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GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC METHOD FOR TRAZODONE AND A DEUTERATED ANALOGUE IN PLASMA

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SUMMARY

A plasma assay method for trazodone and a $^2H_4$ analogue is described which uses gas chromatography—electron-impact selected-ion monitoring mass spectrometry. Etoperidone is used as an internal standard. The analytes are extracted from basic medium into *n*-butyl chloride, then back extracted into aqueous 0.1 *M* hydrochloric acid. The aqueous layer is made basic and re-extracted with *n*-butyl chloride. The solvent is reduced under nitrogen at 35°C and the residue is redissolved in toluene for gas chromatographic—mass spectrometric

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analysis. The ions monitored are *m/z* 231, 235, and 225 for trazodone, [$^2H_4$]trazodone and etoperidone, respectively. Quantitation is in the range 40—1000 ng/ml with acceptable precision and accuracy. The method is suitable for biopharmaceutical studies.

INTRODUCTION

Trazodone, 2-{3-[4-(3-chlorophenyl)-1-piperazinyl]propyl}-1,2,4-triazolo-[4,3-*a*]pyridin-3(2*H*)-one is widely used for the treatment of major depressive episodes [1]. A procedure was required for the identification and measurement of the drug and its $^2H_4$ analogue in plasma for pharmacokinetic and bioavailability studies. Administration of 50 mg of trazodone in tablet form results in plasma concentrations in the range 40—1000 ng/ml over the following 34 h. A high-performance liquid chromatographic (HPLC) [2] and a gas chromatographic—mass spectrometric (GC—MS) [3] method for quantification of trazodone in plasma have been reported; however, neither method was developed and validated for simultaneous quantification of the unlabeled drug and $^2H_4$ analogue.

A recent review by Vink [4] summarizes the rationale for using GC—MS techniques in biopharmaceutical studies. The patient is administered a solution of stable isotope-labeled analogue simultaneously with the unlabeled drug in the solid dosage form. GC—MS is used to selectively measure the plasma concentrations resulting from the solution and solid dosage forms simultaneously being evaluated. In this way, the power of the study to detect differences between dosage forms is greatly increased over a two-way cross-over design, so that a smaller number of clinical tests are required for the same statistical confidence levels. The increase in power of the test is due primarily to a marked decrease in intra-subject variability [5, 6].

The present paper describes a selected-ion monitoring (SIM) GC—MS method for trazodone, its $^2H_4$ analogue and etoperidone, used as an internal standard. The method covers the concentration range 40—1000 ng/ml and has been validated in two laboratories. It has been successfully used in a bioavailability study which is reported elsewhere [7].

EXPERIMENTAL

Materials

The hydrochlorides of trazodone, [$^2H_4$]trazodone (less than 1% [$^2H_0$]-trazodone content) and etoperidone were provided by Bristol-Myers (Evansville, IN, U.S.A.). [$^2H_4$]Trazodone was prepared by starting with [$^2H_6$]-aminopyridine from KOR (Cambridge, MA, U.S.A.), which was converted to [$^2H_4$]bromopyridine by diazotization and bromination. This was converted to [$^2H_4$]triazolopyridinone with perdeuterated semicarbizide hydrochloride and sulfuric acid in [$^2H_1$]2-(2-ethoxyethoxy)ethanol. This was alkylated with 1-chlorobenzyl-4-chloropropyl piperazine.

Toluene (HPLC grade), *n*-butyl chloride (HPLC grade), methanol (pesticide grade), sodium hydroxide (2.0 *M*) and hydrochloric acid (0.1 *M*) were obtained from Fisher Scientific (Pittsburg, PA, U.S.A.) and were used without further purification.

Standard solutions (1 mg/ml of free base) of trazodone, [$^2H_4$]trazodone, and etoperidone were prepared by dissolving the hydrochloride salts in methanol. These standards were diluted to prepare methanolic standards to be used for the preparation of calibration standards in plasma. Calibration standards at concentrations of 1000, 800, and 400 ng/ml were prepared by adding 100 μl of a trazodone—[$^2H_4$]trazodone standard to control plasma in 50-ml volumetric flasks. Of each of these standards 5 ml were added to 50-ml volumetric flasks and diluted to volume with control plasma to give calibration standards at 100, 80, and 40 ng/ml. Control samples at 600 and 60 ng/ml were prepared in the same manner.

Instrumentation

All analyses were performed on a Finnigan MAT 4023 or a 4510 gas chromatograph—mass spectrometer—data system instrument (San Jose, CA, U.S.A.) operated in the electron-impact (EI) mode. The mass spectrometer was operated at 70 eV electron energy and a source temperature of 190°C. Finnigan MAT 4.0 software was used with the INCOS data system to monitor the selected ions, quantitate peak areas, generate calibration lines and calculate concentration values. The mass spectra in Figs. 1—3 were obtained by injecting about 100 ng of each compound into the GC—MS system and scanning 100—500 a.m.u. in 2 sec. Prior to obtaining these spectra, proper tuning of the mass spectrometer was assured by the method of Budde and Eichelberger [8] using decafluorotriphenyl phosphine. For sample analysis, the following selected ions (*m/z*) were repetitively scanned over a unit mass window for 0.21 sec each: trazodone, *m/z* 231; [$^2H_4$]trazodone, *m/z* 235; and etoperidone, *m/z* 225.

The chromatographic column was a 4 m × 0.25 mm I.D. DB-1 fused-silica capillary column (0.25 μm film thickness) from J & W Scientific (Rancho Cordova, CA, U.S.A.). The helium carrier gas linear velocity was 200 cm/sec. The split and septum sweep were set at 30 and 3 ml/min, respectively, and were suspended for the first minute of the run for splitless injection. The column oven temperature was 170°C for 1 min, then it was increased at 15°C/min to a final temperature of 255°C. The injector temperature was set at 250°C, and the separator oven temperature at 255°C.

Sample extraction procedure

A 1-ml volume of plasma sample, control, or standard was pipetted into a silanized glass culture tube (125 × 16 mm). A 20-μl aliquot of the internal standard (etoperidone, 20 ng/μl) was added to each sample, standard, and control sample. These were mixed briefly on a vortical mixer and stood for 5 min. The sample was made basic by adding 0.5 ml of 2 *M* sodium hydroxide and brief vortical mixing.

The sample was extracted by adding 9 ml of *n*-butyl chloride, capping the tube with a PTFE-lined cap, and vortical mixing for 10—15 sec. The sample was centrifuged for 10 min at 2000 *g*. The organic layer was transferred to a second culture tube containing 2 ml of 0.1 *M* hydrochloric acid. The tube was capped tightly, vortically mixed for 20 sec, centrifuged, and the organic layer was removed. The aqueous layer was made basic with 0.5 ml of 2 *M* sodium hydroxide, and 9 ml of *n*-butyl chloride were added. The sample was vortically

mixed for 10—15 sec and centrifuged for 10 min at 2000 *g*. The organic layer was transferred to a 15-ml silanized conical centrifuge tube, and the butyl chloride was evaporated at 35°C under a stream of nitrogen. The residue was dissolved in 1 ml of methanol and transferred to a 3-ml Reacti-Vial. The tube was rinsed with an additional 1 ml of methanol and the rinse was added to the Reacti-Vial. The methanol was evaporated under nitrogen. The residue was dissolved in 200 μl of methanol and briefly vortically mixed. The methanol was evaporated under nitrogen, and the residue was dissolved in 20 μl of toluene. Then 2 μl were injected into the GC—MS system for analysis.

Quality control

Quantification was performed by comparing samples to standards prepared from control plasma spiked over the range 40—600 ng/ml and coextracted with samples.

Inter-assay variability was determined by preparing and analyzing five spiked control plasma samples at two concentration levels on five separate days. Intra-assay variability was evaluated by preparing and analyzing five additional identical samples at the two concentration levels on one of these test days for a total of ten replicates for comparison. Blinded control samples were spiked by one person and blindly analyzed by another. They consisted of blanks plus spikes over a range of 40—600 ng/ml and were analyzed on one day. Throughout the bioavailability study [7], which was conducted using this method, quality control (QC) samples were regularly analyzed. These QC samples consisted of spiked control plasma at 60 and 600 ng/ml analyzed at the same time as a set of samples. Nine samples at each of two concentration levels were analyzed.

Clinical pharmacokinetic studies

In order to evaluate the bioequivalence of trazodone and [$^2H_4$]trazodone, six normal healthy male volunteers were given an oral solution containing 50 mg each of trazodone and [$^2H_4$]trazodone. Blood samples were collected over a 34-h period and stored at −4°C until analyzed. A detailed description of a bioavailability study performed by coadministering trazodone (in liquid or either of two solid forms) and [$^2H_4$]trazodone (liquid) is the subject of a separate report [7].

RESULTS AND DISCUSSION

The low resolution 70-eV EI mass spectra of trazodone, [$^2H_4$] trazodone and etoperidone are presented in Figs. 1—3, respectively. Assignment of the identity of the fragment ions is based on comparison with analogues of the compounds and is not intended to suggest an exhaustive study of fragmentation mechanisms. In some cases ions requiring rearrangements for formation are drawn without special notice for simplicity.

SIM of the base peak of the analytes is not appropriate because trazodone produces a strong response at *m/z* 209 which would interfere with [$^2H_4$]-trazodone. The *m/z* 231 ion for trazodone and the corresponding *m/z* 235 ion for [$^2H_4$]trazodone were found to produce sufficient response for the

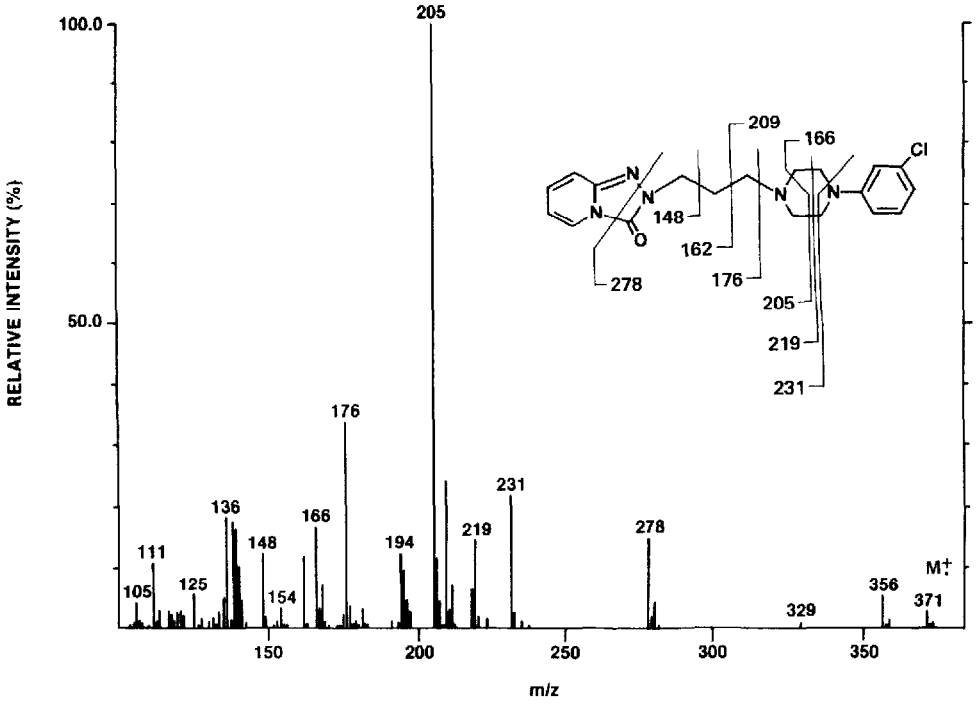

Fig. 1. Electron-impact mass spectrum of trazodone.

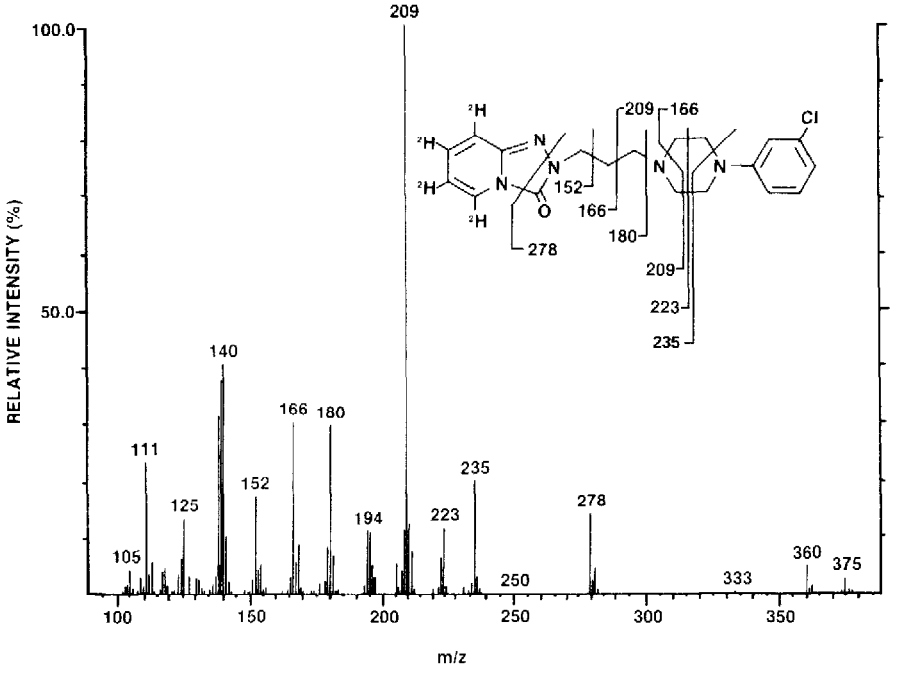

Fig. 2. Electron-impact mass spectrum of [$^2H_4$]trazodone.

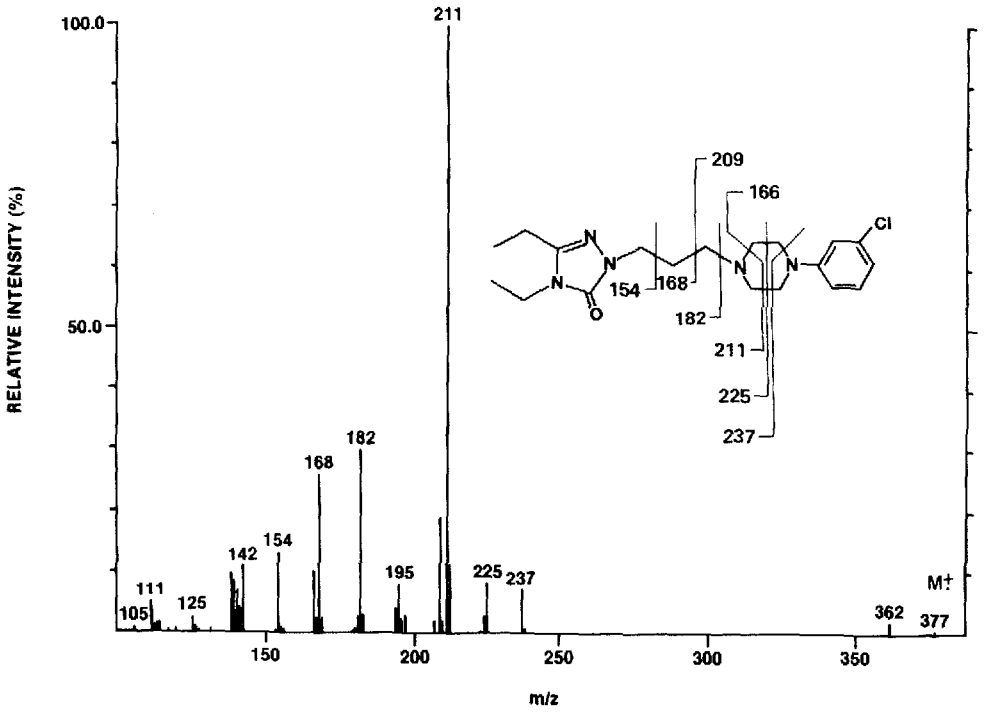

Fig. 3. Electron-impact mass spectrum of etoperidone.

sensitivity requirements of this method. A relatively low intensity ion (*m/z* 225) was also chosen for monitoring etoperidone. The ion resulting from the same fragmentation as chosen for trazodone would have been *m/z* 237 but sample interference was observed at *m/z* 237. Good accuracy and precision were consistently obtained using *m/z* 225.

When fragment ions are chosen for monitoring in an assay there is a possibility of interference from metabolites of the drug which retain the portion of the molecule that produces the chosen fragment ion. In the case of trazodone the known metabolites were either excluded by the selectivity of the extraction procedure or were easily separated from trazodone by the capillary GC. The major metabolites of trazodone in humans have been reported [9]. These were found to be: (1) the piperazinyl-1 oxide, (2) the 7,8-dihydroxy adduct of the triazolopyridinone, and (3) the 4-hydroxyphenyl compound. Only the third metabolite retains the portion of the trazodone molecule which produces the fragment ion chosen for monitoring. This compound separates from trazodone under the capillary GC conditions used for this method (see Fig. 4).

Fig. 5 depicts typical chromatograms of a plasma extract blank to which etoperidone only has been added prior to extraction and a 40 ng/ml plasma standard extract. A non-interfering matrix background 231 a.m.u. peak is produced at 3 min. A non-interfering 235 a.m.u. peak is produced for etoperidone. No endogenous plasma interferences were observed. The GC column was conditioned at the start of each day with a few injections of standard containing all three analytes to eliminate any GC system absorption effects.

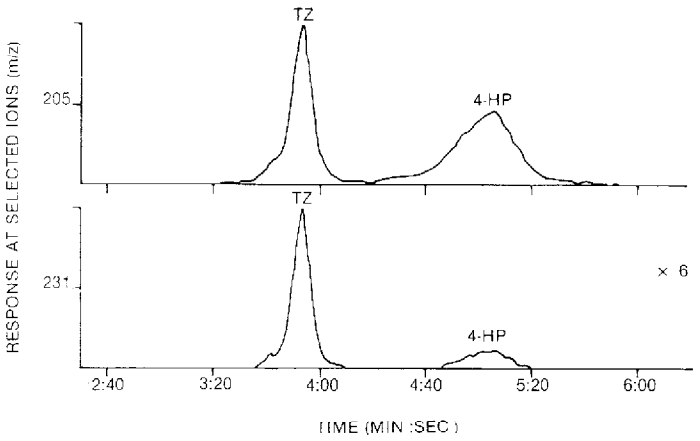

Fig. 4. Chromatographic separation of possible interfering metabolite. Capillary GC conditions were the same as described under *Instrumentation*. Peaks: TZ = trazodone; 4-HP = 3-chloro-4-hydroxyphenyl metabolite (see text).

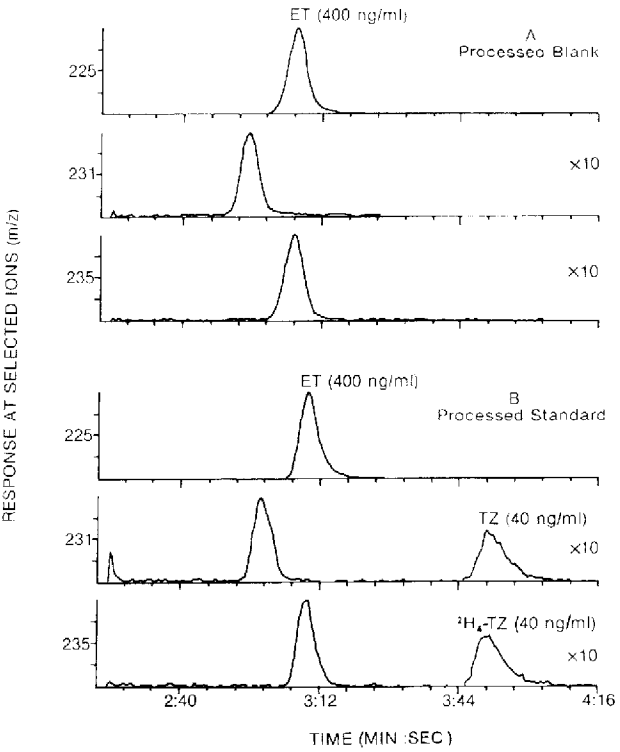

Fig. 5. Selected-ion chromatograms of plasma extracts. (A) Processed blank, spiked with 400 ng/ml etoperidone only; (B) processed standard, spiked with 400 ng/ml etoperidone and 40 ng/ml trazodone and [$^2H_4$]trazodone. Peaks: ET = etoperidone, internal standard; TZ = trazodone; $^2H_4$-TZ = [$^2H_4$]trazodone.

Calibration lines were generated and used by the INCOS data system. An example of the typical linearity of the calibration is presented in Fig. 6. The lower working range of the method is considered to be 40 ng/ml due to the

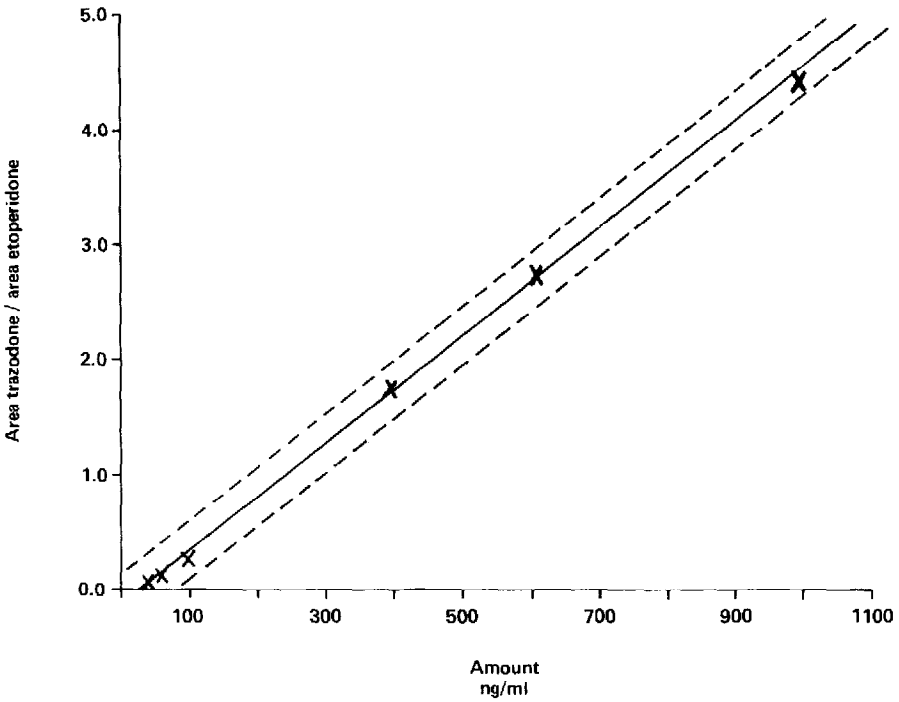

Fig. 6. Trazodone calibration line for a typical day (—); three standard deviations (- - -). Each point represents the mean of two measurements. Correlation coefficient = 0.998.

TABLE I

ACCURACY AND PRECISION OF THE ASSAY

See text for a description of the tests.

Test	Trazodone				[$^2H_4$]Trazodone			
	Lab	Added (ng/ml)	Mean observed (ng/ml)	C.V. (%)	Lab	Added (ng/ml)	Mean observed (ng/ml)	C.V. (%)
Inter-assay	A*	60	60	5.0	A*	60	61	4.9
Inter-assay	A**	600	604	3.1	A**	600	606	4.5
Inter-assay	B	60	66	6.0	B	60	63	4.8
Inter-assay	B	600	594	5.2	B	600	591	5.1
Intra-assay	B	60	59	5.1	B	60	59	4.2
Intra-assay	B	600	616	3.4	B	600	619	3.2
Blinded***	A	40—600	—	16	A	40—600	—	17
Blinded***	B	40—600	—	8.0	B	40—600	—	9.0
QC samples	B	60	63	7.9	B	60	64	9.4
QC samples	B	600	600	4.0	B	600	601	4.7

*One day had three samples.
**Two days had four samples.
***Lab A analyzed twelve samples; Lab B analyzed twenty samples.

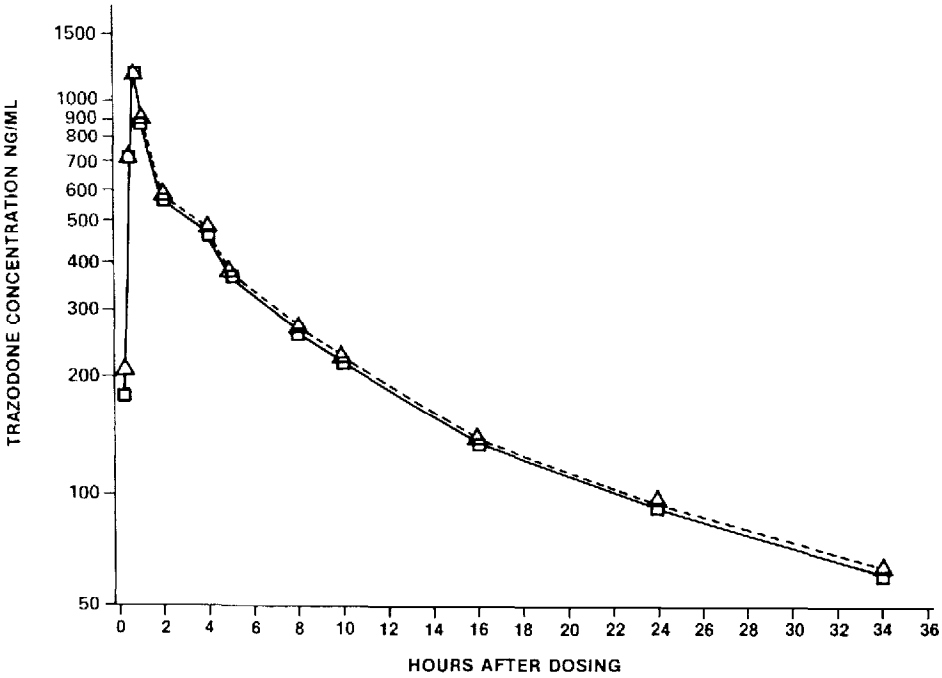

Fig. 7. Plasma concentration versus time profile for trazodone (□), and [$^2H_4$]trazodone (△) orally coadministered in solution to a healthy male volunteer.

low-intensity ions selected. Greater sensitivity could be obtained for trazodone by monitoring its base peak if [$^2H_4$]trazodone was not present in the sample.

Extraction recovery studies over the range 10—4000 ng/ml indicated analyte recoveries ranging from 79% to 96%.

The precision and accuracy of the method was demonstrated in two laboratories. Table I shows the results of this testing. The accuracy (relative difference between observed mean and added concentration) of the method over the range 60—600 ng/ml was 0—10% and the precision (coefficient of variation, C.V.) was 3—17%. The sum of the data in Table I suggests that the method produces acceptable precision and accuracy.

In a test of the bioequivalence of trazodone and [$^2H_4$]trazodone when coadministered as an oral solution, healthy male volunteers were administered a dose of 50 mg and plasma samples were analyzed by the method. Fig. 7 demonstrates for one typical volunteer the equivalent fate of trazodone and [$^2H_4$]trazodone in human plasma. No kinetic isotope effect was observed.

This method has been shown in two laboratories to be accurate and precise in measuring trazodone and [$^2H_4$]trazodone in the range of concentrations resulting from a normal clinical dose. Its utility has been successfully demonstrated in a recent bioavailability study [7]. A preliminary report of this method was previously presented [10].

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