Journal of Chromatography, 222 (1981) 421—428
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 749

DETERMINATION OF CARBIMIDE IN PLASMA BY GAS—LIQUID CHROMATOGRAPHY

CHRISTOPHER W. LOOMIS* and JAMES F. BRIEN

Department of Pharmacology, Queen's University, Kingston, Ontario K7L 3N6 (Canada)

(First received July 8th, 1980; revised manuscript received October 13th, 1980)

SUMMARY

A sensitive and selective method for the measurement of carbimide, the hydrolytic product of calcium carbimide, in plasma is described. The procedure involves extraction with ethyl acetate, derivatization with heptafluorobutyric anhydride and analysis by gas—liquid chromatography with electron-capture detection. The lower limit of sensitivity of the assay is 5.0 ng/ml carbimide in plasma. The overall accuracy of the procedure is 96.1% with a coefficient of variation not exceeding 8.7%. This assay has been used to investigate the time-course of plasma carbimide concentration in the rat following oral administration of calcium carbimide.

INTRODUCTION

Calcium carbimide (CC) is used as a pharmacological adjunct in the treatment of alcoholism [1]. In recent years, detailed studies of the CC—ethanol interaction in man [2—5] and in animals [6, 7] have been conducted. CC inhibits aldehyde dehydrogenase (EC 1.2.1.3), one of the enzymes involved in ethanol metabolism [8, 9]. During the CC—ethanol interaction, blood acetaldehyde concentration is increased, which results in a number of untoward effects (e.g. tachycardia, hypotension), thereby deterring further drinking. One of the shortcomings of these studies on CC has been the inability to measure plasma drug levels due to the lack of a selective and sensitive assay. As a result, it has not been possible to assess the extent of variation in the absorption of CC following oral administration or to determine if there is a correlation between plasma drug concentration and the extent of aldehyde dehydrogenase inhibition, especially during the CC—ethanol interaction.

This paper describes a sensitive and selective method for the measurement of carbimide, the hydrolytic product of CC, in plasma. The procedure involves ethyl acetate extraction, derivatization with heptafluorobutyric anhydride (HFBA) and analysis by gas—liquid chromatography with electron-capture detection (GLC—ECD).

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EXPERIMENTAL

Reagents

Ethyl acetate was certified GC-spectrophotometric grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Stock solutions of carbimide (Aldrich, Milwaukee, WI, U.S.A.) were prepared daily in glass-distilled water. Calcium carbimide and citrated calcium carbimide (Temposil®) were obtained from Lederle (Montreal, Canada). [^{14}C]Carbimide was purchased from ICN Pharmaceuticals (Irvine, CA, U.S.A.). Heptafluorobutyric anhydride was purchased from Supelco (Bellefonte, PA, U.S.A.).

pK_a determination of carbimide

A 0.1 *M* standardized sodium hydroxide solution (Fisher Chemicals, Fair Lawn, NJ, U.S.A.) was used. Carbimide (2.0 mmoles) was dissolved in 100 ml of reverse-osmosis, deionized water. The carbimide solution was acidified with 20 ml of 0.1 *M* hydrochloric acid and then titrated with 0.02-ml aliquots of the sodium hydroxide solution at 21°C. The pK_a of carbimide was determined from the inflection point of the titration curve.

Extraction

A 1.0-ml plasma sample was placed in a FEP-stoppered centrifuge tube (silanized) and the pH was adjusted to 10 with 0.1 *M* sodium hydroxide. After 0.5 g of sodium chloride and 2.0 ml of ethyl acetate were added, the tube was shaken for 2 min on a flat-bed shaker followed by centrifugation at 150 *g* for 3 min. The organic phase was then transferred to a 5-ml reacti-vial (Chromatographic Specialties, Brockville, Canada). The pH of the remaining aqueous layer was readjusted to 10 with 0.1 *M* sodium hydroxide and extracted as previously described with 2.0 ml of ethyl acetate. The organic phase was then transferred to the 5-ml reacti-vial. The combined organic extract was concentrated to dryness with a stream of nitrogen at 50°C and the residue was redissolved in 100 µl of acetonitrile for subsequent derivatization with HFBA and analysis by GLC—ECD.

The efficiency of the procedure for extracting carbimide from plasma using ethyl acetate was determined with plasma standards containing known amounts of [^{14}C]carbimide. A 20-µl aliquot of 0.5 µCi/ml [^{14}C]carbimide (50 µCi/µmole) containing approximately 20,000 dpm was added to each plasma standard. The radioactivity of [^{14}C]carbimide was determined in the ethyl acetate extract by adding 100 µl of the extract to 10.0 ml of ACS scintillation cocktail (Amersham, Arlington Heights, IL, U.S.A.) and liquid scintillation counting in a Searle Mark III counter (Des Plaines, IL, U.S.A.) for 10 min.

Derivatization

The 5-ml reacti-vial, containing the residue of the ethyl acetate extraction dissolved in 100 µl of acetonitrile, was sealed with a mininert valve (Chromatographic Specialties). A 5.0-µl aliquot of HFBA was added through the valve and the contents were mixed for 5 sec using a vortex mixer. The reaction solution was allowed to sit at room temperature (21°C) for 30 min.

The reaction solution was then evaporated to dryness at 45°C with a stream of nitrogen and the residue was redissolved in 100 μl of benzene. A 1.0-μl aliquot of the benzene solution was injected onto the GLC column.

GLC—ECD analysis

A Hewlett-Packard Model 5710A gas chromatograph equipped with a ^{63}Ni electron-capture detector and a 1-mV potentiometric recorder was used. A coiled glass column (1.8 m × 2 mm I.D.) was washed in sequence with distilled water, methanol, acetone, hexane, dried with nitrogen and then silanized with a 10% solution of Dri-Film SC-87 (Chromatographic Specialties) in toluene for 2 h. The column was then rinsed with methanol, dried with nitrogen and heated at 100°C for 1 h. The silanized column was packed with 3% OV-1 on 80—100 mesh Chromosorb W HP (Chromatographic Specialties) and conditioned for 18 h at 220°C with a carrier gas [argon—methane (95:5)] flow-rate of 2 ml/min. For carbimide analysis, the instrumental operating conditions were: injection port temperature, 250°C; column temperature, 185°C; detector temperature, 300°C; carrier gas flow-rate, 20 ml/min.

Gas chromatographic—mass spectrometric analysis

A Biospect (Searle-Scientific Instrument Division, Baltimore, MD, U.S.A.) gas chromatograph—mass spectrometer was used. The mass spectrometer was operated in the chemical ionization mode with methane as the reagent gas and the electron ionization mode, scanning the mass range 70—300 a.m.u. For the gas chromatographic separation, a glass column (1.8 m × 2 mm I.D.) containing 3% SE-30 on 80—100 mesh Chromosorb W (Chromatographic Specialties) was used with a helium carrier gas flow-rate of 15 ml/min. The instrumental operating conditions were: gas chromatograph injection port temperature, 255°C; column temperature, 190°C; mass spectrometer ion source temperature, 210°C; ion source current, 0.2 mA; methane reagent gas pressure, 1 mm Hg.

Aqueous standard curves

Aqueous standards in the concentration range of 5.0—1000 ng/ml were prepared by dissolving known concentrations of carbimide in distilled water; these standards were extracted and analyzed by the procedure outlined above. The peak height of the carbimide signal for each aqueous standard was plotted against the respective carbimide concentration. The concentration of carbimide in a plasma sample was determined by measuring the peak height of the carbimide chromatographic signal and interpolating on the standard curve. The slope and *y*-intercept of the line of best fit, determined by regression analysis, were used for this calculation.

Recovery study

To evaluate the accuracy and precision of the assay, samples were prepared containing 500, 100 and 5.0 ng/ml carbimide in plasma and were quantitated by the GLC—ECD procedure using aqueous standards. The concentration of carbimide measured was divided by the concentration added and a per cent recovery value was determined for each plasma sample. Precision was

assessed by calculating the coefficient of variation for each plasma carbimide concentration.

Hydrolysis of calcium carbimide to carbimide

To test if there is quantitative hydrolysis of CC to carbimide under simulated gastric conditions, 50 mg of CC, a Temposil tablet containing 50 mg of CC and 100 mg of citric acid, and a pulverized Temposil tablet placed in a gelatinous capsule were individually incubated in 50 ml of 0.1 *M* hydrochloric acid (pH 1.14—1.16) at 37°C with gentle mixing. These conditions were selected to mimic the volume and pH of the stomach contents in man. After 1 h, 1.0 ml of the acidic incubate was extracted and the carbimide concentration determined using GLC—ECD to measure the conversion of CC to carbimide.

RESULTS AND DISCUSSION

In the investigation of the CC—ethanol interaction conducted in this laboratory [2—6], it was deemed necessary to determine the variability in the absorption of CC following oral administration and the correlation between plasma drug concentration and the inhibition of hepatic aldehyde dehydrogenase. This required the development of a sensitive and selective analytical procedure. Since the clinically recommended dose of CC in man is 50 mg (0.7 mg/kg for a 70-kg man), plasma concentrations of the drug would probably be low (< 1.0 μg/ml). Therefore the minimum sensitivity of this assay should be in the 1.0—10 ng/ml plasma concentration range.

The compound CC [Ca^{2+} $(N—C \equiv N)^{2-}$] is insoluble in aqueous and organic material. No known solvent will bring about solution of CC without at least partial hydrolysis to calcium hydrogen carbimide [Ca^{2+} $(HN—C \equiv N)_2^-$], or complete hydrolysis to carbimide ($H_2N—C \equiv N$) [10]. Under simulated gastric conditions, it was determined that for 50 mg CC, the Temposil tablet containing 50 mg CC, and the pulverized Temposil tablet containing 50 mg CC, there was conversion to carbimide to the extent of 92%, 57% and 100% in 1.0 h, respectively. For the Temposil tablet, hydrolysis of CC to carbimide was 100% after 10 h.

The assay described involves the quantitative determination of carbimide in plasma as the HFBA derivative using GLC—ECD. A 5.0-μl aliquot of HFBA, representing a molar ratio of HFBA to carbimide of approximately 850: 1 for 1.0 μg carbimide, with acetonitrile as a catalyst has been found to provide optimal derivatization of the most concentrated carbimide samples. With these derivatization conditions, the lower limit of sensitivity of the assay is 5.0 ng/ml in plasma. Larger amounts of HFBA did not improve the sensitivity of the assay nor did HFBA produce any interfering chromatographic signals. The GLC analysis of derivatized carbimide revealed one signal with a retention time of 1.8 min (Fig. 1). There was no interference from endogenous materials in plasma with retention times similar to that of HFBA-carbimide.

Gas chromatographic—mass spectrometric analysis confirmed that this GLC signal represented an HFBA derivative of carbimide. Chemical ioniza-

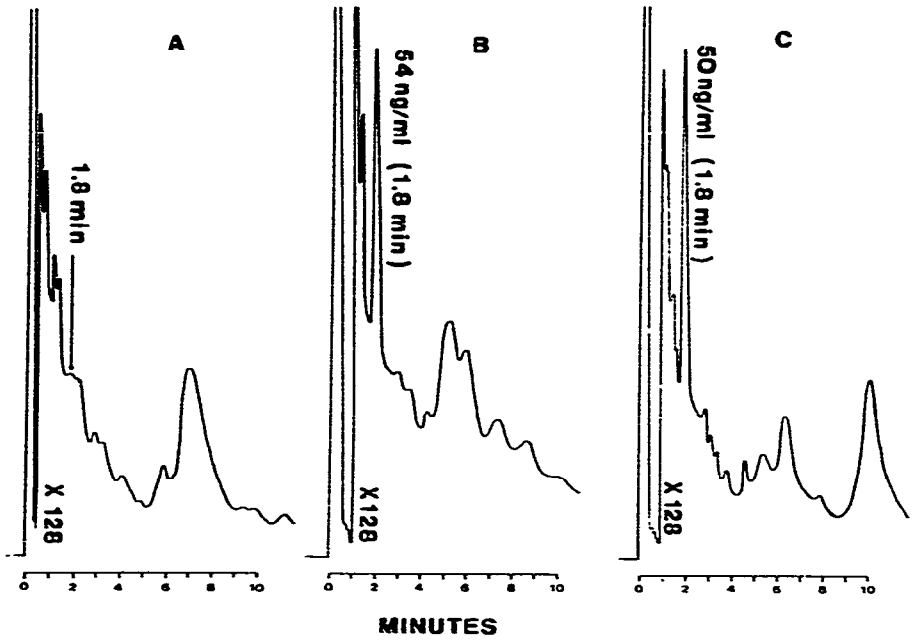

Fig. 1. Chromatograms from carbimide analysis. (A) Blank plasma sample; (B) plasma sample from a rat administered 7.0 mg/kg calcium carbimide orally, containing 54 ng/ml carbimide; (C) aqueous standard containing 50 ng/ml carbimide. The detector sensitivity setting was × 128.

tion produced a quasi-molecular (M + 1) ion at 257 a.m.u.; electron ionization produced the ions 256, 236, 208, 150 and 119 a.m.u. The mass spectral data indicated that the HFBA derivative of carbimide was unicomponent and corresponded to the structure shown in Fig. 2, in which the fragment ions are also tentatively identified. This derivative of carbimide was not expected since the formation of the heptafluorobutyramide would have been predicted. However, the presence of the cyano group on the amino function of carbimide may have compromised the basicity of the amino group due to its negative inductive effect. This could prevent the expected nucleophilic attack of the nitrogen of the amino group on the carboxyl carbon of HFBA, thereby leading to the addition of the heptafluorobutyroxy group to the carbon of the cyano function of carbimide. This carbimide derivative was not heptafluorobutyryl-substituted urea since the retention time of the HFBA-derivatized urea (1.6 min) was different from that of derivatized carbimide (1.8 min) and the molar response with ECD was less for derivatized urea (9 mm peak height per nmole) compared with derivatized carbimide (414 mm peak height per nmole).

For the extraction of carbimide from biological fluids, it was necessary to determine its pK_a value. Using a potentiometric titration procedure [11], the pK_a of the amino function of carbimide was found to be 7.4. At pH 10, the ratio of unionized to ionized carbimide should be in excess of 100:1, thereby facilitating extraction into the organic phase. However, since unionized carbimide is soluble in water and organic solvent, sodium chloride was added to saturate the aqueous phase thereby enhancing the extraction

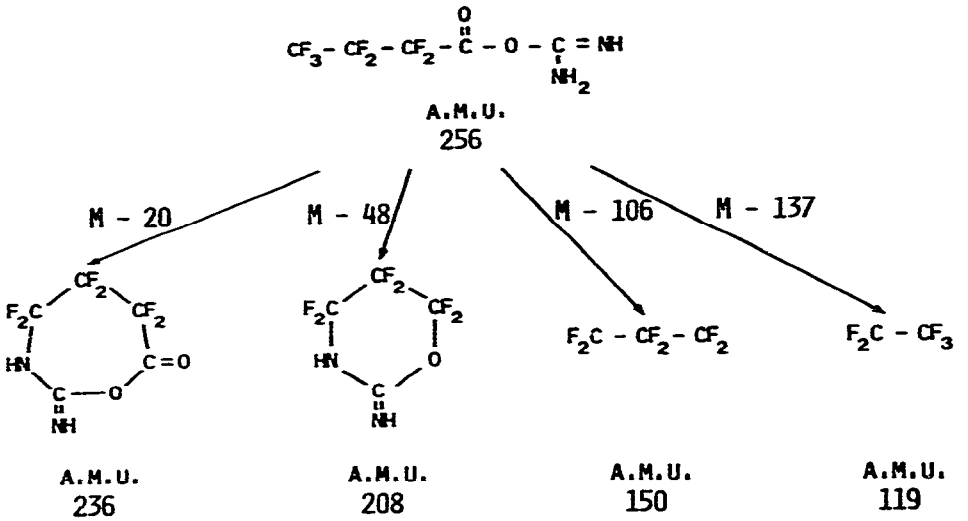

Fig. 2. Chemical structures of HFBA-derivatized carbimide [molecular ion (M) 256 a.m.u.] and fragment ions (236, 208, 150, 119 a.m.u.) as determined by electron ionization mass spectrometry.

of unionized carbimide into ethyl acetate. In order to determine the efficiency of the ethyl acetate extraction step in the assay, [^{14}C]carbimide was used. The absolute efficiency of the ethyl acetate extraction of carbimide was found to be 69.7 ± 3.42 (S.D.) % (n = 102) from plasma and 70.1 ± 4.82% (n = 102) from aqueous solution. The ratio of the efficiency of the ethyl acetate extraction of carbimide from plasma relative to that from aqueous solution is virtually unity.

The ECD response to HFBA-carbimide was linear in the concentration range 5.0—10,000 ng/ml for the GLC analysis of aqueous standards of carbimide. Aqueous standard curves were prepared daily in the range of 5.0—1000 ng/ml for plasma analysis. The data of the recovery study (Table I) indicate that the accuracy of the GLC—ECD procedure for the quantitation of carbimide in plasma, relative to aqueous standards containing known concentrations of carbimide, ranges from 90.8% (5.0 ng/ml) to 105.7% (500 ng/ml) with an overall recovery of 96.1%. The precision of the assay is reflected by the coefficient of variation which did not exceed 8.7% (Table I).

This GLC—ECD assay for carbimide does not include an internal standard. Several compounds were investigated including n-amylamine, n-octylamine, diethylamine, thiourea, β-aminopropionitrile and aminoacetonitrile. However, no compound has yet been found with appropriate chromatographic characteristics. On the basis of the S.D. of the [^{14}C]carbimide extraction efficiency data (3.42%) and the coefficient of variation data for the recovery studies (Table I), there appears to be little within-day and between-day variability in the assay. The aqueous standard curves on different days were also similar, as indicated by the slope values (e.g. range of slope: 0.151—0.168 mm/ng/ml), suggesting little between-day variability in the HFBA derivatization procedure and detector response for HFBA-carbimide.

This GLC—ECD assay has been used to investigate the time-course of plasma carbimide concentration in the rat following oral administration of 7.0 mg/kg

TABLE I

RECOVERY OF CARBIMIDE FROM PLASMA

Carbimide recovery from plasma was determined relative to aqueous standards containing known concentrations of carbimide. Recovery values were the means of five determinations at each plasma concentration.

Carbimide concentration (ng/ml)	Mean recovery (%)	Standard deviation (%)	Coefficient of variation (%)
500	105.7	6.03	5.96
100	91.7	7.97	8.69
5.0	90.8	7.82	8.61

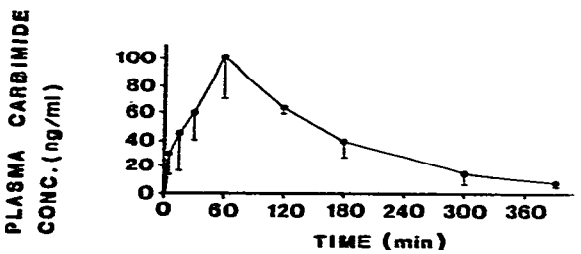

Fig. 3. Time-course of mean plasma carbimide concentration in the rat after oral administration of 7.0 mg/kg calcium carbimide. Each point represents the mean ± S.E.M. of four determinations.

CC (Fig. 3). The procedure allows quantitation of carbimide for at least 6.5 h after drug administration. The method provides a reliable, selective and sensitive procedure for the measurement of carbimide in plasma with a lower limit of sensitivity of 5.0 ng/ml and should permit the evaluation of the pharmacokinetic parameters of carbimide following CC administration to both man and experimental animals.

ACKNOWLEDGEMENTS

This work was supported by the Addiction Research Foundation of Ontario in the form of a Research Scholarship to C.W. Loomis and the Faculty Trust Fund, Faculty of Medicine, Queen's University. The assistance of Dr. B.K. Tang of the Department of Pharmacology, University of Toronto in the mass spectral analysis and Mrs. D. Browne in the preparation of the manuscript is gratefully acknowledged.

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