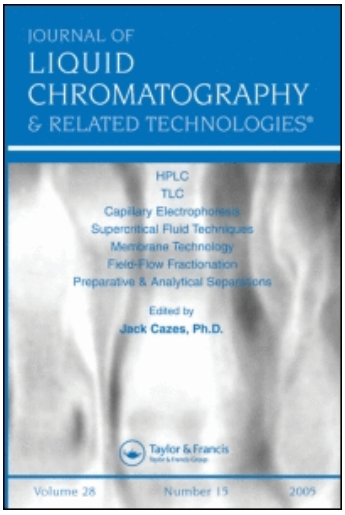

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A Simple HPLC Method for the Determination of Trazodone Human Serum

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A SIMPLE HPLC METHOD FOR THE DETERMINATION OF TRAZODONE IN HUMAN SERUM

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ABSTRACT

A simple HPLC method with minimal sample preparation and good reproducibility for the determination of trazodone in serum is described. Basified serum samples were extracted using ethyl acetate containing diazepam as the internal standard (IS). Chromatography was performed on a cyanopropylsilane column with 15 µL sample injection. The mobile phase consisted of 0.02 M ammonium phosphate, pH 7.5 : acetonitrile (70:30 v/v). The eluent was monitored at 220 nm. The serum standard curve was linear from 10.0 to 8000.0 ng/mL serum. The overall within-run quality control CV was 6.3% for five concentrations (20.0, 40.0, 100.0, 250.0 and 1000.0 ng/mL) and the overall recovery from serum was 85.4%. This method has been applied to the analysis of human serum samples.

INTRODUCTION

Trazodone(2-[3-(4-m-chlorophenyl-1-piperazinyl)propyl]-1,2,4-triazolo-[4,3-a]pyridin-3(2H)-one) is an antidepressant which has a different

mechanism of action and is structurally unrelated to the tricyclic antidepressants or other psychotropic drugs. The drug was first synthesized in 1966, has minimal anticholinergic and cardiovascular effects, and a marked sedative action (1).

Analytical methods for the determination of trazodone in serum include spectroflurometry (2), gas chromatography with flame-ionization, nitrogen-phosphorous selective or mass spectrographic detection (3-6), or high-performance liquid chromatography with ultraviolet, fluorescence or electrochemical oxidation detection (7-12). These methods suffer certain limitations including a) the use of a lengthy extraction procedure (2-3,5,7,12), b) the addition of the internal standard after the extraction step is completed (9), c) the use of toxic or highly flammable extraction solvents (4-7,9,11-12), or d) a limited linear concentration range or a poor limit of detection (2-12).

This paper describes a method for trazodone determination in human serum which does not suffer from the above limitations. Sample preparation is minimized by using a single step extraction with an internal standard. A cyanopropylsilane HPLC column is used in a reverse phase mode with UV detection. The method is linear over a wide concentration range, and therefore is applicable to pharmacokinetic and bioequivalency studies as well as to therapeutic and toxic drug monitoring.

MATERIALS AND METHODS

Instrumentation:

Chromatography was performed with a Hewlett Packard model 1090 liquid chromatograph equipped with a model 1040 diode array detector, an autosampler, and a cyanopropylsilane column, 4.6 mm i.d. x 15 cm long, packed with 5 micron spherical material (Zorbax CN, DuPont). The eluent was monitored at 220 nm with a bandwidth of 25 nm, and the response was recorded on a 3392A Hewlett Packard integrator. The degassed mobile phase was pumped through the column at 2.5 mL/min, and the column compartment was maintained at 40°C.

Reagents:

Trazodone hydrochloride (lot#11676-1) and diazepam (lot#380121) were obtained from Mead Johnson and Hoffmann-La Roche, respectively. A metabolite of trazodone (1-(m-chlorophenyl)piperazine hydrochloride) and ammonium dihydrogen phosphate (reagent grade) were obtained from Aldrich Chemical Co. Sodium hydroxide (reagent grade) and ethyl acetate (spectrophotometric grade) were obtained from J.T. Baker Chemical Co. Methanol (HPLC grade) and ammonium hydroxide (reagent grade) were obtained from Fisher Scientific. HPLC grade acetonitrile was obtained from EM Science.

Drug Solutions:

A 1000.0 µg/mL stock solution of trazodone was prepared in methanol. Working solutions were prepared from the stock solution.

Internal Standard (IS) Solutions:

A 100.0 µg/mL stock solution of diazepam was prepared in methanol. The final extraction solution consisted of a 400 µL aliquot of IS stock solution per liter of ethyl acetate (40 ng diazepam/mL ethyl acetate).

Mobile Phase:

Ammonium dihydrogen phosphate buffer 0.02 M was prepared in deionized distilled water, and the pH was adjusted to 7.5 with ammonium hydroxide. The mobile phase for this assay consisted of 70% buffer and 30% acetonitrile (v/v).

Preparation of Serum Standards:

To 1.0 mL of serum in a 15 mL screw-capped centrifuge tube was added an aliquot of drug solution containing 10.0-8000.0 ng trazodone, 0.5 mL NaOH (0.1M) and 8 mL of extraction solution containing 320 ng of the internal standard. The tubes were vortexed for 20 seconds and centrifuged for 10 minutes at 1100xg. The organic layer was transferred to an evaporating tube (Concentratube®, Laboratory Research Co., Los Angeles, CA). The sample was evaporated to dryness under a gentle stream of nitrogen at 75°C, reconstituted in 100 µL of methanol and transferred to a glass HPLC vial. A 15 µL aliquot was injected onto the column.

Quantification:

Standard curves for serum were constructed ultilizing four replicates at each concentration (10.0-8000.0 ng/mL serum). The peak heights were

integrated and the ratios of trazodone to internal standard were plotted against concentration (ng/mL serum).

Recovery:

The recovery of trazodone from serum was tested for samples containing 20.0-2000.0 ng trazodone/mL serum. These samples were carried through the analysis and the peak heights of drug and internal standard were integrated. Equivalent amounts of trazodone were added to 8 mL aliquots of extraction solution, vortexed, evaporated to dryness, and reconstituted in methanol for injection into the HPLC. The peak height ratios of drug and internal standard were calculated and compared to the ratios obtained from the extracted samples to estimate percent recovery from extraction.

Patient Samples:

Serum samples obtained from patients receiving trazodone were analyzed using this procedure. The amount of drug in the patient samples was calculated utilizing a standard curve prepared daily.

Quality Control (QC) Samples:

Drug-free serum was spiked with known concentrations of a trazodone solution prepared in methanol. Five quality control levels (20.0, 40.0, 100.0, 250.0, and 1000.0 ng/mL) were prepared, aliquoted, and stored at -20°C until needed for use. After the samples were brought to room temperature, the samples were carried through the serum assay. The amount of drug found in the QC samples was calculated by comparison to a standard curve prepared daily.

Interferences:

The possible interference of normal serum constituents was tested by the analysis of drug-free serum samples. The interference of other drugs was tested by direct injection of aqueous or methanolic drug solutions.

RESULTS AND DISCUSSION

The chromatographic conditions chosen for the trazodone assay were selected to give good separation of trazodone and the internal standard from endogenous serum peaks and 1-(m-chlorophenyl)piperazine (CPP), a known metabolite of trazodone. Previous HPLC methods for trazodone

determination used normal phase silica or reverse phase octyl or octadecylsilane columns; however cyanopropylsilane columns are frequently used for the determination and separation of basic drugs. The use of a high efficiency 5 micron particle size cyano column in a reverse phase mode, coupled with a mobile phase consisting of acetonitrile and 0.02M ammonium phosphate, pH 7.5 (30:70 v/v) provided excellent specificity. Baseline resolution of trazodone and the internal standard was achieved from endogenous serum peaks and CPP. Typical chromatograms obtained from serum samples are found in Figure 1. The retention times for the internal standard, trazodone, and CPP are 3.2, 4.8, and 15.7 minutes respectively.

The UV absorbance spectra of trazodone and its metabolite CPP were scanned (200-600 nm) using the HP 1040 diode array detector, and indicated maximum absorbance wavelengths of 214 nm and 210 nm for the drug and CPP respectively. The HPLC effluent was also monitored flurometrically using a Perkin Elmer LS-4 with an excitation wavelength of 230 nm and an emission wavelength of 416 nm. Trazodone gave a fluorescence detector response approximately equivalent to that from the UV detector, but CPP did not fluoresce and the diazepam had limited fluorescence. An ultraviolet absorbance wavelength of 220 nm was chosen for the final assay. It provided high sensitivity and minimized the detector response to the mobile phase solvents, endogenous serum peaks and signal noise seen at 214 nm (the absorbance maximum for trazodone). The serum assay described has a lower limit of determination (10.0 ng/mL serum at a precision level of 22% CV) than those reported in the literature (25.0 ng/mL or higher) (2-6,8-12), and a detection limit of approximately 7 ng/mL serum based on a S/N ratio of three.

Trazodone is readily extracted from serum in a single step using ethyl acetate at alkaline pH resulting in a clean extract without the use of highly flammable or toxic solvents. The overall percent recovery from samples containing 20.0-2000.0 ng trazodone per mL serum was 85.4% using this method. The addition of internal standard to the extraction solution allowed the ratio of the peak height of drug to the peak height of internal standard to be calculated at each serum concentration, and allowed for simple sample processing. Statistical analysis of the data by linear regression indicated excellent linearity and reproducibility in the range of

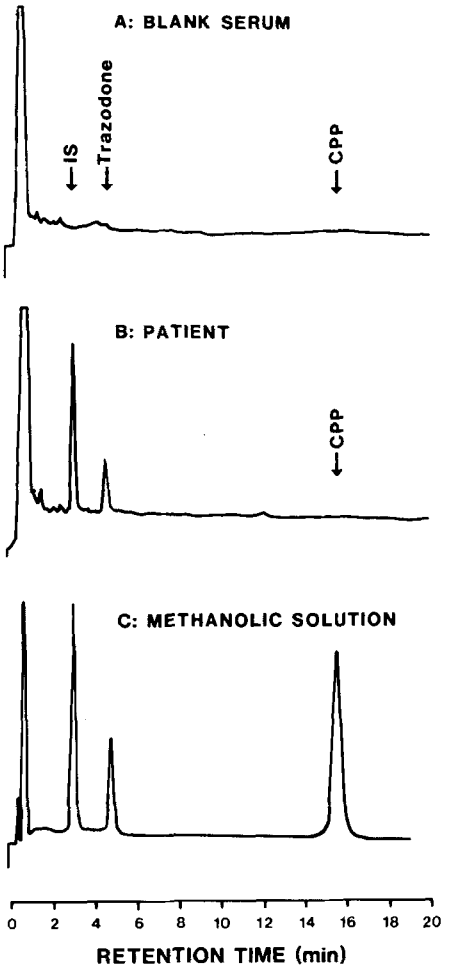

Figure 1. Typical Trazodone HPLC Chromatograms. Key: A, Blank Serum Extract; B, Patient Serum Extract (Equivalent to 278 ng Trazodone/mL Serum); and C, Methanolic Solution Containing Diazepam (IS), Trazodone, and CPP.

TABLE 1. LINEARITY AND PRECISION OF THE TRAZODONE SERUM ASSAY

Trazodone Added (ng/mL serum)	Trazodone Found (ng/mL serum)*	CV (%)
10.0	8.2 ± 1.8	22.0
20.0	23.8 ± 3.7	15.5
40.0	33.8 ± 4.2	12.4
50.0	51.5 ± 1.3	2.5
60.0	58.3 ± 2.4	4.1
100.0	98.5 ± 3.8	3.9
250.0	238.3 ± 6.0	2.5
500.0	501.7 ± 13.9	2.8
1000.0	984.7 ± 36.0	3.7
1500.0	1492.3 ± 55.5	3.7
2000.0	2014.7 ± 51.5	2.6
3000.0	2904.8 ± 14.7	0.5
4000.0	3896.6 ± 31.7	0.8
6000.0	6058.6 ± 96.0	1.6
8000.0	8047.6 ± 79.6	1.0

N=4
Overall CV= 5.3%
Correlation Coefficient = 0.9998
*mean ± standard deviation

10.0-8000.0 ng/mL serum (Table 1). The upper concentration of 8000.0 ng/mL is sufficient to analyze overdose samples without having to reassay the sample after dilution, and the lower limit of 10.0 ng/mL is applicable to bioavailability studies.

This assay has been applied to the analysis of serum samples obtained from patients taking trazodone. A typical serum kinetic profile of trazodone obtained from a volunteer receiving a single 100 mg oral dose is shown in Figure 2. The CPP metabolite was not detected in volunteers receiving a single 100 mg oral dose and therefore was not quantified in this assay.

Quality control samples were analyzed each day with the patient samples. The method showed very good within-run and day-to-day reproducibility (Table 2). These results also demonstrate good stability of trazodone in frozen serum samples as well as the excellent accuracy of the assay.

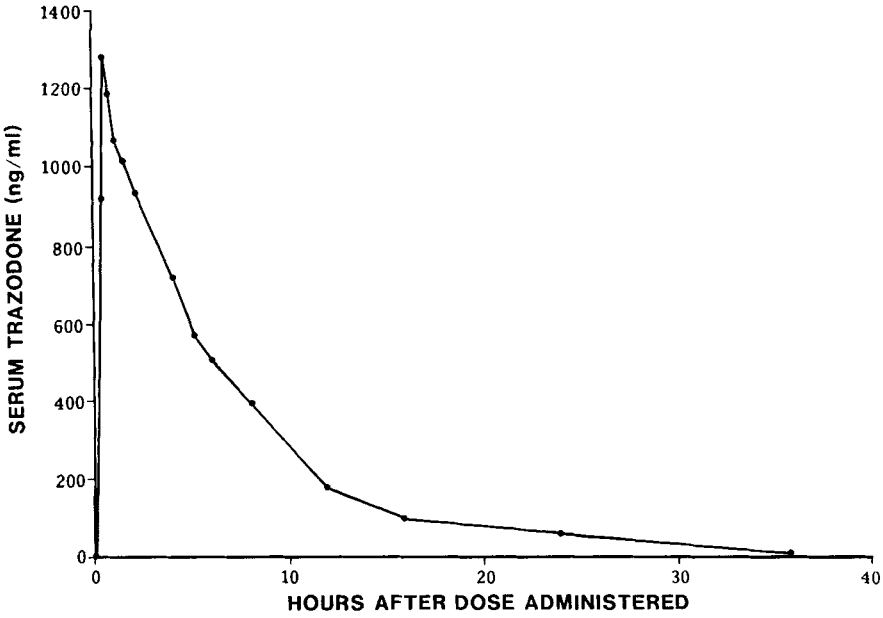

Figure 2. Serum Concentration of Trazodone After a Single 100 mg Oral Dose.

TABLE 2. REPRODUCIBILITY OF THE TRAZODONE SERUM ASSAY

	Quality Control Concentration (ng/mL serum)	Trazodone Concentration Found (ng/mL serum)*	CV%
Within-Run Precision	20.0	24.7 ± 2.6	10.5
	40.0	44.0 ± 3.2	7.3
	100.0	102.2 ± 6.3	6.2
	250.0	244.0 ± 16.0	6.6
	1000.0	1002.3 ± 9.7	1.0
			Overall CV= 6.3%
Day-to-Day Precision	20.0	25.5 ± 3.1	12.2
	40.0	41.5 ± 2.7	6.5
	100.0	98.4 ± 7.2	6.5
	250.0	237.6 ± 15.4	6.5
	1000.0	983.8 ± 29.2	3.0
			Overall CV= 6.9%

* mean ± standard deviation
N=5 (# replicates for within-run precision; # days for day-to-day precision)

No interference from normal serum constituents was observed after extraction and chromatography (Fig. 1A). Several tricyclic antidepressants, benzodiazepine derivatives and cardiac drugs were also injected into the chromatograph to test for interference (Table 3). The results indicate that basic drugs which might be given concomitantly should not interfere with the assay. The tricyclic antidepressants and cardiac drugs eluted after trazodone, while the majority of benzodiazepines eluted earlier.

Table 3. DRUGS TESTED FOR POSSIBLE INTERFERENCE

Drug name	Capacity Factor (k)
Procaine	1.2
Demoxepam	1.8
N-desmethylchlordiazepoxide	2.0
Oxazepam	2.3
Chlordiazepoxide	2.5
Hydroxyethylflurazepam	2.5
N-desmethyldiazepam	3.2
Desalkylflurazepam	3.2
Temazepam	3.5
Lorazepam	3.7
Diazepam (IS)	4.3
Trazodone	7.0
Prazepam	7.8
Lidocaine	8.0
N-acetylprocainamide	10.2
Procainamide	11.3
Mono-n-desalkyldisopyramide	21.5
1-(m-chlorophenyl)piperazine(CPP)	25.2
Amitriptyline	>30.0
Amoxapine	>30.0
Cyclobenzaprine	>30.0
Demethyldoxepin	>30.0
Desipramine	>30.0
Disopyramide	>30.0
Doxepin	>30.0
Flurazepam	>30.0
Imipramine	>30.0
Loxapine	>30.0
Nortriptyline	>30.0
Quinidine	>30.0
Trimipramine	>30.0

CONCLUSIONS

The method for serum trazodone determinations presented in this paper has several advantages over the procedures presently described in the literature. The method provides a wider range of linearity with excellent reproducibility, uses a rapid single step ethyl acetate extraction, and has an equivalent or lower limit of determination than other procedures.

The specificity, simplicity, reproducibility, sensitivity and wide range of linearity for this assay makes it applicable for determining trazodone levels in bioavailability samples, or for monitoring therapeutic and overdose (without dilution) trazodone levels in patients. This method has been used to analyze several hundred human serum samples in our laboratory.

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