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Journal of Pharmaceutical and Biomedical Analysis 29 (2002) 895–900

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

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SPME-LC with UV detection to study delorazepam–serum albumin interactions

Carlo G. Zambonin *, Antonella Aresta

Dipartimento di Chimica, Università degli Studi di Bari, Via Orabona 4, 70126 Bari, Italy

Received 11 March 2002; received in revised form 20 March 2002; accepted 31 March 2002

Abstract

Solid phase microextraction coupled to high performance liquid chromatography with UV detection (SPME/LC-UV) has been employed to study the binding of delorazepam to human serum albumin (HSA) and bovine serum albumin (BSA). The procedure could also be potentially extended to the measurement of partition coefficients between a wide variety of semi- or non-volatile compounds and matrices. The method is solvent free, simple, fast, and drawbacks of the conventional analytical techniques are avoided. Moreover, the matrix did not interfere with the measurement by binding to the fibre and the amount extracted by the fibre was negligibly small; thus it did not disturb the delorazepam–protein binding. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: SPME-LC; Drug–protein interactions; Delorazepam; HSA; BSA

1. Introduction

Protein reversible binding is important in many processes that determine the eventual activity and fate of a drug once it has entered the body. The degree of binding may significantly impact the biological activity of the drug [1,2]. The binding between a drug (D) and a protein (P) can be described by the following equilibrium:

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

This protein binding occurs often with general ligands such as human serum albumin (HSA) [3–7].

Numerous methods [8–16] such as fluorescence spectroscopy, circular dichroism, equilibrium dialysis and ultrafiltration have been frequently used to evaluate protein–drug interactions. However, for compounds highly bound to proteins, the concentration of free drug is frequently low and difficult to detect. Thus, chromatographic [17–21] or electrophoretic [22,23] techniques have been developed for this purpose. These methods are usually based on soluble or immobilised proteins and drugs, that, however, are characterised by intrinsic disadvantages. Large volume samples of the drug and protein to be studied are necessary using the first approach. The use of immobilised ligands in a chromatographic system is able to reuse the same ligands preparation for multiple experiments; however, the immobilisation process

* Corresponding author. Fax: +39-80-544-2026.

E-mail address: zambonin@chimica.uniba.it (C.G. Zambonin).

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PII: S0731-7085(02)00217-0

can affect protein activity through denaturation, improper orientation or steric hindrance of the protein at the sites binding.

Solid-phase microextraction (SPME) was introduced [24] in 1990 and, since then, it has been used in environmental, food and drug studies. This extraction technique has been also applied, coupled to GC, to the measurement of partition coefficients [25,26] due to its ability to determine the distribution of chemicals in aqueous matrices even at low concentration. In particular, SPME-GC has been successfully employed [27,28] to study the binding properties between bovine serum albumin (BSA) and volatile organic compounds. In fact, the amount extracted by the fibre, according to the equilibrium

$$D + SPME \rightleftarrows D - SPME \quad (2)$$

was so small that it did not disturb the binding between the analyte and the protein (see Eq. (1)). Moreover, the matrix did not interfere with the measurement by binding to the fibre. The protein–water partition coefficient of the drug can be determined using the following equation:

$$f_D = [D]_f/[D]_0 = 1/(1 + K_P[P]) \quad (3)$$

where f_D is the freely dissolved fraction of the drug D, $[D]_f$ is the concentration of the freely dissolved chemical, $[D]_0$ is the total concentration (freely dissolved and bound to P), K_P is the protein–water partition coefficient of the drug and [P] is the concentration of the protein P in the aqueous phase.

However, many classes of pharmaceutical products are semi- or non-volatile, and are best analysed by liquid chromatography. Benzodiazepines are a large class of non-volatile drugs commonly used as minor tranquillisers, hypnotics, muscle relaxants and anticonvulsants. Recently, a new SPME-HPLC/UV method for the determination of the benzodiazepine delorazepam was developed in our laboratory [29].

In the present study, SPME coupled to HPLC/UV was employed to study the binding of delorazepam to HSA and BSA and the relevant results have been critically compared.

2. Experimental

2.1. Chemicals

Delorazepam was purchased from Sigma (St. Louis, MO). Methanol stock solutions were prepared and stored in the dark at 4 °C. More dilute solutions were prepared in phosphate buffer (0.1 I, pH 7.4 or pH 3.3) just before use and filtered through a 0.45 mm Millex- HV type filter (Millipore).

BSA fraction V (98% purity) and fatty acid and globulin-free (HSA, 99% purity) were purchased from Sigma, dissolved in phosphate buffer (0.1 M, pH 7.4) at a concentration of 100 μM and stored in the dark at −20 °C.

All organic solvents used (Carlo Erba, Milan, Italy), were HPLC grade. The HPLC mobile phase was filtered through a 0.45 μm membrane (Whatman Limited, Maidstone, UK) before use.

2.2. Apparatus

The SPME-HPLC apparatus consisted of a SPME device, an interface and an HPLC system. The holder and the assembly of the SPME device for manual sampling were purchased from Supelco (Bellefonte, PA). A new fibre was conditioned before use as specified in the literature accompanying the commercial SPME products. The SPME/HPLC interface (Supelco) consists of a standard six-port HPLC Rheodyne valve equipped with a special fibre desorption chamber (total volume: 60 μl) and a bracket for bench top mounting.

The HPLC system used in this study includes an SNC 1000 Vacuum membrane degasser (Thermo Separation Products), a Spectra System Pump, model P2000 (ThermoQuest, San Jose, CA) and a Supelcosil LC 18-DB column (250 × 4.6 mm i.d, particle 5 μm, Supelco, Bellefonte, PA, USA). A 5 μm Supelguard LC-18-DB precolumn (20 × 4.6 mm i.d., Supelco) was used to protect the analytical column. The detector was a HP 1040A photodiode-array spectrophotometer (Hewlett–Packard, Palo Alto, CA) interfaced to an HP 85 computer equipped with an HP dual disk drive and an HP 7470A plotter. A Hewlett–

Packard model HP 3395 laboratory computing integrator directly connected to the analogy output of the photodiode-array detector was also used.

2.3. Chromatographic and detection conditions

The mobile phase consisted of acetonitrile/tridistilled water (65:35, v/v). The flow rate was 1 ml min^{-1} and temperature was ambient. The detection wavelength was 230 nm (4 nm bandwidth) and the reference signal was at 550 nm.

2.4. Solid-phase microextraction

A silica fibre (Supelco) coated with a 60 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) was used. The fibre was appropriate for use with most water-miscible HPLC solvents.

Working solutions were prepared in 20 ml clear vials (Supelco) by dissolving various amounts of delorazepam into 15 ml phosphate buffer (0.1 M, pH 7.4 or 3.3) and submitted to SPME. The extraction was carried out at room temperature for 30 min under magnetic stirring, using a cylindrical-shaped stirred bar (10 × 4 mm) in order to improve mass transfer from the aqueous sample into the fibre coating.

A static desorption of delorazepam from the fibre was carried out in the SPME/HPLC interface. Before transferring the fibre into the desorption chamber, this was flushed and filled by adding 500 μl of acetonitrile. The fibre was then introduced into the desorption chamber and was desorbed for 5 min. The valve was then switched to the "injection" position, and the column separation was initiated. The valve was switched to the "load" position after 60 s and the fibre was removed and cleaned with water for a short time to minimise carryover. The fibre was allowed to dry before the start of the next extraction.

2.5. Protein binding studies

Delorazepam solutions in the concentration range 0.3–1.3 μM in phosphate buffer (0.1 I, pH 7.4 or pH 3.3) were prepared and subjected to SPME-HPLC analysis. Then, 200 μl of a 100 μM buffered albumin solution (HSA or BSA) were then added to each sample (final concentration 1.3 μM) and equilibrated at 37 °C in a water bath for 10 min or 24 h. After equilibrium has been reached samples were subjected to SPME-HPLC analysis in order to determinate the free analyte. The amount bound to the protein was finally calculated as the difference between the initial concentration and that of the free analyte.

2.6. Ultrafiltration

Ultrafiltration was used as reference method. The ultrafiltration system comprises a Centricon YM-50 centrifugal filter device (Millipore Corporation, Bedford, MA) equipped with a regenerated cellulose membrane (50 000 MW) and a AIC 4222 centrifuge (Cecchinato, Mestre, Italy). The centrifugal filter device was made by a sample reservoir containing the membrane, a retentate vial and a filtrate vial. The centrifugal filter devices were prerinsed before use as specified in the manual accompanying the product.

A Perkin–Elmer Lambda 2 UV/vis Spectrophotometer was used for the determination of delorazepam (detection wavelength: 230 nm) before and after the ultrafiltration.

To test the ultrafiltration system for possible artefacts, spectrophotometric determinations of standard solutions of delorazepam in the concentration range 0.8–1.3 μM were performed, at pH 3.3 and 7.4, before and after the filtration. Then, solutions containing delorazepam, in the concentration range 0.8–1.3 μM, and 1.3 μM HSA in phosphate buffer (0.1 I, pH 7.4) and solutions containing delorazepam, in the concentration range 0.5–1.3 μM, and 1.3 μM BSA in phosphate buffer pH 7.4 or pH 3.3 were incubated at 37 °C in water bath for 10 min and then subjected to the ultrafiltration. The absorbance values obtained were directly related to the amount of delorazepam bound to the protein.

3. Results and discussion

The effect of the most important parameters (e.g. extraction time, sample temperature, sample

pH,...) influencing the SPME extraction efficiency and the desorption conditions, as well as validation data of the method, has been already discussed elsewhere [29]. The ideal conditions were applied in the present work to study the binding of delorazepam to HSA and BSA.

SPME is known to be a partition extraction technique. Thus, the amount of drug extracted from the fibre should be so small that it should not disturb the equilibrium between the drug and the protein. In order to confirm this assumption, the fibre–water distribution coefficient K_{fw} and the percentage depletion [27], in equilibrium conditions, were calculated. A $\log K_{fw}$ value of 2.7 and a depletion of 1.9% were obtained, clearly indicating that the amount extracted from the aqueous solution was negligibly small.

Then, the procedure was employed to determine the freely available concentration of the drug in the presence of HSA at pH 7.4, as described in Section 2. Fig. 1 reports an SPME-LC-UV chromatogram relevant (a) to a standard solution of delorazepam, and (b) to the same solution in the presence of HSA. As apparent, a significant difference was observed between the concentration of delorazepam before and after the addition of HSA, clearly showing the occurrence of a protein–drug binding. This behaviour was observed in all the explored concentration range. The unbound fraction of delorazepam was calculated by plotting the total concentration $[D]_0$ and the free concentration $[D]_f$ against each other. The freely dissolved fraction ($f_D = [D]_f/[D]_0$) was given by the slope of the relevant curve, described by the following equation: $y = (0.8536 \pm 0.04)x - (0.0028 \pm 0.01)$. An f_D value of 0.8536 ± 0.04 and a drug–protein partition coefficient K_P of 1.30×10^5 M^{-1} were obtained.

The same experiments were performed in order to study the binding of delorazepam to BSA at pH 7.4. Contrary to the results obtained in the presence of HSA, delorazepam did not seem to interact with the protein in all the explored concentration range. This experimental evidence could be ascribed to the structural differences between HSA and BSA. HSA has been used in more studies of drug binding compared to BSA. Regardless that the two proteins are similar, differences in binding classes have been reported. It is well known that HSA has two main binding sites: the warfarin site (I) and the benzodiazepines and indoles site (II). The binding site II of HSA and the respective one in BSA may differ in the amino acid sequence; this feature could explain the absence of delorazepam–BSA binding.

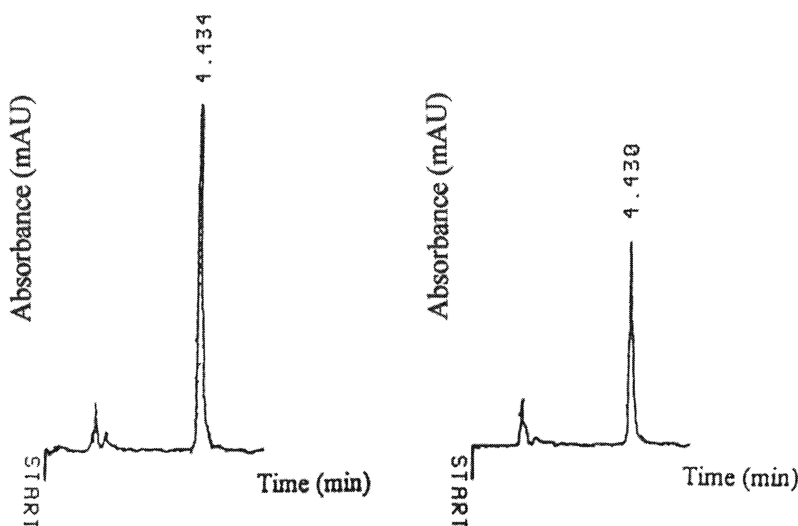

Fig. 1. SPME-LC-UV chromatogram relevant to: (a) a standard solution of delorazepam; (b) the same solution in the presence of HSA. Chromatographic and detection conditions as reported in Section 2. Absorbance axis: 4 mAU full scale.

Table 1
Absorbance values ($n = 3$) obtained before and after the ultrafiltration at different pH values and concentrations of delorazepam

$[D]_0$ (μM)	pH	Before	After		
		D	D	D+HSA	D+BSA
1.3	7.4	0.105 ± 0.002	0.108 ± 0.003	0.084 ± 0.006	0.106 ± 0.007
	3.3	0.104 ± 0.003	0.107 ± 0.004	/	0.082 ± 0.006
1.0	7.4	0.079 ± 0.002	0.081 ± 0.004	0.064 ± 0.007	0.079 ± 0.008
	3.3	0.079 ± 0.002	0.078 ± 0.003	/	0.063 ± 0.007
0.8	7.4	0.060 ± 0.003	0.062 ± 0.005	0.053 ± 0.009	0.059 ± 0.008
	3.3	0.061 ± 0.002	0.060 ± 0.004	/	0.049 ± 0.009

D = delorazepam; $[D]_0$ = initial concentration of delorazepam. HSA and BSA concentration: 1.3 μM. Detection wavelength: 230 nm.

Interactions between drugs and albumin in vitro are influenced by factors such as the pH of the solution, since the dissociated amounts of the analytes can increase (or decrease) according to the pH value; moreover, the protein is not a strictly rigid molecule, thus its tertiary structure can change with the experimental conditions. Consequently, the drug–protein binding was investigated at pH 3.3, since delorazepam [30] is present in an almost completely dissociated form at this pH value. An evident binding between delorazepam and BSA was observed in this case, as demonstrated by the plot of the total concentration $[D]_0$ against the free concentration $[D]_f$, described by the following equation: $y = (0.7890 \pm 0.06)x + (0.0038 \pm 0.01)$. An f_D value of 0.7890 ± 0.06 and a partition coefficient K_P of 2.16×10^5 M^{-1} were obtained.

These experiments were performed assuming, as already reported [25,26], that the matrix did not interfere with the measurement by binding to the fibre or by other mechanisms, causing a lower estimation of the free fraction of delorazepam. An indication of the soundness of data obtained with the SPME method was obtained by the ultrafiltration experiments followed by UV measurements, performed as described in Section 2. Table 1 reports the absorbance values obtained. The insignificant difference between the absorbance values of delorazepam solutions before and after the filtration confirmed that the binding of delorazepam to the ultrafiltration system was not significant. On the contrary, a reduction of absorbance, directly related to the drug–protein binding, was obtained in the experiments performed with solution containing both delorazepam and proteins, with the only exception, as also observed by SPME, of the solutions containing delorazepam and BSA at pH 7.4. As already done by SPME, the unbound fraction of delorazepam was calculated by plotting the total concentration $[D]_0$ and the free concentration $[D]_f$ against each other and drawing the relevant curves. In the presence of delorazepam and HSA at pH 7.4, the equation $y = (0.6779 \pm 0.03)x - (0.1508 \pm 0.03)$ was obtained and an f_D value of 0.6779 ± 0.03 and a partition coefficient K_P of 3.65×10^5 M^{-1} were assessed. In the presence of delorazepam and BSA at pH 3.3, the equation $y = (0.7176 \pm 0.02)x - (0.0844 \pm 0.02)$ was obtained and an f_D value of 0.7176 ± 0.02 and a partition coefficient K_P of 3.00×10^5 M^{-1} were estimated.

As apparent, a good agreement was found between data performed with SPME and ultrafiltration. It is worth noting that using the ultrafiltration method it was not possible to cover the same concentration range explored by SPME due to the poor sensitivity of the UV spectrophotometer at low concentration levels.

4. Conclusions

The present work demonstrates how SPME can be conveniently applied, in conjunction to HPLC/UV, to study the binding of delorazepam to serum albumins. Protein binding constants were

not calculated since BSA has several binding sites [31] and then is nonlinearly related to the freely dissolved concentration of drug.

The procedure could also be potentially extended to the measurement of partition coefficients between a wide variety of semi- or non-volatile compounds and matrices, representing a valid alternative to conventional techniques. In fact the method is solvent free, simple, fast and sensitive and overcomes typical drawbacks of traditional procedures, such as equilibrium dialysis (long equilibration times), ultrafiltration (low sensitivity, especially when working with highly bound drugs), chromatographic and electrophoretic techniques (necessity of soluble or immobilised proteins and drugs).

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