Journal of Chromatography, 421 (1987) 281–289
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3835

DETERMINATION OF 6-(2′-CHLOROPHENYL)-4-HYDROXY-4*H*-IMIDAZO[1,5-*a*][1,4]BENZODIAZEPINE-3-CARBOXAMIDE, A MIXED AGONIST–ANTAGONIST ANXIOLYTIC AGENT, IN HUMAN PLASMA AND URINE BY GAS CHROMATOGRAPHY–NEGATIVE-ION CHEMICAL-IONIZATION MASS SPECTROMETRY

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(First received March 24th, 1987; revised manuscript received June 22nd, 1987)

SUMMARY

A simple, sensitive and specific assay was developed for the determination in plasma and urine of 6-(2′-chlorophenyl)-4-hydroxy-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxamide, compound I, a mixed agonist–antagonist anxiolytic agent. A hexadeuterated analogue of the compound was added to plasma or urine as the reference standard. The titled compound was extracted with benzene at pH 11. Following evaporation of the solvent, the residue was reacted with pentafluoropropionic anhydride in the presence of triethylamine. The derivatizing reagents were evaporated, and the carbonitrile derivative of the analyte was extracted into ethyl acetate at pH 11. The residue remaining after removal of the ethyl acetate was silylated with bis(trimethylsilyl) trifluoroacetamide, and a portion of this solution was analyzed by gas chromatography–negative-ion chemical-ionization mass spectrometry. The mass spectrometer was set to monitor, in the gas chromatographic effluent, the M^- ion of the titled compound and its hexadeuterated reference standard. The ratio of these two ions was calculated and converted to a concentration of analyte using a calibration curve that was generated from the analyses of control plasma fortified with various amounts of analyte and a fixed amount of the hexadeuterated reference standard. The limit of quantitation of the assay was 1 ng/ml for plasma and urine.

INTRODUCTION

6-(2′-Chlorophenyl)-4-hydroxy-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxamide, compound I (Fig. 1), is an imidazobenzodiazepine derivative that shows mixed agonist–antagonist anxiolytic activity in pharmacological tests [1]. A reported high-performance liquid chromatographic assay for the determination

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0378-4347/87/$03.50

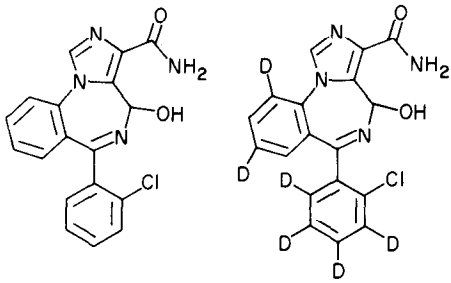

Fig. 1. Structures of I (left) and II (right).

of I in plasma [2] has inadequate sensitivity to evaluate the pharmacokinetic profile of the drug in man at the projected therapeutic dose of 1–5 mg. For this reason, a simple, sensitive and specific gas chromatographic–negative-ion chemical-ionization mass spectrometric (GC–NICIMS) method was developed. The assay uses a hexadeuterated analogue of compound I, compound II (Fig. 1), as the reference standard.

EXPERIMENTAL

Gas chromatography (GC)

The gas chromatograph was a Finnigan (Sunnyvale, CA, U.S.A.) Model 9500 equipped with a 1.9 m × 2 mm I.D. glass column packed with 3% OV-1 on 100–120 mesh Gas Chrom Q (Applied Science Lab., Deerfield, IL, U.S.A.). The column was conditioned overnight at 250°C with methane as the carrier gas at a flow-rate of 10 ml/min. Methane (99% plus, Liquid Carbonic, Chicago, IL, U.S.A.) was used both as the carrier gas (1.7 kg/m^2) and the chemical ionization (CI) reagent gas. The temperature of the oven, injector and transfer lines were 265, 325 and 260°C, respectively. The retention time of compound I was approximately 95 s.

Mass spectrometry (MS)

Mass spectra were obtained using a Nermag (Fairfield, NJ, U.S.A.) R1010C quadrupole mass spectrometer with a Nermag Sidar V2.3 data system. Selected-ion monitoring (SIM) measurements were made with a Finnigan Model 3200 quadrupole mass spectrometer equipped with a Finnigan Promim® peak monitor. Both mass spectrometers were coupled to the gas chromatograph using a divertable direct transfer line. The mass spectrometer was modified for the detection of negative ions as previously described [3], and was tuned to give the maximum response consistent with unit resolution and acceptable peak shape. Methane, at an ion source pressure of 67 Pa, was used as the CI reagent gas. The electron multiplier and conversion dynode were operated at voltages of −2.0 and +2.8 kV, respectively. The Promim peak monitor was set to monitor the ion current at *m/z* 406 and *m/z* 412 in the GC effluent. Each Promim channel was operated at 100 ms dwell time, 0.5 Hz frequency response and 10^{-8} A/V sensitivity. Samples were injected using an automatic liquid sampler under the control of QSIMPS,

Quantitative Selective Ion Monitoring Processing System, which also collected and processed the SIM data [4].

Glassware

Culture tubes (Pyrex 9825, 16 ml) equipped with Teflon®-lined screw caps were used for plasma extraction. Conical centrifuge tubes (Pyrex 8061, 5 ml) were used for the evaporation of the organic extracts. All the tubes were purchased from Ace Scientific (East Brunswick, NJ, U.S.A.). Prior to use, the glassware was treated with PRO SIL28® (PCR, Gainesville, FL, U.S.A.) and finally rinsed with methanol and dichloromethane prior to use.

The autosampler vials (Model 10201) were purchased from Chrompack (Raritan, NJ, U.S.A.) as were the removable glass inserts (Model 10381). The Teflon-lined septa with aluminum caps were purchased from Wheaton Scientific (Millville, NJ, U.S.A.). Solvents were removed at 45°C using a nitrogen evaporator (N-Evap®, Organomation Assoc., South Berlin, MA, U.S.A.). Extractions were done by shaking (60 strokes/min) on a variable-speed reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.). Centrifugations were carried out on a Damon/IEC Model CRU-500 refrigerated centrifuge (Fisher Scientific, Springfield, NJ, U.S.A.) operated at 10°C and 1500 g using a No. 253 rotor.

Chemical and reagents

Compound I, was obtained from Dr. A. Walser, Chemical Research, Hoffmann-La Roche. Compound II was synthesized by Dr. Y.Y. Liu, Isotope Synthesis Group, Hoffmann-La Roche.

Nanograde benzene, methanol, ethyl acetate, heptane, chloroform (without ethanol) and N,N-dimethylformamide (Burdick and Jackson Labs., Muskegon, MI, U.S.A.), Regisil® brand of bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Regis, Morton Grove, IL, U.S.A.) and dibasic sodium phosphate (J.T. Baker, Philadelphia, PA, U.S.A.) were used in sample preparation. The triethylamine (TEA) was from Aldrich (Milwaukee, WI, U.S.A.), and the pentafluoropropionic anhydride (PFPA) was from Pierce (Rockford, IL, U.S.A.).

Collection device

The plasma collection device experiments were performed using human blood as follows. A volume of 30 ml of blood was collected in a 10-ml heparinized vacutainer (Becton-Dickinson, Rutherford, NJ, U.S.A., Cat. No. 6527). The blood was pooled and fortified to give a concentration of 38 ng/ml. Aliquots (5 ml) of the fortified blood were transferred to three vacutainers and three siliconized culture tubes. All the tubes were shaken gently for 30 min and aliquots of 1 ml of sample from each tube were fortified with reference standard and analyzed. The remaining fortified blood was centrifuged to separate the plasma. Plasma (1 ml) from each tube was analyzed the same way as the blood using a separate plasma calibration curve.

Solutions

The analyte stock solution, 1.00 mg/ml compound I, was prepared by dissolving 10 mg of compound I in 10 ml of N,N-dimethylformamide. Aliquots of this stock

solution were diluted with methanol to prepare analyte standard solutions A–E containing 200, 100, 20, 4.0 and 1.0 ng of compound I, respectively, per 50 μl of solution. The reference stock solution (1.00 mg/ml) was prepared by dissolving 10 mg of II in 10 ml of N,N-dimethylformamide. This solution was diluted with methanol to a final concentration of 40 ng per 50 μl (solution F).

A 5% BSTFA solution was prepared by diluting 0.5 ml of BSTFA with 9.5 ml of heptane–ethyl acetate (7 : 3). This solution was prepared immediately prior to use. A 1 *M* sodium phosphate solution was prepared by dissolving 142 g of Na_2HPO_4 in 1000 ml of distilled water. A 5 *M* sodium hydroxide solution was prepared by dissolving 50 g of sodium hydroxide in 250 ml of distilled water. A 1 *M* phosphate buffer, pH 11 solution, was prepared by adjusting the 1 *M* phosphate solution to pH 11 with 5 *M* sodium hydroxide solution. A heptane–ethyl acetate (7 : 3) solution was prepared by diluting 300 ml of ethyl acetate with 700 ml of heptane. A 2% TEA solution was prepared by diluting 0.1 of TEA with 4.9 ml of chloroform (without ethanol). A 5% PFPA solution was prepared by diluting 0.25 ml of PFPA with 4.75 ml of chloroform (without ethanol). These last two solutions were prepared immediately prior to use.

Analytical procedure

The internal calibration samples were prepared in duplicate by transferring 50 μl of one of the analyte standard solutions A–E and 50 μl of solution F into separate culture tubes. A 1-ml volume of analyte-free control plasma or urine was then added to each tube.

For the quality assurance and experimental samples, 50 μl of the reference standard solution F were added to the culture tube followed by the addition of 1 ml of the plasma or urine sample.

A 0.5-ml volume of the 1 *M*, pH 11 sodium phosphate buffer was added to all the tubes, and each rack of the tube was briefly shaken to help mixing. Benzene (6 ml) was added to each of the tubes, and the tubes were tightly capped with Teflon-lined screw caps.

The samples were then extracted by gentle shaking for 20 min followed by centrifugation at 10°C and 1500 *g* for 10 min. Aliquots (5 ml) of the benzene extracts were transferred to 5-ml conical centrifuge tubes and then evaporated to dryness. A 25-μl volume of the 2% TEA solution was added, and the tube was vortexed. Then, 25 μl of the 5% PFPA solution were added, and the tube was vortexed. The solution was allowed to stand at room temperature for 20 min and then evaporated to dryness. Ethyl acetate (200 μl) was added and the tubes were vortexed. Phosphate buffer (100 μl, 1 *M*, pH 11) was added and the tubes were vortexed and allowed to stand at room temperature for 10 min. After centrifugation for 3 min, 180 μl of the ethyl acetate were transferred to another 5-ml conical centrifuge tube. The solution was evaporated to dryness, and 80 μl of the 5% BSTFA solution were added. The tubes were capped and heated to 65–70°C for 60 min. The solutions were cooled and then transferred to 1-ml autosampler vials with 100-μl conical inserts which were sealed with aluminum caps using a hand crimper. The samples were injected into the gas chromatography under

control of QSIMPS. At 45 s after each injection the GC divert valve was turned off to allow the chromatographic effluent to enter the ion source.

The ion ratio of *m/z* 406 (analyte) to *m/z* 412 (hexadeuterated reference standard) in the samples was determined by NICIMS. The concentration of I in experimental samples was calculated from the slope and intercept parameters from a calibration curve ($y=mx+b$) determined using linear regression with a weighting of y^{-1}, where x represents the analyte concentration of the calibration sample and y represents the ion ratio. All processing of the data is automatically done by QSIMPS.

Stability experiments

Bench-top stability was determined by comparing the concentrations of pooled experimental samples allowed to stand at room temperature for 0, 3 and 6 h. The long-term stability at −20°C was determined by duplicate determinations of the same quality assurance sample over period of twenty days (plasma and urine).

Clinical samples

Plasma and urine samples from one healthy male volunteer administered a 1-mg oral dose of I were analyzed using the GC–NICIMS method. Experimental samples were collected in vacutainers (Becton-Dickinson Model 6527) containing sodium heparin as the anticoagulant. The vacutainers for the control plasma samples contained either heparin or EDTA as the anticoagulant.

RESULTS AND DISCUSSION

Compounds I and II could not be chromatographed without derivatization because of their high polarity. The first derivatization step, the conversion of the amide group to a cyano function, has been previously used in this laboratory to derivatize a benzodiazepine structurally similar to I [5]. Surprisingly, this procedure when applied to I did not acetylate the hydroxy at position 4, although it did lead to nitrile formation.

Fig. 2 shows the methane NICI mass spectra of derivatized I and II. Intense molecular anions can be observed at *m/z* 406 and *m/z* 412 for I and II, respectively. If the ion source pressure is too low, the major ion is $[M-90]^-$. Fig. 3 shows the typical selected-ion current profile from the analysis of 1 ml of analyte-free control plasma fortified with 1 ng of I, a sample from a subject pre-dose and a sample from a subject 8 h after administration of I. The limit of quantitation for the assay is 1 ng/ml for plasma or urine.

Based on the calibration data, the overall inter-assay precision in the concentration range 1.0–200 ng/ml was 3.7 and 3.3% for plasma and urine, respectively (Table I). The mean correlation coefficients for the calibration curves were 0.99 for plasma and 0.99 for urine. The overall intra-assay precision (relative standard deviation of the mean ratio of the duplicate analyses) was 11% for both plasma and urine.

A quality assurance sample was prepared by separately pooling plasma and urine samples from subjects given I. The quality assurance sample was analyzed

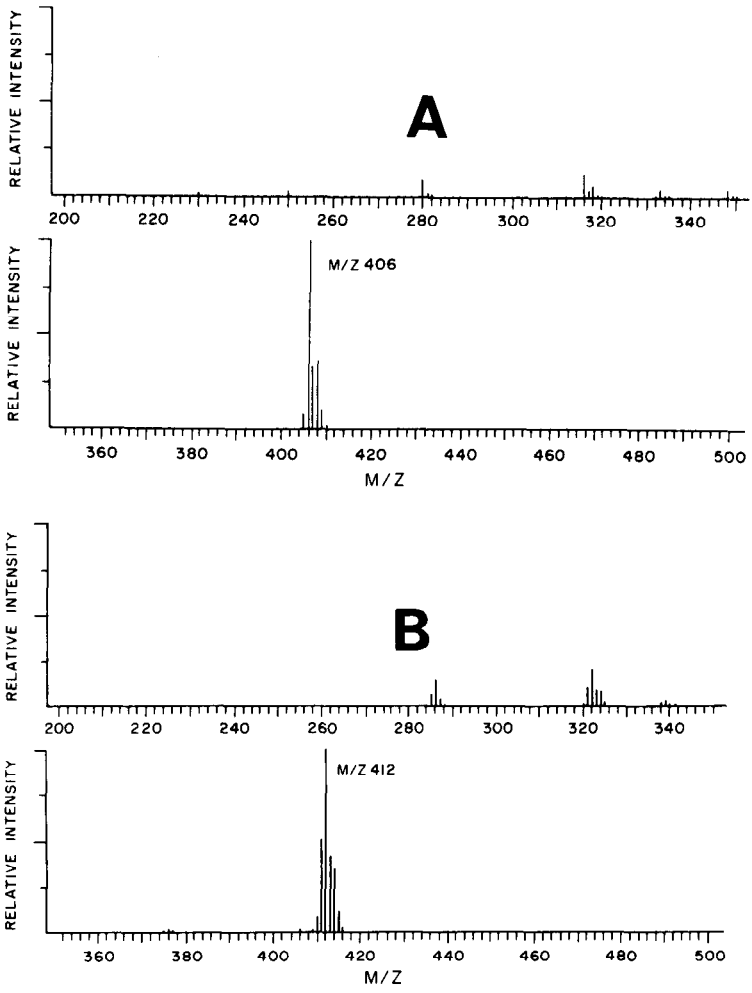

Fig. 2. Methane NICI mass spectra of compounds I (A) and II (B).

in duplicate along with a series of experimental samples. The mean concentration obtained was 26.0 ± 0.6 ng/ml ($n=6$) for the plasma pool and 23.3 ± 0.5 ng/ml ($n=6$) for the urine pool.

The plasma concentration–time curve of I for one male volunteer after administration of a 1-mg dose of the drug is shown in Fig. 4. For this subject the half-life was about 23 h and the volume of distribution was about 60 l. The amount of drug excreted in urine for this subject was 2.6% of the dose.

The measured concentration of I in the blood sample exposed to the vacutainer (38 ± 0.5 ng/ml) was identical to that exposed to the glass container (39 ± 0.5 ng/ml) suggesting that the use of the Model 6527 vacutainer will not bias the analytical results. The concentrations of I in the plasma obtained from the blood were 48 ± 1 ng/ml (vacutainers) and 45 ± 1 ng/ml (glass). Based on the data from the vacutainers experiments, and a hematocrit of 0.52, the red blood cell/plasma ratio for I is 0.6 [6].

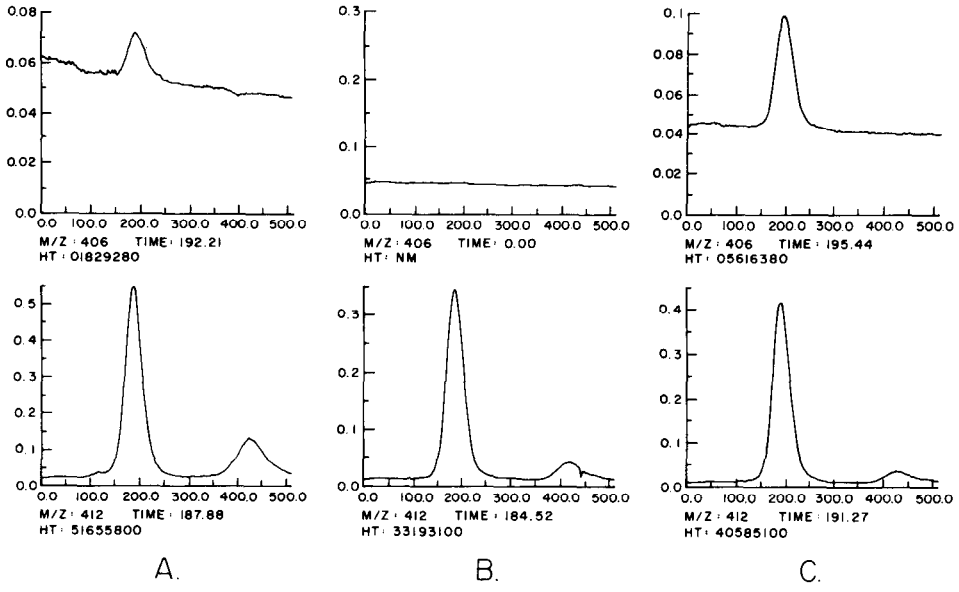

Fig. 3. Selected-ion current profiles from the analysis of plasma from drug-free plasma fortified with 1 ng of compound I (A), a subject pre-dose (B) and plasma from a subject 8 h after administration of 1-mg dose of drug (C). The concentration of I in this last sample was 4 ng/ml. The ordinate is in volts and the abscissa is in scan numbers.

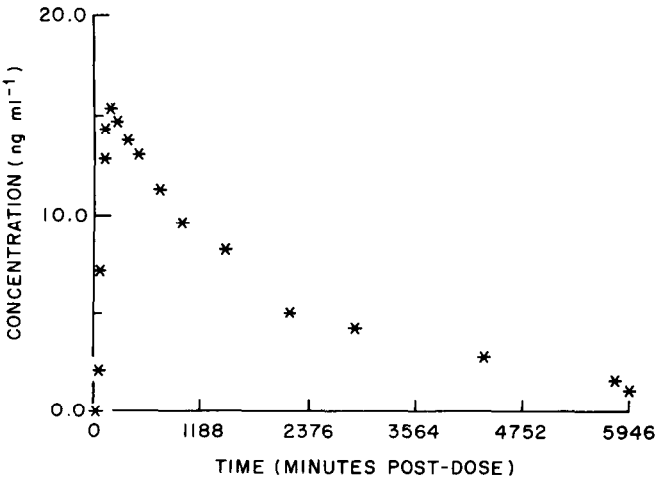

Fig. 4. Plasma concentration–time profile for one individual following administration of 1 mg of compound I.

The average recovery was determined to be 71% for both plasma and urine.

The results of bench-top and long-term stability experiments suggest that I is stable under the stated conditions of temperature and time (Tables II and III). Results of stability studies with I in urine are similar.

To summarize, a simple and relatively rapid GC–NICIMS method has been developed to quantitate a new anxiolytic in human plasma and urine. To date

TABLE I

INTER-ASSAY PRECISION FROM A CONSIDERATION OF THE ASSAY OF THE PLASMA CALIBRATION STANDARDS

All samples were analyzed at regular intervals over a 30-day period. Data from urine calibration standards are comparable. $n = 11$.

Added (ng/ml)	Found (mean ± S.D.) (ng/ml)	Relative standard deviation (%)
200.0	198.9 ± 1.8	0.9
100.0	100.4 ± 1.5	1.5
20.0	20.9 ± 0.8	4.0
4.0	4.3 ± 0.2	5.3
1.0	0.9 ± 0.06	7.0
Overall relative standard deviation		3.7

TABLE II

BENCH-TOP STABILITY OF I IN HUMAN PLASMA

Time (h)	Concentration found (mean ± S.D., $n = 6$) (ng/ml)	Relative standard deviation (%)
0	98.8 ± 2.6	2.7
3	94.4 ± 2.0	2.1
6	100.5 ± 2.5	2.5

TABLE III

LONG-TERM STABILITY OF I IN HUMAN PLASMA

Date	Concentration found (ng/ml)	(Found − mean)/mean · 100 (%)
1/ 8/86	35.83	2.78
1/17/86	35.06	0.56
1/18/86	36.34	4.23
1/17/86	35.27	1.17
1/21/86	34.78	− 0.24
1/21/86	34.73	− 0.37
1/22/86	31.25	−10.33
1/22/86	35.50	1.83
1/22/86	35.55	1.96
1/23/86	35.61	2.16
1/28/86	33.55	− 3.75
Mean	34.86	2.67
S.D. ($n = 11$)	1.39	2.85
R.S.D.* (%)	4.00	

*Relative standard deviation.

over 5000 plasma or urine samples have been analyzed using the method. The method can measure the anxiolytic agent in human plasma over several drug half-lives. Very small amounts of the drug are excreted unchanged in the urine.

ACKNOWLEDGEMENT

The authors wish to thank Ms. Patricia Seymore for her secretarial assistance in the preparation of this report.

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