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Application of solid-phase microextraction in the investigation of protein binding of pharmaceuticals

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Abstract

Protein–drug interactions of seven common pharmaceuticals were studied using solid-phase microextraction (SPME). SPME can be used in such investigations on the condition that no analyte depletion occurs. In multi-compartment systems (e.g. a proteinaceous matrix) only the free portion of the analyte is able to partition into the SPME fiber. In addition if no sample depletion occurs, the bound drug-free drug equilibria are not disturbed. In the present study seven pharmaceuticals (quinine, quinidine, naproxen, ciprofloxacin, haloperidol, paclitaxel and nortriptyline) were assayed by SPME. For quantitative purposes SPME was validated first in the absence of proteins. Calibration curves were constructed for each drug by HPLC-fluorescence and HPLC-UV analysis. SPME was combined to HPLC off-line, desorption occurring in HPLC inserts filled with 200 μL methanol. Binding of each drug to human serum albumin was studied independently. Experimental results were in agreement with literature data and ultrafiltration experiments, indicating the feasibility of the method for such bioanalytical purposes.

Keywords: Protein binding; Quinine; Quinidine; Naproxen; Ciprofloxacin; Haloperidol; Paclitaxel; Nortriptyline; Solid-phase microextraction; SPME

1. Introduction

When a drug reaches the circulation system it can be distributed into the elements of blood (erythrocytes, etc.) or bind to plasma proteins. Blood circulation is a means of drug transfer to different organs where the drug diffuses and is distributed at varying rates. The drug that is not bound to plasma proteins will diffuse to extravascular compartments and tissues where it can then bind to proteins or other components. The free drug concentration is the one correlated to pharmacological action, since this portion is available to reach tissue receptors. In most analytical practices the extraction methods recover the whole drug amount from blood, hence in many therapeutic drug monitoring protocols the total amount (free and bound drug) is finally measured. However, it is of great interest to determine and monitor the protein-free drug concentration in plasma. Several methods have been applied in order to assay protein binding of solutes: spectrofluorimetry, crystallography, surface plasmon resonance, gel filtration, binding assays [1], affinity-frontal chromatography [2] and most frequently equilibrium dialysis, ultrafiltration and microdialysis [3,4]. Each method has its advantages and disadvantages and the results obtained are method and condition specific.

Solid-phase microextraction (SPME) is a relatively new technique, initially developed for sample preparation in the environmental analysis of organic components. Within a decade of practice SPME has gained wide interest in a broad analytical field including food, biological and pharmaceutical analysis. Recently the applicability and the potential of SPME in biopharmaceutical analysis has been covered by comprehensive reviews [5–11]. A very interesting feature is the utility of SPME as a non-depleting extraction mode. Typical extraction recoveries achieved by SPME are lower than 5%. This low recovery is often regarded as a drawback, but in this specific application field, it enables the use of SPME in the measurement of the free analyte concentration. Due to low fiber uptake, analyte depletion from the sample is often negligible. A second advantage is the absence of interferences from the proteinaceuous matrix. PDMS and PA, the most common SPME fibers and the ones used in binding to matrix investigations, are in fact liquid polymers, wherein only small molecules can diffuse and hence be retained. In contrast macromolecules such as proteins and

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polysaccharides can be not extracted because they can not partition into the fiber coating. The amount of drug that is bound to the protein is not extracted as it does not take part into the extraction process. Moreover, due to the low recovery of the method (non-depletion) the equilibrium between the free analyte and the bound to protein analyte is not perturbed. Therefore, the analyte concentration extracted by SPME and measured subsequently is related only to the free analyte concentration in solution.

Vaes et al. have first given the theoretical background and described the use of SPME for the determination of protein free concentrations of four polar compounds: aniline, nitrobenzene, 4-chloro-3-methylphenol and 4-*n*-pentylphenol. Bovine serum albumin (BSA) was used as the model protein. Analyte concentrations were first measured by GC after SPME, and were again measured after the addition of BSA. Results were found to be in agreement to equilibrium dialysis experiments. Binding constants were not calculated, since BSA has several binding sites and binding to these analytes is not known to be equimolar or not [12–14]. The group of Pawliszyn has also studied the binding of alkylbenzenes to BSA [15] and diazepam to human serum albumin (HSA) [16] utilizing SPME-GC. In the latter case two extraction methods were applied. In the first method, extraction was performed in protein solutions of known diazepam concentrations. In the second method the drug was first extracted and next the fiber was desorbed in the protein solution. The amount of analyte left on the fiber was analysed after the system reached equilibrium. Scatchard plots were employed assuming 1:1 molecular interaction between HSA and diazepam. The binding constant (diazepam to HSA) was calculated from the Scatchard plot, although the usage of binding isotherms is more fitting for such calculations. Non equilibrium SPME was also used for the determination of freely dissolved concentration of hydrophobic organic compounds and the study of matrix effects [17]. The aforementioned reports combined SPME with GC for the determination of matrix-free analyte concentrations. Later Zambonin [18] combined SPME with HPLC for the determination of the protein free concentration of delorazepam. Heringa et al., used SPME on disposable PA fibers (5 μm) in order to study the binding of [^{3}H] estradiol to HSA [19]. Following sampling, the fiber was desorbed for 3 h in a scintillation vial and next the radioactivity was measured. The same authors have reviewed the field of the determination of free concentrations, describing the different methods used with emphasis on the application of negligible depletion SPME [20].

In the present communication the application of SPME for the determination of the protein free concentrations of seven common pharmaceuticals is described. Quinine, quinidine, naproxen, ciprofloxacin, haloperidol, nortriptyline and paclitaxel were incubated with HSA solutions and the free amount was extracted by SPME and determined in HPLC. SPME was combined with HPLC off-line; desorption was performed in HPLC inserts filled with the desorption solvent. Initially each drug was assayed independently by SPME and HPLC analysis. Next the protein was added and the protein free concentrations were measured. For three of the pharmaceuticals, the SPME results were compared and found in agreement with ultrafiltration experiments.

1.1. Theory

Drugs bind to proteins by a reversible reaction, where equilibrium is described as:

$$[P] + [D] \overset{K}{\rightleftarrows} [PD]$$

where [P] is the concentration of the free protein, [D] is the free drug concentration and [PD] is the concentration of the drug–protein complex.

At equilibrium the constant K is given as:

$$K = \frac{[PD]}{[P][D]}$$

Human plasma contains more than 60 proteins, but three of them are mainly associated with drug binding: albumin, α−1 acid glycoprotein (AAG) and lipoproteins. Albumin is abundant in plasma accounting for 60% of the total plasma protein content. Albumin binds mostly to acidic (anionic) drugs but also to cationic drugs. At physiological pH, the protein is negatively charged and acidic drugs bind to the N terminal group. Two primary high affinity drug binding sites have been described. At binding site IIA, binding of warfarin, sulfonamides, phenytoin, valproic acid and phenylbutazone is located. Binding site IIIA (benzodiazepine site) is involved for the binding of penicilines and probenecid. The pharmaceuticals studied in the present survey exhibit varying protein binding properties. Naproxen is reported to bind in both binding sites almost exhaustively 99.7%. Quinine is more avidly bound to AAG than to HSA [21]. Reports on the percentage of quinine binding to HSA are concentration dependent but meet to an average of 35%. The percentage of binding to human plasma proteins for the other studied pharmaceuticals is reported to be: 40% for ciprofloxacin, 92% for haloperidol, 92% for nortriptyline, 88–98% for paclitaxel and 87% for quinidine [22]. The binding of paclitaxel to HSA has been the subject of investigations for the identification of the binding site and binding constant but also aiming towards the development of water soluble paclitaxel formulations in the form of albumin conjugates [23–26]. Recent findings indicate that the interaction of paclitaxel with HSA in aqueous solutions results to a partial unfolding of the protein structure and on the non-specific binding of paclitaxel to HSA with an overall binding constant of $1.43 \times 10^4\ M^{-1}$ [23]. Other workers suggested the existence of high affinity specific binding ($K_1 = 2.4 \times 10^6\ M^{-1}$) and an intermediate affinity paclitaxel binding site ($K_2 = 1.0 \times 10^5\ M^{-1}$) [26]. These results although contradicting, indicate rather weak paclitaxel–protein interactions, and could be attributed to the presence of hydrogen bonding interactions between protein donor atoms and the paclitaxel polar groups.

Monitoring of free drug concentrations may be necessary for drugs that exhibit concentration dependent binding over the therapeutic range or in patients with diseases that alter the normal free concentration. Drugs that are highly bound in plasma (higher than 70%) would be candidates for monitoring, because they are most likely to show significant differences under certain conditions [3].

2. Materials and methods

2.1. Chemicals and instruments

SPME fiber holder and fibers (PDMS 100 μm and PA 85 μm) were used as obtained from Supelco (Bellefonte, PA, USA). HPLC analyses were performed using an LKB 2150 pump (Broma, Sweden), a programmable fluorescence detector FASMA 502 Rigas Labs (Thessaloniki, Greece), a Jasco 875 UV detector (Tokyo, Japan), a Rheodyne 7125 injector (Cotati, CA, USA) and a CR 6A integrator from Shimadzu (Kyoto, Japan). Chromatographic data management was achieved using a chromatographic software, developed by Professor P. Nikitas (Department Chemistry, Aristotle University Thessaloniki), running in Visual Basic 6.0.

A Supelco C18 Discovery column 150 mm × 4.6 mm, 5 μm, was used for HPLC analyses of quinine, naproxen and ciprofloxacin. The analysis of haloperidol was performed on a AnalitiCals ERBASIL S C18 250 mm × 4 mm, 10 μm (Carlo Erba, Milan, Italy); the analysis of paclitaxel on a Taxsil-2, 250 mm × 4.6 mm 5 μm (Metachem, Torrance, CA, USA) and the analysis of nortiptyline and quinidine on an Inertsil C18 (250 mm × 4.0 mm, 5 μm from MZ AnalysenTechniek Mainz Germany). HPLC solvents (methanol and acetonitrile) were obtained from Merck (Darmstadt Germany). All aqueous buffers used were prepared with double de-ionised water, analytical reagents and were next filtered though a 0.45 μm filter (Schleicher & Schuell, Dassel, Germany).

HSA (A-3782) 99% pure was obtained from Sigma (St. Louis, MO, USA). Haloperidol and paclitaxel were obtained from Sigma, naproxen was a kind gift from Minerva Pharmaceuticals (Athens, Greece), ciprofloxacin and nortriptyline hydrochloride were kind gifts from Kleva Pharmaceuticals (Athens, Greece) and H. Lundbeck A/S (Copenhagen, Denmark), respectively. Quinine and quinidine were a kind gift from Professor Robert Verpoorte (Leiden University, The Netherlands).

Phosphate buffer (PBS) was prepared as follows: 192 g Na_2HPO_4 and 35.1 g KH_2PO_4 were dissolved in 1600 mL water and the pH was adjusted to 7.4 with the addition of 1 M NaOH. Finally the volume was brought to 2 L by the addition of water.

2.2. HPLC determinations

Each analyte was processed independently; hence a different HPLC protocol was developed for each analyte. Experimental conditions are summarised in Table 1.

2.3. SPME

In the beginning of a day's analysis the fiber was cleaned/regenerated by exposure to 2 mL of MeOH for 4 min and next insertion in the GC injector (250 °C) for 30 min. For the extraction, 4 mL of a solution of the corresponding pharmaceutical in an appropriate buffer was transferred in a 4 mL vial containing a magnetic stirring bar (5 mm × 2 mm). The vial was capped and the sample was agitated with a magnetic stirrer at 700 rpm. Extraction by SPME was allowed to last for 40 min. At the end of that time, the fiber was retracted and was next left exposed in the air for 5 min in order to dry. Finally the fiber was dipped for static desorption (for 4 min) into a transparent glass HPLC insert containing the desorption solvent (200 μL of methanol). The insert had been sealed with parafilm and the SPME needle pierced the paraflim to expose the whole fiber to the desorption solvent. Finally an aliquot (80 μl) of the resulting solution was injected in the HPLC. Analyses were performed at least twice for each sample. Following desorption and prior to the next extraction the fiber was dried in air for 2 min, in order to remove methanol remaining on the fiber coating.

For quantitation purposes, calibration curves were constructed separately for each drug by applying the developed SPME-HPLC scheme to a series of aqueous solutions of each pharmaceutical.

2.4. Protein binding study

For protein binding studies each pharmaceutical was incubated alone with HSA. Quinine, quinidine and nortryptiline were assayed in PBS whereas the other four pharmaceuticals were extracted in 0.9% NaCl. Two milliliters of a solution of HSA (2 mg/mL in PBS, pH 7.4) were mixed in a 4 mL vial with 2 mL of a solution of the pharmaceutical. The solutions were left overnight for incubation at 4 °C. The next day the solutions were left to equilibrate to room temperature and were then subjected to SPME-LC analysis under the developed conditions. Solutions of each pharmaceutical at the same concentrations as in the incubation vials (but without the addition of protein) were also extracted by SPME, in order to have a direct comparison of the response of the total analyte concentration. In this way, quantitative determinations of one analyte (at the same total concentration) were performed with and without the addition of the protein in order to evaluate its protein binding behaviour.

Table 1
HPLC conditions for the analysis of the studied pharmaceuticals

Drug	Detection	HPLC column	Mobile phase
Quinine	Fluorescence, Exc λ = 254 nm, Em λ = 350 nm	Supelco C18 Discovery	Methanol–water–acetonitrile 58:25:17 (v/v/v), pH 3.1
Quinidine	Fluorescence, Exc λ = 280 nm, Em λ = 380 nm	Inertsil C18	0.05 M aqueous ammonium acetate–acetonitrile, 40:60 (v/v)
Naproxen	Fluorescence, Exc λ = 230 nm, Em λ = 350 nm	Supelco C18 Discovery	0.05 M aqueous ammonium acetate–methanol, 45:55 (v/v)
Haloperidol	UV, 210 nm	AnalitiCals ERBASIL C18	0.05 M aqueous ammonium acetate–acetonitrile, 35:65 (v/v)
Cipro	Fluorescence, Exc λ = 300 nm, Em λ = 460 nm	Supelco C18 Discovery	Methanol–water–acetonitrile 58:25:17 (v/v/v), pH 3.1
Nortriptyline	UV, 210 nm	Inertsil C18	0.05 M aqueous ammonium acetate–acetonitrile, 40:60 (v/v)
Paclitaxel	UV, 227 nm	Taxsil-2, Metachem	0.05 M aqueous ammonium acetate–acetonitrile, 35:65 (v/v)

2.5. Comparison of SPME with ultrafiltration

To validate the developed method, comparison with another well-accepted experimental method was performed. Ultrafiltration is used for a variety of purposes in contemporary science, due to its feature as a handy method for the separation of macromolecules. Ultrafiltration is based on the use of an immobilized membrane of a specific porosity within a centrifuge tube. Typically positive pressure or centrifugation is applied to separate the small molecules (free analyte), which end in the filtrate from the large molecules (proteins and bound analyte), which remain on the membrane filter. One milliliter of the incubation solution (containing both HSA and the drug as described in the previous paragraph) was placed in a Microcon YM-50 membrane filter (Millipore, Bedford, MA, USA). The filter has a cut-off level of 50,000 Da which does not allow HSA (MW 69,000) to pass through. The ultrafiltration devices were centrifuged for 5 min at 14,000 rpm (14,000 × *g*) facilitating a rapid passing of the liquid through the membrane. The filtrate was collected and an aliquot (80 μL) was analysed by HPLC. The analyte concentration was compared with the values obtained from the SPME experiments for the same solutions.

3. Results and discussion

3.1. SPME and HPLC determinations

Chromatographic and analytical data together with SPME validation for five of the seven drugs under investigation has already been described by our group [27]. For the two new drugs (nortryptiline and quininidine) a fast and simple HPLC system was found. In the present study HPLC served as the tool for the determination of the pharmaceuticals, hence the simple isocratic elution systems utilised (Table 1), sufficed for these purposes.

The effect of the most important parameters (e.g. extraction time, salt additives, sample pH) influencing the SPME is also discussed in detail in a previous report [27] for five of the seven drugs. The selected conditions for each drug were applied in the present work to study the binding of the drug to HSA. The two new drugs (nortriptyline and quinidine) have not been studied with SPME so far. For both drugs saturation curves were obtained and the distribution constant K_{fs} was determined: $K_{fs\,nortriptyline} = 140.37$, $K_{fs\,quinidine} = 35.12$. Saturation was observed for quinidine in 90 min and for nortriptyline at 60 min. Table 2 summarises basic SPME data of the present study: fiber type, K_{fs} and equilibrium time.

3.2. Protein binding determination by SPME

The procedure was based on the principle that only the free analyte concentration is available to partition into the SPME fiber. SPME may function either as an equilibrium or as a partition extraction technique for a specified time. In the latter case and in certain conditions, the amount of drug extracted from the fiber is often very small, and the equilibrium between the drug and the protein is not disturbed. It is generally accepted [13,17,19,20] that three conditions should be met for the correct application of negligible depletion SPME in matrix-binding studies: (1) there is equilibrium between the bound and the free fraction of the analyte; (2) the binding matrix does not interfere with the extraction and (3) the depletion of the free analyte fraction is negligible. The first two conditions can be rather easily addressed. To verify that the matrix does not interfere with the analysis, SPME was applied to a sample containing only HSA at the selected concentration. No interfering or new peak was observed in the HPLC chromatograms both in fluorescence and UV detection in the applied experimental conditions. On the last condition which is in fact the most critical, there has been a debate, as the extraction yield can never be 0%. Hence researchers have set limits on what they consider as negligible depletion: Gorecki and Pawliszyn set the limit at 1% [28], Vaes et al. [12,13], proposed a higher value of 5%, whereas Parkerton et al. [29] and Poerschman et al. [30], set the limit at 10%. A strategy to meet this condition as reported by Vaes et al. [12], is to use smaller fibers, in order to deliberately lower the extraction yield. The pharmaceuticals analysed in the present study show low distribution constants in SPME (see K_{fs} in Table 2) [27]. In addition, the extraction time selected (40 min) is shorter than the equilibrium time for most of the pharmaceuticals (the time needed to reach equilibrium in SPME sampling from aqueous solutions of the analytes). As a result, for non-protein samples the amount extracted is less than 1% of the total analyte amount in solution. Analyte depletion was calculated dividing the mass found to be sorbed in fibre at the used extraction time by the total initial analyte mass in solution. The corresponding depletion data found is also summarised in Table 2. For proteinaceuous samples, maximum theoretical analyte depletion was calculated in equilibrium conditions. In such samples, depletion due to fiber uptake is more intense, because the free analyte fraction (available for SPME) is restricted due to protein binding. Processing by SPME does not cause analyte depletion in either non-protein or proteinaceuous samples. For two of the pharmaceuticals the situation is rather critical because depletion close to 2% was theoretically calculated in SPME equilibrium conditions: naproxen (2.31%) and nortriptyline (1.86%). These compounds exhibit very strong (close to exhaustive) binding to protein, thus a very small amount will remain free in solution for SPME. However,

Table 2
SPME conditions and basic data for the analysis of the studied pharmaceuticals

Drug	Fiber	Equilibrium time (min)	K_{fs}	Analyte depletion[a] (%)
Quinine	PDMS	120	25.98	0.21
Quinidine	PA	90	35.12	0.36
Naproxen	PA	80	173.7	0.99
Haloperidol	PDMS	240	36.03	0.25
Cipro	PDMS	20	0.08	0.001
Nortriptyline	PA	60	140.4	0.66
Paclitaxel	PDMS	40	51.04	0.68

K_{fs}: distribution constant to the SPME fiber. Equilibrium time is found by performing saturation profile studies (solution-fiber). Analyte depletion is calculated using results obtained after SPME for 40 min sorption from solutions containing no protein.

[a] No protein solutions.

as the sorption time used in the study was much shorter than the time for equilibrium (40 min sorption, compared to 60 min for nortriptyline and 80 min for naproxen SPME equilibrium), lower fiber uptake would occur. This practically means that the depletion attained at 40 min can safely be regarded as negligible as it is lower than 2%. Hence it was decided to include the two compounds in the study.

The concentration of HSA used in this study was 1 mg/mL in the final incubation solution corresponding to 1.45×10^{-5} M. It should be pointed again that this is approximately the 1/20 of the typical human serum content of albumin. The concentration of each pharmaceutical measured by SPME-LC is related only to the free concentration remaining in solution. To calculate free analyte concentrations, calibration curves were constructed by the analysis of protein free samples by SPME-HPLC. These proved to be of adequate linearity: r^2 values ranged from 0.978 to 0.999. Following addition of HSA in solution, the chromatographic peaks obtained were significantly smaller for all the pharmaceuticals and in the whole studied concentration range, clearly indicating the occurrence of protein binding. A typical example can be seen in Fig. 1, where chromatograms of a solution of quinidine without (A), and with the addition of HSA (B) are depicted.

To calculate the unbound fraction of a pharmaceutical X, as suggested by Vaes et al. [12,13], the total concentration $[X]_t$ and the free concentration $[X]_f$ are plotted against each other. The free analyte fraction is given by the slope of the relevant

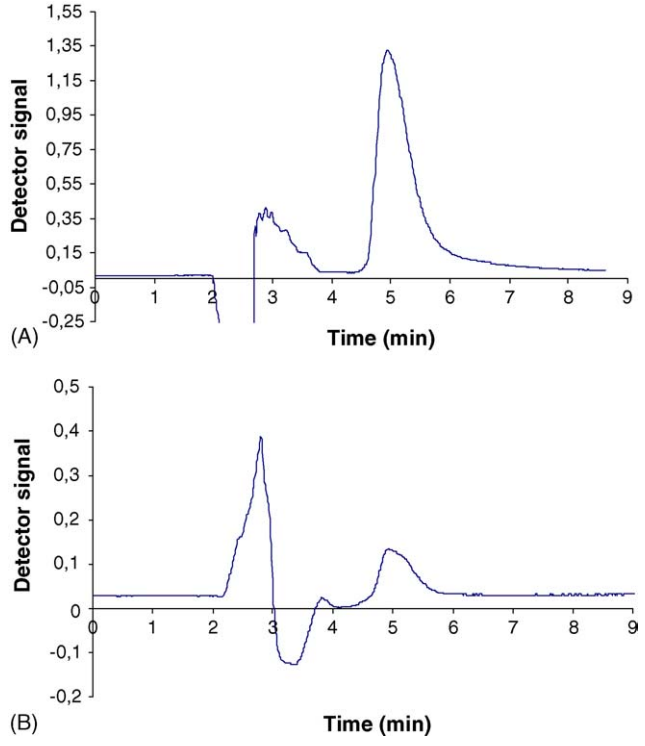

Fig. 1. Chromatographic analysis of the desorption solution resulting from SPME from a quinidine solution (10 μg/L, peak at 5.2 min) without (A) and with the addition of HSA (B). Observe the decrease in the analyte signal due to the binding of quinidine on HSA. Analytical conditions (HPLC and SPME) in text and Table 1.

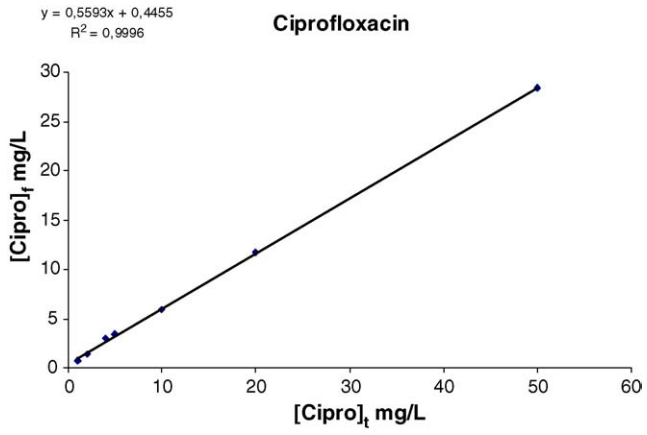

Fig. 2. Plot of the free concentration of ciprofloxacin vs. the total concentration of ciprofloxacin in solution. The slope of the fitted linear curve gives the unbound fraction.

curves ($f_x = [X]_f/[X]_t$). An example of such a graph is shown in Fig. 2, showing the plot of the free fraction versus the total concentration of ciprofloxacin. From the slopes of the above curves the free fraction was calculated to be 56%. The results obtained for the studied pharmaceuticals are summarised in Table 3. For naproxen, paclitaxel and haloperidol, the amount of the free drug was very low for most of the studied concentrations thus no free fraction could be determined by SPME-LC. As a consequence, no such graphs could be plotted for these pharmaceuticals and binding to HSA was regarded to be exhaustive.

The percentage of free quinine in solution was found to be 67%, which means 33% protein binding. This data is in very close agreement with the findings of Wanwimolruk and Denton [21] who report 35% binding of quinine to HSA. In plasma, quinine is reported to bind to proteins to a higher extent: 85–90%. This difference can be attributed to the fact that quinine is more avidly bound to AAG rather than HSA [21]. However the utilisation of protein concentrations other than the real life samples (e.g. plasma) may also (partly) explain the above mentioned differences between the values found in the present study and data from the literature. For quinidine the slope of the free versus total curve gives an overall value of 64% binding to protein. Again the data given for binding to plasma [22] is significantly higher: 87%. Interestingly the experimental findings signify a much stronger binding of quinidine to HSA compared to the direct diastereomer quinine (64% versus 33%). Ciprofloxacin is reported to bind to plasma proteins to 45%. In the present study, ciprofloxacin was found to bind to HSA to 44%. Nortriptyline is reported to bind to plasma to 92% and in the present study binding to HSA was to found to be 78%. From the data in Table 3, one can clearly observe the low deviation values for three of the drugs (quinine, quinidine and ciprofloxacin), whereas for nortriptyline a much higher deviation is observed. This variation is not attributed to the extraction process, but rather to the detection mode. Nortriptyline was detected in the low UV region (210 nm) where the detector signal is not selective. In contrast the other three pharmaceuticals, were assayed by fluorescence, where detection sensitivity and selectivity are much enhanced, resulting to higher accuracy and precision in mea-

Table 3
Summary of the protein (HSA) binding studies of the seven studied pharmaceuticals

Compound	Plot $[X]_f$ vs. $[X]_t$	r^2	f_x	Protein binding (%)
Quinine	$y = (0.6695 \pm 0.0115)x - (0.108 \pm 0.0092)$	0.994	0.67	33 ± 1.71
Quinidine	$y = (0.3552 \pm 0.0082)x - (0.0702 \pm 0.0855)$	0.999	0.36	64 ± 2.34
Naproxen	–		ND	Exhaustive
Nortriptyline	$y = (0.2182 \pm 0.0187)x - (0.7122 \pm 0.4361)$	0.978	0.22	78 ± 8.56
Paclitaxel	–		ND	Exhaustive
Ciprofloxacin	$y = (0.5593 \pm 0.0049)x + (0.4455 \pm 0.1037)$	0.999	0.56	44 ± 0.89
Haloperidol	–		ND	Exhaustive

Analysis was performed SPME-LC. ND: not determined; r^2: correlation coefficient of the linear curve; f_x: the unbound analyte fraction.

surement. This may be a critical point for the correct application of SPME in binding to matrix measurements. In addition calibration should be very carefully considered [20]. The very low amount of analyte that is being extracted may provide a source of errors if experimental conditions are not strictly constant. More sensitive detection may overcome this limitation. This problem could also be addressed by using lower protein concentrations in order to obtain higher free analyte fractions. However, it should be stated again that this may also partly contribute to differences in protein binding. Finally another matter to be addressed in such studies is the net desorption from the unstirred water layer. This phenomenon is described in detail by Oomen et al. [17] and Heringa and Hermens [20]. In the present study no net desorption from the proteinaceuous matrix is expected, as the fiber partition coefficients of the analysed drugs (K_{fs}) are low. Low fiber partition coefficients lead to the expectation that the rate limiting step of fiber uptake is the diffusion through the fiber coating and not through the unstirred water layer. Hence it can be safely anticipated that no net desorption from the protein in the unstirred water layer occurs.

3.3. Comparison of SPME with ultrafiltration

Ultrafiltration experiments were performed for three of the seven studied pharmaceuticals: quinine, naproxen and ciprofloxacin in the whole concentration range studied. For naproxen, protein binding was found to be exhaustive and no free naproxen could be measured in the filtrates even in the high concentration range. For quinine and ciprofloxacin the results obtained from ultrafiltration and HPLC analysis are illustrated in Fig. 3 together with the results of the SPME study. Comparing the results of the two modes an essentially good agreement can be observed. Ultrafiltration offers certain advantages compared with, e.g. equilibrium dialysis: shorter analysis time, commercial available kits of varying molecular weight cut-off and lack of dilution effects. However, ultrafiltration like equilibrium dialysis is not so easily adapted to large number of samples and the utilisation of the ultrafiltration devices may dramatically increase the economic cost of the assay.

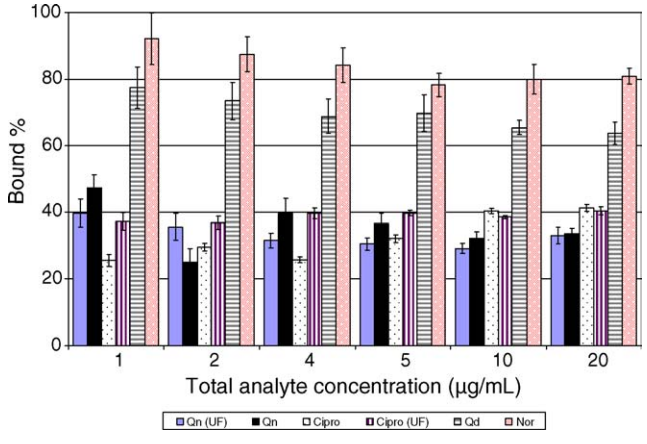

Fig. 3. Fraction bound to HSA for different concentrations for four of the studied pharmaceuticals: Qd: quinidine, Qn: quinine, Cipro: ciprofloxacin, Nor: nortriptyline. UF corresponds to results obtained from ultrafiltration experiments and HPLC analysis. The rest of the results were obtained with SPME and HPLC analysis.

4. Conclusions

It has been shown that SPME provides attractive features in the measurements of protein free drug concentrations, binding to sediments, partition coefficients, etc. The present paper provides further evidence that SPME can be applied, in combination with HPLC, in the study of binding phenomena of small molecules to macromolecules. Determinations of the protein free concentration of seven common pharmaceuticals were performed by SPME combined offline to HPLC. The results obtained were in agreement with the literature and with data obtained by other techniques, illustrating the potential of the method.

The described experimental set-up facilitates an easy and low cost assay on the protein binding properties of pharmaceuticals. The methodology is simple, fast, it covers a satisfactory concentration range and overcomes limitations of conventional methods, such as equilibrium dialysis (long equilibration times), ultrafiltration (low sensitivity, especially in the case of highly bound drugs), chromatographic and electrophoretic techniques (necessity of soluble or immobilised proteins and drugs). However, it should be stressed that the accurate application of SPME in this field requires that matrix effects (fouling of the SPME fiber by the macromolecular matrix) are minimal [20]. This may be the case when working with "academic" solutions of proteins, but in the case of complex biological samples, it should be examined thoroughly as it may alter extraction efficiency to a great extent [7,8,31,32].

The vast majority of the contemporary and especially the novel pharmaceuticals are polar non-volatile compounds mostly analysed by liquid phase separation techniques. Therefore,

the described methodology may prove a useful alternative for the assessment of protein binding behaviour of numerous pharmaceuticals.

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