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QUANTITATIVE ANALYSIS OF 1-(2-PYRIMIDINYL)PIPERAZINE IN PLASMA BY CAPILLARY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A sensitive method for detecting and quantifying 1-(2-pyrimidinyl)piperazine, an important metabolite of buspirone, in human plasma was developed and validated. The range of the method is 0.2—15 ng/ml. The analyte was removed from buffered, sodium chloride-saturated plasma with benzene, extracted into aqueous acid, washed with diethyl ether and reextracted into benzene. The processed extract was derivatized with pentafluorobenzoyl chloride. Instrumental analysis involved on-column injection into a fused-silica capillary gas chromatography column and detection by selected-ion monitoring mass spectrometry.

INTRODUCTION

1-(2-Pyrimidinyl)piperazine (1-PP) is an important metabolite of the new antianxiety drug buspirone [1]. Its structure is shown in Fig. 1. Following administration of 20 mg of buspirone, plasma concentrations of 1-PP reach a maximum of 17 ng/ml [2]. Therefore, a method for quantifying 1-PP in human plasma must have a subnanogram per milliliter detection limit to permit a useful working range. An unpublished plasma assay based on high-performance liquid chromatography (HPLC) with UV detection has a detection limit of 20 ng/ml. Caccia and co-workers [3, 4] reported a method, based on gas chro-

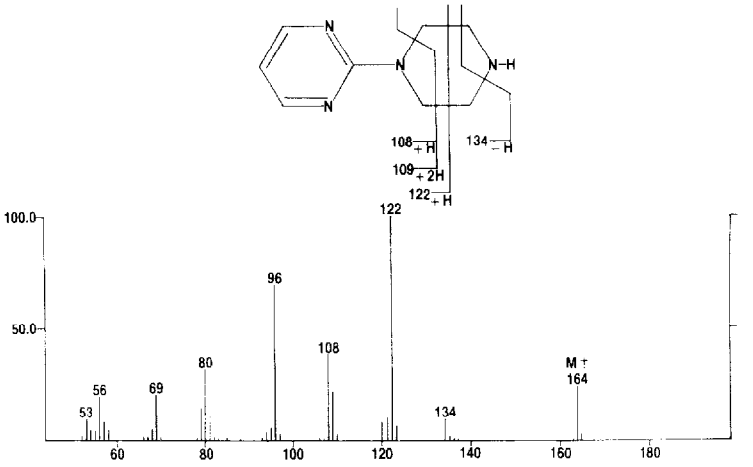

Fig. 1. Structure and electron-impact mass spectrum of 1-(2-pyrimidinyl)piperazine

matography (GC) with electron-capture detection of the heptafluorobutyryl derivative of 1-PP, which was capable of measuring concentrations higher than 50 ng/ml. Therefore, no previous method was capable of measuring concentrations in the required range.

A method for quantifying 1-PP in plasma with a lower limit of quantification of 0.2 ng/ml was developed and the conditions for the method are reported in this paper. The method relies on solvent extraction and clean-up followed by derivatization with pentafluorobenzoyl chloride (PFBOCl) and capillary GC with selected-ion monitoring mass spectrometric (SIM-MS) detection.

EXPERIMENTAL

Materials

The hydrochloride salt of 1-PP (see Fig. 1) and the hydrate hydrochloride salt of 1-(2-pyrimidinyl)piperazine-3,3,5,5-d_4 (d_4-1-PP) were prepared by Bristol-Myers (Evansville, IN, U.S.A.). Benzene and toluene were HPLC grade from Burdick & Jackson Labs. (Muskegon, IL, U.S.A.). Reagent-grade sodium chloride, hydrochloric acid, isopropanol and diethyl ether and methanol (HPLC grade) were from Fisher Scientific (Fairlawn, NJ, U.S.A.). Ethanol was absolute USP grade from U.S. Industrial Chemicals (Houston, TX, U.S.A.). Acetyl chloride and disodium hydrogen phosphate were reagent grade from Mallinkrodt (St. Louis, MO, U.S.A.). Pyridine was silylation grade from Pierce (Rockford, IL, U.S.A.). PFBOCl was 98% pure from Aldrich (Milwaukee, WI, U.S.A.). Deionized water was produced by a Sybron/Barnstead Nanopure II system (Boston, MA, U.S.A.).

Human plasma was obtained from Interstate Blood Bank (Memphis, TN, U.S.A.). The plasma was prepared by centrifugation of Na_2EDTA-treated blood and was stored at nominal −17°C until used.

Primary standards of 1-PP and d_4-1-PP were individually prepared at 100 000 ng/ml. Dilution of these produced working standards at 8—800 ng/ml for 1-PP

and 400 ng/ml for d_4-1-PP. All concentrations are in terms of the free base in ethanol.

Phosphate buffer (0.5 *M*) was prepared by dissolving 1 mol disodium hydrogen phosphate in about 1800 ml water, adjusting the pH to 10.5 with 6 *M* sodium hydroxide and diluting to 2 l. Hydrochloric acid reagent was prepared by diluting to 0.01 *M* with water. A solution of 6.5 m*M* hydrogen chloride in methanol was prepared by dissolving 93 μl of acetyl chloride in methanol and diluting to 200 ml.

Solvent extraction utilized a Roto-Torque rotator set at Hi-6 from Cole Parmer Instrument (Chicago, IL, U.S.A.). Mixing was performed with a standard vortex mixer. Solvent was evaporated with an N-Evap analytical evaporator from Organomation Assoc. (Northborough, MA, U.S.A.). Addition of organic solvents during sample preparation was done with a Lab Industries 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 and derivatizing reagent to extracts was accomplished with a 2.5-ml Hamilton syringe with repeating dispenser (Reno, NV, U.S.A.).

Disposable glass 5-ml pipettes from Corning (Corning, NY, U.S.A.) were used to measure plasma samples and to remove organic solvents during extraction. All extraction and clean-up steps were performed with new disposable 125 × 16 mm Pyrex culture tubes from Corning, and the final extracts were placed in 100 × 13 mm Pyrex culture tubes from Scientific Products. The tubes were capped with reusable PTFE-lined screw caps which were cleaned by soaking and rinsing in isopropanol.

Instrumentation

A Model 4500 gas chromatograph—mass spectrometer from Finnigan MAT (San Jose, CA, U.S.A.) equipped with a conversion dynode electron multiplier was used. The INCOS data system controlled the gas chromatograph and mass spectrometer, monitored selected ions, stored data and integrated areas of peaks on selected-ion chromatograms. The gas chromatograph was enhanced by the addition of an on-column injector from J. & W. Scientific (Rancho Cordova, CA, U.S.A.). It was mounted directly over the standard packed column inlet which was lined with a 6 mm O.D., 2 mm I.D. glass tube and heated to 260°C. Injection of 3 μl of sample over a 2-s period was accomplished using a Model 701RNFS 10-μl on-column syringe from Hamilton with the fused-silica needle extending about 5 cm into the heated injector space after insertion. Between injections the syringe was thoroughly rinsed with methanol which was pulled through the syringe barrel under vacuum and which bathed the outer surface of the needle.

The chromatographic column was an 8 m × 0.25 mm I.D. fused-silica capillary column coated with DB-5 at a film thickness of 0.25 μm from J. & W. Scientific. It was routed directly to the ion source. The velocity of the helium carrier gas was approx. 200 cm/s. The interface oven temperature (between gas chromatograph and mass spectrometer) was 260°C. The temperature of the gas chromatograph was held at 150°C for 1 min following injection; it was then increased at 20°C/min to 300°C.

The mass spectrometer was operated under electron-impact ionization conditions. For the 70-eV full-scan mass spectra of the compounds contained in this report the tuning of the mass spectrometer was standardized by analyzing the mass spectral quality-control compound decafluorotriphenylphosphine according to the method of Eichelberger et al. [5] and achieving the published spectral criteria.

For SIM recordings the electron energy was set at 35 eV. The lens and offset voltages and the ion source magnet position were tuned for maximum response at the 352 a.m.u. ion of perfluorotributylamine while maintaining good ion peak shape and approximately unit resolution with the 353 a.m.u. ion. For sample analyte determination two selected masses were sequentially and repetitively monitored, each for 0.21 s over an 0.25 a.m.u. window, at the mass peak apex. The slected masses were 358 for 1-PP and 362 for d_4-1-PP. The electron multiplier voltage was set at approx. 1400 V. Quantification was based on the area under the chromatographic peak on the appropriate background-subtracted selected-ion chromatogram. A linear regression of ln(concentration) versus ln(area 1-PP/area d_4-1-PP) was used to quantify unknowns.

Procedures

Plasma samples were stored at nominal −17°C until the day of extraction. They were thawed at room temperature and individually mixed. A 2-ml aliquot of each sample was pipetted into a labeled 125 × 16 mm extraction tube.

A set of standards was prepared daily by adding 50 μl of an appropriate ethanolic working standard to blank plasma. This resulted in standards of 0.2, 0.5, 2.0, 5.0 and 15 ng/ml. Each standard was prepared in duplicate. Validation test samples, for characterization of the accuracy and precision of the method, were prepared at 1.0 and 12.5 ng/ml and the concentrations were unknown to the gas chromatograph—mass spectrometer operator. Blank plasma samples were also processed at the same time.

The following extraction process was performed on each tube. Internal standard was added as 50 μl of a solution of 400 ng/ml d_4-1-PP. Approx. 1 g of sodium chloride was added and the sample was mixed. Phosphate buffer (1 ml) was added, followed immediately by mixing. After buffer had been added to all the tubes they were all mixed again. Benzene (6 ml) was added and the tubes were rotated on the Roto-Torque rotator for 10 min. The layers were separated by centrifugation at 600 *g* for 10 min in this step and subsequent centrifugation steps. The organic layer was removed by pipette and placed in a clean 125 × 16 mm tube. The aqueous layer was mixed and reextracted with benzene. The organic layer was separated, removed and combined with the first extract.

The combined benzene layers were extracted by adding 2 ml of 0.01 *M* hydrochloric acid and rotating for 10 min. The layers were separated by centrifugation and the organic layer was removed and discarded by aspiration. To the aqueous layer were added 10 ml of diethyl ether and the tubes were rotated for 10 min. The layers were separated by centrifugation and freezing in a bath of dry ice and isopropanol. The ether layer was decanted and discarded and the aqueous layer was thawed. The aqueous layer was reextracted with diethyl ether and the ether layer was separated and discarded as above. To the thawed

aqueous layer was added approx. 1 g of sodium chloride followed by mixing. Phosphate buffer (1 ml) was added and the solution was mixed. Benzene (6 ml) was added and the tubes were rotated for 10 min. The layers were separated by centrifugation. The organic layer was removed with a pipette, placed in a 100 × 13 mm tube. To the organic extract were added 50 μl of 6.5 m*M* hydrogen chloride in methanol to prohibit volatilization of the analytes. After a brief mixing the solvent was evaporated at 35—45°C under a stream of nitrogen. The wall of the tube was rinsed by adding 0.5 ml of methanol and mixing followed by evaporation of the solvent as above. After adding 100 μl of methanol and briefly mixing, the tubes were stored overnight at nominal —17°C. The next morning the samples were warmed to room temperature, mixed, and the solvent was evaporated.

Derivatization of the samples was accomplished by first adding 50 μl of PFBOCl in toluene (1:5, v/v) to the dried tube with a 2.5-ml syringe and repeating dispenser. Then 0.5 μl of pyridine were added with a 10-μl syringe, the tubes were mixed and the tubes were heated for 10 min at 65°C. After cooling of the samples, 3 μl were injected into the GC—MS system.

The accuracy and precision of the method were characterized by several tests. Intra-assay variability (within-day) was studied by analyzing twelve identical spiked plasma samples at two concentration levels on one day. Inter-assay variability (between-day) was studied by analyzing six identical spiked plasma samples at two concentration levels on three days. The limit of detection was assessed by analyzing ten plasma samples from ten different human subjects which were spiked at 0.05 ng/ml and the same unique ten blank plasma samples on one day for comparison of responses. The recovery of 1-PP from the extraction was assessed by analyzing six extracted spiked plasma samples at two concentration levels (20 and 1 ng/ml) and comparing these to six unextracted standards which were only placed in 100 × 13 mm tubes and submitted to derivatization and instrumental analysis along with the extracted samples. The linearity of the method was studied by analyzing 22 spiked plasma samples over a range of 0.05—25 ng/ml.

A brief study was performed to evaluate the kinetics of the derivatization reaction. 1-PP (20 ng) was placed in each of three tubes and residual solvent was removed under nitrogen. A 50-μl volume of 1:5 (v/v) PFBOCl was placed in each tube. One tube stood at 24°C, one was incubated at 65°C and one, to which were added 0.5 μl of pyridine, stood at 24°C. At specific time points after the addition of reagents an aliquot was withdrawn and injected into the GC—MS system.

RESULTS AND DISCUSSION

The extraction procedure was based on the work of Caccia et al. [3] and the salting out modification of Garattini [2]. In order to achieve the lower limit of detection reported in this paper, additional extraction steps were added to remove interfering sample compounds, the derivatization method was changed and instrumentation was altered from previously published procedures.

Underivatized 1-PP produces a broad tailing GC peak, but derivatization

with PFBOCl yields a sharp peak which elutes rapidly from the GC column despite its greatly increased mass. Another advantage of this derivative is that it has a high molecular ion mass in a region of the GC elution profile where most compounds possess a much smaller mass. This results in chromatograms at the monitored selected ions of 358 and 362 a.m.u., which are relatively free from sample interference and noise. The structure and spectra of the derivatives of 1-PP and d_4-1-PP are shown in Figs. 2 and 3. Assignment of fragment identities was based on comparison to other deuterated analogues and is not intended to indicate the results of an exhaustive fragmentation

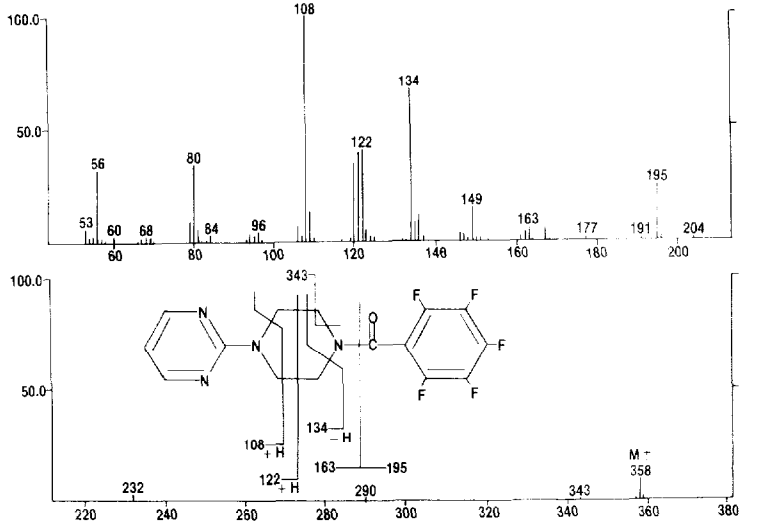

Fig. 2. Structure and electron-impact mass spectrum of 1-pentafluorobenzoyl-4-(2-pyrimidinyl)piperazine.

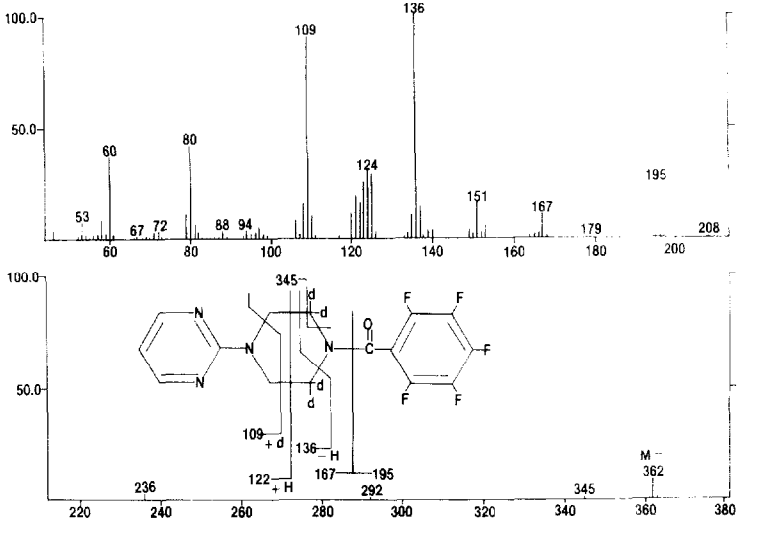

Fig. 3. Structure and electron-impact mass spectrum of 1-pentafluorobenzoyl-4-(2-pyrimidinyl)piperazine-2,2,6,6,-d_4.

mechanism study. Most of the fragmentation occurs within the piperazine ring.

The mass spectrum of the underivatized d_4-1-PP is not shown in a figure. It contained the following major ions (m/z) at the following relative abundances (between parentheses): 168 (20%), 135 (5%), 122 (100%), 110 (15%), 109 (15%), 108 (20%), 98 (50%), 80 (25%), 72 (15%) and 60 (10%).

Early work, in which derivatizations were performed with 1:5 (v/v) PFBOCl in toluene only, indicated that derivatization was incomplete. Pyridine was chosen as an acid scavenger to liberate the free base from the hydrochloride. A brief study was performed to assess the kinetics of the derivative appearance. The results are presented in Fig. 4. Clearly, pyridine has a marked enhancement effect and derivatization is probably complete within minutes at 24°C. The derivatives were demonstrated to be stable for at least six days at nominal −17°C.

Typical chromatograms of spiked and blank plasma extracts resulting from this method are presented in Fig. 5. When a large number of samples over a wide range of concentrations were analyzed, a small increase in the baseline was produced by the blank at the retention time of the 1-PP derivative. This never amounted to greater than one quarter of the response of the 0.2 ng/ml spiked standard and appeared to result from instrumental cross-contamination.

The recovery of the method was assessed by comparing the mean response

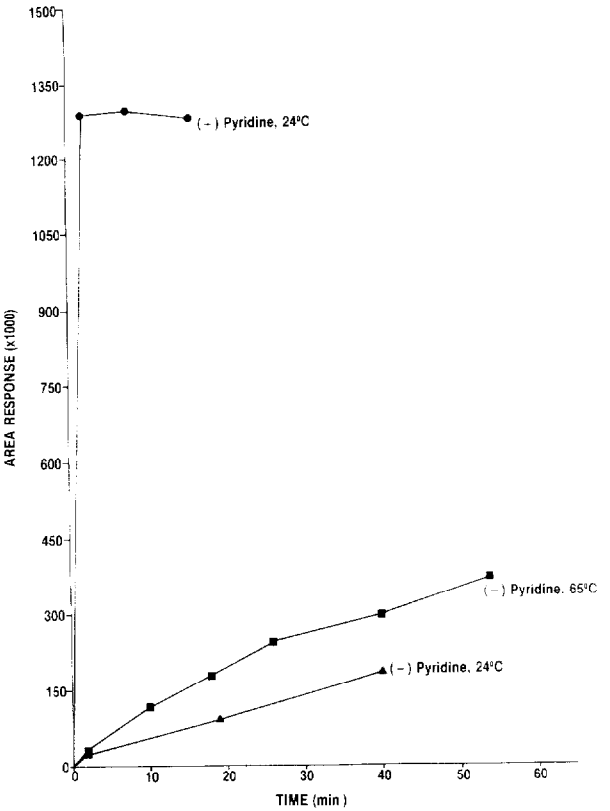

Fig. 4. Appearance of 1-pentafluorobenzoyl-4-(2-pyrimidinyl)piperazine under different reaction conditions.

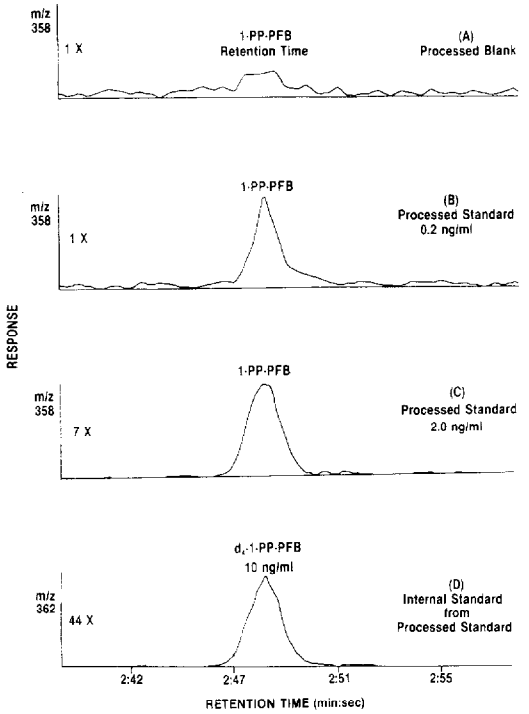

Fig. 5. Selected-ion chromatograms of plasma extracts. (A) Processed blank spiked with 10 ng/ml internal standard only (not shown). (B) Processed standard spiked with 0.2 ng/ml 1-PP and 10 ng/ml internal standard (not shown). (C) Processed standard spiked with 2.0 ng/ml 1-PP and 10 ng/ml internal standard which is shown in D. 1-PP-PFB is the derivative of 1-PP and d_4-1-PP-PFB is the derivative of d_4-1-PP.

for six identical extracted samples versus six identical unextracted samples. At 20 ng/ml, 78% of the 1-PP was recovered and at 1 ng/ml, 59% of the 1-PP was recovered when small volume losses during extraction and clean-up steps are considered.

The limit of detection of 1-PP by the method was assessed statistically by a paired *t*-test of the blank and spiked (0.05 ng/ml 1-PP) unique plasma samples from ten individuals. The probability of the observed difference occurring by chance alone was less than 1.5%. The regular working lower limit of quantitation was chosen as 0.2 ng/ml in order to stay safely within the validated limit of detection and the observed blank responses discussed above but not observed in this study.

The linearity of the method was studied over the range of 0.05–25 ng/ml. A lack-of-fit statistical analysis was performed on the data. A standard line based on area ratio (area 1-PP/area d_4-1-PP) versus concentration was significantly non-linear; however, a standard line based on ln (area ratio) versus ln (concentration) was linear. It had an *F* ratio (mean square lack-of-fit/mean square pure error) [6] of 3.63 with a significance of 0.0240. The equation of the line was ln (area ratio) = 1.234 ln (1-PP concentration) − 2.855. This method of calculating the standard line was chosen for unknown sample quantification.

Data concerning the accuracy and precision of the method are shown in

TABLE I

ACCURACY AND PRECISION OF THE METHOD

Test*	n**	Concentration added (ng/ml)	Mean concentration observed (ng/ml)	Coefficient of variation (%)
Intra-assay	11***	1.0	0.94	12
Intra-assay	12	12.5	14.1	2.5
Inter-assay	6	1.0	1.0	13
	6	1.0	0.91	4.7
	6	1.0	0.90	4.2
	29§	1.0	0.92	8.3
Inter-assay	6	12.5	14.1	3.0
	6	12.5	13.4	0.90
	6	12.5	14.1	1.9
	30§	12.5	13.9	2.7

*For a description of the tests, see under *Procedures*.
**n is the number of data points.
***One data point was rejected because it was 2.5 S.D. from the mean of all twelve data points.
§Combination of data points over three days.

Table I. The tests were discussed under *Procedures*. The accuracy and precision of the method was within 13% of nominal values.

A set of plasma samples were spiked at 1.0 and 12.5 ng/ml and were extracted, cleaned up and stored in methanol at a nominal temperature of −17°C for six days. They produced no significantly different responses than identical samples which were stored overnight at −17°C.

REFERENCES

1 S. Garattini, S. Caccia and T. Mennini, J. Clin. Psychiatry, 43 (1982) 19.
2 S. Garattini, personal communication.
3 S. Caccia, S. Garattini, A. Mancinelli and M. Muglia, J. Chromatogr., 252 (1982) 310.
4 S. Caccia, A. Notarnicola, M.H. Fong and E. Benfenati, J. Chromatogr., 283 (1984) 211.
5 J.W. Eichelberger, L.E. Harris and W.L. Budde, Anal. Chem., 47 (1975) 995.
6 W.J. Dixon and F.J. Massey, Introduction to Statistical Analysis, McGraw Hill, New York, 3rd ed., 1969, p. 200.