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Automated study of ligand–receptor binding using solid-phase microextraction

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ABSTRACT

An automated ligand–receptor binding study was performed for the first time using solid-phase microextraction (SPME) coupled to liquid chromatography–tandem mass spectrometry. A new multi-fibre SPME system, which relies on multi-well plate technology and allows parallel preparation of up to 96 samples was used in order to obtain all data points of the binding curve in a single experiment. The binding of diazepam to human serum albumin was used as the model system in order to evaluate the performance of automated SPME. The time required to establish equilibrium was 30 min; this was verified experimentally by constructing extraction time profiles in the presence and absence of receptor molecules. Fibre constant calibration was used to remove inter-fibre variability from the binding data. Using a simple one-site binding model, a binding constant of $9.1 \times 10^5 \pm 3 \times 10^5$ l/mol was obtained. This result is in excellent agreement with values for equilibrium dialysis and manual SPME procedures reported in the literature. The proposed method can be further extended to study plasma–protein binding or drug binding to whole blood. In comparison to other methods, SPME is simpler, faster and fully automated, can be combined with any analytical detection method, and can be used to directly study complex samples.

1. Introduction

Ligand–receptor binding is the first step in many biological activities. One of the most-studied types of ligand–receptor binding is the interaction between a drug and a protein. Typically, neutral and acidic drugs bind mostly to human serum albumin (HSA), which is the most abundant of the blood plasma proteins, while basic drugs tend to bind to α-glycoprotein [1,2]. Upon drug intake, reversible binding between the drug and protein occurs, a process described by the following equilibrium:

$$D + P \rightleftarrows DP \quad (1)$$

The binding constant, *K*, for this equilibrium is then given by the following equation:

$$K = \frac{[DP]}{[D][P]} \quad (2)$$

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

In pharmacology, it is a well-established fact that only the unbound amount of drug, often referred to as the free concentration, is pharmacologically active and capable of crossing cell membranes. The type and magnitude of the drug–protein interaction significantly affect the distribution, metabolism, elimination, toxicity and biological activity of a drug. Therefore, knowledge of the drug–protein binding is necessary for establishing effective dosages of new drug candidates. In addition, competition between two drugs for the same binding sites may be the underlying cause of adverse drug–drug interactions.

Most of the traditional methods for studying ligand–receptor binding involve the physical separation of free and bound analyte, followed by an analysis step. These methods include equilibrium dialysis [3], ultrafiltration [4] and ultracentrifugation. Equilibrium dialysis is considered the reference method for drug–protein binding, as it is simple and easily temperature-controlled. However, it suffers from very long equilibration times (up to several days) and possible adsorption loss of the analyte to the membrane [1]. Ultrafiltration has been used as a routine method in clinical laboratories due to its simplicity and speed, but it can also suffer from non-specific binding of the drug to membrane, difficulty in controlling the temperature if centrifugation is used, and possible equilibrium shifts, especially for highly bound drugs. Other methods for the determination of drug protein binding include fluorescence spectroscopy [5], microdialysis [6], affinity chromatography [7], capillary electrophoresis [8–10] liquid chromatography [8,11], microdialysis [1] and supported liquid membrane equilibrium extraction [12]. Spectroscopic methods cannot distinguish between binding to one site and binding to multiple sites [5]. In addition, capillary electrophoretic frontal analysis methods suffer from lack

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of precise temperature control, may require a large quantity of protein, suffer from protein adsorption to capillary, and only work for selected buffers. In affinity chromatography, the protein is immobilized on a solid support, which can affect the nature and strength of binding due to steric hindrance, the unavailability of some binding sites, or protein denaturation. However, different ligands can be studied simultaneously, and the protein is reusable. A common disadvantage of all of the abovementioned methods is the lack of automation. In fact, only a few semi-automated binding studies based on 96-well equilibrium dialysis and 96-well filtrate assembly are reported in literature to date [13–15].

SPME is an equilibrium sample preparation method where the amount of drug extracted is proportional to the free concentration. As such, it has become a very useful method for determining drug–protein binding constants and the free concentration of various drugs in biological fluids. In the original applications of SPME to the determination of free concentrations, extractions were performed under conditions of negligible depletion, where it is assumed that the amount of drug (or any ligand) extracted by the fibre is negligible compared with the total amount of drug in the system, so that the equilibrium between the ligand and the protein remains undisturbed [16–21]. Other studies used negligible-depletion SPME to determine tissue-blood partition coefficients [22] and drug-binding equilibrium constants [23,24]. More recently, Musteata and Pawliszyn established the theoretical basis for applying SPME to any equilibrium, regardless of the amount of depletion [25]. Furthermore, SPME was shown to perform well in evaluating drugs with both high and low protein binding [25,26] and to study plasma–protein binding [27]. Some of the advantages of using SPME for such binding studies include the use of a small sample size, short analysis times, and the ability to study samples under any experimental conditions.

The main objective of the current study was to further increase the utility of SPME in binding studies by significantly increasing automation and sample throughput of these experiments. This was achieved by using a new commercial SPME autosampler and by developing appropriate methodology which minimizes the number of experiments required to obtain accurate binding data. Diazepam and HSA were used as the model system for this first automated SPME binding study because of the availability of published data using both traditional and manual SPME methods for this compound.

2. Experimental

2.1. Chemicals and materials

Diazepam and diazepam-D5 were purchased from Cerilliant (Round Rock, TX, United States) as 1 mg/ml methanolic solutions. Acetonitrile (HPLC grade), methanol (HPLC grade), and glacial acetic acid were purchased from Fisher Scientific (Ottawa, Canada). Chemicals required for the preparation of phosphate-buffered saline solution (PBS, pH 7.4) were purchased from EMD Chemicals (Gibbstown, NJ, United States). PBS buffer was prepared by dissolving 8.0 g of sodium chloride, 0.2 g of potassium chloride, 0.2 g of potassium phosphate, and 1.44 g of sodium phosphate in 1 l of purified water and adjusting the pH to 7.4 when necessary. C16-amide coated silica particles (RPA, Ascentis, 5 μm) were obtained as research samples from Supelco (Bellefonte, PA, United States). Type 316 stainless steel wire was purchased from Small Parts Inc. (Miami Lakes, FL, United States). Locktite 349 glue was obtained from R.S. Hughes Company (Plymouth, MI, United States). Polypropylene 96-deep-well plates were purchased from VWR International (Mississauga, Canada). Human serum albumin (HSA) was purchased from Sigma–Aldrich (Oakville, Canada).

2.2. Preparation of extraction phases based on RPA coated silica particles

The preparation procedure for RPA SPME extraction phases was based on the published procedure [28], but it was further optimized and adjusted to produce fibres suitable for use with the PAS Concept 96 SPME Autosampler, as described elsewhere [29]. Initial conditioning of the fibres included overnight exposure to a methanol:water solution (1:1, v/v), followed by a 30-min exposure to purified water, and a 30-min exposure to an acetonitrile:water mixture (1:1, v/v). In subsequent uses, the fibres were only conditioned for 30 min using the methanol:water mixture (1:1, v/v).

2.3. PAS concept 96 autosampler

The PAS Concept 96 autosampler (PAS Technology, Magdala, Germany) automates all of the steps in SPME using a multi-fibre SPME device and multi-well plate technology. It performs parallel SPME preparation of up to 96 samples. All SPME steps (fibre positioning, extraction time, internal standard addition, and desorption time) are computer-controlled via Concept software, thus eliminating the need for user intervention except for the placement of sample solutions in the multi-well plate. All experiments were performed at $25 \pm 2\,^{\circ}C$.

2.4. Extraction time profile experiments

The extraction time profiles (5–90 min) for diazepam were obtained in the presence and absence of 25 μM HSA in order to determine the time required for the binding between diazepam and HSA to reach equilibrium. The experiments were performed using agitation of 850 rpm. The data used to construct the time profiles with and without receptor was obtained simultaneously (in wells 1–6 without receptor, and in wells 7–12 with receptor).

2.5. Fibre constant calibration

Working standards (0.1–500 ng/ml) were prepared from 1 mg/ml diazepam stock solution using acetonitrile:water (1:1, v/v) as the diluent. They were kept refrigerated when not in use. These standards were injected directly into LC–MS/MS and used for the calibration of instrument response.

The fibre constants for all fibres used in the study were determined in the range of 1–250 ng/ml diazepam in PBS buffer. Total of five calibration standards was used to construct individual calibration curves for each fibre. The organic content of all standard solutions was kept constant at 1% methanol, as the presence of organic modifier can affect the equilibrium under study. The extraction was performed from 1.0 ml of sample solution using the PAS Concept 96 Autosampler for 30 min (equilibrium) at an agitation rate of 850 rpm. The fibres were then desorbed at 850 rpm for 30 min using 1.0 ml of desorption solvent (acetonitrile:water, 1:1, v/v), followed by LC–MS/MS analysis as described in section 2.7. An individual calibration curve was constructed for each fibre by plotting the amount extracted versus standard concentration. Finally, a simple non-weighted linear regression model was applied to the individual calibration curves. The calculated slope from the line of best fit was equal to the fibre constant for that fibre.

2.6. SPME procedure for the determination of diazepam-human serum albumin binding

The automated binding experiment was performed using the method of multiple-standard solutions [25], by extracting seven PBS standard solutions containing various amounts of diazepam,

which ranged from 0.2 μM to 7 μM, while keeping the HSA concentration constant at 25 μM. These concentrations were selected to cover a wide range of binding stoichiometries, ranging from 1:100 to about 1:3 (drug:ligand). SPME was performed with the automated multi-fibre autosampler using 1.0 ml of these standard solutions for 30 min at 850 rpm agitation. The fibres were then desorbed at 850 rpm for 30 min using 1.0 ml of desorption solvent (acetonitrile:water, 1:1, v/v), followed by LC–MS/MS analysis as described below. The pairs of corresponding free and total drug concentrations were then calculated for each sample. Free drug concentration was calculated by determining the amount of diazepam extracted by each fibre and dividing it by the fibre constant for the particular fibre used in the extraction of each standard solution. Total concentration of the drug after extraction was then calculated by subtracting the free concentration, determined by SPME, from the original concentration of the drug present in each well. The binding ratio (B), the ratio of the amount of bound drug to the amount of protein present in the well, was calculated by dividing the bound drug concentration after SPME extraction by the total protein concentration. Non-linear regression of free diazepam concentration versus B was performed on the final data set in SigmaPlot 2004 for Windows (version 9.0) software. The data was fit to a one-site ligand–receptor binding model to extract the binding constant (K_1) for the site with the highest affinity for diazepam because this is the most pharmacologically relevant binding constant [15].

2.7. LC–MS/MS analysis

All analyses were performed using a LC–MS/MS system consisting of a CTC-HTS PAL autosampler with a cooled sample tray, a Shimadzu 10AVP LC with dual pumps (Shimadzu LC10ADvp) and a system controller (SCL10Avp) and Applied Biosystems API3000 tandem mass spectrometer equipped with a TurboIonSpray source. Analyst software (version 1.4.1) was used for data acquisition and processing. The column used for analyte separation was Symmetry Shield RP18 (2.1 mm × 50 mm, 5 μm particles, Waters, Millford, MA, United States). Chromatographic conditions are reported elsewhere [30]. Sample injection volume was 20 μl. Samples were injected in duplicate and kept at 5 °C on the autosampler while waiting for analysis. Total chromatographic run-time was 5.0 min and included the re-equilibration of the analytical column. Diazepam was analyzed in positive ion MRM mode by monitoring the transition 285.0 to 153.9. The MS conditions used were: nebulizer gas = 6, curtain gas = 10, CAD gas = 12, ionspray voltage 5300 V, declustering potential = 92 V, focusing potential = 120 V, entrance potential = 7.5 V, collision energy = 39 V, cell exit potential = 10 V and source temperature set to 400 °C.

3. Results and discussion

When SPME is used to study ligand–receptor binding, two equilibria are established in the sample solution: (i) equilibrium between the ligand and the receptor and (ii) equilibrium between the SPME coating and free ligand concentration. The amount extracted by the SPME fibre is directly proportional to the free concentration, as shown in Eq. (3), which is valid for both absorptive and adsorptive (if far from saturation) coatings

$$[\mathrm{D}] = C_{\mathrm{free}} = \frac{n}{f_{\mathrm{c}}} \tag{3}$$

where n is the number of moles of drug extracted from the solution, and f_{c} is the fibre constant that represents the product between the fibre volume and K_{es} (equilibrium distribution constant between sample and extraction phase) for absorptive coatings, or the product of K_{A} (adsorption equilibrium distribution constant between sample and extraction phase) and the surface area for solid coatings [25]. The fibre constant can be determined simply by the extraction from standard solutions in the buffer, where the total concentration of drug is considered to be equal to the free drug concentration, as no proteins or matrix are present.

3.1. Optimization of SPME parameters

A RPA bonded silica particle coating was chosen in this study because it is easy to prepare, reusable, and has a high extraction capacity for diazepam and short equilibration times [29]. The structure of this coating is a C16 chain with an embedded amide group. The absolute recovery of diazepam from PBS buffer solutions using this coating is ~30% which allowed the use of significant depletion conditions during the binding study. Coatings with such high fibre constants are preferred for the study of drugs with high binding constants, such as diazepam, in order to ensure that the amount of drug extracted by the coating is sufficiently high so that instrumental sensitivity is adequate to accurately determine the amount of drug extracted by the coating. Acetonitrile:water (1:1, v/v) was chosen as the desorption solvent because it was compatible with direct injection into the LC–MS/MS. Desorption time was selected as 30 min in order to ensure efficient analyte desorption from the coating. With these optimized desorption conditions, diazepam carryover was 0.3–1.7% which is considered negligible for quantitative analysis. Prior to subsequent extraction, however, this small amount of carryover was eliminated completely during a 30-min fibre pre-conditioning step performed prior to each sample analysis. The coating was found to be re-usable for >15 times with no loss of extraction efficiency [29], and longer-term reusability studies are currently on-going.

3.2. Equilibrium time

Diazepam and HSA were chosen as the model system for the ligand–receptor binding study, because this system has been well-studied using various traditional methods as well as manual SPME [3,9,10,23,25,31,32]. The determination of ligand–receptor binding using SPME should be performed using extraction times long enough to establish two simultaneous equilibria: (1) the equilibrium between the drug and the protein and (2) the equilibrium between the free concentration of the drug and the SPME fibre. In order to evaluate the time required to establish both equilibria, an extraction time profile for diazepam was constructed in the absence (Fig. 1a) and presence of protein (Fig. 1b). The time required to reach equilibrium is defined as the time when there is no further increase in the amount extracted. Equilibrium was reached in 30 min for both time profiles, as shown in Fig. 1; therefore, 30 min was selected as the extraction time for the automated ligand–receptor binding study. Theodoridis reported the determination of free concentration and percent drug–protein binding for several drugs to HSA using pre-equilibrium SPME, but such an approach is only feasible if equilibrium under study is established more rapidly than SPME equilibrium or if the drug is pre-incubated with the protein prior to SPME extraction [33]. One advantage of SPME over some of the traditional binding methods is that the time required to reach equilibrium between the ligand and the receptor can be verified experimentally, similar to equilibrium dialysis. In contrast, chromatographic and electrophoretic methods typically assume rapid local equilibrium or "instantaneous equilibration" [8], while ultrafiltration and ultracentrifugation methods assume slow equilibrium. Such assumptions may be invalid and can lead to erroneous results.

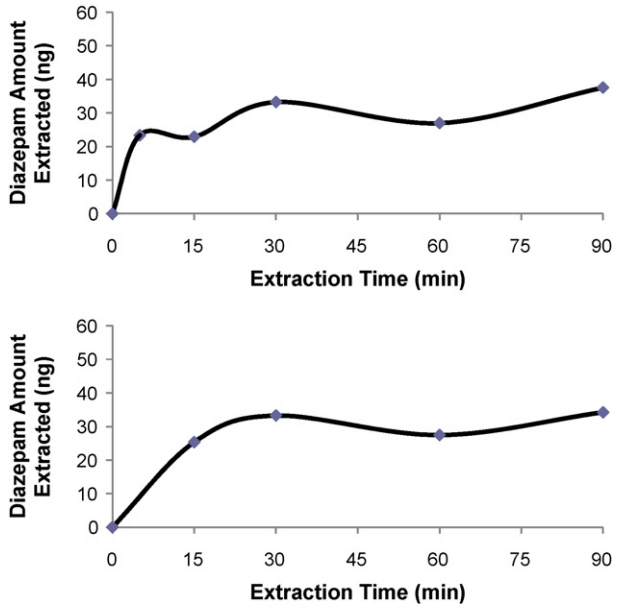

Fig. 1. Extraction time profiles ($n = 6$ fibres) for diazepam at 850 rpm agitation in absence and presence of HSA. (a) 100 ng/ml standard solution of diazepam in PBS buffer pH 7.4. (b) 1.8 μM standard solution of diazepam with 25 μM HSA in PBS buffer pH 7.4.

3.3. Fibre constant calibration

The performance of the multi-fibre SPME–LC–MS/MS system for automated ligand–receptor binding studies was evaluated using the method of multiple standard solutions for the determination of binding parameters [25]. The original studies [16,21,23–26] used a single fibre for sequential extractions from multiple standard solutions containing known concentrations of ligand and receptor; however, in this study, multiple fibres were used to perform the SPME procedure in parallel, thus significantly enhancing throughput. Therefore, the determination of fibre constant for each fibre was necessary in order to accurately determine the exact free concentration of drug in each standard used for the binding study. The fibre constant is the product of the coating volume and the distribution constant between the coating and diazepam. The determination of the fibre constant was performed for diazepam under identical conditions; therefore, the differences observed are due mainly to slight differences in the volume of coating on each fibre. The use of individual fibre constants allowed for the elimination of inter-fibre differences in extraction capacity from the binding data thus permitting an accurate determination of binding constants. The results obtained for the seven fibres which were used in the binding study are shown in Table 1. According to Eq. (3), the slope of the linear regression is equal to the fibre constant.

Table 1
Fibre constant for each of seven fibres employed in the automated binding study

Analyte	Slope	Standard error of slope	Intercept	Standard error of intercept	r^2
1	0.337	0.003	−0.317	0.404	0.9998
2	0.375	0.001	0.0480	0.139	1.000
3	0.288	0.002	−0.0965	0.236	0.9999
4	0.369	0.006	−0.564	−0.741	0.9994
5	0.351	0.002	−0.314	0.232	0.9999
6	0.314	0.001	−0.00393	0.161	1.000
7	0.297	0.002	−0.164	0.0215	0.9999

Summary of linear calibration curves acquired in the range of 1–250 ng/ml diazepam in PBS buffer ($n = 5$ standard solutions).

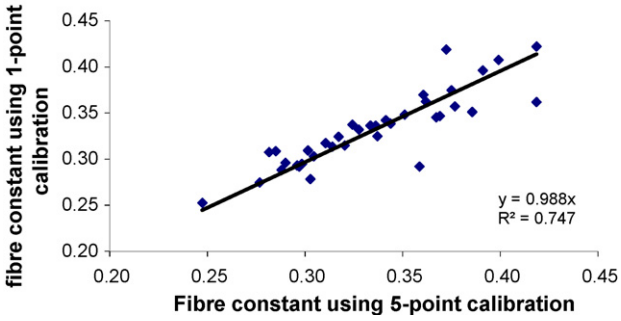

Fig. 2. Comparison (linear regression analysis) of the determination of fibre constant using 5-point calibration versus 1-point calibration.

As expected from theory, the *Y*-intercept passes through origin at 95% confidence interval in all cases. The regression coefficients (r^2, linear least-squares, non-weighted) for the determination of fibre constants were ≥0.9994 in all cases, indicating excellent performance of all the fibres. The differences in the magnitude of fibre constant (slope as shown in Table 1) for each individual fibre can be used to calculate the inter-fibre variation for the seven fibres shown in Table 1 (mean = 0.33, S.D. 0.03, %R.S.D. = 10). The inter-fibre variation of 10% is significant, so the fibre constant calibration was employed in order to eliminate this inter-fibre variation from binding results in the current study. Inter-fibre variability for all $n = 96$ fibres was also evaluated and found to be 12% for the extraction of diazepam from PBS buffer [29]. In future, further improvements to the coating procedure and/or availability of commercial coatings can further improve inter-fibre variability and possibly eliminate the need to employ fibre constant calibration.

In the next experiment, it was investigated whether one-point calibration can yield accurate values for fibre constant, as this would significantly reduce the number of experiments required for binding analysis. One-point fibre constant calibration was performed using a single diazepam standard with concentration of 100 ng/ml and using Eq. (3) to calculate f_c. The results for fibre constant obtained using a 5-point calibration were plotted against the fibre constant obtained using a 1-point calibration, as shown in Fig. 2 for a total of 36 fibres. The slope of the regression line was 0.988, and there was no significant difference between the two data sets using Student's *t*-test ($t = 0.311$). To conclude, one-point fibre constant calibration is sufficient to remove inter-fibre variability from analytical data. However, the standard concentration selected for this determination should fall approximately in the middle of fibre linear range, since the use of very low standard concentrations (close to limit of quantitation) can result in poor accuracy.

The ability of fibre constant calibration to correct for inter-fibre variation was evaluated using triplicate determinations of diazepam free concentration. This assay was performed at three different total concentrations of diazepam (low, medium and high concentration) while keeping the HSA concentration constant at 25 μM. The results of this experiment are summarized in Table 2. Excellent precision was obtained as shown by R.S.D. values ranging from 6.8% to 13%.

3.4. Automated binding study

With the HSA and diazepam binding data acquired in a single automated experiment, a binding curve was constructed as shown in Fig. 3 to determine the binding parameters for this interaction. One of the main theoretical models for drug–protein binding, the multiple equilibrium site-oriented model, assumes that ligand binding is independent of events at other sites, which means that

Table 2
Summary of results for assay of free concentration using SPME, performed at three total diazepam concentrations 55 ng/ml (0.2 μM), 225 ng/ml (0.8 μM) and 1800 ng/ml (7 μM)

Assay	Free diazepam concentration (ng/ml)	Free diazepam concentration (ng/ml)	Free diazepam concentration (ng/ml)
1	12.0	46.5	403.4
2	10.6	42.0	324.8
3	10.7	40.6	419.0
Mean (n = 3):	11.1	43.0	382
S.D. (n = 3)	0.76	3.1	50
%R.S.D.	6.8	7.2	13
Total diazepam concentration (ng/ml)	55	225	1800

HSA concentration was kept constant at 25 μM for all determinations.

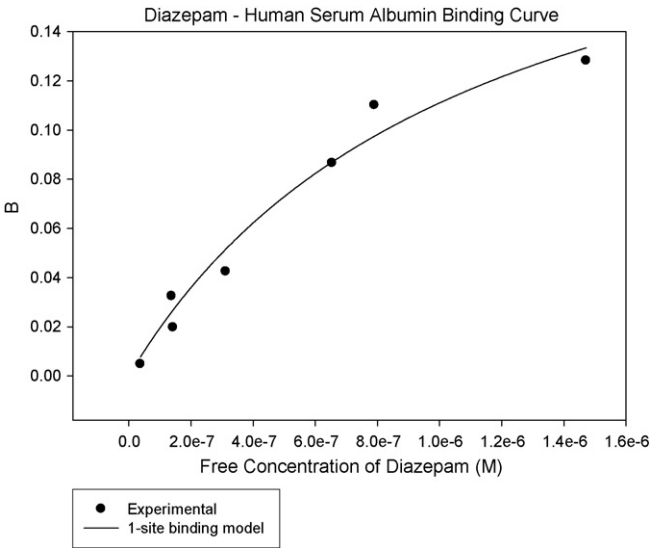

Fig. 3. Experimental binding curve for the interaction between diazepam and HSA, fitted to one-site binding model.

K and n_i at each site are independent and constant [25]

$$r = \sum \frac{n_i K_{ai}[D]}{1 + K_{ai}[D]} \quad (4)$$

where n_i is the number of binding sites, K_{ai} the association constant of the ith binding class (also known as site-binding constant), [D] the free concentration of drug and r is the mole of bound ligand per mole of total protein.

A good fit between the data and the one site-binding model was obtained as indicated by regression coefficient of 0.991. The binding parameters obtained in current study are summarized in Table 3, where they are also compared with values found in the literature. The results of this study agree very well with those using manual SPME and the equilibrium dialysis method within experimental error. Equilibrium dialysis is the main method chosen for this comparison, because it is considered the most accurate and reliable. Based on the good agreement between the results of the proposed automated SPME–LC–MS/MS and those obtained by equilibrium dialysis, the automated SPME was shown to be suitable for automated ligand–receptor binding studies. It is important to note that Eqs. (1)–(4) are valid for any type of ligand–receptor binding (not just drug–protein binding). Eq. (3) is valid for all such binding studies using SPME, regardless of the analytical method used for the detection, provided that (i) the extraction time used is equal to or longer than the time required to reach equilibrium and that (ii) the matrix does not interfere with the measurement by binding to the fibre.

The experimental error in the determination of binding constant in current study was slightly high at 30%, but better than obtained for equilibrium dialysis. It is believed that is caused by using limited number of data points (seven), but this can be further improved in future by increasing the number of data points in the binding curve to 12–15. The results for manual SPME–LC study [25] which used 14-points show much lower experimental error.

An examination of HSA–diazepam binding values in literature reveals significant variation in experimental binding constants. The results obtained from electrophoretic techniques show great variation from accepted equilibrium dialysis results. Ostergaard et al. attribute the discrepancy in the results in their study to insufficient number of data points with r values <1 [9]. In general, the variation in binding results obtained by various methods can be attributed to the differences in the analytical methods used as well as to the differences in experimental parameters during the binding studies, such as different temperatures, pH, ionic strengths, albumin fractions, and binding models used. For example, a recent study demonstrates a large impact of pH on the unbound (free) fraction of basic drugs, and shows that the results obtained can be highly variable unless strict pH control is used during binding studies [15]. Most of the studies reported in Table 3 used HSA concentrations of 10–50 μM which is not close to physiological conditions, in order to ensure that the free concentration of drug is sufficiently high to be analytically detectable. Current study employed similar HSA concentration (25 μM), so that the results can be compared easily to existing literature. In future, additional automated SPME studies employing physiological concentration of HSA can be performed in

Table 3
Summary of diazepam–HSA binding experimental results using various methods

Technique	Diazepam–HSA binding parameters, experimental results	Reference
Automated multi-fibre SPME–LC	$K = 9.1 \times 10^5 \pm 3 \times 10^5$ l/mol	Current study
Manual SPME–GC	$K = 10.2 \times 10^5$ l/mol (method 1, large volume) $K = 12.3 \times 10^5$ l/mol (method 2, small volume)	[23]
Manual SPME–LC	$K_1 = 17.6 \times 10^5 \pm 6.32 \times 10^4$ l/mol, $n_1 = 1$	[25]
Equilibrium dialysis	$K_1 = 17.49 \times 10^5 \pm 6.26 \times 10^5$ l/mol, $n_1 = 1$	[3]
Equilibrium dialysis	$K_1 = 11.59 \times 10^5$, $n_1 = 1$	[31,32]
Capillary electrophoresis—frontal analysis	$K_1 = 0.32 \times 10^5$ l/mol, $n_1 = 1.6$	[10]
Capillary electrophoresis—frontal analysis	$K_1 = 2.1 \times 10^5$ l/mol, $n_1 = 1.5$	[9]

All data are shown in scientific notation with the exponent 10^5 in order to facilitate the comparison across the methods.

order to yield improved and physiologically more-relevant binding data since albumin aggregation at high protein concentrations may cause drug displacement [10]. In fact, one advantage of SPME is that it can be used to perform binding studies in any desired medium including whole blood.

Despite the importance of receptor–ligand binding studies for drug development and discovery, none of the traditional methods have achieved full automation and high-throughput. Only semi-automated methods, based on ultrafiltration and equilibrium dialysis for the determination of plasma–protein binding have been reported [13–15,34]. Therefore, the availability of automated SPME can play an important role in this field. The 96-well equilibrium dialysis apparatus proposed by Banker et al. requires 8 h to reach equilibrium for 10 selected drugs including diazepam [14], while proposed automated SPME technique is considerably faster with 1 h sample preparation time.

4. Conclusions

Further improvements to the PAS Concept 96 SPME autosampler are currently under way and include the addition of temperature-control in order to allow binding studies under physiological conditions. The use of SPME in ligand–receptor binding studies permits great deal of flexibility in comparison to traditional methods as it allows the experiments to be performed under any desired conditions (e.g., any buffer or real physiological matrix, any concentration of ligand and receptor) and permits analysis using any suitable detection method. In the future, the methodology presented in current study can be easily applied to investigate other interactions, such as drug-plasma protein binding or affinity binding of drug candidates to desired biomolecular targets. The ability to prepare up to 96 samples simultaneously allows for the acquisition of binding data for 8–12 compounds in a single experiment, depending on the number of points used in each binding curve. This represents a very significant increase in sample throughput over manual SPME techniques and traditional binding methods.

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