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Direct determination by capillary electrophoresis of cardiovascular drugs, previously included in liposomes

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Abstract

The lipophilicity of some cardiovascular drugs was determined by capillary electrophoresis (CE). Mexiletine, amlodipine and indapamide, the drugs considered, were in contact with liposomial vescicles for 2, 4 or 6 h. After the contact time the drugs, penetrated into liposomial vesicles, were determined by CE using phosphate buffer (pH 6.3 or 7.4) or borate buffer (pH 9). The lipophilicity of three drugs was determined considering the drug percentage penetrated into liposomial vesicles. The found lipohilicity order was amlodipine > mexiletine > indapamide.
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1. Introduction

Drug lipophilicity plays a pivotal role to promote the passage through biological membranes and the access to the site of action so modulating the pharmacokinetic and pharmacodynamic phenomena which happen in vivo. At present, lipophilicity is currently expressed as the logarithm of the partition coefficient between an organic solvent and an aqueous phase ($\log P$) [1]. However, this parameter has often proved inadequate to predict the partition of ionized compounds in biological membranes, mainly due to the fact that electrostatic interactions, that take place between solutes and membrane phospholipids, are not mimicked by partition systems in organic phase [2–5]. The use of phospholipids as partition phase seems able to produce data better mimicking the interactions between membranes and ionized analytes. These data can be achieved by performing two experimental techniques, namely the measure of partition in phospholipid vesicles, i.e. liposomes, and the measure of HPLC chromatographic capacity factors ($\log k'$) on phospholipid stationary phases, the so-called immobilized artificial membrane (IAM phases) [6,7].

This system is advantageous in many respects, mainly due to the fact that it is fast, reproducible, and easy to perform; however, to date, only phosphatidylcholine analogues are marketed as HPLC columns and the study of interaction with other kind of phospholipids (e.g. phosphatidyl ethanolamine, phosphatidyl serine, and sphingomyelin derivatives) or with cholesterol cannot be performed by HPLC.

This limitation encouraged us to revise other methods for the determination of partition coefficient in phospholipids. Indeed, the direct determination of partition coefficient in phospholipidic membranes has been one of the main problems in the determination of partition in liposomes. The purpose of this work was the study of an analytical method to quantitate a drug inside a cellular membrane. Liposomes may simulate some properties of biolog-

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ical membranes and constitute a model system to investigate their behaviour [8–15]. Further liposomes are non toxic, biodegradable and can include many compounds [16]. Therefore if a drug is kept in contact with liposomes for a known time, its partition between lipidic and aqueous phases can be controlled by determining the drug amount inside the liposomes. Liposomes are vesicles obtained by dispelling phospholipids in water at a temperature higher than that corresponding to transfer of phospholipid from the gel state to the liquid crystal state. The liposome vesicles obtained have an hydrophobic part (inside) and an external hydrophilic part. By changing the phospholipid fatty acid and the working conditions like pH, ionic strength and temperature, the membrane characteristics (solidity and permeability) will change. In the partition studies the unilamellar vesicles are preferred because of their similarity to the biological membranes.

In this paper we propose the use of the CE to determine a drug directly into liposome and then determine its lipophilicity in phospholipids.

2. Experimental

2.1. Reagents and chemicals

Indapamide, L-phosphatidylcholine dimyristoil (DMPC), cholesterol (CHOL) were purchased from Sigma (Milan, Italy).

Mexiletine hydrochloride was purchased from Boehringer-Ingelheim Italia s.p.a. Amlodipine was a kind gift from Department of Pharmaceutical and Toxicological Chemistry of University of Naples Federico II.

All other chemicals used were provided from V.W.R International (Milan, Italy) and were all of analytical or HPLC grade, water included.

2.2. Apparatus

The analyses were carried out by a Hewlett-Packard^{3D} CE apparatus (Waldbronne, Germany) equipped with a linear UV–vis diode array detector and an autosampler. The instrument was controlled and the data were evaluated by a ChemStation and a computer HP-KAYAK XM 600 Pentium 3. The experiments were carried out in an uncoated fused silica capillary (total length 55 cm, effective length 48 cm, i.d. 50 μm) purchased by Composite Metal Services (Hallow, Wores, UK).

2.3. Liposomial vesicles preparation

The unilamellar vesicles were obtained by means of the “film” method [18,19]. In a test tube DMPC and CHOL (1/1) were solubilized both together in a mixture of methanol/chloroform (1/3). The organic phase was removed at room temperature under reduced pressure to form a thin film of dry lipid on the inner wall of the test tube. The residue of organic solvents was then removed at 10^{-1} mmHg.

The film was hydrated by adding 5 ml of 0.01 M HEPES buffer (*N*-(2-hydroxyethyl)piperazine-*N*′-(4-etansulphonic acid)) at pH 7.4. The dispersion was vortexed for 20 min and then sonicated for 3 min at 60 °C (Vibracells-VCX 400 (Sonics)) equipped with an exponential microprobe operating at 23 kHz and an amplitude of 6 mm).

2.3.1. Vesicles purification

The vesicle dispersion was transferred in a visking tubing (36/32S.I.C.) and purified by an exhaustive dialysis against buffer and then by gel-filtration on Sephadex G75 using a glass column (50 cm × 4 cm). HEPE buffer was used as eluent (dead volume 34 ml and sample volume 13 ml).

2.4. Analytical procedures

The purified liposomial vesicles were divided in fourth parts: a blank and three samples, each having a volume of 1.5 ml. Each sample was mantained in contact with the drug solution for 2, 4 or 6 h (T_2, T_4, T_6). The drug solutions were prepared at the following concentration ranges:

Mexiletine	10 mg/ml, 2 mg/ml, 0.12 mg/ml
Amlodipine	10 mg/ml, 2,58 mg/ml, 0.1 mg/ml
Indapamide	0.9 mg/ml, 0.16 mg/ml

The drug solutions were prepared by adding respectively to:

Mexiletine,	300 μl of 50 mM phosphate buffer pH 7.4
Amlodipine,	300 μl of 50 mM phosphate buffer pH 6.3
Indapamide,	300 μl of 50 mM borate buffer pH 9

The used drug/liposome ratio was 1/2. At the end of each contact time (T_2, T_4 and T_6) three samples of 1.5 ml were prepared in the singular eppendorff and vortexed (30,000 rev/min) for 20 min. The supernatants were separated from the liposomial vesicles and the external part of liposomial vesicles were accurately washed by picking up the washing waters.

Then from each sample with the same drug concentration, but different contact time, nine samples were obtained, gathered in three groups:

A. Three samples (T_2, T_4 and T_6) containing liposomal vesicles. These samples allowed to determine the drug molecules which got in through the phospholipidic membranes in the established time.
B. Three supernatants obtained from the centrifugation of the samples A.
C. Three samples obtained from the washing waters of samples A.

From the analyses of A–C the drug amount got into liposomial vesicles, the drug amount remained in the supernatant and the drug found in the washing waters were obtained.

2.4.1. Electrophoretic conditions

The analyses were carried out in an uncoated fused-silica capillary. The other analytical conditions were: applied voltage 20 kV, working temperature 25 °C, The samples were injected by hydrodynamic mode, applying a pressure of 50 mbar for 3 s. The analytes were detected at a λ value of 200 nm.

3. Results and discussion

CE resulted to be a good technique to determine the drug inside the liposomial vesicles and then to know its lipophilicity. To carry out this determination three series of mexiletine, amlodipine and indapamide samples at different concentration were kept in touch with empty liposomial vesicles for a fixed time: 2, 4 or 6 h. Each drug was solubilized in 50 mM phosphate buffer (pH 7.4 or 6.3) or in 100 mM borate buffer (pH 9) and injected in the run buffer (Fig. 1). During the contact time the analytes will cross, or not, the vesicle membrane according to their lipophilicity. The liposomial vesicles injected in the electrophoretic capillary allows the determination of the analyte inside the liposome. Knowing the volumes of liposomal vesicles and watery phases, it is possible to determine the drug/partition coefficient between the two phases.

As run buffer 50 mM phosphate buffer (pH 6.3) for mexiletine, 50 mM phosphate buffer (pH 7.4) for amlodipine and 50 mM borate buffer (pH 9) for indapamide were used. The liposomes, purified as described in Section 2, were divided in three groups each of one formed by four samples: a reference blank and three samples containing the liposomes and the drug solution in the ratio 1 to 2. Drug solution and liposomes of each group remained in contact for 2, 4 or 6 h. At the end of this contact time, the mixtures drug/liposomes were vortexed (30,000 rev/min) and the supernatant was divided from the liposomes. The liposome samples, after several washings, were directly injected in the capillary. As it can be seen in Fig. 2 mexiletine, amlodipine and indapamide maintained their nature of cations or anions and then the migration order, but increased their migration times:

From 2.00 to 2.02 min	Mexiletine
From 2.05 to 2.2 min	Amlodipine
From 2.60 to 3.3 min	Indapamide

Considering the liposomes like a chromatographic pseudo stationary phase, the increase of the analytes migration time means that the drugs interact with the liposomes. Also the signal corresponding to the electroosmotic flow was retarded because the viscosity of the running buffer was changed.

To know the real drug amount in the liposomial vesicles we made three determinations:

- The determination of drug inside the liposomes, considering the three different contact times (T_2, T_4, T_6).
- The determination of the drug in the supernatant of the T_2, T_4, T_6 samples.
- The determination of drug in the washing waters of each sample.

The results, summarized in the Tables 1–3, show a good correspondence between the drug weighed and the drug recovered from the liposomes, supernatants and washing waters.

Two parameters influence the drug amount included in the liposomial vesicles: the drug concentration and the contact time between drug and liposomes. Actually these parameters influence the balances generated at the external and internal surfaces of liposomic membrane.

The optimum of concentration for the drugs investigated was between:

Table 1
Mexiletine

Time	Drug found in the liposomial vesicles (mg)	Drug found in the supernatant (mg)	Drug found in the washing waters (mg)	Drug % in the liposomes
(A) 1 ml of mexiletine solution (0.12 mg/ml) in contact with 1 ml of liposomial vesicles				
2 h	0.005	0.105	0.0105	4.13
4 h	0.006	0.09	0.024	6.00
6 h	0.004	0.108	0.0083	3.75
(B) 1 ml of mexiletine solution (2 mg/ml) in contact with 1 ml of liposomial vesicles				
2 h	0.031	1.97	0.0075	1.53
4 h	0.050	1.95	–	2.50
6 h	0.022	1.98	0.0075	1.08
(C) 2.5 ml of mexiletine solution (10 mg/ml) in contact with 2.5 ml of liposomial vesicles				
2 h	0.0375	24.9	0.062	0.15
4 h	0.057	24.88	0.055	0.23
6 h	0.0375	24.9	0.062	0.15

Table 2
Amlodipine

Time	Drug found in liposomes (mg)	Drug found in the supernatant (mg)	Drug found in the washing waters (mg)	Drug % in the liposomes
(A) 1 ml of amlodipine solution (0.1 mg/ml) in contact with 1 ml of liposomial vesicles				
2 h	0.006	0.079	0.015	6.00
4 h	0.007	0.078	0.015	6.70
6 h	0.006	0.081	0.014	5.70
(B) 1 ml of amlopdipine solution (2.58 mg/ml) in contact with 1 ml of liposomial vesicles				
2 h	0.14	2.64	0.018	5.00
4 h	0.17	2.60	0.030	6.10
6 h	0.12	2.65	0.034	4.20
(C) 1 ml of amlodipine solution (8.57 mg/ml) in contact with 1 ml of liposomial vesicles				
2 h	0.042	8.478	0.05	0.49
4 h	0.024	8. 526	0.02	0.28
6 h	0.030	8.043	0.01	0.35

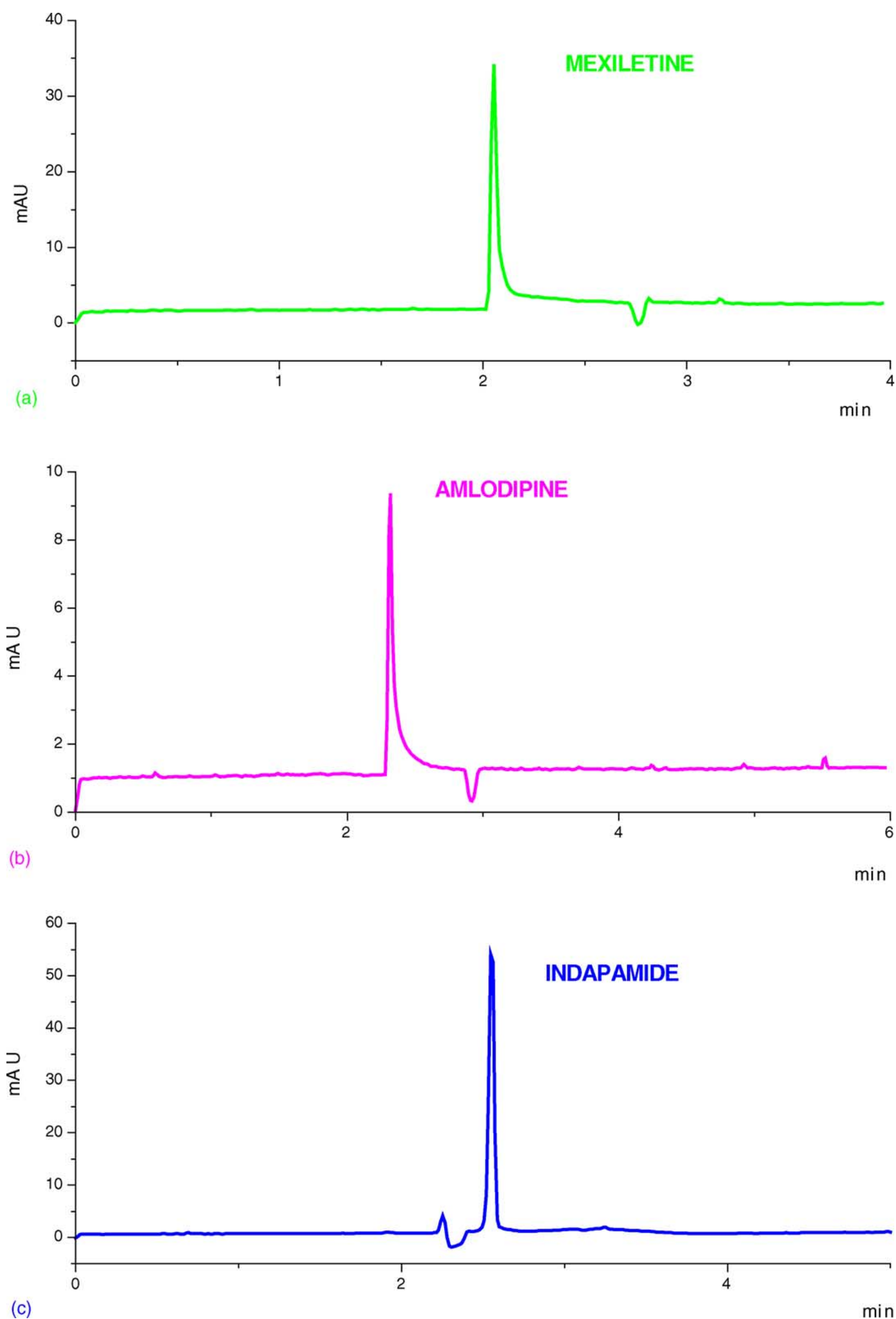

Fig. 1. Electropherograms of (a) mexiletine (0.12 mg/ml) in H_2O–B.G.E.: 50 mM phosphate buffer (pH 7.4). Applied voltage: 20 kV; temperature 25 °C; injection time: 50 mbar × 3 s. (b) Amlodipine (0.1 mg/ml) in MeOH–B.G.E.: 50 mM phosphate buffer (pH 6.3). The applied voltage: 20 kV; temperature 25 °C; injection time: 50 mbar × 3 s. (c) Indapamide (0.16 mg/ml) in MeOH–B.G.E.: 50 mM borate buffer (pH 8.9). Applied voltage: 20 kV; temperature 25 °C; injection time: 50 mbar × 3 s.

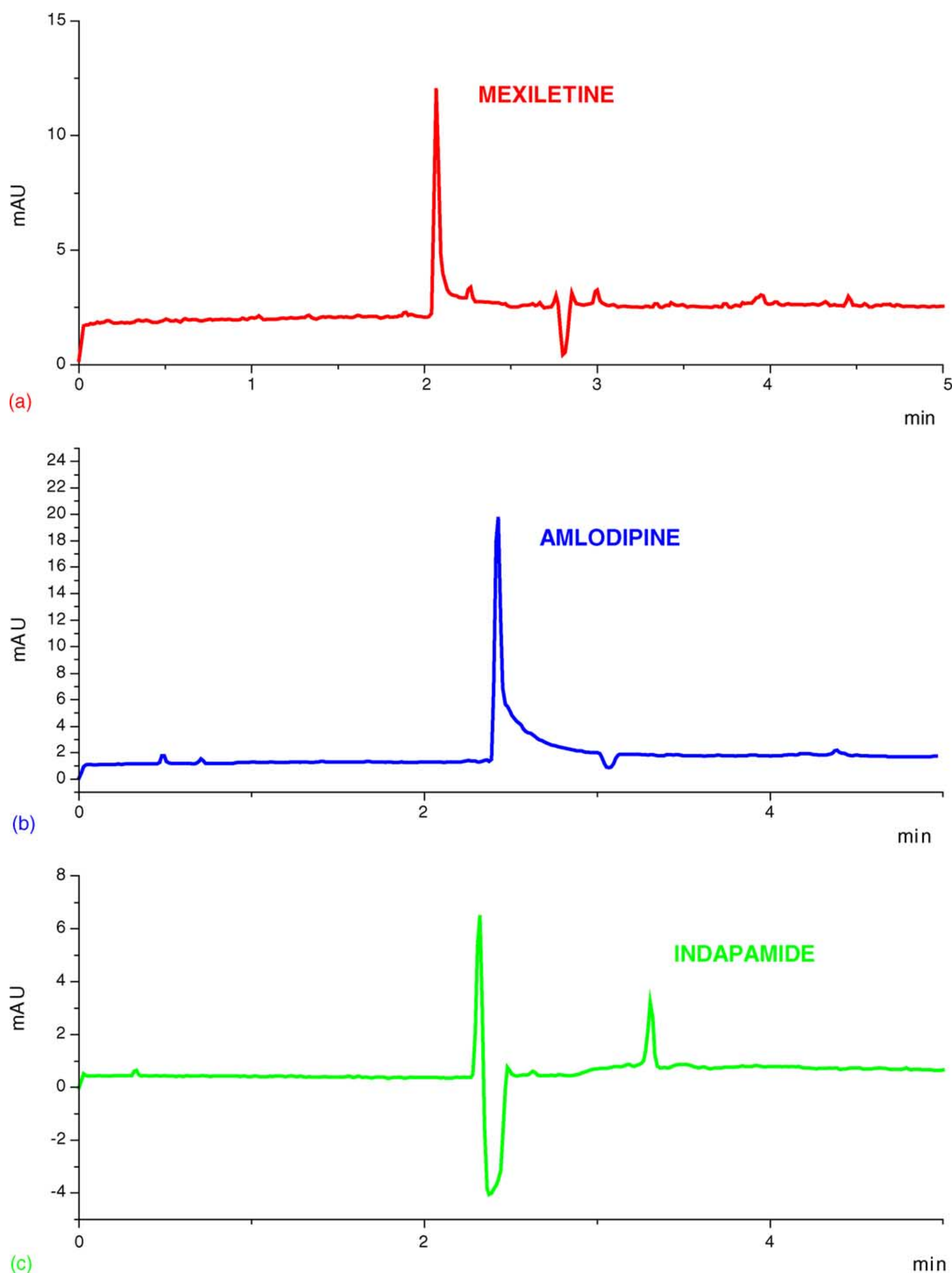

Fig. 2. Electropherograms obtained from each sample after the contact between drug and liposomial vesicles: (a) mexiletine (0.12 mg/ml) in H_2O–B.G.E.: 50 mM phosphate buffer (pH 7.4). Applied voltage: 20 kV; temperature 25 °C; injection time: 50 mbar × 3 s. (b) Amlodipine (0.1 mg/ml) in MeOH–B.G.E.: 50 mM phosphate buffer (pH 6.3). Applied voltage: 20 kV; temperature 25 °C; injection time: 50 mbar × 3 s. (c) Indapamide (0.16 mg/ml) in MeOH–B.G.E.: 50 mM borate buffer (pH 8.9). Applied voltage: 20 kV; temperature 25 °C; injection time: 50 mbar × 3 s (20 kV, 25 °C, injection: 50 mbar × 3 s).

0.12 mg/ml ⇒ 2 mg/ml — For mexiletine

0.1 mg/ml ⇒ 2.58 mg/ml — For amlodipine

0.16 mg/ml ⇒ 0.9 mg/ml — For indapamide

For our drugs the optimal contact time resulted to be 2 h for indapamide and 4 h for mexiletine and amlodipine.

Considering the drug percentage penetrates into liposomes, the lipophilicity order was amlodipine > mexiletine > indapamide (Fig. 3). The same lipophilicity order was obtained analyzing amlodipine, mexiletine and indapamide by IAM-HPLC.

The CE method here proposed yields the same scale of interaction as found by IAM-HPLC technique [17,18]. Indeed,

Table 3
Indapamide

Time	Drug found in liposomial vesicles (mg)	Drug found in the supernatant (mg)	Drug found in the washing waters (mg)	Drug % in the liposomes
(A) 1 ml of indapamide solution (0.16 mg/ml) in contact with 1 ml of liposomial vesicles				
2 h	0.006	0.154	–	3.80
4 h	0.004	0.156	–	2.40
6 h	0.006	0.155	–	3.40
(B) 1 ml of indapamide solution (0.9 mg/ml) in contact with 1 ml of liposomial vesicles				
2 h	0.033	0.836	0.032	3.70
4 h	0.015	0.864	0.021	1.60
6 h	0.017	0.875	0.008	1.80

IAM surfaces are formed by a phospholipid monolayer, quite similar to liposomial membranes and both systems allow a direct measure of the partition coefficient of a drug between a lipophilic and an aqueous phases. However, in IAM-HPLC analyses the results may be affected by silanophilic interactions, analogously to that observed with all silica-based stationary phases. Furthermore, IAM columns can work in an only narrow pH range (pH 3–7). These problems do not occur in HPCE because the separation of analytes take place in an uncoated capillary. Here analytes run in the central part of the capillary without contact with its internal side, according to the electroosmotic flow.

In conclusion the determination by CE of a drug included in the liposomes is a method direct, very easy, accurate and precise. However, this method presents two disadvantages:

The preparation of liposomes is long and laborious [19].
The liposomes reproducibility is very scarce.

Actually it is practically impossible to have two liposomial vesicles preparations one the exact copy of the other. Consequently, a variation of the amount of lipophilic solutes in the liposomial vesicles could occur making not reliable the investigation.

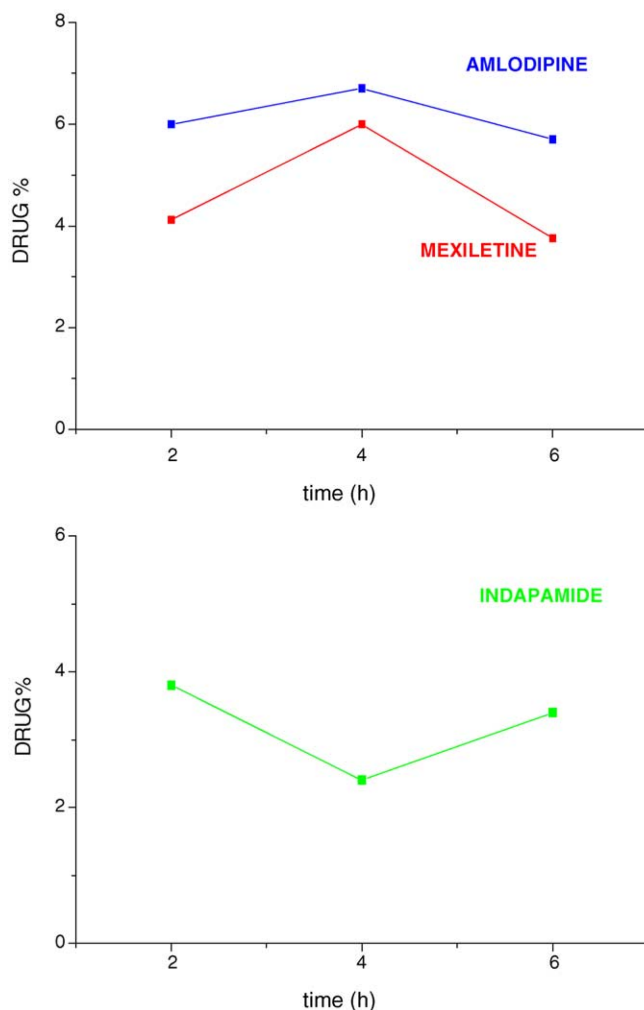

Fig. 3. Optimal contact time between drug and liposomial vesicles: indapamide 2 h, mexiletine and amlodipine 4 h.

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