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Determination of drug–protein binding using supported liquid membrane extraction under equilibrium conditions

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Abstract

A technique for determination of drug–protein binding based on a membrane extraction technique termed "equilibrium sampling through membrane (ESTM)" is presented. It involves the establishment of an equilibrium between an aqueous buffer and either a blood plasma sample or a matched buffer, both containing the drug. Analysis of the aqueous buffer in the two cases gives the drug–protein binding. The principle bypasses some sources of systematic error found with common techniques for this measurement based on e.g. ultrafiltration, as it senses the equilibrium conditions without disturbing the sample. The technique is applied to some local anesthetic drugs as model substances and two alternative ways for the evaluation are presented. Results with these evaluation methods are compared with literature values for the drug–protein binding of these compounds. It is found that the drug–protein binding values obtained are lower than literature values, which is attributed to reduced systematic error.
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Keywords: Drug–protein binding; Local anesthetics; Supported liquid membrane extraction; Equilibrium extraction; Equilibrium sampling through membrane (ESTM)

1. Introduction

The binding of a drug with blood proteins is an important process in determination of the activity and fate of a pharmaceutical agent once it has entered the body. The drug binding to specific plasma transport proteins (human serum albumin—HSA, α_1-acid glycoprotein—AAG, lipoproteins, etc.) is an integral part of different intermolecular interactions in a living organism [1,2]. These bindings can be important in determination of the overall distribution, excretion, activity and toxicity of a drug.

The techniques used for determination of drug–protein binding in vitro are based on one of the following procedures: separation of free and protein-bound fractions and determination of the concentration of free ligand, or detection of a change in a physicochemical property of the complexed ligand and binding protein [3,4].

The evaluation of the binding of drugs with plasma proteins is complicated because of low analyte concentration (0.1–10 ng mL^{-1} level), very low free drug concentration (protein binding can reach 99%), sample matrix complexity, and sample volume limitation. Thus, the sample preparation is crucial in the analysis of drugs in biological samples and includes both analyte preconcentration and sample clean-up [5].

The most frequently employed methods for determination of protein binding are conventional methods such as dialysis, ultrafiltration and ultracentrifugation [2]; chromatographic methods such as affinity chromatography [6,7] and high-performance size-exclusion chromatography [8–11], and capillary electrophoresis [12–14]. Because of the limitations of the applied methods, including long periods of analysis, nonphysiological conditions (pH, presence of or-

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ganic solvents), low concentrations of the free drug (even under the detection limit), complicated processes of automation, etc. [1,2] there has been continuing research to find better, faster and more convenient approaches for the analysis of drug–protein binding.

For the determination of drugs in biological matrices several studies have been reported on alternative extraction techniques, including supported liquid membrane (SLM) extraction [15–17] and liquid phase micro extraction [5,18,19]. In the SLM technique, aqueous samples containing different drugs are pumped at one side of a microporous membrane, and the drugs are extracted into an organic solvent immobilized in the membrane pores and then into an acceptor solution on the other side of the membrane. Either very thin flat membranes or hollow fiber membranes can be used for this purpose. The advantages of SLM extraction over other methods for drug determination are small sample volumes, short analysis times, low consumption of organic solvent for extraction, possibilities to work under physiological conditions and an excellent possibility for connection with various analytical instruments, possibly with automation of the whole process [20]. There are a number of applications of the SLM extraction method connected with GC, HPLC and CE in the analysis of drugs in blood plasma samples, such as analysis of Amperozide and its metabolite [21], various local anesthetics [22] etc. In these applications it was seen that drug–protein binding decreased the extraction efficiency when plasma samples were extracted compared to water solutions. From this difference reasonable values of the drug–protein binding ratio could be calculated [21,22]. Essentially the same principle was applied to the analogous problem of metal-humic acid binding in environmental applications [23].

Another principle for measurement of interactions by SLM extraction is the principle of equilibrium extraction, based on the work on incomplete trapping by Chimuka et al. [24]. This principle permits measurements of unbound fractions of the analyte without disturbing any equilibria and has potential applications both in biological and environmental problems. This study is the first application of SLM extraction under equilibrium conditions to determination of drug–protein binding, using local anesthetics as model compounds. To carry this out, the physiological conditions were simulated and the local anesthetics were extracted from pure, undiluted plasma. The conditions in the acceptor phase were adjusted to achieve equilibrium extraction with incomplete trapping and to avoid any influence on the equilibrium between the protein–drug complex and the free drug in plasma.

2. Theory

SLM extraction consists of two processes: extraction of an analyte from the donor phase into an organic solvent situated in membrane pores, and a simultaneous back-extraction from the organic phase into an aqueous acceptor phase, where the analyte is converted to a non-extractable form and thus trapped.

The overall mass transfer process under steady-state conditions consists of three mass transfer processes: the mass transfer in the donor phase, in the membrane (organic solution) phase and in the acceptor phase [25]. The overall mass transfer coefficient is given by the equation:

$$\frac{1}{k} = \frac{1}{k_D} + \frac{1}{k_M K_D} + \frac{K_A}{k_A K_D} \quad (1)$$

where the three terms refer to the donor, membrane and acceptor phases, respectively, and the symbols are defined in the list of symbols. Jönsson et al. have developed in detail the mass transfer theory for analytical enrichment and sample preparation using SLM extraction in a flow system with stagnant acceptor phase [26]. The presence of the analyte in extractable and nonextractable forms in both the donor and the acceptor phase are included in this theory through the parameters α_D and α_A, respectively, which signify the fraction of analyte in extractable form. The overall mass transfer coefficient depends on these parameters and can be expressed as:

$$\frac{1}{k} = \frac{\alpha_D}{k_D} + \frac{1}{k_M K_D} + \frac{\alpha_A K_A}{k_A K_D} \quad (2)$$

Further, the rate of the mass transfer is proportional to the concentration difference, ΔC, over the membrane, which can be written as [24]:

$$\Delta C = \alpha_D C_D K_D - \alpha_A C_A K_A \quad (3)$$

where C_D is the mean concentration of analyte in the donor phase, approximately equal to the average value of initial total concentration of analyte in the donor phase (C_I) and the concentration of analyte in the donor waste accumulated from the start of the experiment (C_W).

In most applications of SLM extraction, the conditions are set so that the second term in Eq. (3) is negligible ($\alpha_A \approx 0$) and α_D is close to 1. For basic compounds, this means that the acceptor pH should be at least 3.3 pH units below the pK_a of the basic compound and the donor pH above the same pK_a [26]. Then the analyte will be trapped in the acceptor phase and C_A increases gradually during the extraction, usually up to values well over C_D. The rate of mass transfer is constant; the enrichment factor C_A/C_D increases linearly with time and C_A is proportional both to C_I and extraction time. These are the conditions of complete trapping, which are usually attempted for sample preparation and sampling. There are many applications for this situation [27–29] and this is not further discussed here.

However, when $\alpha_A > 0$, i.e. with higher acceptor pH values (for basic compounds) the second term in Eq. (3) will increase as the extraction proceeds, and the rate of mass transfer will decrease and eventually approach zero. Then extraction finishes and the entire system is in equilibrium. At these conditions, $C_I = C_D = C_W$. If the extraction is performed in

a flow system, so the donor is continuously replenished, the equilibrium conditions in the donor represent the situation in the original sample; any equilibrium in which the analyte participates is undisturbed. Alternatively, if the extraction is performed in a batch or recycling mode, it is necessary that the volume of sample be large enough that the analyte concentration is not influenced by the extraction, which will approximate the situation of undisturbed equilibria.

According to Eq. (3) the maximum concentration enrichment factor is reached at equilibrium and this can be expressed by:

$$E_{e(max)} = \left(\frac{C_A}{C_I}\right)_{max} = \frac{\alpha_D K_D}{\alpha_A K_A} \tag{4}$$

When the extraction conditions are set so that K_D can be assumed to be close to K_A (if the ionic strengths and compositions of the donor and acceptor phase are similar), then the maximum concentration enrichment factor is approximated by:

$$E_{e(max)} = \frac{\alpha_D}{\alpha_A} \tag{5}$$

If the studied analyte is a weak base or a weak acid (all local anesthetics investigated in the work reported herein are weakly basic amines), and there are no complexing reactions (e.g. protein binding), the fraction of analyte in extractable (i.e. non-charged) form, depends on the pH both in the donor and in the acceptor phase [26]:

$$\alpha_i = \frac{K_a}{[H^+]_i + K_a}, \quad i = A, D \tag{6}$$

Further, for a drug in blood plasma, equilibrium between the protein–drug complex and the free drug in plasma is established. The reversible interaction of the drug (A) and plasma proteins (P) can be described generally by the equation:

$$P + mA \Leftrightarrow PA_m \tag{7}$$

As these drug–protein binding reactions (transport protein–drug) are non-covalent, the equilibrium can easily be changed with changing conditions, for example pH-changes.

If blood plasma spiked with a drug is in contact with the organic phase in the SLM contactor, two equilibrium reactions occur: the equilibrium between free drug and plasma proteins (Eq. (7)), and the equilibrium between free drug in extractable form and in the organic phase. Additionally, for acidic or basic drugs, there is the pH-dependent equilibrium between charged and non-charged (extractable) form of the drug.

When the conditions for donor-controlled extraction with complete trapping are established, the extraction of free drug into the organic phase influences (and shifts) the equilibrium between the protein drug complex and the free drug in the plasma. Jönsson et al. included this phenomenon in the theory of mass transfer kinetics for analytical enrichment [24] and studies of drug–protein binding [21] have been performed in this way. Although giving reasonable results, this principle could be questioned as the drug–protein equilibrium is shifted. On the other hand, there is a potential possibility to study the kinetics of the drug–protein binding (not published).

If, instead, extraction is performed until equilibrium is reached, the drug–protein equilibrium is not affected; in fact it becomes possible to sense the position of this equilibrium without influencing it. To reach this situation, it is necessary that the total concentration of the drug in the plasma sample in equilibrium with the membrane and acceptor does not change significantly during the extraction. In other words, the plasma sample should have enough volume compared to the membrane and acceptor, so the total amount of drug in the latter phases is negligible compared to that in the plasma sample. This can be achieved either by using miniaturized membrane equipment, or, as in the work reported herein, by pumping the plasma sample past the membrane, so that when equilibrium is established, the membrane is in equilibrium with an undisturbed plasma sample.

When equilibrium between all phases is established, the α_A from Eq. (6) is still valid, but α_D has to be modified in order to include the effects of the protein binding:

$$\alpha_D = \alpha_P \, \alpha_{Dd} \tag{8}$$

where α_P is the fraction of drug not bound to protein, and α_{Dd} is the uncharged fraction of the non-bound drug due to the dissociation equilibrium. The latter is calculated directly from Eq. (6) with $i = \mathrm{Dd}$. Thus, for equilibrium extraction of a drug–protein solution (i.e. plasma containing drug), the following is valid

$$E^P_{e(max)} = \frac{\alpha_P \, \alpha_{Dd} \, K^P_D}{\alpha_A \, K_A} \tag{9}$$

Subscript and superscript p refer to conditions in the plasma solution. As α_A and α_{Dd} can be easily calculated if pK_a is known, determination of C_A after attainment of equilibrium permits the calculation of the free drug concentration in plasma using SLM extractions under equilibrium conditions and incomplete trapping of analyte, and thereby an estimation of the drug–protein binding ratio is enabled. This additionally requires either the assumption that the partition coefficients K_D and K_A are equal, or, for the best accuracy, knowledge of their values.

A very simple estimate of the drug–protein binding ratio can be obtained by measuring C_A after equilibrium extraction of drug solutions with the same total drug concentrations, C_D, from both plasma (C^P_A) and aqueous buffer (C_A). From Eqs. (4) and (9), we obtain:

$$\alpha_P = \frac{C^P_A \, \alpha_D \, K_D}{C_A \, \alpha_{Dd} \, K^P_D} \tag{10}$$

If it can further be assumed that $\alpha_D = \alpha_{Dd}$ and $K_D = K^P_D$, necessitating a proper matching for the pH and ionic strength between the aqueous buffer and the plasma solution, we arrive

Table 1
The main characteristics of studied local anaesthetics

Name	pK_a		Extraction from phosphate buffer		Extraction from citrate buffer		Protein binding (%)
			K_D	K_A	K_D	K_A	
Prilocaine	8.0[a]	7.9[b]	29.4	7.1	18.4	4.2	55[c]
Ropivacaine	8.9[a]	8.1[d]	124	15.8	111	17.9	90[e]
Lidocaine	8.5[a]	7.8[f]	32.2	7.1			40–60[c]
Bupivacaine	8.9[a]	8.1[b]	280	42.7			95[e]
Mesocaine	8.6[a]		115	14.5			50–70[g]

Partition coefficients are determined as described in the text, and given at the lowest pK_a value for each compound.
[a] Calculated by the program ACD/pK_a DB (Advanced Chemistry Development Inc., Toronto, Canada).
[b] Ref. [31].
[c] Ref. [32].
[d] Ref. [33].
[e] Ref. [34].
[f] Ref. [35,36].
[g] Estimated, T. Arvidsson, personal communication.

to the following simple relation:

$$\alpha_P = \frac{C_A^P}{C_A} \tag{11}$$

3. Experimental

3.1. Chemicals and reagents

The local anesthetics studied (Prilocaine, Lidocaine, Ropivacaine and Bupivacaine) were obtained as hydrochloride salts from Astra Pharmaceutical Production (Södertälje, Sweden). Mesocaine was synthesized at Astra Pain Control (Södertälje, Sweden). The main characteristics of the studied compounds are given in Table 1.

Stock solutions (200 mg dm^{-3}) were prepared in water and stored at 4 °C. Aqueous working solutions were prepared daily from the stock solution.

The donor solution contained 0.5–2.5 mg dm^{-3} of individual local anesthetics in buffer solution. Two buffer solutions were used in the experiments: 0.067 mol dm^{-3} phosphate buffer and citrate buffer both at pH = 7.53. Citrate buffer contained approximately the same concentration of components of anticoagulants (0.01 mol dm^{-3} citric acid, 0.07 mol dm^{-3} sodium citrate, 0.01 mol dm^{-3} sodium dihydrogenphosphate and 0.08 mol dm^{-3} glucose) as their corresponding concentrations in plasma.

Blank plasma was obtained from the blood center, Lund University Hospital (Lund, Sweden) and kept frozen at −20 °C. The plasma was thawed, centrifuged and kept at room temperature during the day of analysis. The spiked plasma solutions were obtained by adding appropriate amounts of drug solution to the blank plasma.

The acceptor solutions were either 0.067 mol dm^{-3} phosphate buffer in the pH range from 4.0 to 7.0 or citrate buffer at pH = 7.0.

The microporous poly(tetrafluoroethene) PTFE membrane (TE 35 membrane filter, Schleicher & Schuell GmbH, Dassel, Germany) was impregnated by soaking in di-hexyl ether with 5% tri-octylphosphine oxide (TOPO) for at least 20 min.

3.2. Equipment

An automatic sample processor ASPEC, Model 232 (Gilson S. A., Villiers-le-Bel, France) was employed for the on-line-extraction (see Fig. 1). It consisted of an autosampling injector, two dilutors (Gilson, Model 401) and a six-port switching valve. This equipment is described in more details in Refs. [21,30]. The extraction of the target substances is realized in a membrane module, which consists of two blocks (one made of PVDF and another of PTFE) with identical channels, dimensions 0.1 mm × 2.5 mm × 40 mm, with a nominal volume of 10 μL. This type of membrane module is described in more details in Refs. [20–22,24,30].

The HPLC system consisted of an isocratic pump (Kontron Instruments HPLC Model 422), a column (250 mm × 4.6 mm, 3 μm, Ace 3 C_{18}, Advanced Chromatography Technologies, Scotland), LC-detector (LC spectrophotometer, Lambda-Max Model 480, Waters) at 210 nm. A mobile phase consisting of 30% methanol and 70%

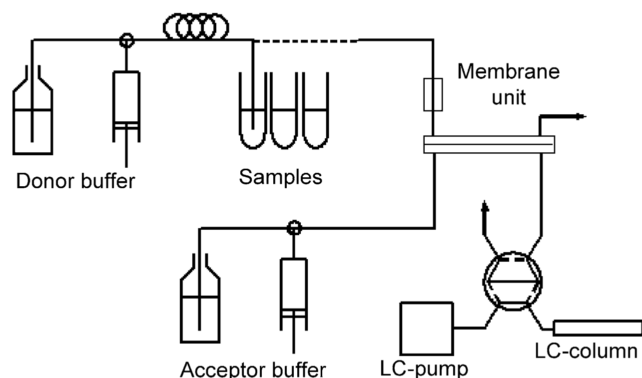

Fig. 1. Schematic of the setup for supported liquid membrane (SLM) extraction. Redrawn after ref. [21].

0.025 mol dm^{-3} phosphate buffer at pH 2.5 was pumped with a flow rate of 0.8 cm^{3} min^{-1}.

3.3. Operation

A new membrane was mounted in the membrane contactor and washed with donor and acceptor solution at a flow rate of 200 μL min^{-1} until 5 cm^{3} of the buffer had passed on both sides. The same procedure was applied for washing the membrane between the extraction cycles.

Different volumes (0.25–8 cm^{3}) of donor phase (drug solution in buffer or plasma) were pumped through the donor channel at constant flow rate (35 μL min^{-1}). The acceptor phase was stagnant during the extraction process. When the whole sample had passed through the membrane contactor, acceptor solution (100 μL) was pumped through the acceptor channel to transfer the whole enriched plug into the injection loop and subsequently to the HPLC column.

3.4. Determination of partition coefficients

The partition coefficient for the analytes between the donor aqueous phase and organic phase, as well as the partition coefficient between organic phase and acceptor aqueous phase, were determined using batch extraction as described in reference [24]. The partition coefficient was determined for each local anesthetic separately. The donor solution was shaken in an extraction funnel with 5% TOPO in dihexyl ether (in 5:1 phase volume ratio) for 15 min at room temperature. The mixture was left to stand and the aqueous and organic phases were separated. The concentration of analyte in the aqueous donor phase was measured before (C_1) and after (C_2) extraction using the HPLC system described above and K_D was calculated according to:

$$K_D = \frac{C_{org}}{C_2\,\alpha_D}; \quad C_{org} = (C_1 - C_2)\frac{V_{aq}}{V_{org}} \qquad (12)$$

where C_{org} is the calculated concentration in the organic phase, and V_{aq} and V_{org} are the volumes of the phases.

Then, the analyte was re-extracted from the organic phase into the acceptor aqueous phase (in 1:2 volume ratio) for 15 min at room temperature. The concentration of analyte was measured in the acceptor (C_3) after separation of the phases and K_A was calculated as:

$$K_A = \frac{C'_{org}}{C_3\,\alpha_A}; \quad C'_{org} = \left(C_{org} - C_3\frac{V'_{aq}}{V_{org}}\right) \qquad (13)$$

where C'_{org} and V'_{aq} refer to the second extraction.

4. Results and discussion

4.1. Optimization of the SLM extraction

For dissociable compounds, the pH of the sample has a large influence on the equilibrium between protein-bound and free analytes in plasma [36]. Since the drugs are mostly bound to plasma proteins in their non-charged form, decreasing pH reduces the protein binding for basic compounds due to the increase in the cationic form of the drug [35]. On the other hand, this might be counteracted by electrostatic binding of the cationic form of the drug to anionic sites on the proteins. However, it is known [37] that acidosis decreases the protein binding of drugs, influencing their pharmacological activity. As the pH of blood plasma is typically 7.5, the protein–drug binding would not be maximal in untreated, native plasma sample. In view of this, and to be able to study the drug–protein binding at native conditions, the pH of donor solutions, i.e. buffer solutions of drug and spiked plasma, was held constant and equal to 7.53 in all the experiments.

As discussed above, the extraction efficiency of the SLM extraction is also controlled by the acceptor pH, which is a limiting factor in attainment of high extraction efficiency. The acceptor pH should be at least 3.3 pH units below the pK_a of the basic compound in order to achieve complete trapping in the acceptor solution, i.e. the equilibrium will not be attained within reasonable time periods [26]. For higher pH values less enrichment is expected and equilibrium will be attained after relatively short time. The values of pK_a for the investigated local anesthetics are given in Table 1. Two different values of pK_a for some drugs are calculated using the program ACD/pK_a DB (Advanced Chemistry Development Inc., Toronto, Canada) and from the literature. The values of pK_a from the literature are specified at 37 °C [37].

The time variation of the enrichment factor, E_e for Prilocaine at different pH of acceptor phase, with the same ionic strength of the donor and acceptor phase (0.067 mol dm^{-3} phosphate buffer) is shown in Fig. 2. The donor flow rate was constant in all the experiments (0.035 cm^{3} min^{-1}) and the acceptor phase was stagnant during the extraction. The enrichment factor decreases with increasing pH of the acceptor phase, as the fraction of analyte in active form in the

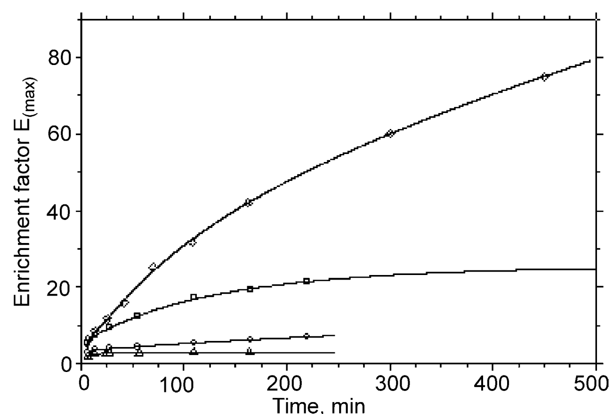

Fig. 2. The time variation of enrichment factor for Prilocaine, E_e at different acceptor phase pH values. The ionic strengths of the donor and acceptor phases are the same, 0.067 mol dm^{-3} phosphate buffer, C_I = 2.5 mg dm^{-3}. The donor flow rate was constant, 0.035 cm^{3} min^{-1}, and the acceptor phase was stagnant with pH$_A$ as follows: ◇ = 4.8, □ = 6.0, ○ = 6.5, △ = 7.0.

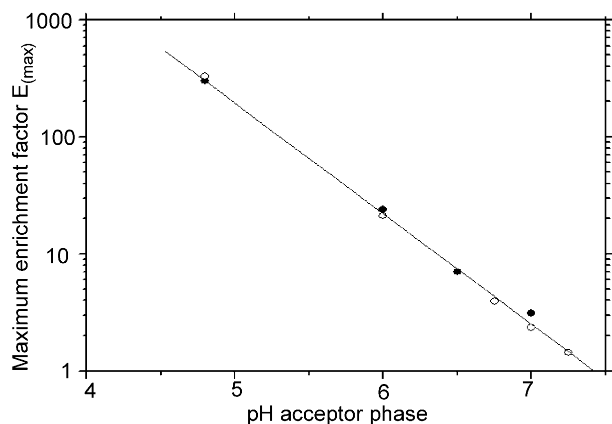

Fig. 3. Dependence of maximum enrichment factor, $E_{e(max)}$, for Prilocaine on acceptor pH. ● = experimentally obtained values, ○ = calculated values of $E_{e(max)}$ using Eq. (4).

acceptor phase, α_A, increases from 0.0006 to 0.0909 with increasing acceptor pH from 4.8 to 7.0. It is evident from Fig. 2 that the time required for the establishment of steady-state conditions also depends on acceptor pH. For example, in the case of the incomplete trapping (acceptor pH equal to 7.0) equilibrium conditions were established in a very short time (10 min), while for acceptor pH equal to 4.8, equilibrium conditions were not established during the longest extraction time (450 min). At the plateau, the concentrations (more precisely: the activities) of uncharged analytes in the acceptor and donor phases are equal, so the system reaches equilibrium and the flux ceases.

Fig. 3. shows the dependence of the maximum enrichment factor for Prilocaine on the acceptor pH. The experimental values are obtained from SLM extraction of Prilocaine at constant flow rate (0.035 cm^3 min^{-1}) and the same ionic strength of donor and acceptor phase (0.067 mol dm^{-3} phosphate buffer). The acceptor pH was changed from 4.8 to 7.0 in different experiments. In the cases of the low acceptor pH when the equilibrium was not established, the maximum enrichment factor was obtained by mathematical extrapolation of the experimental values. Also, for comparison, the values of the maximum enrichment factor calculated using Eq. (4) and experimentally obtained values of K_A and K_D (Table 1) are shown in Fig. 3. It is evident from Fig. 3 that the experimental data and the calculated values agreed very well.

4.2. *Extraction of local anesthetics at equilibrium conditions from buffer solution and plasma*

The influence of the type of buffer solution on extraction of local anesthetics (Prilocaine and Ropivacaine) was investigated and results are presented in Figs. 4 and 5. Different concentrations of the local anesthetic were extracted from different buffer solutions. The ionic strength of the donor and acceptor buffer solutions was equal; the pH of the donor was constant and equal to 7.53, which is the same as the pH of plasma. The pH of the acceptor was 7.0 so that incomplete trapping was accomplished. See Fig. 2. Two different buffer systems were used: 0.067 mol dm^{-3} phosphate buffer and citrate buffer. Both buffers contained similar concentration of components as their corresponding concentrations in plasma in order to better approach the condition $K_D = K_D^P$.

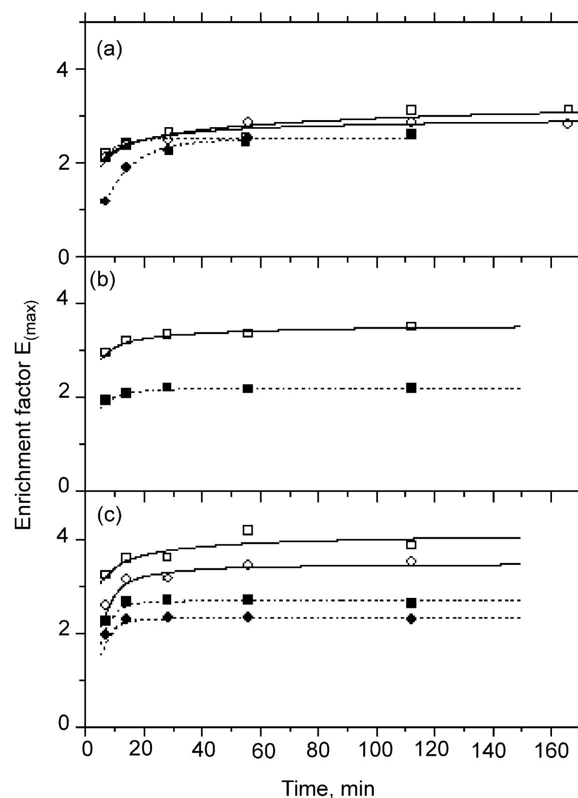

Fig. 4. The time variation of the enrichment factor for Prilocaine. The total Prilocaine concentrations in donor phase were: (a) 2.5 mg dm^{-3}, (b) 1.0 mg dm^{-3}, and (c) 0.5 mg dm^{-3}. The donor flow rate was constant, 0.035 cm^3 min^{-1}, the acceptor phase was stagnant. □ = donor and acceptor: phosphate buffer, ■ = donor: spiked blood plasma, acceptor phosphate buffer, ○ = donor and acceptor: citrate buffer, ● = donor: spiked blood plasma, acceptor citrate buffer, in all cases $pH_D = 7.53$ and $pH_A = 7.00$, concentrations of both buffers was 0.067 mol dm^{-3}.

Fig. 4a and c shows time dependences of the enrichment factors in Prilocaine extraction for phosphate and citrate buffers. It is evident that there is a slight difference between values of the enrichment factors for Prilocaine obtained by the extractions from phosphate and citrate buffers. The difference is slightly higher at the lower concentration (0.5 mg dm^{-3}) of Prilocaine in the donor phase.

Extraction of Prilocaine from plasma that was spiked with Prilocaine is also shown in Fig. 4. It is evident from Fig. 4b and c that the enrichment factor is lower for Prilocaine extraction from plasma than from buffer. However, in the case of the highest studied Prilocaine concentration (2.5 mg dm^{-3}), shown in Fig. 4a, the differences of the enrichment factors between Prilocaine extraction from plasma and the buffer solution are less significant. This could be attributed to high concentration of Prilocaine and relatively weak protein binding.

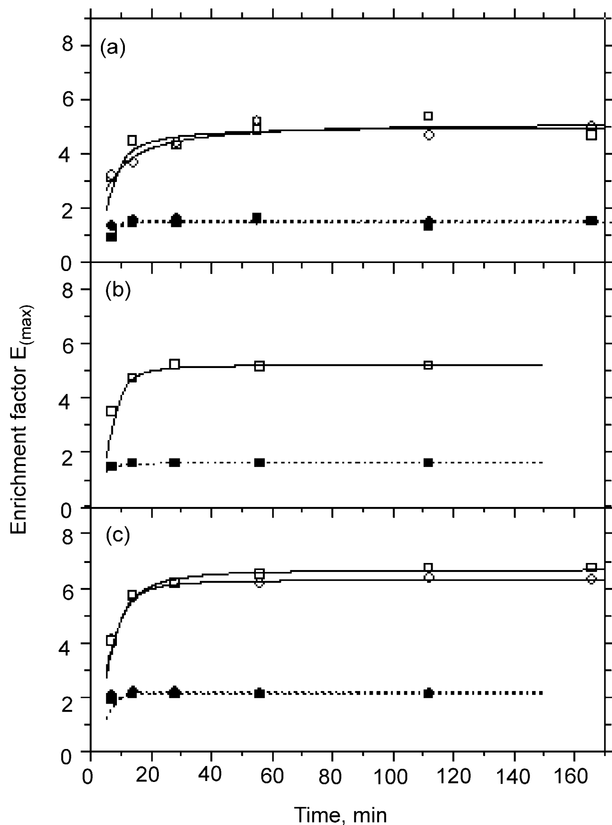

Fig. 5. The time variation of the enrichment factor for Ropivacaine. The total Ropivacaine concentrations in the donor phase were: (a) 2.5 mg dm^{-3}, (b) 1.0 mg dm^{-3}, (c) 0.5 mg dm^{-3}. Notation and other conditions are the same as in Fig. 4.

Fig. 5a and c shows the time dependence of the enrichment factors in Ropivacaine extraction for phosphate and citrate buffers. As in the case of the Prilocaine extraction, a slightly higher enrichment factor is obtained for extraction of Ropivacaine from phosphate buffer than from citrate buffer. Fig. 5 also shows the extraction of Ropivacaine from spiked plasma at different concentrations of Ropivacaine in donor phase. It is clear that under the given conditions, for all studied concentrations of Ropivacaine, the enrichment factor is significantly lower for the extraction from plasma than from any applied buffers, signifying a stronger protein binding of this drug than of Prilocaine.

The dependencies of the drug extractions on drug concentrations in the donor phase can be seen in Figs. 4 and 5. The enrichment factor decreases slightly from 3.8 to 2.9, and from 6.7 to 5, for Prilocaine and Ropivacaine, respectively, when increasing of the drug concentration in the donor solution from 0.5 to 2.5 mg dm^{-3}.

The extractions of three more local anesthetic amines were studied. The time dependencies of the enrichment factor for Lidocaine, Bupivacaine and Mesocaine are shown in Figs. 6–8, respectively. As observed for Prilocaine and Ropivacaine extractions, the enrichment factors of the other local anesthetics were lower from plasma compared with phosphate buffer solution. The ratio of the enrichment factors be-

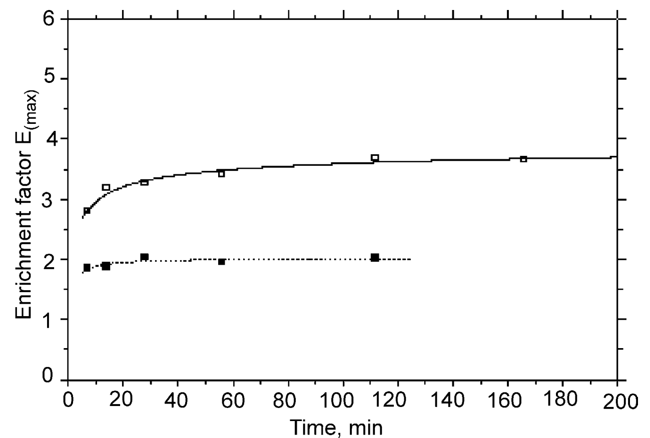

Fig. 6. The time variation of the enrichment factor for Lidocaine. The total Lidocaine concentration in the donor phase was 0.5 mg dm^{-3}. The donor flow rate was constant, 0.035 cm^3 min^{-1}, and the acceptor phase was stagnant. The acceptor was a phosphate buffer with $pH_A = 7.0$; □ = the donor was a phosphate buffer with $pH_A = 7.0$; ■ = the donor was spiked blood plasma.

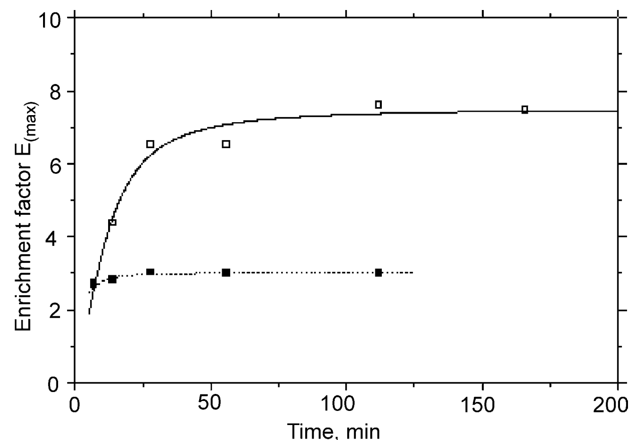

Fig. 7. The time variation of the enrichment factor for Bupivacaine. The total Bupivacaine concentration in the donor phase was 0.5 mg dm^{-3}. Other conditions were the same as for Fig. 6.

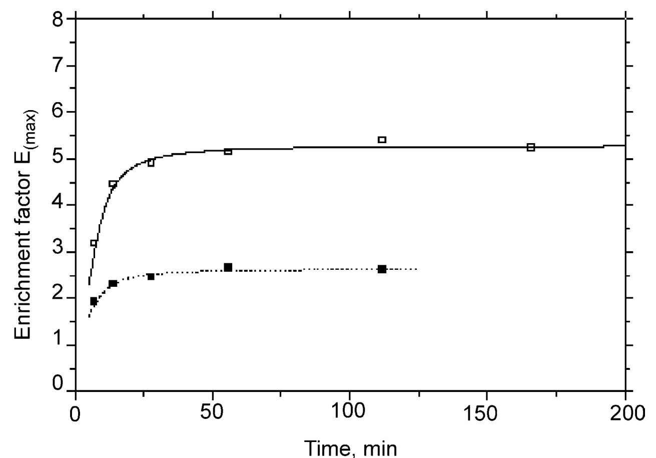

Fig. 8. The time variation of the enrichment factor for Mesocaine. The total Mesocaine concentration in the donor phase was 0.5 mg dm^{-3}. Other conditions were the same as for Fig. 6.

tween the drug extractions from the buffer and plasma depends on the protein binding of each drug. It is also clear from Figs. 4–8 that the maximum enrichment factor depends on the pKa of the compounds. For example, for the more basic local anesthetics Ropivacaine and Bupivacaine, $E_{e(max)}$ are 6.6 and 6.7, respectively, while for the less basic local anesthetics Prilocaine and Lidocaine, $E_{e(max)}$ are 4 and 3.7, respectively.

4.3. Determination of protein-binding of local anesthetics

α_1-Acid glycoprotein is the major protein involved in amine binding in serum. All basic drugs such as local anesthetic studied in this paper bind to AAG. The AAG concentration in serum is relatively low (0.5–1.0 g dm^{-3} in the adult) and depends on the age and health status of the body (for example AAG concentration increases during inflammatory processes). In a number of pathological states a decrease in the plasma protein binding of drugs is observed; for example acidosis markedly decreases the affinity of local anesthetics. The local anesthetics bind more or less specifically to HSA. Although the affinity of local anesthetics to HSA is less than to AAG, the enormous binding capacity of HSA renders this protein important in the binding equilibrium process: when binding to AAG is saturated, HSA continues to bind these drugs [37].

Experimental data on SLM extraction of local anesthetics from the buffers and plasma solutions (Figs. 4–8) were used to calculate protein binding values. It is presumed that in the case of SLM extraction in a flow system under equilibrium ($\Delta C = 0$, see Eq. (3)) and incomplete trapping conditions ($K_A \cong K_D$, see Eq. (3)), free drugs in plasma are in equilibrium with those in the acceptor and the drug–protein equilibrium is not disturbed as discussed in the Theory section above. For more accurate results and to test the condition $K_A \cong K_D$, the partition coefficients of the studied local anaesthetics were experimentally determined (Table 1). It is also presumed that ionic strength of plasma is similar to the ionic strength of the buffers used, so that $K_D^P = K_D$.

The fraction of drug in extractable form in the donor (α_D) and acceptor phase (α_A) in buffer was calculated using Eq. (6) and these values obtained for the studied compounds are shown in Table 2.

The protein bindings are calculated on the basis of the experimental data on the extraction from plasma and buffer using Eq. (9) and the fraction of drug not bound to protein (α_p) is easily estimated. The obtained values are given in Table 2 as protein binding (PB) expressed in percent; PB = 100 × $(1 - \alpha_p)$. If it assumed that α_{Dd} (in plasma) is equal to α_D (in buffer) and the partition coefficient between donor phase and organic phase for the studied drugs is the same for the extraction from buffer and plasma, then Eq. (11) can be applied for the calculation of α_P, which is presented in the same way. It is observed that the final result is independent of the pK_a values used for the calculation.

The obtained results for protein binding using Eqs. (9) and (11) are similar, in most cases with Eq. (11) giving slightly lower values. This indicates that the assumptions leading to Eq. (11) are reasonable. Thus, the simple procedure suggested by Eq. (11) involving only a comparison of the equilibrium enrichment factor of the drug in blood plasma and a matched buffer, provides similar results as the more complicated procedure according to Eq. (9) involving determination of partition coefficients (which also adds to uncertainty). Obviously, this is a statement that needs further validation.

The obtained drug–protein binding values are the same when citrate buffer was used compared to phosphate buffer using both equations.

The protein binding values obtained for the most hydrophobic drugs (Ropivacaine and Bupivacaine) are (20–35%) lower than the literature values, while for the other drugs the agreement is better. The literature values are mainly obtained using the ultrafiltration technique, which can be suspected to have a tendency to provide systematically high protein binding values. This could happen as the protein–drug equilibrium is shifted during the ultrafiltration procedure when the relative concentration of drug in contact with the protein is decreased. Another reason is that if some amount of drug is absorbed to the filter, this amount will in the

Table 2
The parameters obtained in SLM extraction under the equilibrium conditions of the five local anesthetics and the calculated protein bindings (PB)

Name	pK_a	α_A	α_D	Extraction from phosphate buffer		Extraction from citrate buffer		PB(%) literature value (see Table 1)
				PB (%) (Eq. (11))	PB (%) (Eq. (9))	PB (%) (Eq. (11))	PB (%) (Eq. (9))	
Prilocaine	8.0	0.091	0.253	35	45	33	50	55
	7.9	0.112	0.299		45		50	
Ropivacaine	8.9	0.012	0.041	68	76	66	71	90
	8.1	0.074	0.212		76		71	
Lidocaine	8.5	0.031	0.097	44	57			40–60
	7.8	0.137	0.349		57			
Bupivacaine	8.9	0.012	0.041	58	57			95
	8.1	0.074	0.212		57			
Mesocaine	8.6	0.024	0.078	50	70			50–70

All measurements were made at a total drug concentration of 0.5 mg dm^{-3}.

Table 3
The dependence of protein binding on the total concentration of local anesthetics in donor phase

Name	C_I (mg dm^{-3})	Extraction from phosphate buffer		Extraction from citrate buffer	
		PB (%) (Eq. (11))	PB (%) (Eq. (9))	PB (%) (Eq. (11))	PB (%) (Eq. (9))
Prilocaine	2.5	19	41	12	43
	1.0	36	41		
	0.5	35	45	33	50
Ropivacaine	2.5	70	83		
	1.0	73	81		
	0.5	68	76	66	71

common procedure decrease the measured concentration in the filtrate and therefore be counted as bound. Therefore, the values obtained here could be more accurate as they are based on a true equilibrium and would be insensitive to absorption in the membrane.

Also, the influence of the total concentration of two local anesthetics (Prilocaine and Ropivacaine) on drug–protein binding was investigated. The obtained results are given in Table 3. In the case of Prilocaine for which the protein binding is relatively weak, α_P varies with the total concentration of Prilocaine in the donor phase, while in the case of Ropivacaine α_P is practically constant.

5. Conclusions

The presented technique using equilibrium membrane extraction for measurement of drug–protein binding has provided encouraging results. The equilibrium technique suggests a negligible bias in contrast to the more dynamic techniques commonly applied. Providing that a matched (regarding pH and ionic strength) reference buffer is used, the calculations are conceptually very simple, without need for pK_a and partition coefficient data. The experimental format used for this preliminary study is somewhat complicated; a simplified format is presently under development.

Nomenclature

- C_A concentration of analyte in acceptor phase
- C_D mean concentration of analyte in donor phase
- C_I initial total concentration of analyte in donor phase
- C_{org} calculated concentration of analyte in organic phase in LLE experiments
- C_W concentration of analyte in the donor waste accumulated from the start of the experiment
- C_1, C_2, C_3 measured concentrations of analyte in LLE experiments for determinations of partition coefficients
- E_e concentration enrichment factor
- k overall mass transfer coefficient
- k_A mass transfer coefficient in the acceptor phase
- k_D mass transfer coefficient in the donor phase
- k_M mass transfer coefficient in the membrane phase
- K_a dissociation constant of the analyte
- K_A partition coefficient between acceptor phase and organic phase
- K_D partition coefficient between donor phase and organic phase
- K_D^P partition coefficient between donor protein solution and organic phase
- V_{aq} volume of aqueous phase in LLE experiments
- V_{org} volume of organic phase in LLE experiments

Greek letters

- α_A fraction of analyte in extractable form in acceptor phase
- α_D fraction of analyte in extractable form in donor phase
- α_P fraction of analyte not bound to protein in donor phase

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