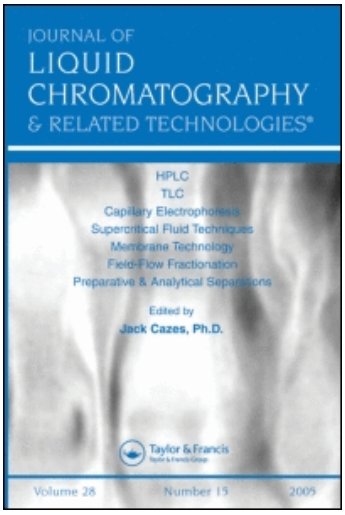

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A Semi-Automated 96-Well Solid Phase Extraction and High Performance Liquid Chromatographic Determination of a Selective GABA-A Receptor Agonist in Human and Rat Plasma Using Fluorescence Detection

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A Semi-Automated 96-Well Solid Phase Extraction and High Performance Liquid Chromatographic Determination of a Selective GABA-A Receptor Agonist in Human and Rat Plasma Using Fluorescence Detection

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ABSTRACT

A semi-automated, 96-well solid phase extraction (SPE) method combined with high performance liquid chromatography (HPLC) was developed, validated, and implemented to demonstrate its high sample throughput utility for the determination of a selective gamma amino-butyric acid (GABA)-A receptor agonist in human and rat plasma. A Tomtec Quadra 96 system was utilized to perform the typical 96-well SPE steps, such as plate conditioning and washing, sample loading, drug and internal standard elution, dilution, and liquid transfers.

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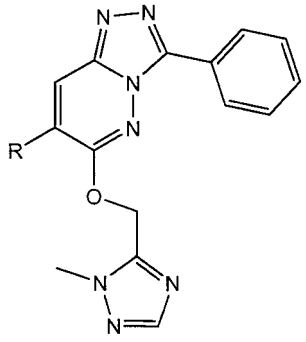
The extracted and diluted eluents were analyzed by HPLC with fluorescence detection with excitation and emission wavelengths at 256 and 447 nm, respectively. Precision and accuracy for standard curve and quality control samples in human and rat plasma were all within acceptable limits. Post-dose plasma samples from three rats were prepared and analyzed using this semi-automated 96-well SPE method. Full details of the analytical methodology, method validation, and the results of analyses of rat plasma samples are presented.

Key Words: 96-Well solid phase extraction; HPLC; Fluorescence detection; Selective GABA-A receptor agonist; Human and rat plasma.

INTRODUCTION

It is estimated that over 70 million people suffer from anxiety. However, due to the adverse effects associated with currently available therapeutic options, only a small minority of all of these patients are actively treated. Benzodiazepines are the most effective class of therapy for anxiety disorders, which include panic disorder, obsessive-compulsive disorder, post-traumatic stress syndrome, and generalized anxiety disorder. Although they are effective, benzodiazepines are associated with sedation, cognitive impairment, muscle relaxation, and the potential for addiction and withdrawal symptoms, including rebound anxiety and insomnia. Since benzodiazepines are non-selective gamma amino-butyric acid (GABA)-A receptor agonists, it is hypothesized that an effective non-sedating anxiolytic drug can be developed by designing a subtype selective GABA-A receptor agonist.[1–4]

Compound **I** (7-cyclobutyl-6-(2-methyl-2*H*-[1,2,4]triazo-3-yl-methoxy)-3-phenyl-[1,2,4]-triazolo-[4,3-*b*]-pyridazine, Fig. 1) was previously shown to be a selective GABA-A $\alpha_{2,3}$ partial agonist in animal species. A sensitive and selective analytical method, with a limit of quantification of <1 ng/mL for the determination of **I** in biological fluids, was required to potentially support the clinical studies necessary to develop this drug candidate. High performance liquid chromatographic (HPLC) methods with native fluorescence detection have been shown to be highly competitive with HPLC with tandem mass spectrometric detection, in terms of selectivity and sensitivity.[5–13] Several semi-automated methods, utilizing Tomtec Quadra 96 workstation and 96-well plate techniques for sample preparation and extraction prior to HPLC analysis, have also been reported to improve assay reproducibility and sample throughput.[14–20]



I, R = Cyclobutyl

ISTD, R = Cyclopentyl

Figure 1. Chemical structures of **I** and **ISTD**.

In this paper, we describe the development and application of a semi-automated 96-well solid phase extraction (SPE) method for the quantification of **I** in human and rat plasma based on HPLC with fluorescence detection.

EXPERIMENTAL

Materials

Compound **I** and the internal standard (**ISTD**, Fig. 1) were obtained from Merck Neuroscience Research Center (Terlings Park, UK). Heparinized human and rat control plasma were obtained from Sera Tech Biologicals (North Brunswick, NJ) and from in-house sources, respectively. Acetonitrile (ACN), methanol (MeOH), formic acid, and ammonium hydroxide were all obtained from Fisher Scientific (Springfield, NJ). Trifluoroacetic acid and triethylamine were purchased from Sigma (St. Louis, MO). Water was deionized through a Millipore Milli-Q-filtered system (Milford, MA). 96-Well SPE plates with mixed MP3 phase were purchased from Ansys Diagnostics, Inc. (Lake Forest, CA). 96-Well micro titer plates (0.65 and 2 mL) for Tomtec Quadra 96 (Hamden, CT) system validation and sample preparation, respectively, were obtained from PerSeptive Biosystems (Framingham, MA).

Instrumentation

Tomtec Quadra 96 workstation was utilized for semi-automated 96-well SPE. The HPLC system included a Perkin-Elmer Series 250 pump (Norwalk, CT) and a Leap Technologies (Chapel Hill, NC) CTC A200SE autosampler with 100-μL sample loop. A BDS Hypersil C_{18} analytical column (10 cm × 4.6 mm, 5 μm) protected by a column inlet-filter (5 μm), both purchased from Keystone (Bellefonte, PA), was utilized for chromatographic separation. The fluorescence detector employed was from Perkin-Elmer LC 240 (Norwalk, CT) with excitation and emission wavelengths set at 256 and 447 nm, respectively. Data collection, integration, and report generation were performed on a PE-Nelson Access*Chrom (Norwalk, CT) system. A Qiagen 4K15C centrifuge (Valencia, CA) was used for 96-well plate centrifugation.

Chromatographic Conditions

The mobile phase was prepared by pre-mixing 0.1% trifluoroacetic acid in water and ACN (67 : 33, v/v), and adjusting the pH = 3 with triethylamine. The mobile phase was delivered isocratically through the analytical column at a flow rate of 1.2 mL/min.

Preparation of Standard Curve and Quality Control Samples

Stock solutions of **I** and **ISTD** were prepared in ACN. All subsequent dilutions for both compounds were made in ACN : water (50 : 50, v/v). Working solutions of **I** were prepared at concentrations of 2, 1, 0.4, 0.2, 0.1, 0.04, 0.02, and 0.01 μg/mL, and the **ISTD** solution was prepared at a concentration of 650 ng/mL. An additional stock standard solution, independent of those used for preparation of working standards, was used to prepare quality control (QC) samples. QC samples were prepared by spiking control plasma with the separately prepared working standard solutions of **I**. Three concentrations of QC samples—low QC (LQC, 3 or 0.5 ng/mL), middle QC (MQC, 30 or 5.5 ng/mL), and high QC (HQC, 75 or 10 ng/mL) were made in human and rat plasma, respectively. The lower concentration QC samples were prepared by diluting the higher concentration QC samples with control plasma. All QC samples were divided into 1.25 mL aliquots in polypropylene tubes and stored at −20°C until they were processed together with unknown samples. An eight-point calibration curve was obtained daily by analyzing control plasma samples spiked with 50 μL of each working

standard solution. One set of QC samples (HQC, MQC, and LQC) was analyzed immediately after the analysis of standard curve samples, and the other after the analysis of the dosed plasma samples.

Sample Preparation and Analysis

Frozen plasma samples were thawed to room temperature and vortex-mixed vigorously for 15 sec on a vortex-mixer. Dosed plasma (100–1000 μL) diluted with control make-up plasma (900–0 μL) and 50 μL of **ISTD** (650 ng/mL) were pipetted into a 96-well plate. After a brief mixing, the plate was placed onto the Tomtec Quadra 96 system for 96-well SPE. The detailed 96-well SPE steps used for the extraction of **I** from plasma are detailed in Table 1.

Precision, Linearity, Accuracy, and Selectivity

The precision of the method was determined by replicate analyses ($n = 5$) of control plasma (human or rat), containing **I** at all concentrations utilized for constructing the calibration curves. Standard curve samples were prepared and assayed daily, along with QC and unknown plasma samples. The linearity of each standard curve was determined by plotting the peak height ratios of **I** to **ISTD** vs. concentrations of **I**. The unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by weighted $(1/y)$ linear regression of the standard curve. The accuracy (%) of the method was expressed as [(mean determined concentration)/(nominal concentration)] × 100. Assay selectivity was assessed by analyzing control and pre-dose plasma samples.

Table 1. 96-Well SPE steps using Tomtec Quadra 96 system.

1.	Sequentially wet the plate with 400 μL of MeOH, 400 μL of H_2O, and 400 μL of 0.5% formic acid in H_2O (v/v).
2.	Load 1 mL of plasma mixture onto the plate and apply low vacuum (<3 psi).
3.	Wash the plate with 3 × 333 μL of 0.5% formic acid in water (v/v).
4.	Wash the plate with 3 × 333 μL of MeOH.
5.	Centrifuge the plate at 850 *g* for 5 min.
6.	Elute with 2 × 100 μL of 60% ACN/2% NH_4OH in water (v/v).
7.	Centrifuge the plate at 850*g* for 5 min with collection tubes underneath.
8.	Dilute the eluent with 200 μL of 0.5% formic acid in water (v/v).
9.	Vortex-mix and transfer the mixture to HPLC autosampler vials.

Data Acquisition and Analysis

Peak heights for **I** and **ISTD** were measured and automatically processed using a PE-Nelson Access*Chrom laboratory data system. Concentrations of **I** in QC and unknown plasma samples were calculated from the daily standard curve obtained by least-square linear regression of peak height ratios of **I** to **ISTD** vs. concentrations.

RESULTS AND DISCUSSION

Spectroscopic Properties of I

In order to establish the best conditions for the sensitive detection of **I**, a series of studies evaluating the spectroscopic properties of the molecule were performed. The ultraviolet (UV) absorption spectrum of **I**, dissolved in a mixture of phosphate buffer (10 nM)/ACN (50 : 50, v/v), indicated the presence of two absorption bands with maxima at <210 and 256 nm. The position of the maxima of these UV bands were found to be independent of pH values tested (pH = 3.7 and 10). The molar absorption coefficient (ε) at 256 nm was 21,400 $M^{-1}\,cm^{-1}$. Compound **I** exhibited strong fluorescence with emission maximum wavelength at 447 nm.

Optimization of Chromatographic and 96-Well SPE Conditions

A variety of analytical columns with different packing materials and of different dimensions and different mobile phases were investigated to achieve the best chromatographic separation conditions for the extracted plasma samples. ACN was found to be a better organic mobile phase modifier than MeOH, giving sharper peaks with shorter retention times for **I** and **ISTD**. A BDS Hypersil C_{18} column was finally selected, providing the acceptable peak shapes and sufficient retention of analytes. In addition, the utilization of the BDS Hypersil C_{18} column provided chromatographic separation of a number of endogenous interfering substances from both **I** and **ISTD**. A column inlet-filter was used and changed daily to protect the analytical column.

An Ansys 96-well method development plate, including a variety of packing phases such as C_2, C_8, C_{18}, end-capped C_{18}, and MP3 (a mixed phase containing reverse phase and cation ion exchange packings), was used to select the best phase for extraction. The MP3 phase was finally chosen because it offered a cleaner background and relatively high recoveries of **I** and **ISTD**. Other SPE parameters, such as the compositions of different

solutions used for washing and analytes elution, centrifugation speed, and number of addition/elution were also investigated and optimized.

Validation of Tomtec Quadra 96 System

A method based on liquid transfers of fluorescein working solution (50 ng/mL) and water was used to determine the precision and accuracy of the Tomtec Quadra 96 system, respectively. Fluorescein working solution was placed in one of the trays and an empty 96-well cytoplate in another. A program was written to transfer defined volumes of fluorescein working solution to the empty plate. The plate was then tested for measuring fluorescence solution and precision was determined for each and all the probes combined. In a separate experiment, the container containing deionized water was placed in one of the trays and the rack containing empty weighed microtubes was placed in another. A different program was written to transfer defined volumes of water from the container to the pre-weighted microtubes. The individual microtube was weighed again after the program has been run. The transfer volume of the water was calculated by dividing the determined weight by the density of water at the temperature of the room, and accuracy was determined for each probe and for all the probes combined. Results presented in Table 2 have shown acceptable pipetting capability of Tomtec Quadra 96 system.

Recovery

The recovery of the 96-well SPE extraction method was determined by comparing the responses of neat standards containing **I** with those of extracted

Table 2. Liquid transfer validation of Tomtec Quadra 96 system.

Volume transferred (μL)	25	50	100	150
Precision (CV%)[a]	<2.0	<1.0	0.5	0.5
	Mean[b] calculated volume (μL)	Programmed volume (μL)	Accuracy[c] (%)	
Test #1	78	75	104.1	
Test #2	174	175	99.4	

[a]Precision is expressed as coefficient of variation, $n = 12$.
[b]$n = 12$.
[c]Accuracy is expressed as [(mean calculated volume)/(programmed volume)] × 100.

plasma standards. The mean recovery for **I** both in human and rat plasma over the entire concentration range of the assay was >85%. The recovery of the **ISTD** was found to be comparable with that of **I**.

Assay Selectivity

No peaks eluting at the retention times of **I** and **ISTD** were detected in samples from five different lots of human or rat control plasma, and pre-dose rat samples that were processed in accordance with the procedure described in "Sample Preparation", confirming assay selectivity. Representative chromatograms of human and rat control plasma, human and rat plasma spiked with **I** and **ISTD**, and post-dose rat plasma samples are shown in Fig. 2.

Assay Validation

The assay was validated over the concentration range of 0.5–100 ng/mL and 0.5–20 ng/mL for **I** in human and rat plasma, respectively. The lower

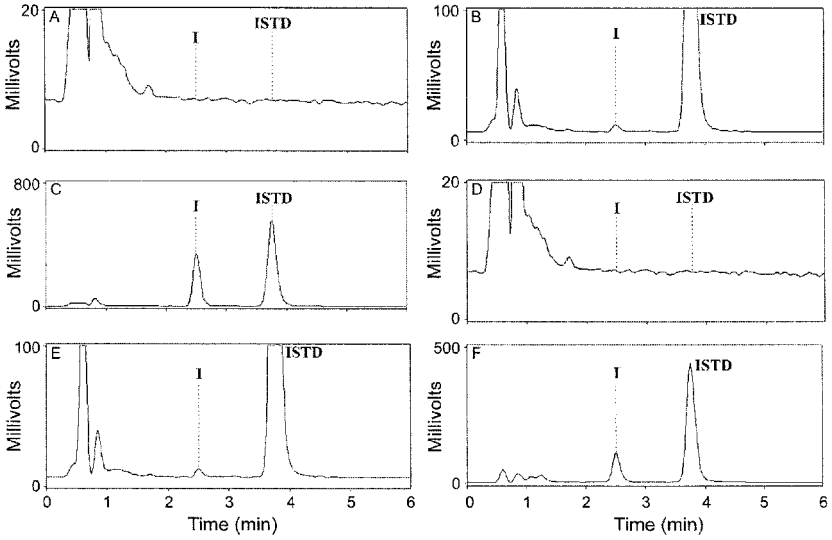


Figure 2. Representative chromatograms of: (A) human control plasma; (B) human control plasma spiked with **I** (0.5 ng/mL) and **ISTD** (32.5 ng/mL); (C) human control plasma spiked with **I** (20 ng/mL) and **ISTD** (32.5 ng/mL); (D) rat control plasma; (E) rat control plasma spiked with **I** (0.5 ng/mL) and **ISTD** (32.5 ng/mL); (F) post-dose rat plasma (at 0.333 hr) contained 33.02 ng/mL of **I** and 32.5 ng/mL of **ISTD** after receiving p.o. administration of 3 mg/Kg of **I**.

limit of quantification (LLOQ), defined as the lowest concentration on the standard line for which acceptable accuracy ($100 \pm 10\%$) and precision (coefficient of variation, CV $<11\%$) were obtained, was 0.5 ng/mL in both matrices. Plots of the peak height ratios of **I** to **ISTD** vs. drug concentrations were found to be linear when weighted (weighting factor $= 1/y$) least-squares regression was applied.

Assessments of the intra-day variability of the assay were conducted in five different lots of human or rat control plasma spiked with **I**, over the respective calibration range of 0.5–100 or 0.5–20 ng/mL. The resulting assay precision and accuracy data are presented in Table 3. QC samples (3, 30, and 75 ng/mL in human plasma; 0.5, 5.5, and 10 ng/mL in rat plasma) were prepared and analyzed using this semi-automated 96-well SPE method. The results shown in Table 3 indicate that the intra-day variability of the assay were $\leq 10.6\%$ CV. The accuracy was within $\pm 4.0\%$ of the nominal values in both matrices.

Table 3. Precision and accuracy of replicate analyses ($n = 5$) of **I** in standard curve and QC samples.

Nominal Concentration (ng/mL)	Accuracy[a] (%)		Precision[b] (%)	
	Human plasma	Rat plasma	Human plasma	Rat plasma
Standards ($n = 5$)				
0.5	104.0	98.8	8.8	10.6
1	102.6	101.2	3.0	3.1
2	99.0	98.3	0.8	0.6
5	96.4	102.4	3.9	0.8
10	100.5	100.8	1.1	0.7
20	97.7	99.3	2.9	2.8
50	101.4		1.4	
100	100.0		1.5	
QCs ($n = 5$)				
LQC (3[c], 0.5[d])	99.0	97.5	0.9	5.7
MQC (30[c], 5.5[d])	103.9	104.0	2.5	3.5
HQC (75[c], 10[d])	98.5	96.8	1.1	2.9

[a]Accuracy is expressed as [(mean determined concentration)/(nominal concentration)] $\times$ 100.
[b]Precision is expressed as coefficient of variation (CV%, $n = 5$).
[c]Prepared in human control plasma.
[d]Prepared in rat control plasma.

In order to assess freeze–thaw stability of **I**, human QC samples ($n = 5$ at each concentration) of **I** were subjected to three freeze–thaw cycles consisting of a thaw to reach room temperature and then refreezing to -20°C. These samples, together with a set ($n = 5$ at each level) of QC samples that were not subjected to additional freeze–thaw cycles, were then defrosted and analyzed. In all cases, the results for the QC samples that were subjected to additional freeze–thaw cycles were practically unchanged confirming freeze–thaw stability of **I** in human plasma. The developed assay was also validated for quantifying **I** in human urine in the same concentration range (0.5–100 ng/mL) as the plasma assay. The selectivity of the urine assay was also confirmed by observing no interfering peaks eluting at the retention time of **I** or **ISTD**.

The semi-automated extraction method performed by Tomtec Quadra 96 workstation allowed the preparation of 96 samples in one batch, greatly simplifying the sample preparation. Ninety-six plasma samples were prepared, extracted, and ready for the HPLC analysis in less than 4 hr.

Analysis of Preclinical Samples

The assay was utilized for the determination of **I** in rat plasma samples after p.o. administration of **I**. Under the conditions utilized, no endogenous peaks eluting at the retention time of **I** or **ISTD** were observed. The selectivity of the method was further illustrated by the fact that all predose rat plasma samples were free of interfering peaks. Representative plasma concentrations from rats after receiving p.o. dose (3 mg/kg) of **I** are presented in Table 4.

Table 4. Plasma concentrations (ng/mL) of **I** in rats following p.o. administration of 3 mg/kg of **I**.

Rat #	Pre-dose	0.333 hr	0.5 hr	1 hr	4 hr	6 hr
1	ND[a]	No sample	No sample	5.94	No sample	1.24
2	ND	No sample	13.24	No sample	No sample	No sample
3	ND	33.02	No sample	No sample	3.22	No sample

[a]Not detected.

CONCLUSION

A semi-automated HPLC method with fluorescence detection using Tomtec Quadra 96 system has been developed and validated for the determination of **I** in human and rat plasma. The method has been demonstrated to be sensitive, selective, precise, and accurate for the quantification of **I** in rat plasma samples after p.o. administration of **I**.

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