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CAPILLARY GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC DETERMINATION OF BUSPIRONE IN PLASMA

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SUMMARY

Methods for determining buspirone plus a deuterated analogue and for buspirone alone in plasma samples are described. Analytes are prepared from plasma by liquid extraction into *n*-butyl chloride and subsequent back-extraction clean-up steps. Instrumental analysis involves selected-ion monitoring gas chromatography—mass spectrometry with fused-silica capillary chromatography. Quantification is in the range 0.05—10 ng/ml with acceptable accuracy and precision.

INTRODUCTION

Buspirone, 8-{4-[4-(2-pyrimidinyl)-1-piperazinyl]butyl}-8-azaspiro[4.5]-decane-7,9-dione, is an important new anti-anxiety drug whose properties have been reviewed [1, 2]. Its structure is indicated in Fig. 1.

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Following administration of the normal clinical dose of 5 mg, plasma concentrations reach a maximum of less than 5 ng/ml, so an assay method of low detection limit was required. Unpublished plasma assays based on high-performance liquid chromatography (HPLC) using ultraviolet detection are capable of measuring concentrations as low as 10 ng/ml, and radioimmunoassay techniques measure down to 0.05 ng/ml; however, this method is not suitable for general use due to the limited availability of antiserum with suitable specificity. Caccia et al. [3] reported a gas chromatographic (GC) method using flame-ionization detection (FID) capable of measuring concentrations greater than 200 ng/ml. Therefore, no previous chromatographic method is capable of measuring concentrations in the required range. Our research required development of two different high-sensitivity methods. Gas chromatography—mass spectrometry (GC—MS) was chosen for instrumental analyses in both methods. First, a method was required to simultaneously quantify buspirone and a stable-isotope-labeled analogue for use in pharmacokinetic studies involving coadministration of the two isotope analogues in different dosage forms. Such an approach has been shown [4] to have statistical advantages for pharmacokinetic studies. We refer to this method as method 1. A second method was required for quantification of buspirone alone at a lower detection limit. This report refers to this method as method 2.

This paper describes validated extraction and instrumental analysis conditions for determination of buspirone and a [$^2H_4$]buspirone analogue (method 1) and buspirone alone (method 2). The methods utilize a fluorinated buspirone internal standard, GC—MS instrumentation, on-column injection, capillary GC, electron-impact (EI) ionization, and selected-ion monitoring (SIM). The range of determination for method 1 is 0.2—10 ng/ml and for method 2 is 0.05—5 ng/ml.

EXPERIMENTAL

Materials

The hydrochlorides of buspirone (MJ 9022, see Fig. 1), [$^2H_4$]buspirone (MJ 9022-992, see Fig. 2), 5-fluorobuspirone (MJ 14594, see Fig. 3), and 5-hydroxybuspirone (MJ 14131, see Fig. 6) were synthesized by the Pharmaceutical Research and Development Division of the Bristol-Myers Company (Evansville, IN, U.S.A.). Descriptions of the syntheses of the latter three compounds are in preparation [5]. Toluene (distilled in glass) and *n*-butyl chloride (HPLC grade) were from Burdick & Jackson Labs. (Muskegon, IL, U.S.A.). Methanol (HPLC grade), diethyl ether (ACS-reagent grade) and isopropanol (ACS-reagent grade) were from Fisher Scientific (Fairlawn, NJ, U.S.A.). Ethanol (absolute, USP grade) was from U.S. Industrial Chemicals (Houston, TX, U.S.A.). Deionized water was obtained from a Sybron/Barnstead Nanopure II system (Boston, MA, U.S.A.). Boric acid (ACS-reagent grade) was from J.T. Baker (Phillipsburg, NJ, U.S.A.). Hydrochloric acid (ACS-reagent grade) and sodium hydroxide (certified ACS grade) were from Fisher Scientific. All reagents were used without further purification. Human plasma was obtained from Ohio Valley Blood Services (Evansville, IN, U.S.A.). The

plasma was obtained by centrifugation of EDTA-treated blood. Twelve units of bulk plasma were combined for use and stored at −17°C.

A secondary standard solution of combined buspirone and [$^{2}H_{4}$]buspirone (method 1) or buspirone alone (method 2) at 10 μg/ml in ethanol was prepared from 100 μg/ml individual primary standards of the hydrochloride salts of each compound in ethanol. Aliquots of this standard were diluted to 50 ml in ethanol to obtain tertiary standards with final concentrations of 1.2—400 ng/ml. The standards were stored at 4°C for use in preparing daily standard curves. In a similar manner a 40 ng/ml tertiary standard in ethanol of 5-fluorobuspirone, for use as the internal standard, was prepared from a primary 100 μg/ml standard. All concentrations were in terms of the free base.

Borate buffer was prepared by dissolving boric acid in deionized water, adjusting the pH to 8.5 with 6 *M* sodium hydroxide and diluting to 0.5 *M*. Hydrochloric acid reagent was prepared by diluting to 0.01 *M* in deionized water.

Solvent extraction utilized a Roto-Torque rotator from Cole Parmer Instrument (Chicago, IL, U.S.A.). Solvent was removed with a N-Evap analytical evaporator from Organomation Assoc. (Northborough, MA, U.S.A.) with a water bath temperature of 40—45°C and nitrogen gas stream. Additions of organic solvent during sample preparation were made with a Labindustries repipet from American Scientific Products (McGraw Park, IL, U.S.A.). Addition of aqueous reagents utilized Gilson Distrivar repeating pipettes from Rainin Instrument (Woburn, MA, U.S.A.). Addition of internal standard to samples was accomplished with a 5- or 2.5-ml Hamilton syringe with repeating dispenser (Reno, NV, U.S.A.).

Plasma samples were measured with disposable glass pipettes from Corning (Corning, NY, U.S.A.). All extraction steps were performed in new disposable 16 × 125 mm pyrex culture tubes from Corning. The tubes were closed with reuseable PTFE-lined screw-caps which were cleaned by soaking and rinsing in isopropanol.

Instrumentation

General. All analyses were performed on a Model 4500 gas chromatograph—mass spectrometer from Finnigan MAT Instruments (San Jose, CA, U.S.A.) equipped with a PPINICI enhancement. An INCOS data system was used to control the gas chromatograph and mass spectrometer, monitor selected ions, store data, integrate areas of peaks on selected-ion chromatograms, generate calibration lines and calculate concentration values.

The gas chromatograph was enhanced by the addition of an on-column injector from J & W Scientific (Rancho Cordova, CA, U.S.A.) mounted directly over the 230°C packed-column inlet which was lined with a 0.6 cm O.D. and 0.2 cm I.D. glass tube. Injection of sample over a 2-s period was via a 5-μl on-column syringe No. 701RNFS from Hamilton with the fused-silica needle protruding about 5 cm into the 230°C heated space upon insertion. Between injections the syringe was thoroughly rinsed with methanol which was pulled through the syringe barrel under vacuum and bathed the outer surface of the needle.

The chromatographic column was a 0.25 mm I.D. fused-silica capillary column coated with DB-1 at 0.25 μm film thickness from J & W Scientific

routed directly to the ion source. Helium carrier gas was used. The interface oven between the gas chromatograph and mass spectrometer was 230°C.

The mass spectrometer was operated under EI conditions. For 70-eV scanning of mass spectra of the compounds contained in this report the mass spectrometer was tuned to yield ion intensities and unit mass resolution which was balanced across the scanned mass range. This was checked by analyzing the mass spectral quality-control (QC) compound bis(pentafluorophenyl)-phenyl phosphine under these conditions according to the method of Eichelberger et al. [6] and achieving the published spectral criteria. For 30-eV SIM experiments the lens and offset voltages and the ion source magnet position were tuned for maximum response at the 264-a.m.u. ion of perfluorotributyl amine while maintaining good ion peak shape and approximately unit resolution with the 265-a.m.u. ion. The electron energy of 30 eV was found to produce maximum response and minimum background. For SIM determination of the sample analytes the selected masses were repetitively monitored. Each mass was scanned for 0.42 s over a 0.25-a.m.u. window at the mass peak apex. Electron multiplier voltage was approximately 1300 V. Quantification was based on the area under the chromatographic peak on the appropriate background subtracted selected-ion chromatogram.

Method 1. Specific conditions for method 1 were as follows: 1 μl of sample was injected into the gas chromatograph. The capillary column was 3 m long and was coated with DB-1. Helium carrier gas was at a velocity of 200 cm/s. The GC temperature was held at 150°C for 1 min following injection, then was increased at 20°C/min to 250°C. The mass spectrometer was set to alternately monitor the selected masses 265 and 269 for the period of time specified above.

Method 2. Specific conditions for method 2 were as follows: 3 μl of sample were injected into the gas chromatograph. The capillary column was 8 m long and was coated with DB-5. Helium carrier gas was at a velocity of 100 cm/s. The GC temperature was held at 180°C for 0.1 min following injection, then was increased at 20°C/min to 280°C. The mass spectrometer was set to monitor mass 277 only for the period of time specified above.

Procedures

General. A standard curve was prepared daily with each set of samples by pipetting 2-ml aliquots of plasma into extraction tubes and adding 50 μl of an appropriate ethanolic standard in the range 1.2—400 ng/ml. Each concentration level was prepared in duplicate. These standards were then extracted along with the test samples. Validation spikes for characterization of method accuracy and precision were prepared in multiples in the same manner as the standard curve.

QC samples were prepared close to the time of clinical sample collection and were stored with the samples under identical conditions to assure the integrity of sample storage and analytical methodology. When a set of clinical samples was withdrawn from storage and analyzed, then a duplicate set of QC samples were also withdrawn. QC samples were originally made by adding standards in ethanol to a bulk volume of control plasma, stirring and aliquoting 2-ml volumes into individual tubes for storage.

Plasma samples were extracted as follows: samples, QC samples and plasma for the standards and validation spikes were removed from the −17°C storage, thawed at room temperature, and vortically mixed. A 2-ml aliquot of each sample was pipetted into a labeled extraction tube. Internal standard (5-fluorobuspirone) was added to each tube as an ethanol solution. The tubes were vortically mixed for 3 s and 0.2 ml of borate buffer was added. As borate was added to each tube, the tube was immediately mixed, then all tubes were again mixed after borate had been added to all the tubes. The aqueous phase was extracted with 10 ml of *n*-butyl chloride for 10 min on a Roto-Torque rotator with a setting of 6 out of 10 in the "high" mode. The layers were separated by centrifugation at 600 *g* for 10 min, then freezing of the aqueous layer in a bath of dry ice and isopropyl alcohol. The organic layer was decanted into a new tube and extracted with 2.0 ml of 0.01 *M* hydrochloric acid for 10 min on the rotator. The layers were separated by centrifugation and freezing as above. The organic layer was decanted and discarded. The aqueous layer was thawed and washed with 10 ml of diethyl ether for 10 min on the rotator. The layers were separated by centrifugation as above and then the (upper) ether layer was removed by aspiration (method 1) or freezing and decanting as above (method 2). This ether extraction was repeated once. The pH of the aqueous phase was adjusted to 8.5 by adding 2 ml of borate buffer and it was extracted with 10 ml of butyl chloride on the rotator as above. The layers were separated by centrifugation and freezing as above. The organic layer was transferred to a smaller 13 × 100 mm screw-cap tube and taken to dryness under nitrogen. The procedure for this transfer was different for methods 1 and 2. Refer to the specific procedures below. The dried residue was rinsed to the bottom of the tube with 500 μl of methanol which was again removed under nitrogen. The extract was stored overnight at −17°C in 100 μl of methanol. The next morning the methanol was removed under nitrogen and the sample was redissolved in 20 μl of toluene for GC—MS analysis.

The accuracy and precision of the method was characterized by several tests. Intra-assay variability, or single day errors, were studied by analyzing multiple identical spiked plasma samples at two concentration levels on one day. Inter-assay variability, or long-term errors, were studied by analyzing multiple identical spiked plasma samples at two concentration levels on each of three days. Instrumental variability, or single sample instrument-induced errors, were studied by replicate injections of a single 1 ng/ml spiked plasma sample on day 1 of the inter-assay study. QC samples for method 1, prepared as described above, were analyzed in duplicate at two concentration levels on 25 work days when samples from a clinical bioavailability study were analyzed. These reflect the very-long-term reproducibility of method 1; analogous QC data are not available for method 2.

An evaluation of the effectiveness of two capillary column injection techniques was conducted prior to choosing on-column injection for this method. A 2-μl volume of a 1 ng/μl solution of buspirone was injected both by on-column technique using the J & W Scientific injector and by splitless techniques using the injector supplied by Finnigan MAT for the Model 9610 gas chromatograph. The mass spectrometer and column conditions were those specified above for method 2, except the injector temperature was 270°C for

splitless injections. The splitless injector was set-up according to the instructions and specifications of the manufacturer, which included: split flow-rate 50 ml/min, sweep flow-rate 7 ml/min, and suspension of sweep and split for 0.7 min after injection. Five replicate injections were made by each technique.

Method 1 was used to analyze samples from a bioavailability study in which normal healthy volunteers were simultaneously given both 20 mg of [$^2H_4$]-buspirone in an oral solution and 20 mg of buspirone in a tablet formulation. Blood samples were collected at specified times and stored at −17°C until analyzed. A pharmacokinetic analysis of the data provided evidence for the bioavailability of the tablet formulation [7].

Method 1. Specific procedures for method 1 were as follows: a standard curve of 0.2—10 ng/ml was used, except for inter- and intra-assay studies when standards extended up to 25 ng/ml. QC samples were at 0.5 and 3.0 ng/ml, while blanks contained no buspirone. Internal standard was added as 100 μl of a 40 ng/ml standard to give a final concentration of 2.0 ng/ml. For the final transfer of the organic layer to the 13 × 100 mm tube, the whole organic layer was first transferred to a clean 16 × 125 mm tube and taken to dryness under nitrogen. The residue was transferred to the 13 × 100 mm tube by rinsing the larger tube twice with 1 ml of methanol and drying under nitrogen.

Method 2. Specific procedures for method 2 were as follows: a standard curve of 0.05—5 ng/ml was used. Internal standard was added as 50 μl of a 40 ng/ml standard to give a final concentration of 1.0 ng/ml. For the final transfer of the organic layer to the 13 × 100 mm tube, half of the layer was first decanted into the tube, it was taken to dryness under nitrogen, then the other half was decanted into the same tube and also taken to dryness under nitrogen.

RESULTS AND DISCUSSION

The 70-eV EI mass spectra of buspirone, [$^2H_4$]buspirone and 5-fluorobuspirone are presented in Figs. 1—3. Assignment of fragment identities was supported by comparison to many buspirone analogues and are not intended to indicate the results of an exhaustive fragmentation mechanism study. Some ions require rearrangements which were not depicted for the sake of simplicity. Most of the primary fragmentation occurs in conjunction with the piperazine ring. For method 1, the choice of 265 a.m.u. for SIM of buspirone and 5-fluorobuspirone, and 269 for [$^2H_4$]buspirone SIM was necessitated by the low relative abundance of the molecular ion and the sample matrix background interference with the base peaks (177, 181) and with the 281 a.m.u. ion of [$^2H_4$]buspirone. For method 2, mass 277 was found to be optimal for SIM because there is much less GC column bleed background noise vis-a-vis 265 a.m.u., thus increasing the signal-to-noise ratio and decreasing the detection limit. Despite the necessity of monitoring these low-intensity ions for heavily fragmenting compounds, sufficient ion current was produced to quantify 40 pg injected into the instrument for method 1 and 15 pg for method 2.

To obtain such extreme sensitivity it was necessary to strictly control the cleanliness of reagents, glassware and apparatus used in the analysis. A careful

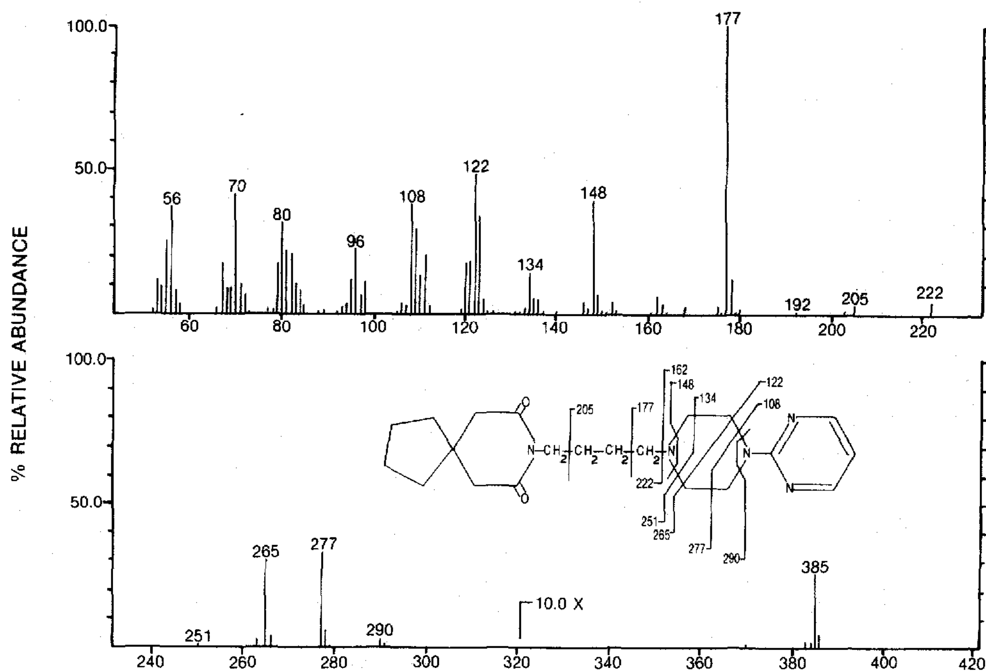

Fig. 1. Electron-impact mass spectrum and structure of buspirone.

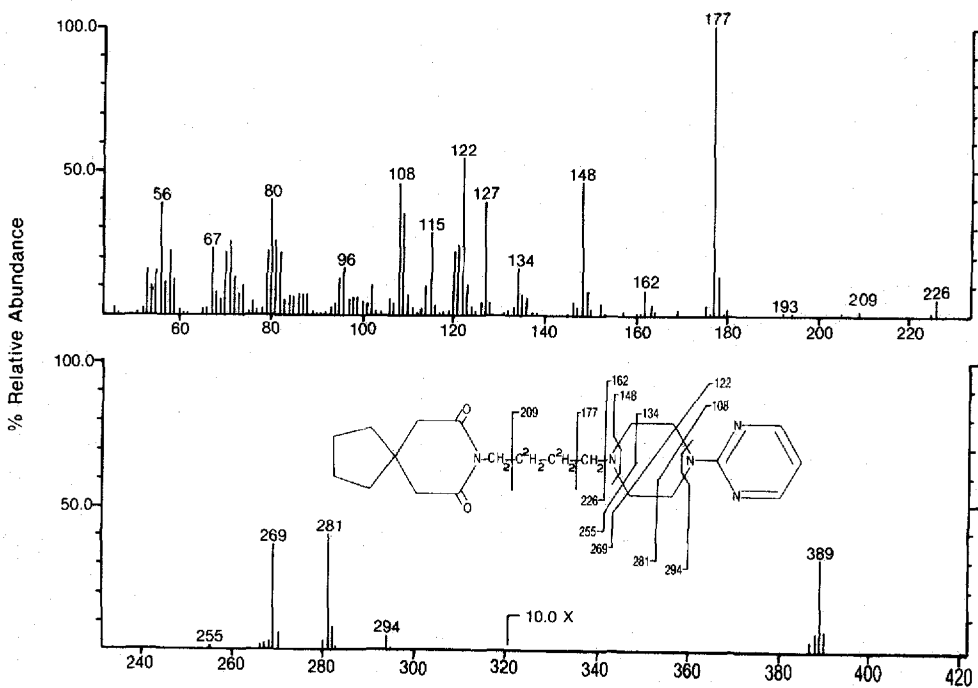

Fig. 2. Electron-impact mass spectrum and structure of [$^2H_4$]buspirone.

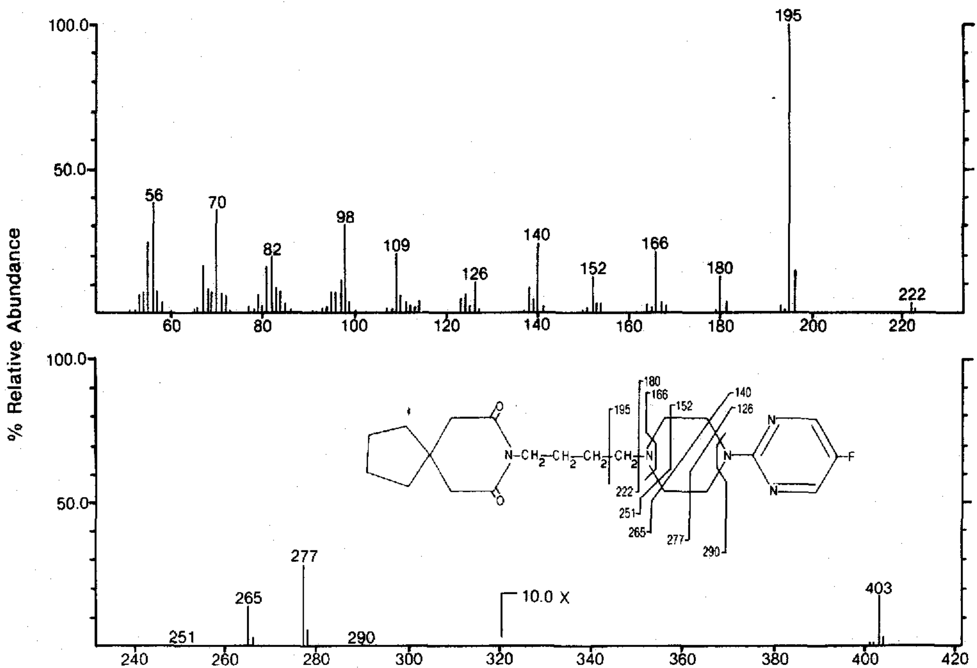

Fig. 3. Electron-impact mass spectrum and structure of 5-fluorobuspirone.

stepwise study was necessary to test the contribution of each reagent and apparatus to background interferences which would overwhelm the selected-ion chromatograms of the sub-ng/ml samples. Of particular importance was the use of new clean disposable glassware, because washed glassware produced interfering residues. Some sources of water and some lots and grades of reagents were avoided due to interferences. The GC on-column injection syringe was thoroughly rinsed with methanol between sample injections. Interfering peaks were produced by the column as the injection temperature was increased above 230°C. Electron energy was optimized at 30 eV for reduction of instrumental background noise and maximization of ion current. The back-extraction clean-ups in the procedure serve to remove interfering sample matrix which would decrease the sensitivity of the method.

Typical chromatograms of spiked and blank plasma extracts resulting from this careful control of sample preparation are presented in Fig. 4 for method 1 and Fig. 5 for method 2. In Fig. 4 both mass chromatograms for each sample are normalized to the largest GC peak in the 265-a.m.u. selected-ion chromatogram so the 269-a.m.u. chromatogram is the same scale. No endogenous plasma interferences were observed in the blanks. Under normal conditions the system responses and peak width were stable over several days of operation. When peaks were observed to broaden the situation could be corrected by holding the column temperature at 290°C for 10 min.

The technique of on-column injection into a heated area of the column is unconventional but served several purposes. Mounting of the on-column injector is difficult on the Finnigan 9610 gas chromatograph without severe alteration of the instrument or installation in an inconvenient location. With

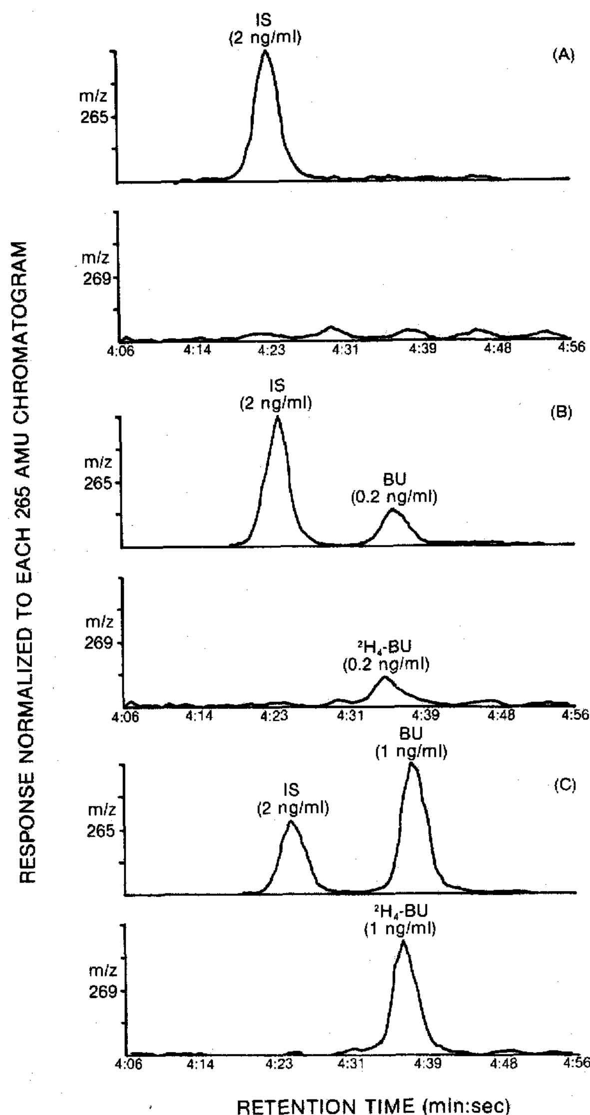

Fig. 4. Selected-ion chromatograms of plasma extracts (method 1). (A) Processed blank, spiked with 2 ng/ml internal standard only. (B) Processed standard, spiked with 2 ng/ml internal standard and 0.2 ng/ml buspirone and [$^2H_4$]buspirone. (C) Processed standard, as above except 1 ng/ml. Peaks: IS = 5-fluorobuspirone, internal standard; BU = buspirone; $^2H_4$-BU = [$^2H_4$]buspirone.

mounting above the heated packed-column injector port, no carry-over cross-contamination of samples was produced. No loss of chromatographic resolution was observed compared to splitless injection. In a brief comparison study between splitless and on-column injection, using the same column and buspirone test solution, the on-column technique yielded several times greater response. Table I presents the results of this study. The precision of response of repetitive injections of a sample indicated greater precision of on-column injection. The on-column injector also adds one more section of inert sample

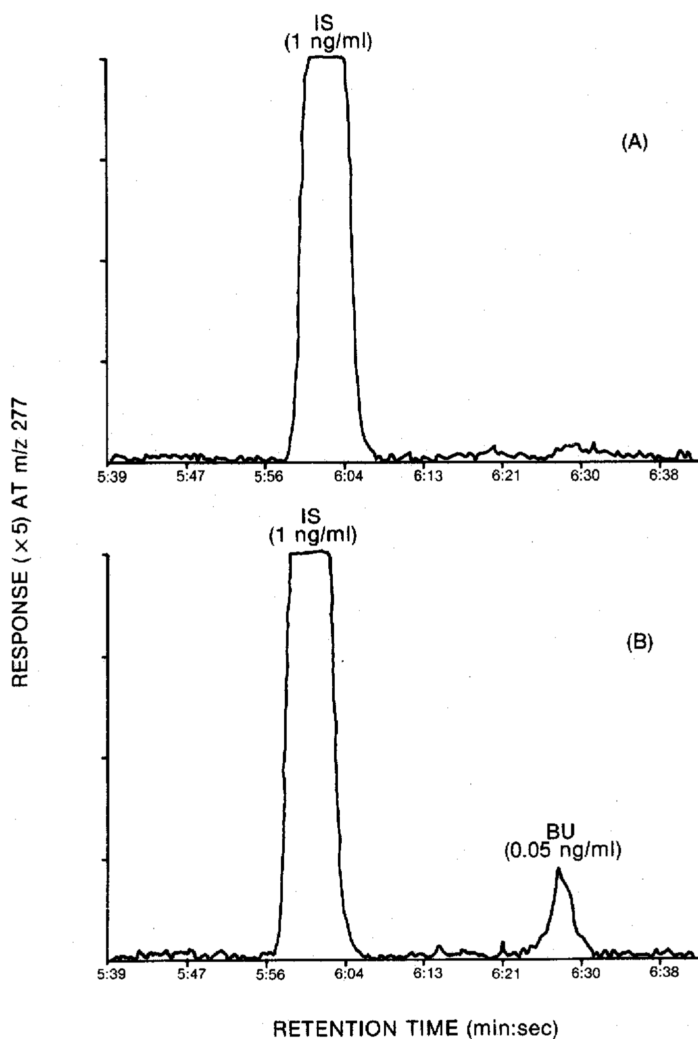

Fig. 5. Selected-ion chromatograms of plasma extracts (method 2). (A) Processed blank, spiked with 1 ng/ml internal standard only. (B) Processed standard, spiked with 1 ng/ml internal standard and 0.05 ng/ml buspirone. Peaks: IS = 5-fluorobuspirone, internal standard; BU = buspirone.

TABLE I

COMPARISON OF ON-COLUMN AND SPLITLESS INJECTION

Technique	Mean response*	Coefficient of variation (%)
On-Column	$1.2 \cdot 10^6$	5.9
Splitless	$2.5 \cdot 10^5$	25

*Mean area response of five replicate injections of 2 ng buspirone.

contact area, replacing the glass and metal of the splitless injector. Another advantage of the on-column injector is the observed lack of silicone compound interference from the septum of the splitless injector. Probably the high boiling point of buspirone and high flow-rates used in these methods make hot on-column injection possible for this application.

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 which produces the chosen fragment ion. In the case of buspirone the only known metabolite of this kind is 5-hydroxybuspirone and it is separated from buspirone under the chromatographic conditions used in these methods as shown in Fig. 6.

Good linearity of response for method 1 was produced over 0.2—10 ng/ml. Similar results were produced for method 2 over 0.05—5 ng/ml. The general equation for method 2 was area buspirone/area internal standard = 0.93 × concentration buspirone — 0.03; the standard error of the slope was 0.009 and of the intercept was 0.02. The correlation coefficient was 0.999. Recovery of the extraction ranged from 73 to 91% over the concentration range 0.05—5 ng/ml. Sample extracts were stable for at least fourteen days when stored in 100 μl of methanol at —17°C.

Extensive precision and accuracy data are collected in Table II for method 1 and Table III for method 2. Part of the precision variability is probably

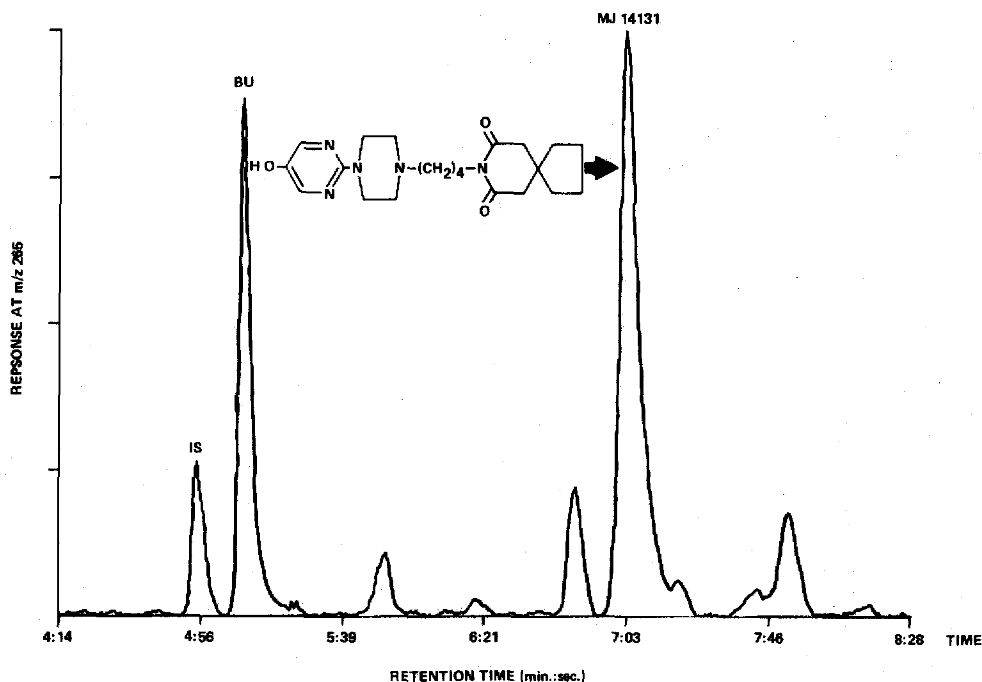

Fig. 6. Separation of a possible interfering metabolite (method 1). IS is 5-fluorobuspirone (1 ng injected), BU is buspirone (2 ng injected) and MJ 14131, 5-hydroxybuspirone, is the possible interfering metabolite with structure shown (2 ng injected). All compounds were unextracted standards.

TABLE II

ACCURACY AND PRECISION OF METHOD 1

Test*	n**	Concentration added (ng/ml)	Mean concentration found (ng/ml)		Coefficient of variation (%)	
			Buspirone	[$^2H_4$]Buspirone	Buspirone	[$^2H_4$]Buspirone
Intra-assay	14(1)	1.0	0.83	0.87	4.4	10
Intra-assay	14(1)	20	22	22	13	13
Inter-assay	9(1)	1.0	0.85	0.84	3.9	8.1
	9(1)	1.0	0.88	0.81	7.1	9.8
	9(1)	1.0	1.2	1.1	6.9	4.3
	27(3)***	1.0	0.97	0.91	17	15
Inter-assay	9(1)	20	21	20	13	13
	9(1)	20	23	23	11	10
	9(1)	20	21	21	7.7	7.6
	27(3)***	20	22	22	12	11
Instrumental	8(1)	1.0	1.2	1.1	3.2	7.6
Quality controls	50(25)	0.50	0.50	0.49	15	13
Quality controls	50(25)	3.0	2.8	2.8	9.8	11

*See text for a description of the tests.
**n is the number of data points over (x) days.
***Combination of the three days above.

TABLE III

ACCURACY AND PRECISION OF METHOD 2

Test*	n**	Concentration added (ng/ml)	Mean concentration found (ng/ml)	Coefficient of variation (%)
Intra-assay	10	0.10	0.089	7.8
	10	0.10	0.090	23
Intra-assay	10	4.0	4.6	6.2
	10	4.0	4.2	10
Inter-assay	6	0.10	0.099	9.7
	6	0.10	0.12	12
	6	0.10	0.090	9.1
	5	0.10	0.12	8.0
	5	0.10	0.094	10
	5	0.10	0.11	6.2
	33***	0.10	0.10	14
Inter-assay	8	4.0	4.5	13
	6	4.0	4.8	4.0
	6	4.0	4.6	5.7
	6	4.0	4.2	6.0
	5	4.0	4.3	5.1
	7	4.0	4.0	6.6
	38***	4.0	4.4	9.3

*See text for a description of the tests.
**n is the number of data points over one day.
***Combination of the six days above.

introduced by the instrument and part during sample preparation. The data indicate good integrity and reproducibility over a large number of samples and time period.

Method 1 has been used for the collection of data from clinical bioavailabili-

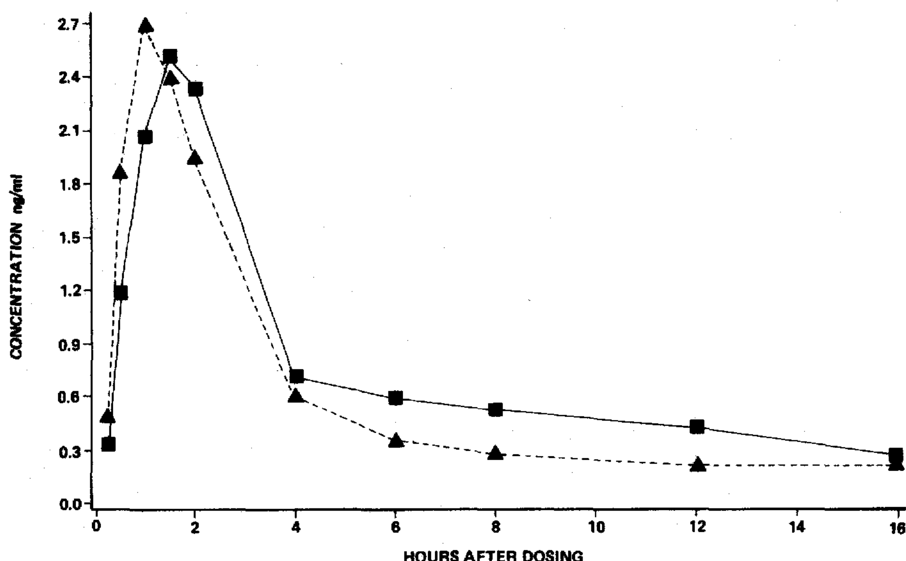

Fig. 7. Pharmacokinetic data from a bioavailability study in which [$^2H_4$]buspirone (▲) in a liquid solution and buspirone (■) in a tablet formulation were simultaneously administered and subsequently quantified by method 1.

ty studies in which tetradeuterated and unlabeled buspirone in separate formulations were simultaneously administered to test subjects. A typical example of the resulting pharmacokinetic data for one subject in one study is shown in Fig. 7. A detailed description of the study is the subject of a separate report [7].

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