylic acid, phenytoin and ketoprofen (Fig. 6). At this pH acetylsalicylic acid eluted before salicylic acid (compare the elution order at pH 7.5, see Fig. 2). After inserting the liposome plug the elution order of the test analytes remained the same, however the running times were increased by 1.0–3.0 min, the largest increase concerning the ketoprofen peak (All other conditions remained the same as with the experiments done at pH 7.5, the whole drug sample was bound to the liposomes) (Fig. 7). While at pH 7.5 the differences in the run time between the bound and unbound fractions were quite large with the salicylic and acetylsalicylic acid (over 22.2 and 25.6 min, respectively) and very small with ketoprofen (with which the bound and unbound part made a single tailing peak, see Fig. 3), at pH 9.2 complexation with liposomes increased the run time by 1–3 min (Table 1). This increase can be ascribed to the interaction of individual solutes with the liposomes, as the endo-osmotic flow remained unaltered after the insertion of the liposome plug (40.2–40.4 min).

At pH 9.2, no peaks of unbound drugs were observed. All compounds investigated (except propranolol, which was not investigated further) interacted with the liposome plug. In order to obtain

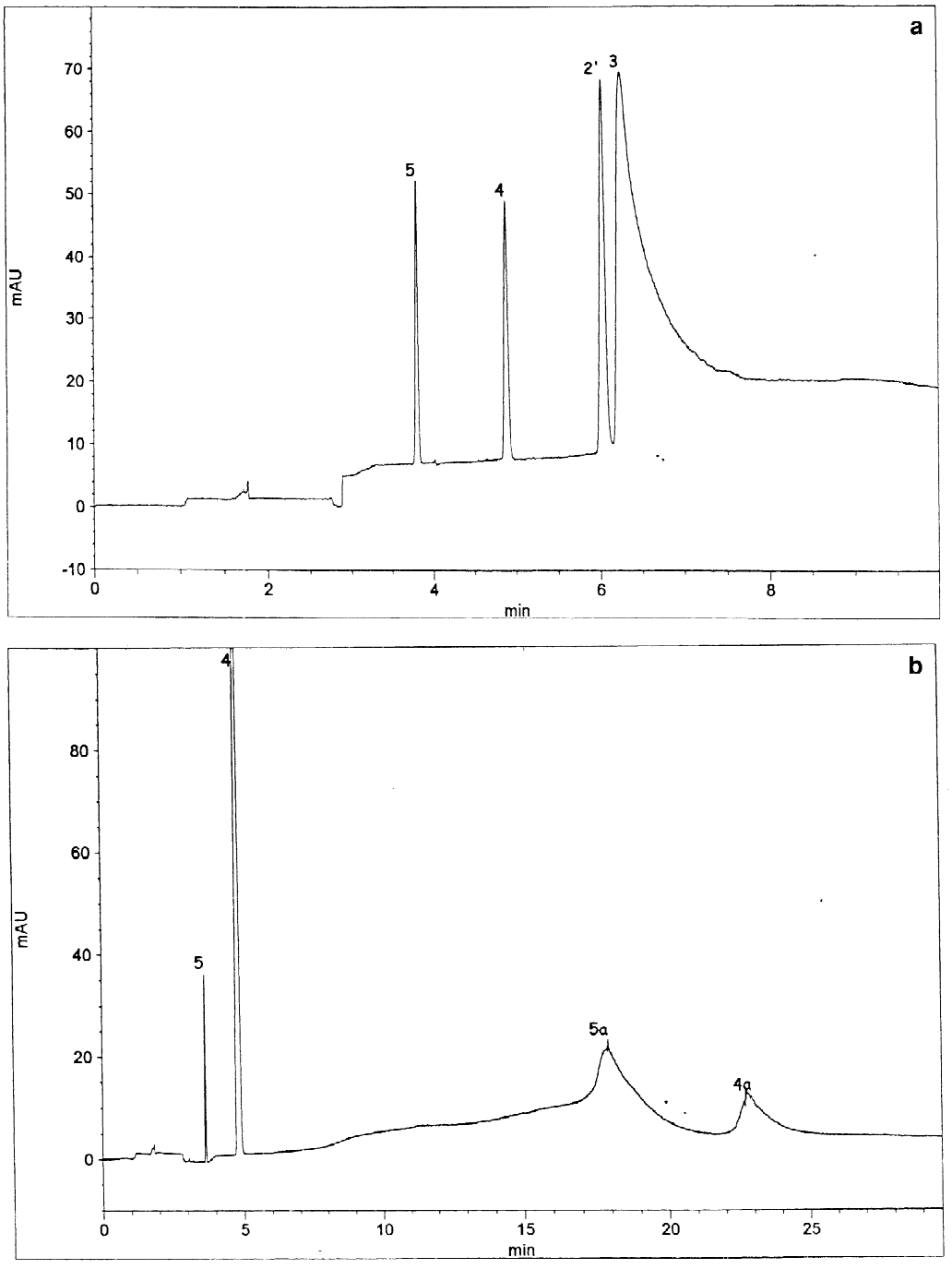

Fig. 4. (a) Capillary electrophoresis in polyimide coated capillary as in Fig. 3; negative polarity (i.e. cathode at the injection point, 10 kV); 90-s injection plug of liposome suspension. Peak identification: 2′=phenytoin degradation product; 3=ketoprofen, 4=acetylsalicylic acid; 5=salicylic acid (the unbound drugs section of the electropherogram). (b) Acetic and acetylsalicylic acid. Electrophoresis under the conditions specified in 4a (negative polarity, cathode at the injection point). Peak identification: 4=acetylsalicylic acid (unbound), 5=salicylic acid, 4a=liposome bound fraction of acetylsalicylic acid; 5a=liposome bound fraction of salicylic acid. (c) Identification of the phenytoin degradation product 2′=phenytoin degradation product unbound, 2′a=bound phenytoin degradation product.

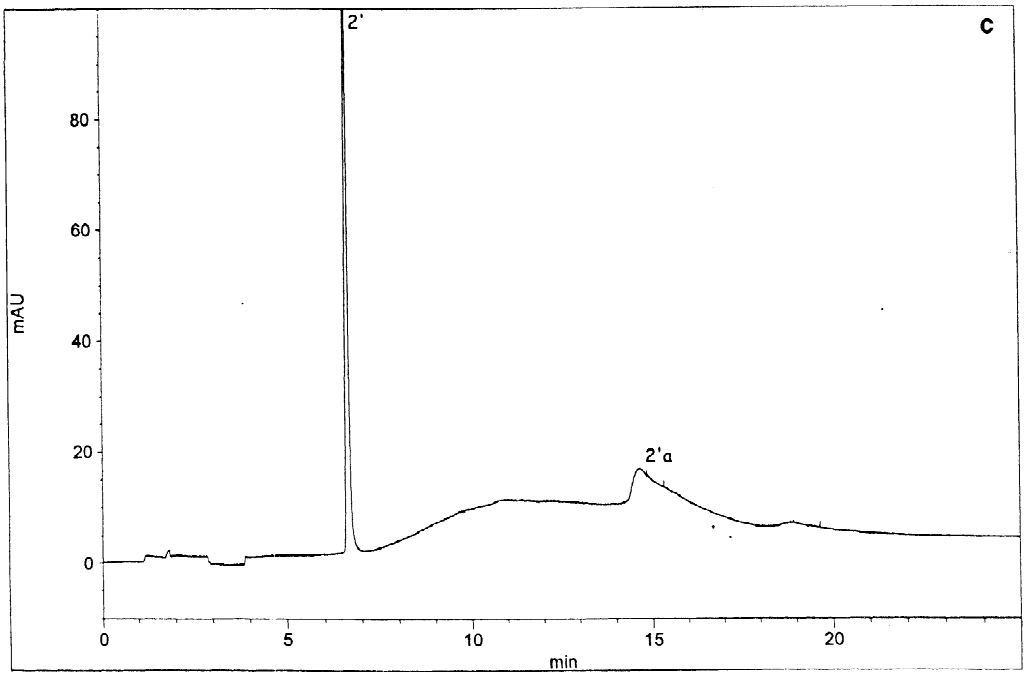

Fig. 4. (*continued*)

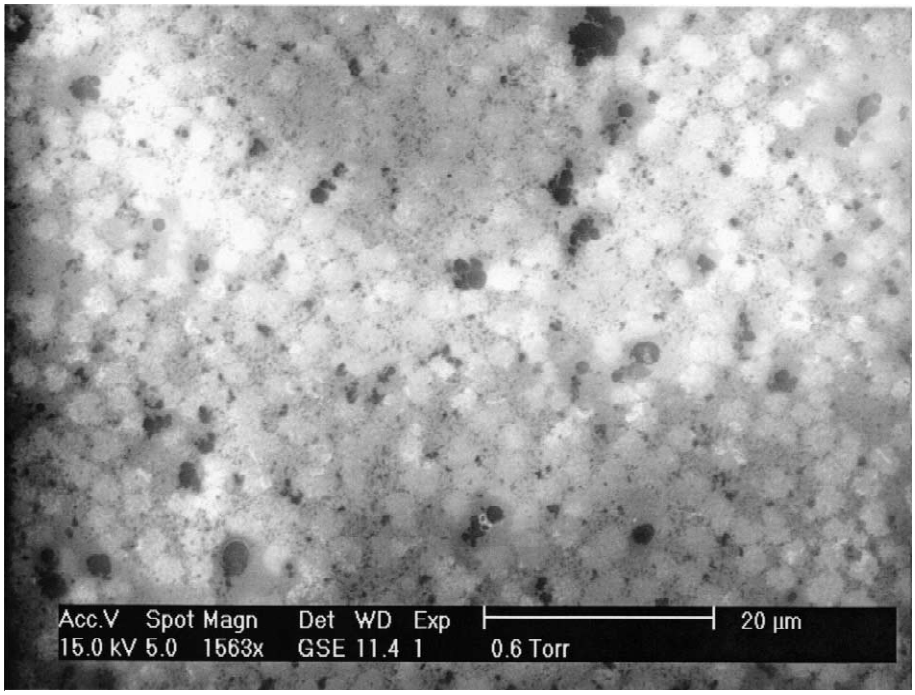

Fig. 5. Picture of the liposome suspension used for the plug injection was obtained with the scanning electron microscope (low vacuum). Magnification: ≈1600×; individual liposomes which passed the 1-μm filter are 1.78–2.86 μm in diameter.

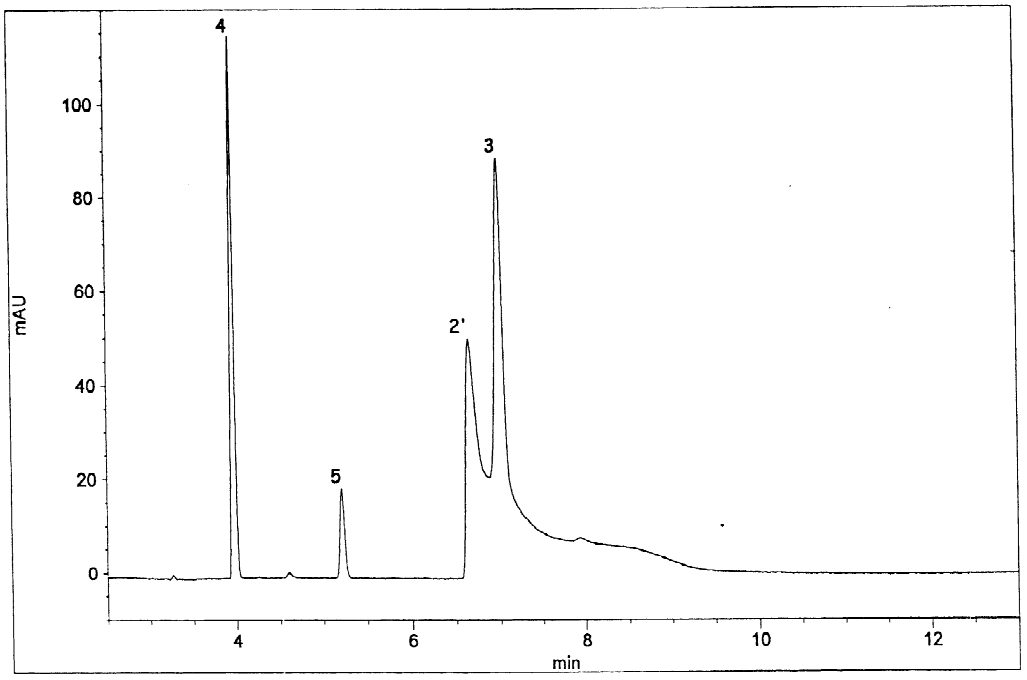

Fig. 6. Electrophoretic separation in phosphate buffer pH 9.2; conditions and peak identification the same as in Fig. 2a (2′=phenytoin degradation product; 3=ketoprofen; 4=acetylsalicylic acid; 5=salicylic acid). No liposomes present.

analogous data for propranolol (a basic drug), the polarity of the system should be reversed (all the above described experiments were carried out with negative polarity at the inlet side). Phenytoin was readily degraded to its hydrolysis product, which moved both in the presence and absence of liposomes ahead of the ketoprofen peak.

When comparing the data presented with those published by Zhang et al. [9] several conclusions can be drawn. The $\Delta(\Delta G^{\circ})$ for salicylic acid (if acetylsalicylic acid is taken as the reference compound) is by 0.2 kJ/mol lower (about 17%). This can be ascribed to the fact that in our case the system possessed an endoosmotic flow which, being rather small, is not accessible to accurate measurement. Of course, the reported value was obtained at pH 7.5 while that reported in the present communication was obtained at pH 9.2. At a pH closer to the experiments done by Zhang et al. [9] we got a binding close to the acetylsalicylic acid, i.e. much lower than that obtained in the quoted paper which probably reflects the difference in the nature of liposomes used and/or the residual endoosmotic flow of the capillary. In spite of all efforts we were unable to find commercially available capillaries that would be completely devoid of endoosmotic flow. It should be emphasized that no difference in the residual endoosmotic flow was observed at pH 9.2 whether the liposome plug was present or not. Consequently in studies of this type two facts should be respected: (i) the degree of similarity of liposomes used and (ii) the presence of the residual endoosmotic flow in capillaries used.

From the practical applicability of the results obtained, it must be respected that if the drugs become irreversibly bound to liposomes (lipid bilayers) no uptake across cell layers will take place and no effect elsewhere in the body will occur. If the release is show the drug bound liposomes may be adequate; however, partitioning into the lipid bilayer would better described the situation.

4. Conclusions

Using polyimide coated capillaries with minimized

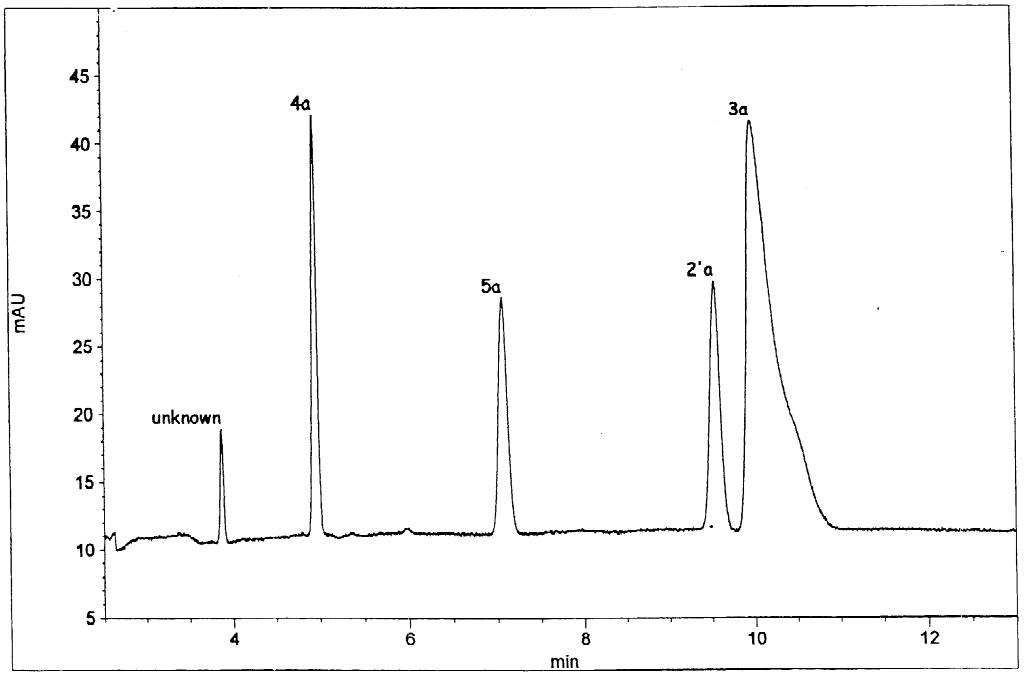

Fig. 7. Electrophoresis of the test mixture in the presence of the liposome plug at pH 9.2; the peaks represent liposome bound species; 2′a=(bound) phenytoin hydrolysis product; 3a=ketoprofen; 4a=acetylsalicylic acid; 5a=salicylic acid.

endoosmotic flow (not detectable at pH 7.5 and ~40 min at pH 9.2) it was possible to reveal the interaction of model mixture of test drugs with POPC liposomes. However, the separations (tests) had to be run separately for basic and acidic/neutral drugs. Only the acidic drugs could be studied in more detail. For this reason all experiments were run with reversed polarity (i.e. negative pole at the injection site). The interaction was investigated at two different pHs, namely 7.5 and 9.2. At pH 7, two clusters

Table 1
The difference in the analyte migration times at pH 7.5 and 9.2 and the relative (to acetyl salicylic acid) free energy values for drug (analyte)–liposome interaction[a,b]

Compound	pH 7.5			pH 9.2			
	Analyte migration time (min)		$\Delta(\Delta G^\circ)$ (kJ/mol)	Analyte migration time (min)		$\Delta(\Delta G^\circ)$ (kJ/mol)	$\Delta(\Delta G^\circ)$ (corrected for EOF)[b]
	Without liposomes	With liposomes		Without liposomes	With liposomes		
Salicylic acid	3.70	27.0	−0.56	5.25	7.06	−0.75	−0.93[c]
Acetyl salicylic acid	4.91	29.40	0	3.96	4.95	0	0
Phenytoin degradation product	Not estimated	Not estimated	Not estimated	6.65	9.54	−1.32	−1.76
Ketoprofen	6.48	9.48	−1.12	6.99	9.98	−1.25	−1.79

[a] Calculation according to Eq. (5) in Ref. [9], i.e. $\Delta(\Delta G^\circ) = -RT \ln\left[\left(t_2^{(+)}/t_2^{(-)} - 1\right)\left(t_1^{(+)}/t_1^{(-)} - 1\right)^{-1}\right]$, where the subscript 2 refers to the studied drug and the subscript 1 refers to the compound to which the calculation is related (acetyl salicylic acid). Plus and minus in the superscript refer to the presence (+) and (−) and absence (−) of liposomes, respectively.

[b] Calculations based on Eq. (2) in Ref. [9], i.e. $\Delta G^\circ = -RT \ln\left[\varnothing^{-1} v^{(-)}\left(v^{(+)} - v^{EOF}\right)^{-1} - 1\right]$ and done simultaneously for the compound investigated (subscript 2) and reference (subscript 1); see footnote a; EOF=40 min.

[c] As compared to −1.13 (see Ref. [9]) in a system reportedly free of the endoosmotic flow and run at pH 7.5.

of peaks appeared on the electropherogram, the faster corresponding to the portion of unbound drugs, the more slowly moving corresponding to the bound fraction. At pH 9.2, no free (liposome unbound) drugs were revealed. At pH 7.5, no interaction of phenytoin with the liposome plug could be revealed and bound/unbound ketoprofen yielded a single tailing peak only; at pH 9.2, clear-cut results for all acidic and neutral drugs present in the test mixture (i.e. acetylsalicylic acid, salicylic acid, ketoprofen and phenytoin) were obtained, however phenytoin at this pH was readily converted into its degradation product the binding of which was clearly demonstrated. The relative free energy binding $\Delta(\Delta G^{\circ})$ at pH 9.2 was close at least with salicylic acid to that reported previously [9] provided that the value of the residual endoosmotic flow was introduced into the calculations.

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