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Application of solid-phase extraction in the determination of U-82217 in rat serum, urine and brain

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ABSTRACT

The techniques of solid-phase extraction (SPE) were applied in the analytical method development for the determination of U-82217, 3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5-[(4-methoxyphenyl)methyl]-imidazo[1,5-*a*]quinoxalin-4(5*H*)-one, in rat serum, urine and brain. Samples of serum, urine or brain homogenate containing U-82217 were loaded on C_{18} SPE columns and eluted with acetonitrile (300 μl). The prepared samples were analyzed by reversed-phase HPLC using an ODS column with a mobile phase of acetonitrile–water (45:55, v/v) containing 0.12% of acetic acid (pH 6.0 ± 0.1). The UV absorbance of the column effluent was monitored at a wavelength of 318 nm. The absolute extraction recovery from serum, urine and brain samples was *ca.* 90%. Linear calibration graphs were obtained over the ranges 5 ng/ml–20 μg/ml (serum), 20 ng/ml–20 μg/ml (urine) and 50 ng/g–200 μg/g (brain). The intra- and inter-assay precision and accuracy were all found to be <13% at the concentrations evaluated. The strategy in SPE development and the application of this method to the determination of U-82217 in rat serum and brain for a pharmacokinetic study are also discussed.

INTRODUCTION

Solid-phase extraction (SPE) methods have been extensively used in the extraction of chemicals from biological samples. Highly purified and concentrated isolates for chromatographic analysis can be achieved by the selective extraction with desired sorbents to yield chromatograms with minimal interferences and improved sensitivity [1]. Therefore, the SPE method is useful in bioanalytical method development. The method for the determination of U-82217 in rat serum, urine and brain is a typical example of the application of SPE in biomedical analysis. U-82217, 3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5-[(4-methoxyphenyl)methyl]-imidazo-[1,5-*a*]quinoxalin-4(5*H*)-one, is under preclinical evaluation as a potential hypnotic compound. U-82217 is an oxadiazole-substituted imidazobenzodiazepine compound which has demonstrated reduced physical dependence-inducing properties

compared to benzodiazepine agonists, minimal amnesia-inducing effects and high potency on locomotor-based hypnotic tests in animal models [2]. A number of analytical methods have been reported for the determination of other imidazobenzodiazepines in biological samples [3–7]. Most of them were for plasma samples and used a liquid–liquid extraction procedure for isolating the compounds of interest from the biological matrix. An HPLC method with UV detection using an SPE procedure for the determination of U-82217 in rat serum, urine and brain was therefore developed to support the evaluation of oral bioavailability and pharmacokinetic characteristics and initial toxicokinetic properties of U-82217 in animal models. In this paper the analytical method development and validation for the determination of U-82217 in biological matrices, particularly the development of SPE procedures, are discussed.

EXPERIMENTAL

Reagents and materials

U-82217 and the internal standard (I.S.),

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U-80447 (Fig. 1) were provided by Upjohn (Kalamazoo, MI, USA). HPLC-grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI, USA) and analytical-reagent grade acetic acid, ammonia solution and potassium phosphate (dibasic) from Mallinckrodt (Paris, KY, USA). Purified water was produced with a Milli-Q reagent water system (Millipore, Bedford, MA, USA).

Chromatography

The liquid chromatographic system consisted of a Waters (Milford, MA, USA) M-6000A pump, a Kratos (Ramsey, NJ, USA) Spectroflow 783 variable-wavelength UV detector set at 318 nm, a Perkin-Elmer (Norwalk, CT, USA) ISS-100 Autosampler fitted with a 200-μl sample loop, and an ODS column (250 × 4.6 mm I.D., 5 μm particle size) (Jones Chromatography, Littleton, CO, USA) protected by a Pelliguard ODS guard column (50 × 2.1 mm I.D., 32 μm particle size) (Whatman, Clifton, NJ, USA). The mobile phase was a mixture of 450 ml of acetonitrile, 550 ml of water and 1.2 ml of acetic acid with an apparent pH of 6.0 ± 0.1 adjusted with ammonia solution, and was then filtered and degassed with helium prior to use. The chromatographic system was operated at ambient temperature (21–23°C) with an eluent flow-rate of 1.0 ml/min. Quantification was accomplished based on the peak-height ratio of drug to I.S. using a Harris computer system.

Serum extraction

Twelve C_{18} SPE columns (100 mg/1.0 ml) (Varian, Harbor City, CA, USA) placed on the vacuum extraction manifold (Supelco, Bellefonte, PA, USA) were prewashed with one column volume of

U-82217　　　　　　　　　**I.S.**

Fig. 1. Structures of U-82217 and the I.S. (U-80447).

acetonitrile followed by one column volume of 0.1 M K_2HPO_4 solution. Unknown serum samples (1ml), each mixed with 50 μl of 10 μg/ml I.S. solution, were loaded onto SPE columns with a vacuum of 86 kPa. After vacuum aspiration for 5 min at *ca.* 27 kPa, the SPE columns were rinsed with 100 μl of acetonitrile–water (30:70,v/v) followed by 2 ml of 0.1 M K_2HPO_4 solution (86 kPa). The columns were dried with vacuum aspiration (*ca.* 27 kPa) for 10 min. U-82217 and the I.S. were then eluted from the column with 300 μl of acetonitrile by applying a slow uniform pressure to the top of the column using nitrogen (about 0.2 kg/cm^2). Each eluate was collected in a 2-ml autosampler vial and mixed with 200 μl of purified water. A 50-μl volume of the mixture was injected into the HPLC system for analysis.

Urine extraction

A 1-ml volume of unknown urine sample mixed with 100 μl of 10 μg/ml I.S. solution was transferred to the prewashed SPE column as described above. The SPE column was rinsed with 150 μl of acetonitrile–water (30:70, v/v) under gravity flow (without vacuum). The remainder of the SPE procedure was the same as described for serum except that 700 μl instead of 200 μl of water were mixed with the 300 μl of acetonitrile extract for final HPLC analysis.

Brain extraction

Unknown brain samples were prepared by homogenizing accurately weighed rat brain sample (*ca.* 200 mg) in a 5-ml grinding chamber with 1 ml of acetonitrile–water (50:50, v/v). The homogenate was combined with 1 ml of water used for rinsing the grinder piston and vortex mixed for 30 s. A 1-ml volume of the brain homogenate was transferred into a 1.5-ml micro centrifuge tube, mixed with 50 μl of I.S. working solution (10 μg/ml) and centrifuged at 750 g for 1 min in a Brinkmann (Westbury, NY, USA) Model 5415 micro centrifuge. The supernatant of brain homogenate was then transferred into a C_{18} SPE column. The remainder of the SPE procedure was the same as that for the serum extraction.

Validation

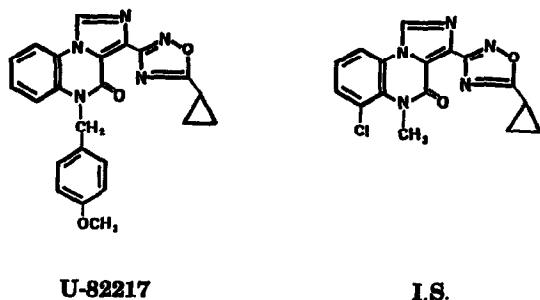The assay validation was similar to the analytical

method validation procedures described by Shah *et al.* [8] for bioavailability, bioequivalence and pharmacokinetics studies. Briefly, to determine the linear range of this method, freshly prepared fortified standards of U-82217 were analyzed on four different days. The limit of quantification (LOQ) was estimated by analyzing fortified serum samples at the presumed LOQ in five replicates to determine if it had acceptable precision and accuracy ($<20\%$). The precision and accuracy of the method were evaluated at three concentrations (low, medium and high). The intra-assay precision was determined by analyzing five fortified serum samples at each concentration on the same day, and the inter-assay precision was obtained by analyzing one fortified serum sample at each concentration on four different days. The absolute extraction recovery was determined at three concentrations for U-82217 and the I.S. Samples at each concentration were extracted as outlined for unknown samples and analyzed in four replicates. The peak heights of the extracted samples were compared with those of unextracted external reference standards containing the corresponding concentrations. In all instances, the means, standard deviations (S.D.) and relative standard deviations (R.S.D.) were calculated. A *p*-value of <0.05 was considered significant in statistical analysis.

RESULTS AND DISCUSSION

Solid-phase extraction development

Extraction is still the one of the most important preparation procedures for the assay of drugs in biological samples. An efficient extraction method can improve the assay precision and accuracy and also the sensitivity. It is well known that proteins are major components that must be removed from biological samples before chromatographic analysis. The solid-phase extraction technique has been proved to be one of the best approaches for removing large amounts of protein from biological samples. Owing to their high molecular masses, most proteins are exposed to a minimum of active functional groups on the sorbent surface and pass unretained, particularly through non-polar SPE columns. The retained proteins can be easily eluted by rinsing the SPE column with water or buffer solutions. Therefore for this application, C_8, C_{18} and

phenyl SPE columns were evaluated to determine their extraction efficiencies. Among these, the C_{18} column proved to be the most satisfactory phase as far as the extraction recovery of U-82217 was concerned. Further, a C_{18} column provided relatively cleaner background of chromatographic profiles with minimum impurity front from endogenous components of biological samples compared with phenyl and C_8 columns. In general, the smaller volume of solvent used for the elution of the compounds of interest from the SPE column produced cleaner extracts if an adequate recovery of U-82217 and the I.S. can be achieved. The 100 mg of sorbent were sufficient to retain the compounds and provided efficient elution with a minimum volume of solvent (300 μl) so that the compounds could be concentrated for HPLC without the need for evaporation. Approximately 80% extraction recovery was obtained when using 300 μl of acetonitrile to elute U-82217 and the I.S. The recovery was further improved to 90% when the SPE column was conditioned with 2 ml of 0.1 M K_2HPO_4 prior to the elution step, which provided a basic environment to facilitate complete elution of the compounds of interest. A large impurity front from endogenous urine components was observed following the extraction procedure developed for serum samples. When the SPE column was rinsed with 150 μl of acetonitrile–water (30:70, v/v) under gravity flow, most of the endogenous urine components were eliminated without reducing the extraction recovery. The solvent used for extracting U-82217 from brain was acetonitrile–water (50:50 v/v), 1 ml of which was sufficient to obtain an extraction recovery greater than 90%. The extract was then purified through the SPE column, yielding a clean chromatogram with no interfering peaks at the retention volumes of U-82217 and the I.S.

The average absolute extraction recoveries were evaluated for both U-82217 and the I.S. at concentrations of 0.01, 0.5 and 10 μg/ml for serum, 0.02, 0.5 and 10 μg/ml for urine and 0.1, 5 and 100 μg/g for brain. The overall average ($n=4$) extraction recoveries for U-82217 ranged from 93.5 ± 3.0 to $95.6\pm2.6\%$, 91.2 ± 3.3 to $94.0\pm3.5\%$ and 88.7 ± 6.1 to $95.4\pm4.8\%$, for serum, urine and brain, respectively, with no perceivable dependence on the recoveries as a function of analyte concentration. The extraction recovery for the I.S. was great-

er than 88% in all instances. Typical chromatograms after extraction of fortified serum, urine and brain standards along with a serum blank are shown in Fig. 2.

Linearity and sensitivity

Linear calibration graphs were obtained over the concentration ranges 5 ng/ml–20 μg/ml, 20 ng/ml–20 μg/ml, and 50 ng/g–200 μg/g, for serum, urine and brain, respectively, with correlation coefficients greater than 0.999 and intercepts not significantly ($p > 0.05$) different from zero. The linear regression equations were $y = (0.1239 \pm 0.0026)x - (0.0309 \pm 0.0132)$, $y = (0.1270 \pm 0.0018)x - (0.0364 \pm 0.0240)$ and $y = (0.1301 \pm 0.0032)x - (0.0781 \pm 0.0792)$ ($n = 4$) for serum, urine and brain standard curves, respectively, where y is the peak-height ratio of drug to I.S. and x is the drug concentration. The LOQ at which the precision (R.S.D.) and accuracy (bias) were acceptable (<20%) were 5 and 20 ng/ml for serum and urine, respectively, and 50 ng/g for brain, based on using 1 ml of serum or urine and 200 mg of brain for extraction.

Precision and accuracy

As listed in Table I, intra-assay ($n = 15$) and inter-assay ($n = 12$) precisions ranging from 3.5 to

8.9%, 3.6 to 8.6% and 3.3 to 11.7%, for serum, urine and brain, respectively, were obtained at the three concentrations studied. The accuracy was $\leqslant 10.0\%$ in all instances. The system precision, determined by injecting a prepared sample five times, was found to be not greater than 0.8% in most instances.

Application

The proposed method was applied to the determination of U-82217 concentrations in serum and brain samples collected from a pilot pharmacokinetic study in rats. The study was conducted using 66 male Sprague–Dawley rats with approximate masses of 250 g. The rats were randomized into four dose groups using body masses. The vehicle group, consisting of three rats, received a single oral dose of control article and were killed for blood sampling 1 h after dosing. The remaining three groups, each containing 21 rats, received a single oral dose of U-82217 at levels of 10, 30 or 100 mg/kg. The control article (vehicle: 98.35% purified water, 1.25% Avicel 591, 0.2% sorbic acid and 0.2% polysorbate) and the U-82217 suspension formulation were administrated orally by gastric intubation. Three rats were killed for blood and brain sampling at each of 0.5, 1, 2, 4, 8, 16, and 24 h after dosing. The blood samples were allowed to clot at room temperature
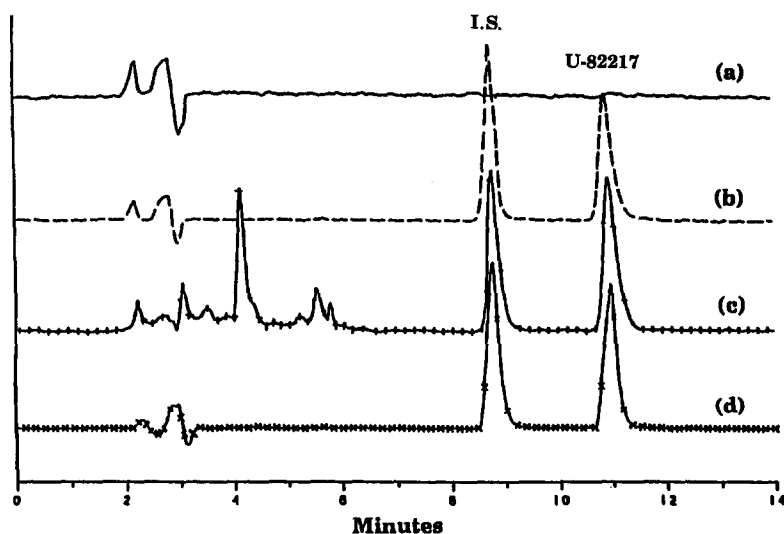

Fig. 2. Typical chromatograms after extraction of (a) rat serum blank, (b) rat serum blank fortified with 0.5 μg/ml each of U-82217 and I.S., (c) rat urine blank fortified with 1 μg/ml each of U-82217 and I.S. and (d) rat brain sample fortified with 5 μg/g of U-82217 and 0.5 μg/ml of I.S. Detection wavelength, 318 nm.

TABLE I

INTRA- AND INTER-ASSAY PRECISION AND ACCURACY

Sample	Theoretical concentration	Intra-assay ($n = 5$)		Inter-assay ($n = 4$)	
		Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
Serum	10 ng/ml	7.4	8.0	6.8	10.0
	500 ng/ml	3.5	1.6	4.0	−4.4
	10 μg/ml	3.9	2.0	8.9	1.0
Urine	20 ng/ml	8.6	−1.5	10.4	5.5
	500 ng/ml	4.4	−4.2	3.6	−4.9
	10 μg/ml	3.8	5.0	5.2	−2.0
Brain	100 ng/ml	4.9	−1.6	11.7	4.2
	5 μg/ml	4.2	−4.0	8.1	−4.0
	100 μg/ml	5.0	3.2	3.3	−1.9

for 15–20 min and the serum was harvested by centrifugation, then transferred into a clean tube. Brain samples were frozen on dry-ice immediately and serum and brain samples were stored at −20°C until analysis.

The average serum concentration–time curves of each dose level for U-82217 are shown in Fig. 3. The low systemic serum concentration of U-82217 (<0.12 μg/ml for all doses) in rats might reflect poor absorption owing to the low water solubility of this drug (0.4 μg/ml). The concentration of U-82217 in brain at dose levels of 10 and 30 mg/kg were below the level of detection except for one

brain sample that contained 52 ng/g of U-82217 at 2 h after a dose of 30 mg/kg. The brain concentrations were all less than 0.17 μg/g at a dose level of 100 mg/kg. These results agree with the U-82217 serum concentrations, which suggested poor oral bioavailability of U-82217 in rats.
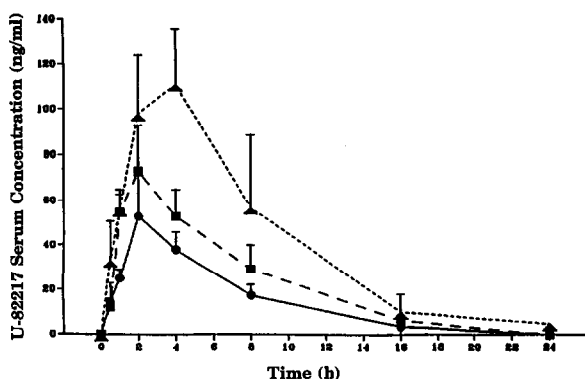
Fig. 3. Average serum concentration–time profiles of U-82217 in rats after single oral administration of (●) 10 mg/kg, (■) 30 and (▲) 100 mg/kg of U-82217.

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