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Short Communication

Determination of 3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5-(1-methylethyl)imidazo[1,5-*a*]quinoxalin-4(5*H*)-one in serum by high-performance liquid chromatography

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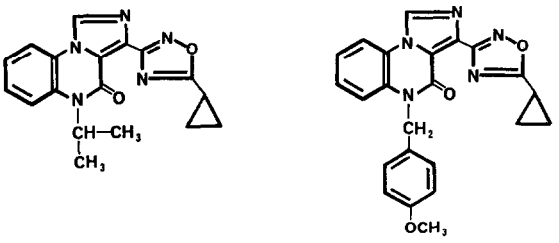
ABSTRACT

A high-performance liquid chromatographic assay with solid-phase extraction (SPE) for the rapid and sensitive quantitation of 3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5-(1-methylethyl)imidazo[1,5-*a*]quinoxalin-4(5*H*)-one (I, U-78875) in serum is described. The validation results indicated that the present method had excellent intra- and inter-assay precision ($\leq$ 9.5%, mean ± S.D. = 3.9 ± 3.0%, n = 25) and accuracy ($\leq$ 10.0%, mean ± S.D. = 3.0 ± 2.9%, n = 25), as well as improved sensitivity (2 ng/ml, using a 100-μl injection). Each chromatographic run is only 10 min and the organic solvent for the extraction of I and internal standard (U-82217) from serum was only 300 μl. The application results obtained from the SPE method were in good agreement with the advanced automated sample preparation method.

INTRODUCTION

3-(5-Cyclopropyl-1,2,4-oxadiazol-3-yl)-5-(1-methylethyl)imidazo[1,5-*a*]quinoxalin-4(5*H*)-one (I, U-78875) (Fig. 1), an anxiolytic drug candidate, is being developed. Compound I exhibits anxiolytic activities in animal models comparable to diazepam, alprazolam and other benzodiazepines, but lacks the side-effects normally associated with benzodiazepines [1] and is undergoing clinical testing for safety and efficacy.

To evaluate I oral bioavailability and pharmacokinetic characteristics in animals, a high-performance liquid chromatographic (HPLC) method using Varian Advanced Automated Sample Processor (AASP) system has been developed. This assay has a minimum quantifiable level (MQL) of 3.6 ng on-column (36 ng/ml) using a 100-μl serum sample [2]. Although the AASP method has been validated and applied to the assay of biological samples from several preliminary pharmacokinetic studies, the reliability of the AASP system to process large volumes of samples has not been established as some mechanical difficulties have



I IS

Fig. 1. Chemical structures of I and I.S.

been experienced [2]. As an alternative to this method and without relying on the AASP, a simple and rapid HPLC method using solid-phase extraction (SPE) has been developed. The present SPE method has been validated and showed excellent reproducibility as well as improved sensitivity. The utility of this method was demonstrated by determination of concentrations of I in serum samples collected from a preliminary pharmacokinetic study in dogs. The validation results as well as the characteristics of the chromatography and sample preparation of the SPE method are described in this paper.

EXPERIMENTAL

Chemicals and reagents

Compound I and the internal standard, 3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5-[(4-methoxyphenyl)methyl]imidazo[1,5-*a*]quinoxalin-4(5*H*)-one (I.S., U-82217), were provided by The Upjohn Company (Kalamazoo, MI, U.S.A.) (Fig. 1). All organic solvents were of HPLC grade and purchased from Burdick and Jackson (Muskegon, MI, U.S.A.). Other chemicals were of analytical reagent grade. Purified water was produced by a Millipore-Q reagent water system (Millipore, Bedford, MA, U.S.A.).

Standards

Stock solutions of I and I.S. were prepared in acetonitrile–water (3:7, v/v) to give concentrations of 200 and 100 μg/ml, respectively. The analytical reference standard solutions containing 1 ng/ml to 20 μg/ml I and 500 ng/ml I.S. were prepared by diluting stock solutions with acetonitrile–water (3:7, v/v). Serum control standards were prepared by aliquoting appropriate volumes of I stock solution (200 μg/ml) and working solutions (100 ng/ml and 1 and 10 μg/ml), and 50 μl of I.S. working solution (10 μg/ml) to 1 ml of control dog serum (drug-free) to produce a concentration series the same as the analytical reference standards.

Chromatography

The HPLC system consisted of a Spectra-Physics SP 8810-10 isocratic pump with a Spectra-Physics SpectraChrom-011 variable-wavelength UV–VIS detector (San Jose, CA, U.S.A.), a Perkin-Elmer ISS-100 autosampler (Norwalk, CT, U.S.A.) and a Kipp & Zonen Model BD-41 dual-channel recorder (Delft, The Netherlands). The analytical column employed was an ODS column (250 mm × 4.6 mm I.D., 5 μm particle size) (Jones Chromatography, Littleton, CO, U.S.A.) linked with a guard column (Pelliguard ODS, 50 mm × 2.1 mm I.D., 32 μm) (Whatman, Clifton, NJ, U.S.A.). The mobile phase used for the isocratic reversed-phase chromatography was acetonitrile–water (48:52, v/v) containing 5 ml of triethylamine (TEA) per liter of mobile phase with a final pH of 6.0 ± 0.1 adjusted with acetic acid. The chromatographic system was operated at 21–23°C with an eluent flow-rate of 1.0 ml/min. The UV absorbance of column effluent was monitored at 315 nm with 0.001 a.u.f.s. sensitivity.

Sample preparation

Twelve C_{18} SPE columns (100 mg per 1.0 ml, Analytichem International, Harbor City, CA, U.S.A.) placed on the vacuum extraction manifold (Supelco, Bellefonte, PA, U.S.A.) were prewashed with one column volume of acetonitrile followed by one column volume of 0.1 *M* K_2HPO_4 solution. A 1-ml volume of each unknown serum sample was mixed with 50 μl of I.S. working solution (10 μg/ml) and then transferred onto individual SPE columns. The columns were washed by 100 μl of acetonitrile–water (3:7, v/v) followed by 2 ml of 0.1 *M* K_2HPO_4 solution and were dried with vacuum aspiration (200 mmHg) for 10 min. Compound I and 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 gas (about 0.2 kg/cm^2). Each eluate was mixed with 700 μl of purified water, and 100 μl of the mixture were injected onto the HPLC system for analysis.

RESULTS AND DISCUSSION

HPLC characteristics

The chromatographic system with an analytical column of C_{18} and a mobile phase of acetonitrile–water with TEA as modifier effected a complete separation of I and I.S. from the endogenous serum constituents. The peak shape and symmetry of I and I.S. improved significantly when TEA was added to the mobile phase. Variations in the composition and pH of the mobile phase were then explored. An increase in the percentage of acetonitrile or TEA or an increase in pH of the mobile phase from 4 to 7 resulted in a decrease of the retention times of I and I.S. The mobile phase of acetonitrile–water (48:52, v/v) containing 5 ml of TEA per liter of mobile phase (pH 6) was the best eluent on the C_{18} reversed-phase column and resulted in optimal separation and peak shape as well as adequate capacity factor (k') for I and I.S. Under these chromatographic conditions,

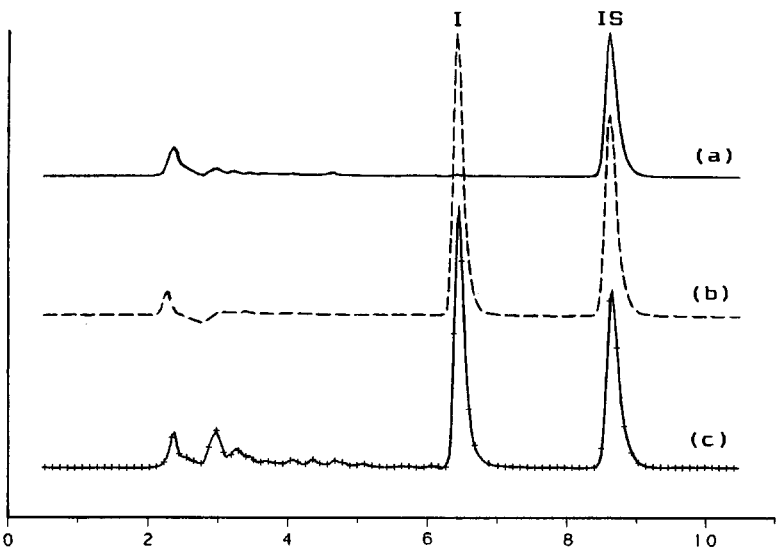


Fig. 2. Typical chromatograms of extracts of (a) serum blank with I.S., (b) serum sample spiked with 0.5 μg/ml each of I and I.S., and (c) serum sample collected from the dog administered 30 mg/kg I orally.

the retention times for I and I.S. ranged from 6.2 to 6.6 (k' = 1.9) and 8.6 to 9.0 min (k' = 3.0), respectively.

Fig. 2 shows typical chromatograms of an extract of blank dog serum with I.S., dog serum spiked with 0.5 μg/ml each of I and I.S., and a serum sample collected from a dog administered with I (30 mg/kg) orally. No significant interfering peaks were observed in the chromatogram of dog serum blank and post-dosed dog serum samples.

Linearity

Ten fortified serum standards and ten analytical reference standards containing 2 ng/ml to 20 μg/ml I were analyzed on four different days. Calibration curves obtained by least-squares linear regression showed good linearity between peak-height ratios and concentrations from 2 ng/ml to 20 μg/ml. The average slopes (n = 4) were 0.2954 ± 0.0088 and 0.2936 ± 0.0085 for the fortified serum and analytical reference standards, respectively, with correlation coefficients greater than 0.999. The 95% confidence intervals of the intercepts included the origin and the coefficients of variation (C.V.) of the slopes were less than 5%. No significant differences were observed between fortified serum standard curves and reference standard curves.

Limit of quantitation

The low limit of quantitation (LLQ) of this method was evaluated by analyzing serum samples at the presumed minimum quantifiable levels in five replicates

TABLE I

INTRA- AND INTER-ASSAY PRECISION AND ACCURACY

Precision = C.V. = S.D./mean × 100; accuracy = percentage deviation = (measured − theoretical)/theoretical × 100.

Theoretical concentration (ng/ml)	Concentration measured (mean ± S.D.) (ng/ml)	Precision (%)	Accuracy (%)
Intra-assay (n = 5)			
1	1.3 ± 0.2	13.3	30.0
2	2.1 ± 0.2	9.5	5.0
5	5.1 ± 0.2	4.7	2.0
10	10.1 ± 0.3	3.0	1.0
500	494.1 ± 5.5	1.1	−1.1
10 μg/ml	10.3 ± 0.3 μg/ml	2.8	3.1
Inter-assay (n = 4)			
1	1.4 ± 0.2	15.4	40.0
2	2.2 ± 0.2	9.1	10.0
5	5.2 ± 0.1	1.0	4.0
10	9.7 ± 0.3	3.1	−3.1
500	495.0 ± 7.4	1.5	−0.2
10 μg/ml	10.3 ± 0.3 μg/ml	3.5	1.0

[3]. As shown in Table I, a concentration of 1 ng/ml could be detected, but the quantitation was inadequate because of the poor assay precision and accuracy at this level. The intra- and inter-assay precision (C.V.) and accuracy (percentage deviation) values ($\leq 10\%$) were acceptable at concentrations ≥ 2 ng/ml (Table I). Therefore, the LLQ for this method is 2 ng/ml, based on an injection volume of 100 μl.

Since this method produced a clear chromatogram at the elution position of I and I.S., the sensitivity can be improved by increasing the injection volume. The peak height of I increased proportionally with the increase of injection volume from 50 to 200 μl at the three concentrations evaluated (10 and 500 ng/ml and 10 μg/ml). Thus, an LLQ of 1 ng/ml can be reached by injecting 200 μl of prepared sample to the chromatographic system.

Extraction recovery

The absolute extraction recoveries were determined for I at concentrations of 10 and 500 ng/ml and 10 μg/ml, and for I.S. at a concentration of 500 ng/ml by comparing the peak heights of extracted fortified serum standards with those obtained from direct injection of a reference standard with same concentration of I or I.S. The average recoveries (Table II) for I at each concentration ranged from 92.9 to 94.3%. An overall recovery (mean ± S.D.) of 93.7 ± 2.3 (n = 15,

TABLE II

ABSOLUTE EXTRACTION RECOVERY OF SERUM STANDARDS

Concentration	Extraction recovery (mean ± S.D., n = 5) (%)	
	I	I.S.,
10 ng/ml	94.3 ± 1.6	
500 ng/ml	94.0 ± 1.3	95.3 ± 2.6
10 μg/ml	92.9 ± 3.5	
Overall	93.7 ± 2.3	95.3 ± 2.6

concentration-independent) and 95.3 ± 2.6 (n = 5, at 500 ng/ml) were obtained for I and I.S., respectively.

The extraction recoveries were also evaluated using different volumes of serum sample. The results showed a proportional increase in the amount of I extracted, when the sample volume increased from 0.1 to 1 ml (Table III). Therefore, a 0.1-ml serum sample can also be used for extraction when only a small sample volume is available.

Several SPE columns such as C_8, phenyl and C_2 were also evaluated without showing increased or similar recovery compared to the C_{18} column. To limit the elution of non-polar serum endogenous components, 300 μl of elution solvent were used for testing the extraction recovery with different organic solvents [4]. Of these solvents, acetonitrile was found to be the solvent of choice with respect to recovery and purity of the extracts. The drying of the SPE column for 10 min prior to the elution of analytes is an important step to achieve reproducible extraction recovery since only 300 μl of solvent were used for elution. Reconstitution of the sample extracts in smaller volume and injection of a large portion of the sample can improve the assay sensitivity (LLQ).

TABLE III

RECOVERY OF SAMPLE USING DIFFERENT VOLUMES FOR EXTRACTION

Concentration	Amount recovered (mean ± S.D., n = 5)		
	1-ml sample	0.5-ml sample	0.1-ml sample
50 ng/ml	50.8 ± 1.4 ng	24.8 ± 0.8 ng	5.1 ± 0.3 ng
500 ng/ml	495.1 ± 13.2 ng	242.5 ± 6.3 ng	48.9 ± 0.7 ng
10 μg/ml	10.0 ± 0.2 μg	5.1 ± 0.1 μg	1.0 ± 0.2 μg

Accuracy and precision

The intra- and inter-assay precision and accuracy of the method were evaluated at several concentrations of I in serum [5]. The intra-assay precision was determined by analyzing five fortified serum samples at each concentration on the same day. The inter-assay reproducibility was obtained by analyzing one fortified serum sample at each concentration on four different days. Results from the analysis on each day are summarized in Table I. The intra-assay precision and accuracy ranged from 1.1 to 9.5% and −1.1 to 5.0% (n = 25), respectively. The inter-assay precision and accuracy ranged from 1.5 to 9.1% and −3.1 to 10.0% (n = 20), respectively.

Applicability of the SPE method

The validity of the method described above was confirmed by determining concentrations of I in serum samples from a preliminary pharmacokinetic study and comparing serum levels to those obtained by the previous method (AASP). Blood samples were taken from four dogs that received a single oral dose of 30 mg/kg. Serum concentrations of these samples had been determined using the AASP method two months earlier [6]. The average concentration–time profile of the four dogs is shown in Fig. 3. Good correlation was observed by the linear regression over all points (n = 47) measured by the present and AASP methods, which yield a slope of 1.04 and correlation coefficient of 0.956 with an intercept not significantly different from zero ($p > 0.05$). Only 2 of the 47 points were outside the 95% confidence interval. In addition, the drug in serum samples was stable for at least two months when stored at −20°C.

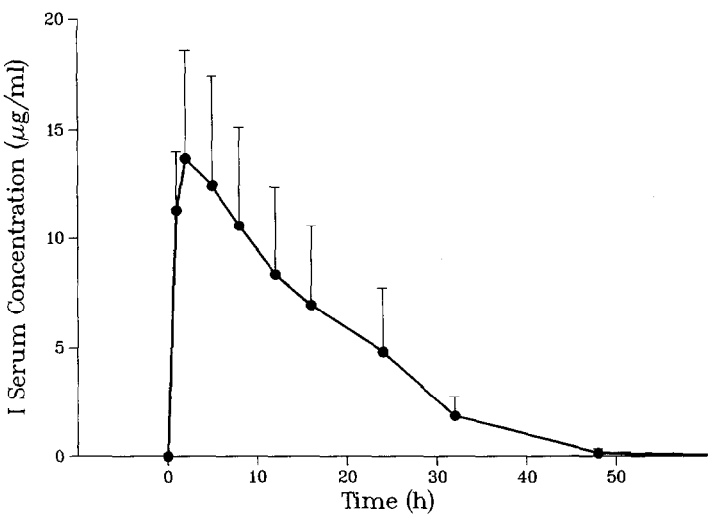


Fig. 3. Average (n = 4) serum concentration–time profile following a single oral administration of 30 mg/kg I to the dog.

CONCLUSION

An HPLC assay with SPE for the rapid and sensitive quantitation of I in serum is described. Each chromatographic run is only 10 min and the organic solvent for the extraction of I and I.S. from serum was only 300 μl. The validation results indicated that this method had excellent intra- and inter-assay precision and accuracy, as well as improved sensitivity.

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