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DETERMINATION OF IMIDAZOBENZODIAZEPINE-3-CARBOXAMIDE, A NEW ANXIOLYTIC AGENT, IN HUMAN PLASMA BY GAS CHROMATOGRAPHY—NEGATIVE CHEMICAL-IONIZATION MASS SPECTROMETRY

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SUMMARY

A method is described for measuring imidazobenzodiazepine-3-carboxamide, a new anxiolytic agent, in human plasma. A tetradeuterated analogue of the analyte is used as the internal standard. The drug and its internal standard are (1) extracted from plasma at pH 9 with benzene containing 20% 1,2-dichloroethane, (2) derivatized with pentafluoropropionic anhydride in the presence of triethylamine and (3) the nitrile derivative of the analyte and internal standard are analyzed by gas chromatography (GC)—negative chemical-ionization mass spectrometry (CIMS) using methane as both GC carrier gas and CI reagent gas. The mass spectrometer is set to monitor the intense $(M—HCl)^-$ ions of imidazobenzodiazepine-3-nitrile and its tetradeuterated analogue at *m/z* 316 and *m/z* 320, respectively. Quantitation of an experimental plasma sample is based on the comparison of the *m/z* 316 to *m/z* 320 ion ratio in each sample to that obtained from the analyses of control plasma spiked with various amounts of the drug and a fixed amount of internal standard. The limit of quantitation of the method is approximately 100 pg ml^{-1} of plasma and the precision (relative standard deviation) at a plasma concentration of 1 ng ml^{-1} is 4%.

INTRODUCTION

Imidazobenzodiazepine-3-carboxamide, 8-chloro-6-(2′-chlorophenyl)-4H-imidazo[1,4]benzodiazepine-3-carboxamide [1], is currently undergoing testing as an anxiolytic agent. The compound is significantly more potent than diazepam in animal tests used to detect anxiolytic properties [2].

A published high-performance liquid chromatographic procedure [3] for imidazobenzodiazepine-3-carboxamide with a limit of quantitation of 50 ng/ml is too insensitive to measure the less than 2 ng/ml concentrations of imidazo-

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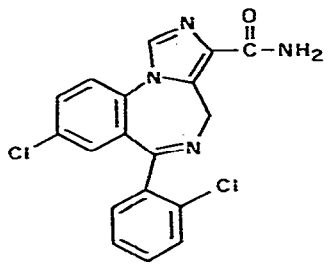
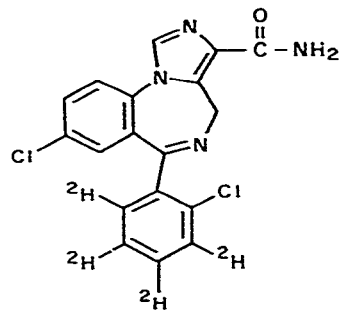


Imidazobenzodiazepine-3-carboxamide



Tetradeuterated imidazobenzodiazepine-3-carboxamide

benzodiazepine-3-carboxamide found in human plasma following a therapeutic 0.2-mg oral dose of the drug.

This paper reports a sensitive, specific and relatively simple gas chromatographic (GC)—negative chemical-ionization mass spectrometric (CIMS) procedure for imidazobenzodiazepine-3-carboxamide. The method is based on the conversion of imidazobenzodiazepine-3-carboxamide to a more easily chromatographed nitrile derivative. Chromatographic peak shape is improved by using a WSCOT capillary GC column. The assay features the use of a tetradeuterated analogue of imidazobenzodiazepine-3-carboxamide as internal standard.

EXPERIMENTAL

Equipment and operating conditions

Gas chromatograph. A Finnigan Model 9500 gas chromatograph was equipped with a WSCOT CP™ SIL 8 capillary column (24.3 m × 0.48 mm I.D.) obtained from Chrompack, Santa Fe Springs, CA, U.S.A. Methane (0.2 kg m^{-2}) was used as carrier gas. The injector, column, interface oven and transfer line were operated at 325, 320, 250 and 250°C, respectively. The retention time of derivatized imidazobenzodiazepine-3-carboxamide was 115 sec. Prior to use, the column was conditioned overnight at 300°C with a 2 ml min^{-1} flow of nitrogen and then by several daily injections of the reconstituted residue from the ethyl acetate extract of drug free plasma.

Mass spectrometer. A Finnigan Model 3200 quadrupole mass spectrometer was adjusted to give the maximum response consistent with reasonable ion peak shape and unit resolution. The modifications to the instrument to permit the detection of negative ions have been described [4]. Methane, at an ion source pressure of 67 Pa, was used as reagent gas. To avoid "ghosting", the MS tuning and GC conditions were optimized using the response from the injection of μg amounts of 8-deschloro-imidazobenzodiazepine-3-carboxamide into the GC—MS system. The voltage across the continuous dynode electron multiplier was —2.0 kV and the conversion dynode was operated at +2.5 kV.

Peak monitor. A Finnigan Promim® with a Rikadenki recorder was used to set the mass spectrometer to monitor *m/z* 316 and *m/z* 320 in the GC effluent. Each Promim channel was operated at 100-msec dwell time, a 0.5-Hz frequency response and a gain of 10^{-8} A/V.

Glassware. Culture tubes (16 ml, Pyrex 9825), provided with Teflon®-lined screw caps, were used for plasma extractions. Conical centrifuge tubes (5 ml, Pyrex 8061) were used for the derivatization procedure and for the evaporation of the final extract. All tubes were washed with detergent and water, were treated with Siliclad® (Clay Adam, Parsippany, NJ, U.S.A.) and, prior to use, were rinsed with methanol and dichloromethane (Fisher Scientific).

Solvent evaporator. Solvents were removed at 60°C with a nitrogen evaporator (N-Evap, Organomation Associates).

Shaker. Extractions were performed by shaking (60 strokes min^{-1}) on a variable speed reciprocating shaker (Eberbach) for 20 min.

Centrifuge. A Damon/IEC Model CRU-500 refrigerated centrifuge was operated at 1600 *g* and 10°C.

Chemicals

Imidazobenzodiazepine-3-carboxamide and 8-deschloro-imidazobenzodiazepine-3-carboxamide were obtained from Dr. W. Scott, Hoffmann-La Roche, Nutley, NJ, U.S.A. Tetradeuterated imidazobenzodiazepine-3-carboxamide was prepared by Dr. Yu-Ying Liu, Hoffmann-La Roche. Nanograde benzene, methanol, ethyl acetate, N,N-dimethylformamide, chloroform, hexane and 1,2-dichloroethane were purchased from Burdick and Jackson Labs.

Solutions

Imidazobenzodiazepine-3-carboxamide. A 1 mg ml^{-1} stock solution was prepared in N,N-dimethylformamide. This solution was diluted with methanol to give solutions A, B, C and D containing 0.1, 0.5, 1 and 5 ng of imidazobenzodiazepine-3-carboxamide per 50 μl of solvent, respectively.

Tetradeuterated imidazobenzodiazepine-3-carboxamide. A 1 mg ml^{-1} stock solution was prepared in N,N-dimethylformamide. This solution was diluted with methanol to give solution E containing 10 ng of tetradeuterated imidazobenzodiazepine-3-carboxamide per 50 μl of solvent.

20% 1,2-dichloroethane in benzene. 200 ml of 1,2-dichloroethane were diluted to 1000 ml with benzene.

2% triethylamine in chloroform. Triethylamine (0.1 ml) was diluted with 4.9 ml of chloroform. This solution was prepared immediately prior to use.

Molar borate buffer (pH 9). Boric acid (61.8 g) and potassium chloride (74.7 g) were dissolved in 1000 ml of distilled water. This solution was then used to titrate a solution of 106 g of sodium carbonate in 1000 ml of distilled water to pH 9.

0.25 M borate buffer (pH 9). One volume of 1 *M* borate buffer pH 9 was diluted with 3 volumes of distilled water.

Procedure

Calibration curve samples are prepared in duplicate by spiking 1 ml of drug-free control plasma with 0 or 50 μl of either solution A, B, C or D (0, 0.1, 0.5, 1 or 5 ng of imidazobenzodiazepine-3-carboxamide, respectively). A volume of 50 μl of solution E (10 ng of tetradeuterated imidazobenzodiazepine-3-carboxamide) are added to both calibration curve and experimental samples and each resulting mixture is vortexed briefly. A volume of 1 ml of 1 *M*

pH 9 borate buffer is added and the samples are extracted with 5 ml of 20% 1,2-dichloroethane in benzene. The samples are centrifuged for 10 min, 4 ml of the benzene extract are transferred to a 5-ml centrifuge tube and the benzene is evaporated. The residue is dissolved in 50 μl of the 2% triethylamine in chloroform solution, and 20 μl of pentafluoropropionic anhydride are added. The solution is vortexed and allowed to stand at room temperature for 15 min. The solution is evaporated to dryness, the residue is dissolved in 0.5 ml of hexane and 0.2 ml of 0.25 *M* pH 9 borate buffer is added. The mixture is vortexed briefly and then centrifuged. As much hexane as possible is transferred to another 5-ml centrifuge tube, the solvent is evaporated, and the residue is reconstituted in 40 μl of ethyl acetate. Aliquots (3—5 μl) of this solution are injected into the gas chromatograph—mass spectrometer with the mass spectrometer set to monitor *m/z* 316 and *m/z* 320 in the effluent of the gas chromatograph. Thirty sec after injection the divert valve of the gas chromatograph is turned off and 15 sec later the ionizer is turned on.

The ion ratio of *m/z* 316 to *m/z* 320 from an unknown sample is converted to a known amount of imidazobenzodiazepine-3-carboxamide using a calibration curve. The calibration curve is constructed by fitting the *m/z* 316 to *m/z* 320 ion ratios from the calibration curve samples to their respective concentration values using a linear least squares computer program. The resulting slope

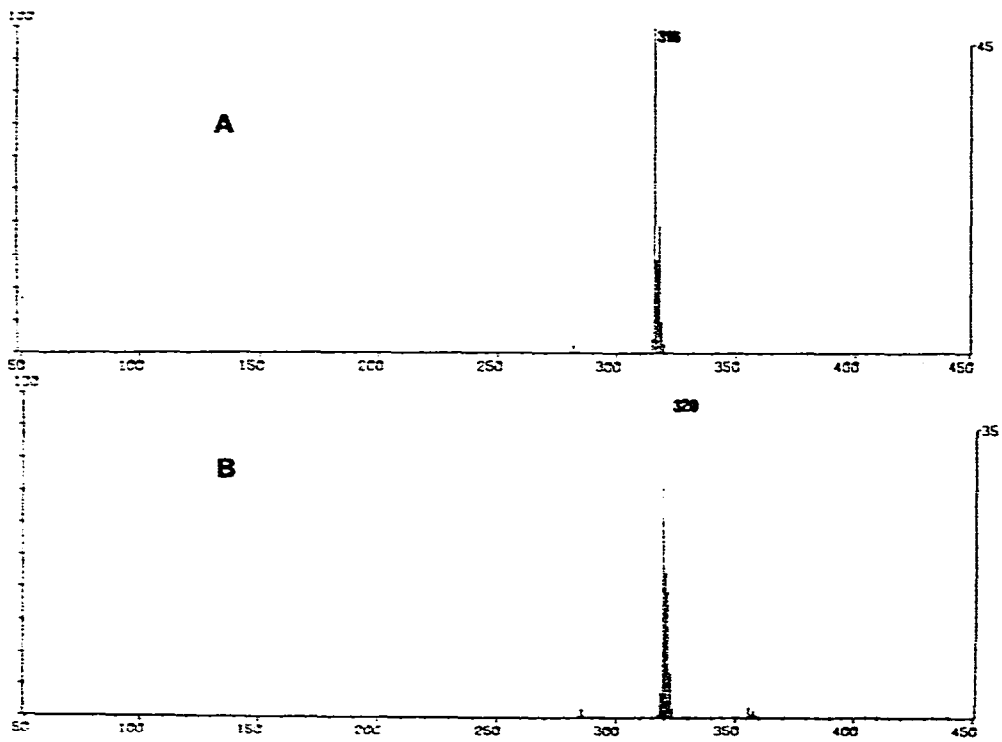


Fig. 1. Methane negative chemical-ionization mass spectra of the nitrile derivatives of imidazobenzodiazepine-3-carboxamide (A) and tetradeuterated imidazobenzodiazepine-3-carboxamide (B).

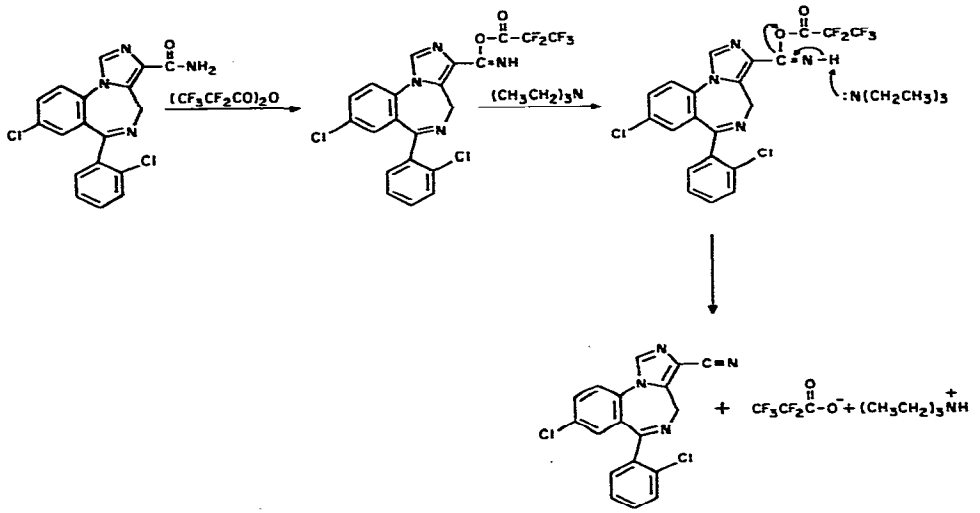
(m) and intercept (b) values are then used to calculate the amount (x) of imidazobenzodiazepine-3-carboxamide corresponding to each ion ratio (R) in experimental samples by the equation: $x = (R-b)/m$.

Clinical samples

A healthy, male volunteer did not eat anything for 7.5 h prior to receiving a 0.2-mg oral dose of imidazobenzodiazepine-3-carboxamide. Aliquots (10 ml) of whole blood were drawn at −0.25, 0.5, 1, 2, 3, 4, 6, 9, 12, 18, 24, 36, 48, 60 and 72 h post dosing into a Vacutainer® 6527 containing heparin (Becton-Dickinson). The blood was centrifuged for 30 min and the resulting plasma was isolated and stored at −10°C until analyzed.

RESULTS AND DISCUSSION

Several years ago while searching for a volatile derivative of imidazobenzodiazepine-3-carboxamide we discovered that treatment of the drug with pentafluoropropionic anhydride and triethylamine converted the primary amide function of imidazobenzodiazepine-3-carboxamide into a nitrile function. The conversion is quantitative using even picogram amounts of imidazobenzodiazepine-3-carboxamide. A possible mechanism for this reaction is shown below.



Gal et al. [5] and Stogniew and Callery [6] have recently used a similar reaction to convert the amide functions of disopyramide and gabamide into their respective nitrile derivatives.

Analysis by thin-layer chromatography and direct insertion probe-MS of reacted imidazobenzodiazepine-3-carboxamide suggest that nitrile formation occurs in the reaction tube and not in the injection port of the gas chromatograph.

The methane negative CI mass spectra of the nitrile derivative of imidazobenzodiazepine-3-carboxamide and tetradeuterated imidazobenzodiazepine-3-carboxamide are shown in Fig. 1. Neither mass spectra shows an intense molecular

anion at either *m/z* 352 or *m/z* 356, respectively. However, intense $(M-HCl)^-$ fragment ions occur at *m/z* 316 for imidazobenzodiazepine-3-carboxamide and at *m/z* 320 for tetradeuterated imidazobenzodiazepine-3-carboxamide. Negative CI was used, in spite of the disadvantage of requiring the monitoring of a fragment ion, because an assay with a low limit of quantitation was required. Previous work in this laboratory with other 1,4-benzodiazepines have shown that negative CI provides extremely efficient ionization of this class of compounds [4, 7, 8]. Also, the loss of HCl is not expected to occur on metabolism of imidazobenzodiazepine-3-carboxamide and specificity is accordingly maintained.

Typical selected ion current profiles from the analysis of 1 ml of either control plasma spiked with 0.1 ng of imidazobenzodiazepine-3-carboxamide (A) or plasma from a subject either 15 min before (B) or 72 h (C) after receiving a 0.2-mg oral dose of imidazobenzodiazepine-3-carboxamide are shown in Fig. 2. The small response at *m/z* 316 in selected ion current profile B is from undeuterated imidazobenzodiazepine-3-carboxamide in the tratradeuterated imidazobenzodiazepine-3-carboxamide and "ghosting" from previous injections. Using 5 ng of tetradeuterated imidazobenzodiazepine-3-carboxamide as internal standard, this response typically represents 0.03 ng ml^{-1} of imidazodiazepine-3-carboxamide. The limit of quantitation for this assay is considered to be three times the response at *m/z* 316 in the selcted ion current profiles from the calibration curve plasma samples spiked only with tetradeuterated imidazobenzodiazepine-3-carboxamide, and is typically 0.1 ng ml^{-1}.

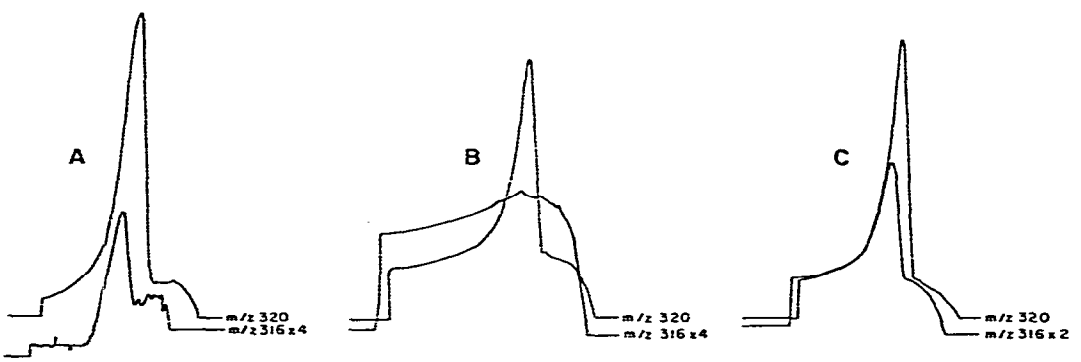


Fig. 2. Selected ion current profiles from the analysis of 1 ml of either control plasma spiked with 0.1 ng of imidazobenzodiazepine-3-carboxamide (A) or plasma from a subject either 15 min before (B) or 72 h after (C) receiving a 0.2-mg oral dose of imidazobenzodiazepine-3-carboxamide. All samples were spiked with 2.5 ng of tetradeuterated imidazobenzodiazepine-3-carboxamide. The measured concentration of imidazobenzodiazepine-3-carboxamide in the 72-h post dose sample was 0.64 ng/ml.

Assay precision and recovery of imidazobenzodiazepine-3-carboxamide were determined by spiking 1-ml plasma samples with either 0.1, 0.5, 1.0 or 5.0 ng of authentic compound and analyzing the samples using the procedure described. The relative standard deviations (n = 4) of the determinations were 17%, 9%, 3% and 0.5%, respectively. The mean recovery (± S.D.) of imidazobenzodiazepine-3-carboxamide from these samples, based on the responses to

injection of external standard solutions containing known amounts of derivatized imidazobenzodiazepine-3-carboxamide, was 42 ± 6%.

The plasma concentration—time curve for imidazobenzodiazepine-3-carboxamide in a male volunteer following a 0.2-mg dose of the drug is shown in Fig. 3. Assuming that distribution is complete after 6 h, the elimination half-life of the drug in this volunteer is 39 h.

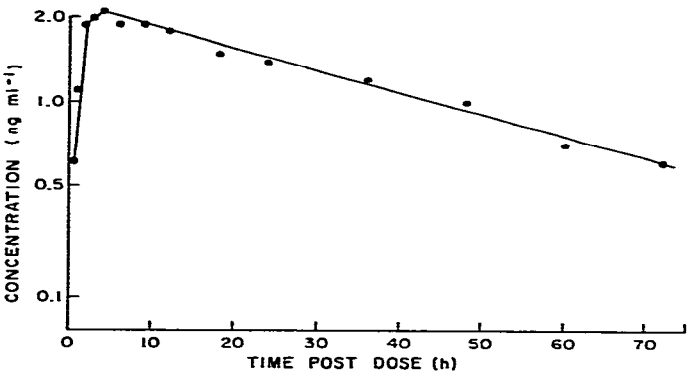


Fig. 3. Plasma concentration—time curve for a male volunteer who had received a 0.2-mg oral dose of imidazobenzodiazepine-3-carboxamide.

CONCLUSION

A sensitive and specific GC—MS procedure has been described which can measure imidazobenzodiazepine-3-carboxamide in human plasma for up to 72 h following a 0.2-mg dose of the drug.

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