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Affinity electrochromatography of acidic drugs using a liposome-modified capillary

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Abstract

Liposomes can be effectively deposited on the inner surface of a capillary wall by flushing the electrophoretic system with a liposome suspension followed by air-drying of the capillary and removal of the excess of loosely bound liposomes by a 0.1 *M* NaOH wash. It was demonstrated that capillaries prepared in this way could be used for studies of analyte (drug)–liposome binding. The results were expressed as free binding energy changes [$\Delta(\Delta G^0)$] relatively to an arbitrarily selected standard (acetylsalicylic acid). The results were compared to [$\Delta(\Delta G^0)$] changes obtained from binding studies effected by capillary electrophoresis using a stable liposome plug in a capillary with minimized endoosmotic flow. Good agreement of data reported in the literature (without correction for the residual endoosmotic flow), our previous data obtained in a similar way (however, after the correction for the residual endoosmotic flow) and data obtained by the immobilized liposome affinity electrochromatography reported in this communication was achieved.

Keywords: Affinity electrochromatography; Coated capillaries; Electrochromatography; Liposomes; Acetylsalicylic acid; Ketoprofen; Phenytoin; Propranolol; Salicylic acid; Warfarin

1. Introduction

Open tubular capillary electrochromatography was designed to exploit interactions of separated analytes with an inner surface that has been subjected to some sort of modification (for review see [1]). Successful applications to a number of compounds can be found in the literature. The chemical nature of separated analytes is very broad indeed (for instance tetracyclines and their degradation products [2,3], tryptamine and serotonin [4,5], aspartame [6], purine/pyrimidine bases [7,8], caffeine and theophylline [7]). As far as the capillary wall modifiers are concerned octadecyl and diol modified surfaces [4,5], cyanopentoxy (nematic type) liquid crystals [7] or expanded porphyrins [8–10] have been reported and have given good results with particular sets of model compounds.

Coating of the capillary wall offers altered selectivity of the system based on mutual interactions of the analytes with the wall modifier.

All the examples of open tubular electrochromatography mentioned so far refer to situations where modification of the capillary wall was effected through the immobilization of a chemical entity. However, capillary electrochromatography can be also exploited to reveal the interaction of a particular analyte with supramolecular structures provided that

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they can be attached to the capillary wall. Typically, liposomes (phospholipid bilayer vesicles) appear suitable candidates for detailed studies, as they are capable of encapsulating a wide range of solutes (drugs); this property was made use of for controlled delivery of enzymes, drugs, hormones and DNA into cells [11]. They can also be used to modify biodistribution of e.g. plasma membrane lipids and enhance the interaction of drugs with cells. Owing to the increasing interest in the use of liposomes in pharmaceutics, reliable and fast analytical techniques capable of revealing the interaction of drugs with liposomes are needed. Three approaches can be found in the literature: (i) chromatography using (proteo)liposomes immobilized in gel beads [12–14], (ii) liposome capillary electrophoresis [15] in which liposomes are added to the background electrolyte to constitute the hydrophobic pseudophase in a way similar to micellar electrokinetic chromatography and (iii) capillary electrophoresis using capillaries with a minimized electroosmotic flow and a liposome plug in which the drug sample is, depending on its structure, more or less retained [15,16].

It was the aim of the present study to exploit the possibility of immobilizing the liposomes at the inner wall of the bare fused-silica capillary and to apply the electrochromatographic operational mode to reveal the drug–liposome interactions.

2. Materials and methods

Hewlett-Packard capillary electrophoresis model 3D equipped with HP CHEMSTATION Rev. A.06.03(509) was used throughout the experimental part of this work. All buffer components were purchased from Merck (Darmstadt, Germany) and were of the analytical-reagent grade or the highest purity available. Model drugs were obtained from the GlaxoSmithKline (GSK, Verona, Italy) stock (for the formulae see Fig. 1). β-Oleoyl-γ-palmitoyl L-α-phosphatidyl choline (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* buffer (pH 7.5) to yield a concentration of 3.25 m*M* and sonicated in a water bath at 4 W for 20 min. The suspension was

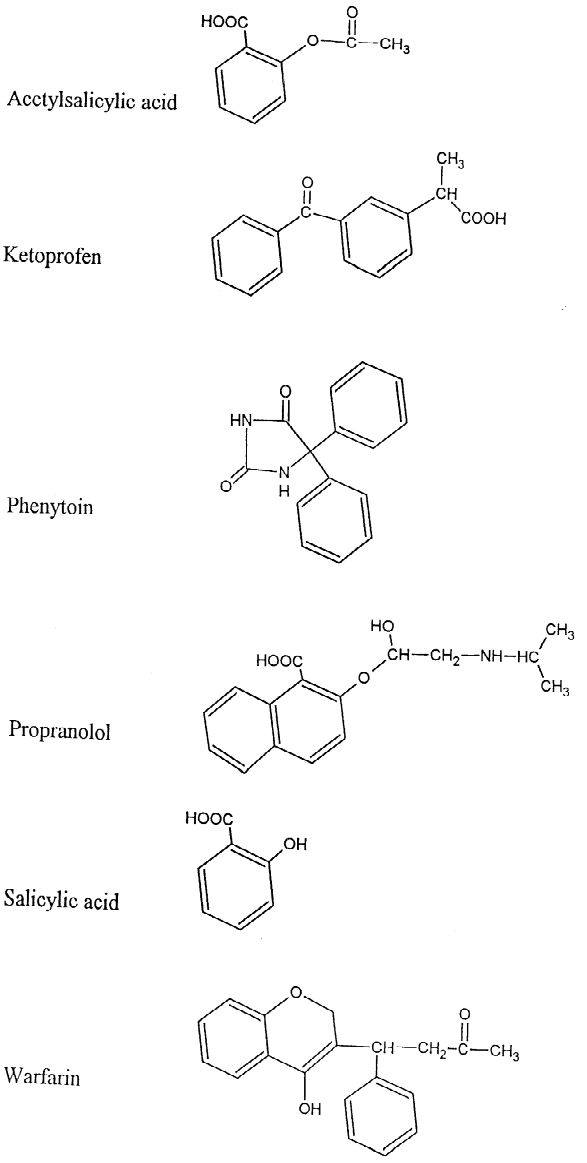

Fig. 1. Structures of the drugs investigated.

filtered (0.1 μm inorganic membrane filter, Anotop 10, Whatman, Maidstone, UK) and the suspension thus obtained was directly injected into the capillary. (If reused, the suspension was sonicated again, however at a reduced time—10 min only).

A bare silica capillary, 40.0 cm (7.5 cm to the detector)×50 μm I.D. was purchased from Composite Metal Services (Hallow, UK) and used for

comparative experiments. Liposome loading (using an identical capillary) was done with the suspension prepared as described above; the capillary was flushed with air for 5 min at room temperature; before use the freshly modified dry capillary was rinsed for 5 min with 0.1 *M* NaOH to remove loosely adhering liposomes. Next the capillary was washed with water and after obtaining a straight baseline the capillary was conditioned with 50 m*M* buffer and was ready for sample injection.

In order to avoid a large scatter of analyte migration times (in particular with late eluting peaks) on which the calculation of free energy changes is based, short capillaries had to be used. Because of the spatial arrangement of the capillary cartridge used in our capillary electrophoresis apparatus, sample injections were done at the capillary end close to the detector and the 7.5 cm available path between the injection site and the detector window were exploited for the separation. The long part of the capillary between the detector's window and the distant (grounded) end contributed to the endoosmotic flow and was not involved in the separation process.

For the experiments with an unmodified capillary, positive polarity was attached to the injection point (reversed polarity in terms of the standard arrangement); on the contrary, with the liposome modified capillary negative polarity was attached to the injection point (see Fig. 2 for a schematic view of the experimental set-up). Polarity reversal for the experiments with the liposome loaded capillary was needed in order to obtain reasonable running times for the endoosmotic flow markers, as, naturally, with liposome loaded capillary the endoosmotic flow was considerably slowed down.

Concentration of the injected drugs was routinely 150 μg/ml. All runs were executed at 10 kV and the capillary column was thermostated to $T = 295$ K.

3. Theory

An interaction between the separated solutes and the entities immobilized on the inner capillary wall surface can be characterized best by the change in the free binding energy, $[\Delta(\Delta G^0)]$. So far this approach has been used for evaluating interactions between a migrating solute and a background electrolyte modifier (interaction of leukovorin) stereoisomers and bovine serum albumin, see Ref. [17]) or between an immobilized plug of particles and a set of model drugs and peptides in a capillary with minimized endoosmotic flow (see Ref. [15]). However, as no silica capillary can be treated in such a way as to abolish the endoosmotic flow completely the above approach was developed further in Ref. [16].

The spatial arrangement of the electrokinetic process during which the interacting counterparts are brought together can vary considerably. For instance, the migrating solutes can interact with the capillary wall modified by the interacting counterparts. Then, by comparing the migration times in an untreated and a treated capillary the change in the free interaction energy for a set of solutes can be calculated.

The observed migration time (t_{obs}) of a given species is determined by the distance it travels from the injection point and its net velocity according the equation:

$$t_{obs} = \frac{l}{v_{obs}} \quad (1)$$

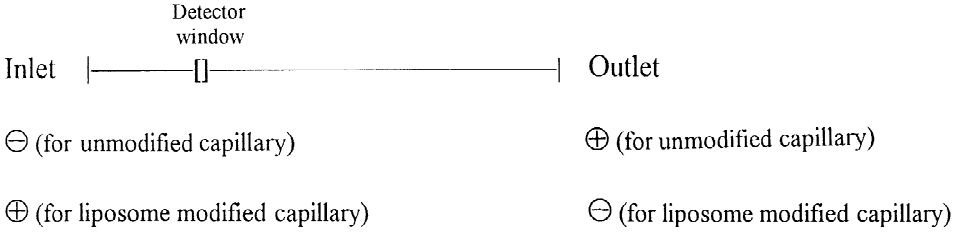

Fig. 2. Schematic view of the capillary electrophoresis arrangement.

where l is the effective length of the capillary (from the injection point to the detector's window) and v_{obs} is the net velocity; in our case this magnitude can be visualized as a sum of two components, namely the electromigration velocity (v_E) and the velocity of the endoosmotic flow (v_{EOF}):

$$v_{obs}^{-} = \frac{l}{v_E^{-} + v_{EOF}^{-}} \quad (2)$$

where minus in the superscript refers to the experiment done in the absence of the interacting counterpart (liposomes). If the liposomes are present the velocity is changed by a factor R which specifies the fraction of time the analyte spends by binding to the liposomes. (The calculation is a small variation of that published in Ref. [17]. Consequently it holds:

$$v_E^{+} = Rv_E^{-} \quad (3)$$

and

$$\frac{1}{t_E^{+}} = R \cdot \frac{1}{t_E^{-}} \rightarrow \frac{t_E^{-}}{t_E^{+}} = R \quad (4)$$

where t_E^{+} and t_E^{-} are the electrophoretic migration times in the presence $\oplus$ and absence O of liposomes. Because

$$v_{obs}^{+} = \frac{1}{v_E^{+} + v_{EOF}^{+}} \quad (5)$$

the change in the EOF caused by liposome coating is also adequately respected.

As demonstrated in Ref. [17] the magnitude R is related to the capacity factor k' by:

$$R = \frac{1}{1+k'} \Rightarrow k' = \frac{1}{R} - 1 \quad (6)$$

and keeping in mind that $k' = K\phi$ where ϕ is the phase ratio, one gets the basis for free energy calculation. However, the phase ratio ϕ is difficult to assess and, consequently, it appears reasonable to calculate the change of free energy [$\Delta(\Delta G^0)$] relatively to an arbitrarily chosen standard.

This strategy has been used for the first time by Zhang et al. [15]. Acetylsalicylic acid was selected as reference and in order to be able to compare our results with the published data, it was used in the present study as reference as well.

For obtaining the [$\Delta(\Delta G^0)$] value the following equation was used:

$$\Delta(\Delta G^0) = -R_g T \ln \frac{k'_x}{k'_a} \quad (7)$$

where R_g is the gas constant, T the absolute temperature (taken as 295 in all calculations) and k'_x and k'_a are the capacity factors for the investigated compound (subscript x) and reference standard (subscript r), respectively.

4. Results and discussion

Using the routine, standard arrangement (40 cm, effective length 7.5 cm capillary, inlet at the distant anodic end) the investigated set of drugs resulted in a electrophoretic profile shown in Fig. 3A; when the approach described in Materials and methods was used (i.e. using reversed polarity and injection at the end close to the detector's window) the pattern shown in Fig. 3B was obtained. The difference regarding the quality of separation was only in a partial overlap of the ketoprofen and warfarin peaks which, as no quantitation of individual peaks was needed in our study, was unimportant. All other solutes were baseline separated. On the other hand the SD ($n=6$) of the migration time decreased from ± 0.22 to ± 0.11 min (calculated for the salicylic acid peak) when the shorter capillary was used.

In the next stage the same capillary was loaded with the liposome suspension by the procedure described in Materials and methods. After the liposomes were introduced a rinsing wash with 0.1 *M* KOH was necessary in order to remove loosely bound liposomes. If this step was omitted and the test mixture was directly injected, all the analytes eluted in a single broad peak. After the KOH rinse, the separations shown in Fig. 4 were obtained (three subsequent runs). There are several points to be emphasized. First of all, even after the washing procedure some liposomes are loosely bound and appear on the electrochromatogram as a system peak (the last one of the set). This peak comigrates with the EOF (as demonstrated by injecting thiourea or methanol) and can be used as an EOF marker. Second, only two well-separated peaks and a peak fused with the system peak were discerned in the

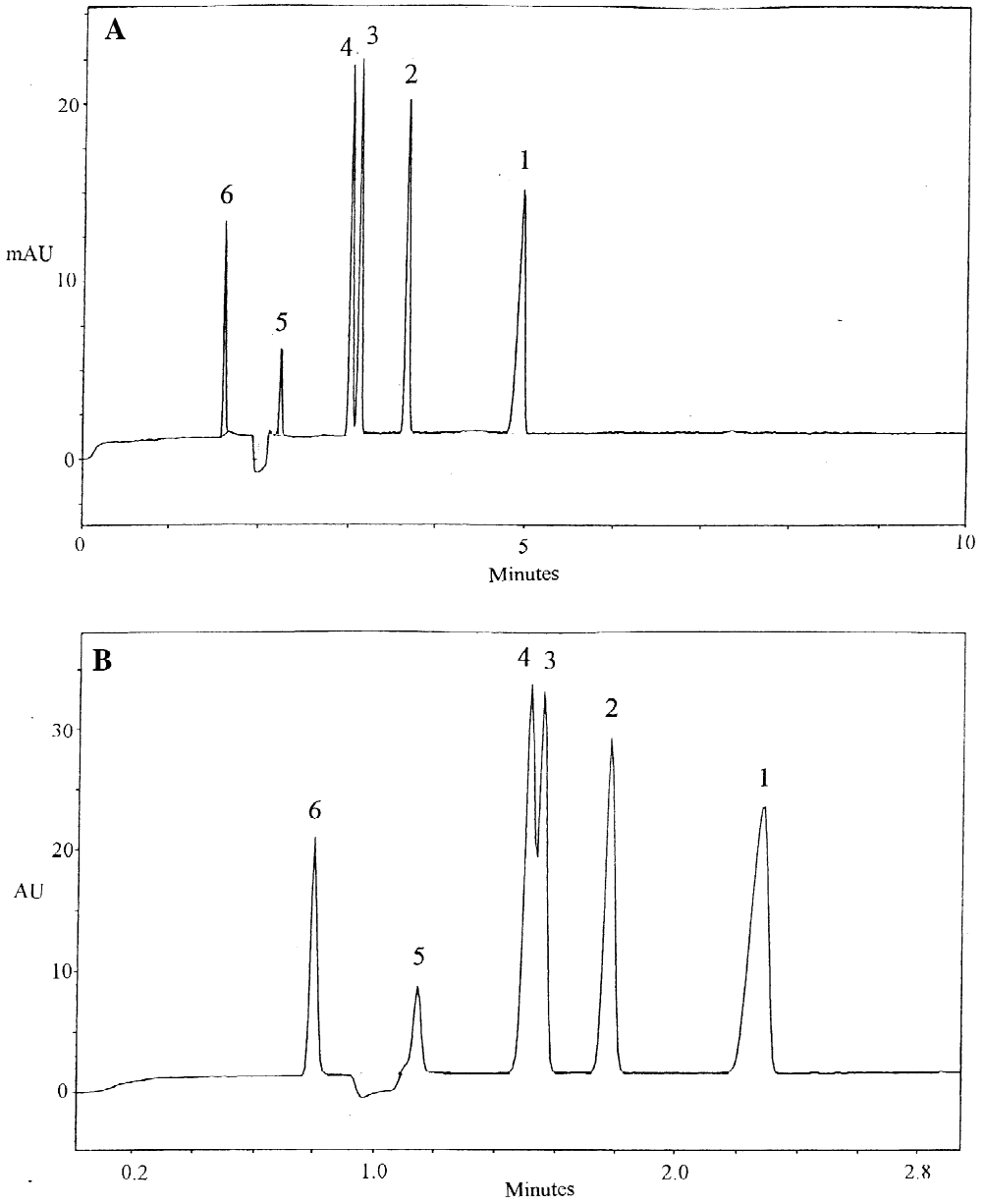

Fig. 3. Separation of the standard mixture in a bare-silica capillary. (A) A typical result obtained with a 40 cm (7.5 cm effective length) capillary. Standard polarity arrangement (minus polarity at the detector end, injected at the distant end of the capillary to which plus pole was attached). (B) A typical result obtained with the short capillary (plus pole attached to the detector end, injection at the capillary end close to the detector). Peaks: 1 = salicylic acid; 2 = acetylsalicylic acid; 3 = ketoprofen; 4 = warfarin; 5 = phenytoin; 6 = propranolol.

first and second run. The reason was that acetylsalicylic and salicylic acid comigrated in the first (fastest) peak while the second peak represented both ketoprofen and warfarin. The shoulder in front of the system peak was due to the presence of phenytoin. The propranolol peak, because of its basic nature, did not appear on the electrochromatogram. In the third run the phenytoin peak was not discerned

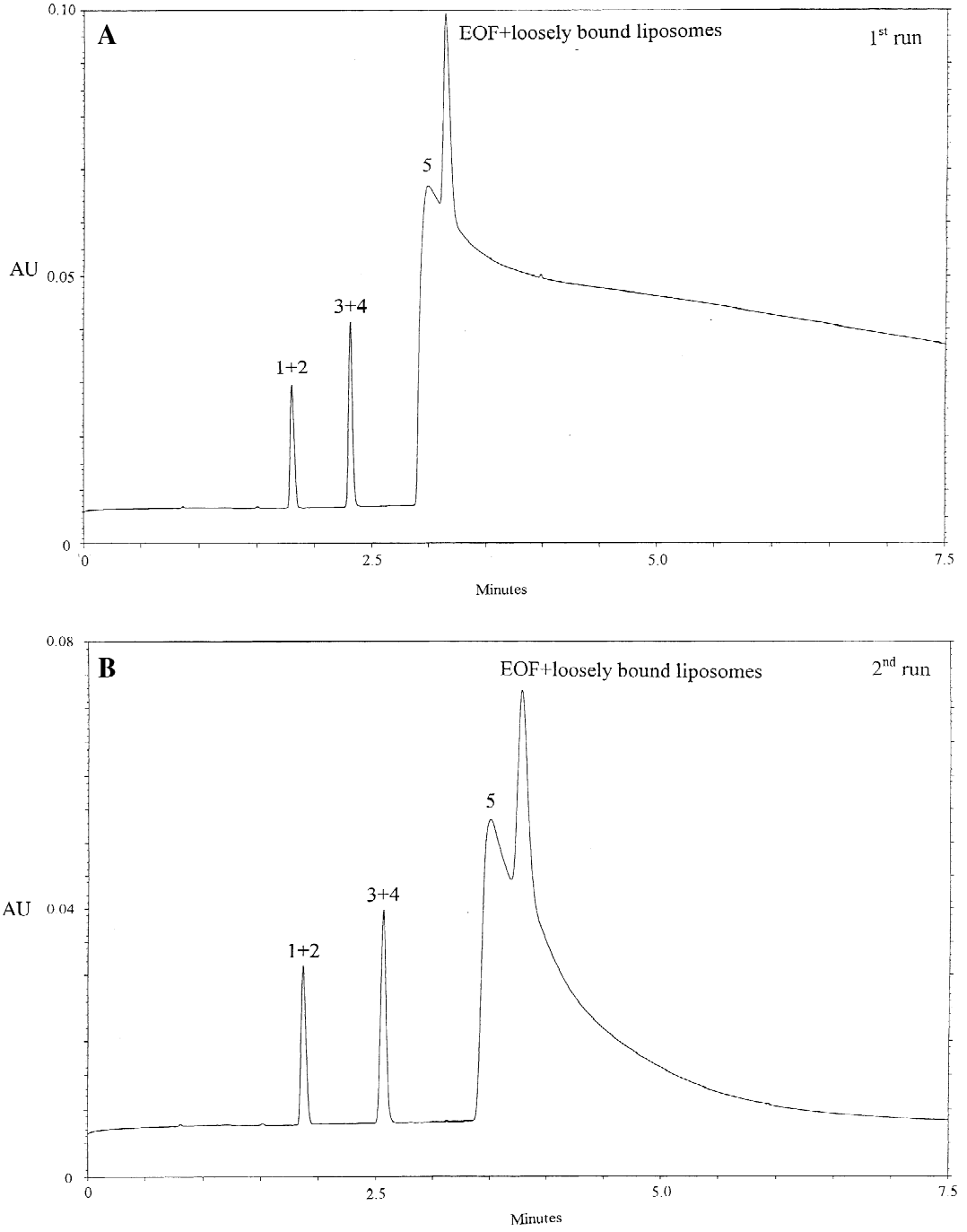

Fig. 4. Three subsequent runs (1–3) of the standard drug mixture in liposome-modified capillary. Peak identification as in Fig. 3 EOF, electroosmotic flow.

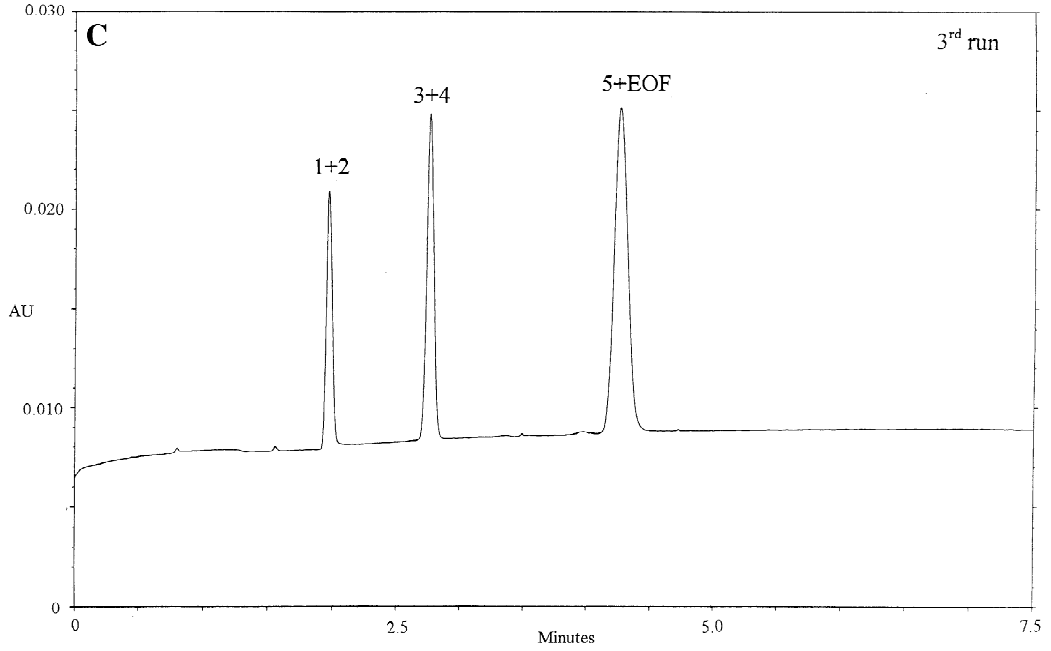

Fig. 4. (*continued*)

from the system peak and, consequently, no calculation of the binding energy [$\Delta(\Delta G^0)$] could be done. The poor selectivity of the system is caused by the interaction of individual solutes with the liposome layer. However, if the method is standardized in such a way that after every run the capillary is rinsed with a liposome suspension flush, the first run can be repeated with a less than $\pm$4% variability (SD) of the migration time of the first and about $\pm$5.5% of the last peak (phenytoin) of the set. The poor selectivity that results in fused peak formation represents no problem in calculations. The actual migration times for a typical set of the first three runs shown in Fig. 4 is summarized in Table 1. The respective values for R and the capacity factor k' are summarized in Table 2, while Table 3 compares the calculated $\Delta(\Delta G^0)$ values obtained with the demonstrated three subsequent runs with the values obtained by the plug method without endoosmotic flow correction (taken from Ref. [15]) and with the correction for endoosmotic flow (taken from Ref. [16]). Typically for salicylic acid the value of $\Delta(\Delta G^0)$ is larger in the coated capillary method as compared to the liposome plug method by 5 or 21% depending on whether in the plug method the correction for the residual endoosmotic flow was taken into consideration or not. With ketoprofen the difference is plus 13% in comparison with the plug method provided that in the plug method calculation the residual endoosmotic flow was adequately considered.

Propranolol, which behaved as a cation in control experiments (bare-silica capillary) was difficult (if at all possible) to detect in liposome loaded capillaries and was deleted from the test mixture in experiments with liposome-loaded capillaries (it was probably co-eluted with the system peak). It is possible to conclude that the experimental arrangement described in this report is capable of revealing the relative free energy values [$\Delta(\Delta G^0)$] changes for acidic drugs.

5. Conclusions

A simple technique based on liposome immobilization effected by drying of a liposome flushed capillary and subsequent washing with 0.1 M NaOH (to remove loosely bound liposomes) was elaborated

Table 1
Actual migration times in three successive runs in a liposome coated capillary as compared to the migration times in a bare capillary; for experimental arrangement see Materials and methods

Compound	Uncoated capillary		Liposome coated capillary					
	t^-_{obs} (min)	t^-_E (min)	1st run		2nd run		3rd run	
			t^+_{obs}	t^+_E	t^+_{obs}	t^+_E	t^+_{obs}	t^+_E
Acetylsalicylic acid	1.77	−2.40	1.94	−4.74	2.00	−4.1	2.10	−4.02
Salicylic acid	2.28	−1.84	1.94	−4.74	2.00	−4.1	2.10	−4.02
Warfarin	1.51	−3.10	2.45	−9.68	2.70	−8.77	2.90	−8.54
Ketoprofen	1.55	−2.98	2.45	−9.68	2.70	−8.77	2.90	−8.54
Phenytoin	1.14	9.66	3.12	−63.93	3.63	−52.40	4.39	NA[a]
Endoosmotic flow	1.02	–	3.28	–	3.90	–	4.39	

Symbols used: t^-_{obs}, observed migration time in the uncoated capillary (standard polarity); t^-_E, electrophoretic migration time in the uncoated capillary (standard polarity); t^+_{obs}, observed migration time in the liposome coated capillary (reversed polarity); t^+_E, electrophoretic migration time in the liposome coated capillary (reversed polarity).
[a] NA, not available; in the third run the phenytoin peak comigrates with the endoosmotic flow.

Table 2
Calculated values for the R magnitude and the capacity factor in three subsequent runs in a liposome coated capillary

Compound	$R = t^-_E/t^+_E$			$k' = (1/R) - 1$		
	1st run	2nd run	3rd run	1st run	2nd run	3rd run
Acetylsalicylic acid	0.50	0.58	0.59	1.0	0.72	0.69
Salicylic acid	0.38	0.44	0.45	1.63	1.27	1.22
Warfarin	0.32	0.35	0.36	2.12	1.85	1.77
Ketoprofen	0.30	0.33	0.34	2.33	2.03	1.94
Phenytoin	0.15	0.18	NA[a]	5.66	4.55	NA[a]

k', capacity factor; for other symbols used see the footnote to Table 1.
[a] NA, not available; the phenytoin peak is fused with the endoosmotic flow and the t^-_E/t^+_E value as well as k' cannot be calculated.

Table 3
Differences in free energy of interaction between drugs and liposomes in capillary electrochromatography with immobilised liposomes. Experimental data are compared to results reported in Refs. [15] and [16]

Compound	$-\Delta(\Delta G^0)$[a] (kJ/mol)			According to Ref. [15][c]	According to Ref. [16][d]
	1st run	2nd run	3rd run		
Salicylic acid	−1.19	−1.37	−1.39	−1.13	−0.93
Warfarin	−1.84	−2.31	−2.30	−2.05	Not reported
Ketoprofen	−2.07	−2.69	−2.52	Not reported	−1.79
Phenytoin	−4.25	−4.52	0[b]	Not reported	

[a] Relatively to acetylsalicylic acid (see Ref. [15]).
[b] Cannot be calculated as the peak of phenytoin comigrates with the EOF peak in liposome modified capillary.
[c] Results obtained with the liposome plug placed in a capillary with minimised endoosmotic flow; no correction for the residual endoosmotic flow considered.
[d] Results obtained with the liposome plug placed in a capillary with minimised endoosmotic flow (similarly to comment No. 3), correction for residual endoosmotic flow considered.

to serve as an effective approach for liposome immobilisation.

It was demonstrated that liposome modified capillary can be used for revealing analyte (drug) binding to these molecular structures. Apparent migration times of the analyte, of a reference compound and apparent migration times of the electroosmotic flow are the only data needed to be able to calculate the relative change in free binding energy [$\Delta(\Delta G^0)$]. Good agreement (within the range 5–21% rel.) between the published data [15], our previous experiments (done with a liposome plug [16]) and the present results were obtained.

Acknowledgements

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