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DETERMINATION OF COCAINE IN PLASMA BY AUTOMATED GAS CHROMATOGRAPHY

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SUMMARY

A rapid and sensitive gas chromatographic method is described for the determination of cocaine in plasma. A close structural analogue of cocaine, *m*-toluylecgonine methyl ester, is used as an internal standard. The simplicity of the extraction scheme and the use of an automatic sampler makes the method convenient for large numbers of samples generated in pharmacokinetic studies.

INTRODUCTION

Despite the interest in cocaine, both as a drug of abuse and as a useful local anesthetic, relatively little is known about its pharmacokinetics and metabolism in humans. This is in part because until recently sufficiently sensitive methods [1] for cocaine determination in biological fluids have been lacking.

In studies of cocaine pharmacokinetics in humans, we required a method of quantitation suitable for large numbers of samples and sensitive enough to measure plasma concentrations several hours after a single dose. Published methods with adequate sensitivity for plasma include gas chromatography (GC) with nitrogen—phosphorus [2, 3] or electron-capture [4] detection and gas chromatography—mass spectrometry (GC—MS) [1, 5]. GC—MS is a highly selective, sensitive technique; however, the expensive instrumentation and highly trained personnel required are serious drawbacks for routine applications. High-performancce liquid chromatography [6—8] has been used for the quantitation of cocaine in biological fluids, but the sensitivity is inadequate for low concentrations in small plasma samples.

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In recent years, the nitrogen—phosphorus detector has been extensively utilized in the GC determination of basic drugs, including cocaine [2, 3]. The very high selectivity of this detector for organic compounds containing nitrogen and/or phosphorus minimizes interference from extraneous substances and frequently allows determination of subnanogram quantities. This paper describes a sensitive, precise method for the determination of cocaine in plasma using GC with nitrogen—phosphorus detection. A close structural analogue of cocaine is used as an internal standard. The extraction scheme has been designed so that large numbers of samples may be processed simultaneously and analyzed in large batches by automated GC. The assay is considerably faster and more convenient than previously reported methods.

MATERIALS AND METHODS

Chemicals and reagents

Cocaine hydrochloride was from Mallinckrodt (St. Louis, MO, U.S.A.). The internal standard, *m*-toluylecgonine methyl ester, was synthesized as described below. Aqueous reagent solutions were prepared from analytical reagent grade chemicals and water distilled in glass. Isoamyl alcohol and *tert.*-amyl alcohol were reagent grade; *n*-butyl acetate and toluene were from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Each new bottle of solvent was checked by GC for interfering substances.

Instrumentation

GC used a Hewlett-Packard Model 5711A Instrument, configured for on-column injection and equipped with nitrogen—phosphorus detectors. Flow-rates for carrier gas (nitrogen), detector air and hydrogen were 30, 50 and 5 ml/min, respectively. Columns were 1.8 m × 2 mm I.D. glass, packed with 3% OV-101, 0.1% potassium hydroxide on 100—120 mesh Chromosorb W HP (Alltech Assoc., Deerfield, IL, U.S.A.), conditioned overnight at 250°C. Chromatograms were recorded on a Hewlett-Packard 3390A plotter/integrator interfaced with a Hewlett-Packard 7672A automatic sampler. The automatic sampler parameters were as follows: number of sample pumps, 3; number of sample prewashes, 3; number of solvent postwashes, 5; injection volume, 5 μl. A toluene—isoamyl alcohol (90:10) postwash reduced ghosting to non-detectable levels.

Analyses were at column oven temperatures ranging from 200 to 220°C, depending upon the column condition. Clean separation of cocaine and internal standard was achieved with the retention of internal standard relative to cocaine of 1.35.

Preparation of glassware

Screw-top glass culture tubes (13 × 100 mm) and autosampler vials were washed with a phosphate-free detergent, soaked overnight in 4 *M* hydrochloric acid, rinsed several times with water and then dried at 80°C. The PTFE-lined tube caps were soaked overnight in dilute hydrochloric acid, rinsed with distilled water and dried. Autosampler vials were further soaked overnight in a 0.1% tetrasodium EDTA solution and then oven-dried.

Extraction procedure

Plasma (1 ml) and internal standard (100 ng in 100 μl of 0.01 *M* sulfuric acid) were pipetted into culture tubes and briefly mixed. Samples were alkalinized with a potassium carbonate—bicarbonate buffer pH 9.5 (0.5 ml, 1 *M*). Two ml of toluene—*tert.*-amyl alcohol (90:10) were added, the tubes were fitted with PTFE caps, and vortex-mixed for 6 min with a multiple tube vortex mixer. The tubes were centrifuged at 0°C for 15 min at 700 *g* to break emulsions and then placed in a dry ice—acetone bath to freeze aqueous layers. Organic layers were poured into clean tubes containing 0.5 ml of 0.1 *M* sulfuric acid, which were then vortex-mixed, centrifuged and frozen as above. The organic phase was poured off and discarded, the aqueous layer was thawed and then washed with 2 ml toluene—*tert.*-amyl alcohol (90:10). The extraction could be interrupted at this point if necessary and the samples stored frozen. Buffer, pH 9.5 (0.5 ml, 1 *M*) and butyl acetate (0.5 ml) were added, the tubes were vortex-mixed, centrifuged, and frozen in dry ice—acetone to separate the aqueous layers. The butyl acetate extracts were poured into EDTA-treated autosampler vials, which were left uncapped and loaded into the autosampler cassette for GC analysis.

Calibration procedure

Stock solutions (1 mg/ml as the base) of cocaine hydrochloride and of internal standard, *m*-toluylecgonine methyl ester hydrochloride, were prepared in 0.01 *M* sulfuric acid and stored frozen. Fresh cocaine solutions were prepared every four weeks. These solutions were diluted to appropriate concentrations with 0.01 *M* sulfuric acid and added (as 100-μl aliquots) to blank plasma obtained from drug-free volunteers to provide several concentrations spanning the range 0—500 ng/ml cocaine and 100 ng/ml internal standard.

The samples were taken through the extraction procedure and analyzed by GC as described above. Standard curves were constructed by plotting the peak height ratio of cocaine versus internal standard, and were linear over the range 0—1000 ng/ml. A standard in the middle of the expected concentration range was reinjected and the computing integrator was calibrated using the internal standard method.

After several runs, we found aqueous standard curves were always identical to those obtained from spiked plasma and, consequently, aqueous standard curves were used for calibration in future runs. As controls, plasma spiked with 50, 100 and 500 ng/ml cocaine as well as blank plasma were included in each run.

Sample collection and storage

Cocaine undergoes both spontaneous [9, 10] and enzymatic hydrolysis [11] in plasma at physiologic pH. Special precautions are necessary to avoid losses. Blood (approximately 6 ml) from experimental subjects was drawn into heparinized tubes on ice containing 0.2 ml of saturated sodium fluoride to inhibit plasma esterases [5, 12]. The tubes were immediately vortexed and placed back on ice until centrifuged at 0°C to separate the plasma. Immediately following centrifugation, the plasma samples were frozen and stored at −10°C until analysis. Blank plasma used for standards and control samples was like-

wise spiked with sodium fluoride (0.2 ml, saturated) prior to adding cocaine. As a check for stability during storage, samples were reanalyzed over periods ranging from two weeks to one year.

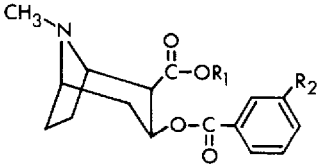
Synthesis of the internal standard, m-toluylecgonine methyl ester hydrochloride

A solution of ecgonine methyl ester hydrochloride [13] (235 mg, 1 mmol in 25 ml water) was made basic with potassium carbonate and extracted with 50 ml of methylene chloride. The extract was evaporated to dryness on a rotary evaporator, reconstituted in 10 ml of toluene, and refluxed for 3 h with *m*-toluic anhydride (300 mg). The mixture was cooled, extracted with 50 ml of 0.5 *M* sulfuric acid, and the resulting aqueous extract washed with two 25-ml portions of diethyl ether. Potassium carbonate was added to bring the pH to 10, and the product was extracted with two 25-ml portions of diethyl ether. The extract was dried over anhydrous potassium carbonate and evaporated to give an oil, which was purified by column chromatography on silica gel (Merck, 70—230 mesh, 6 × 1.5 cm column), eluting with ethyl acetate—methanol—58% ammonia (85:10:0.5). Fractions (4 ml) were taken and monitored by thin-layer chromatography (TLC) for product. The impurities, which included unreacted ecgonine methyl ester, had lower R_F values and were readily separated. Those fractions containing product were combined and evaporated with a rotary evaporator. Isopropyl alcohol (2 ml) was added followed by 3 drops of concentrated hydrochloric acid, which produced an acidic solution, pH < 2 by damp, universal pH paper. The solution was slowly diluted with anhydrous diethyl ether, with stirring and scratching with a glass rod, which caused the product to crystallize. The salt was collected by filtration, washed with anhydrous diethyl ether and air-dried to give 55 mg of white crystalline powder, m.p. 180—181°C. The product was homogeneous by TLC (silica gel) and GC (OV-101). A microanalysis for carbon, hydrogen and nitrogen was within ± 0.2% of theory.

RESULTS AND DISCUSSION

Hydrolytic instability of cocaine and internal standard

The ease with which cocaine hydrolyzes, particularly under alkaline conditions, presents special problems in its quantitative analysis. Cocaine is a basic compound, and extraction from aqueous solution into organic solvents must be carried out at alkaline pH, a condition which will lead to some unavoidable losses. For example, Fletcher and Hancock [9] found that even at



1, $R_1 = CH_3$; $R_2 = H$

2, $R_1 = CH_3$; $R_2 = CH_3$

3, $R_1 = n\text{-}C_3H_7$; $R_2 = H$

Fig. 1. Structures of cocaine (1), *m*-toluylecgonine methyl ester (2), and benzoylecgonine propyl ester (3).

pH 8, cocaine was hydrolyzed to the extent of 17% in 1 h at room temperature. At pH 9.4 the loss was 41%.

In chromatographic assays, errors due to loss of the analyte can often be minimized by the use of an internal standard with similar chemical and physical properties. The site of lability in the cocaine molecule is the methyl ester grouping, which is hydrolyzed to the carboxylic acid derivative, benzoylecgonine [9, 10]. For this reason, we synthesized an internal standard, *m*-toluylecgonine methyl ester (2, Fig. 1) which maintains this critical functionality. During the alkaline extraction steps, hydrolysis of this close structural analogue proceeds at a rate similar to that of cocaine hydrolysis. This results in constancy of the ratio of the two substances following extraction even if significant hydrolysis has occurred. Aliquots of a solution containing 100 ng/ml of both compounds in pH 9.5 buffer extracted at 15, 30, 60, 90 and 120 min gave peak height ratios that differed by only ± 2% from the mean, although the decrease in magnitude of peak heights was about 30% over the 2-h period. Consequently, allowing samples to stand in alkaline solution does not lead to serious errors. To obtain maximum sensitivity, however, the samples should be extracted without delay. Previously reported methods for the GC determination of cocaine have utilized either unrelated compounds or the propyl ester analogue (3, Fig. 1) as internal standards [1—3]. These compounds would be expected to be stable or less vulnerable to hydrolysis than cocaine.

Stability of stored plasma samples

Due to the hydrolytic lability of cocaine, special precautions were necessary for sample storage. Blood was drawn into cold tubes containing sodium fluoride to inhibit hydrolysis catalyzed by pseudocholine esterases [5, 12] and immediately centrifuged in the cold to separate the plasma. Samples were stored frozen at −10° C until analyzed. To check stability, split samples were reanalyzed at intervals from one week to three months. In addition, a batch of plasma was spiked with cocaine, stored frozen, and analyzed on successive runs as a control. The data (Table I) indicated that losses are insignificant for storage periods as long as one year, if the samples are kept frozen. We observed that a sample repeatedly thawed and frozen over a period of several weeks lost significant amounts of cocaine.

Extraction

The extraction scheme simplifies the handling of large numbers of samples to make efficient use of the automatic sampler. Organic solvents liquid at −80° C facilitate separation of aqueous layers by freezing in a dry ice—acetone bath. A multi-tube vortex mixer allowed the simultaneous extraction of 24 samples. Using this simple extraction procedure (Fig. 2), one technician can extract as many as 60 samples in a day.

Gas chromatography

The GC analysis was carried out on a 1.8-m column packed with 3% OV-101 and 0.1% KOH on 100—120 mesh Chromosorb W. Baseline separation of cocaine and the internal standard was readily achieved, and co-extracted

TABLE I

STABILITY OF COCAINE IN FROZEN PLASMA

Sodium fluoride added to plasma (10 mg/ml) prior to addition of cocaine. Set 1 was outdated plasma from a local blood bank; sets 2, 3 and 4 were freshly prepared plasma specimens from three drug-free volunteers.

	Set 1	Set 2	Set 3	Set 4
Concentration given (ng/ml)	70.0	85.0	85.0	85.0
Concentration found, mean	70.2	88.9	84.3	81.4
Number of samples	8	6	6	5
Standard deviation	6.68	5.30	10.4	5.20
Coefficient of variation	9.5	6.0	12.3	6.4
Time period* (month)	18	2	2	2

*Time between spiking and analysis of last sample. All samples within a given set were analyzed on different days.

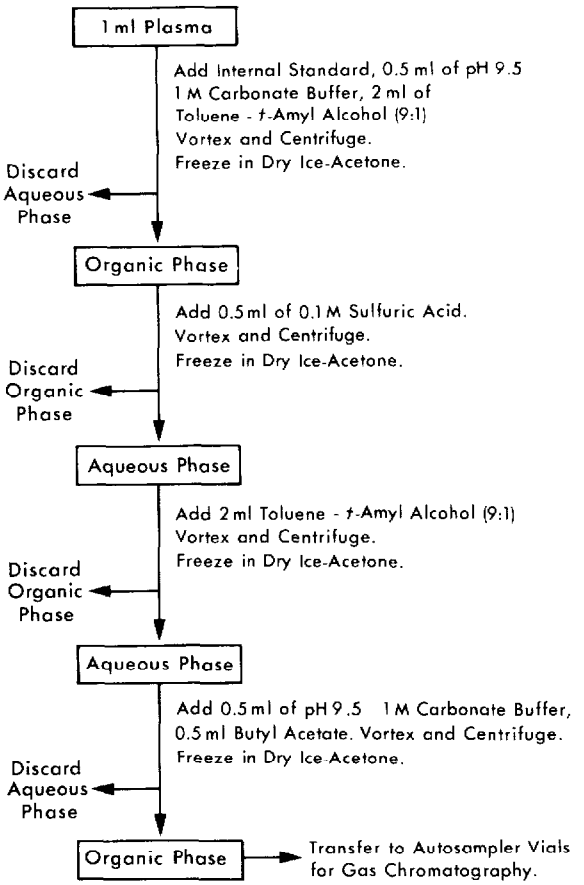


Fig. 2. Flow diagram of the extraction procedure.

endogenous substances did not lead to interfering peaks (Fig. 3). The cleanness of the extracts allowed injection of samples at short intervals (run time = 5 min), and automated analysis could proceed unattended. Some care was necessary to avoid extraneous peaks from solvent impurities or contaminated glassware. An occasional bottle of butyl acetate contained a substance with a retention time similar to cocaine. Consequently, all solvents were checked by GC before using. The caps for autosampler vials were another source of interfering peaks, presumably derived from the PTFE-lined rubber septa. This problem was solved by using a high boiling solvent (butyl acetate, b.p. 127° C) to minimize evaporation and by leaving the vials uncapped. All glassware was meticulously acid-washed including several rinses with distilled water prior to oven-drying at 80° C.

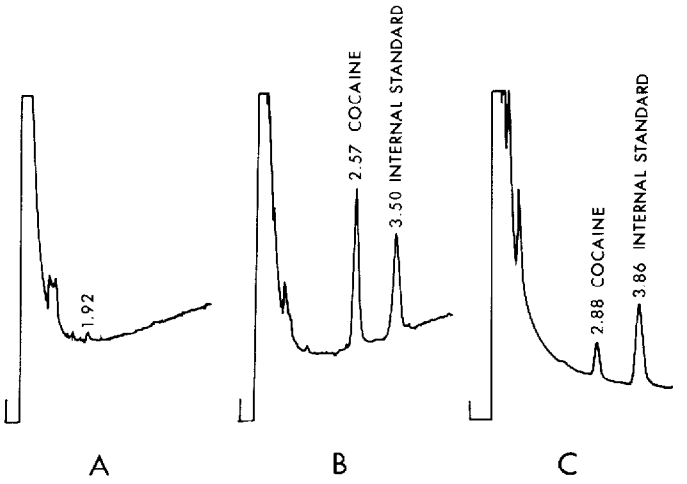


Fig. 3. Gas chromatograms obtained from extracts of human plasma. (A) Extract of drug-free plasma; (B) extract of plasma spiked with 100 ng/ml cocaine and 100 ng/ml internal standard; (C) extract of plasma containing 30 ng/ml cocaine from a subject following intravenous injection.

Initially, we encountered difficulty with losses due to adsorption on the surfaces of the borosilicate glass autosampler vials. After standing several hours, the peak heights for both cocaine and the internal standard were greatly diminished when the sample was reinjected. We had previously encountered a similar problem with nicotine [14], and found that the addition of basic substances (ammonia or triethylamine) or base-washing the vials helped somewhat, but did not eliminate the problem. On the assumption that metal ions on the glass surface could form a complex with tertiary amines, such as nicotine or cocaine, we added a final rinse with a chelating agent, 0.1% aqueous ethylenediamine tetraacetic acid tetrasodium salt (EDTA). This resulted in a two-fold increase in peak heights compared to identical samples in untreated vials. Reinjection of samples after 24 h resulted in no significant change in peak heights, which meant that samples could be run overnight on the autosampler.

Precision and sensitivity

Within-run precision was determined by running in duplicate randomly chosen samples from clinical studies. All samples were given code numbers. The person carrying out the extraction and GC had no knowledge of which samples were duplicates. As can be seen from Table II, duplicates agreed quite closely, the average variation being < 4%. Between-run precision was determined analogously (Table III).

No difficulty was encountered in measuring cocaine at concentrations as low as 3 ng/ml. Good precision was obtained for duplicates both within-run and between-run for concentrations in the range 0—25 ng/ml.

TABLE II

WITHIN-RUN PRECISION: PERCENT DEVIATION FOR DUPLICATE ANALYSES

Concentration range (ng/ml)	0—25	25—50	50—100	100—200	200—400
Number of samples	7	8	10	26	13
Mean concentration (ng/ml)	14.0	34.6	74.3	156	250
Mean percent deviation*	3.6	2.0	3.0	2.5	2.5

*Computed by determining percent deviation from the mean for each pair, and then averaging the deviations for all pairs within the specified concentration range.

TABLE III

BETWEEN-RUN PRECISION: PERCENT DEVIATION FOR DUPLICATE ANALYSES

Concentration range (ng/ml)	0—25	25—50	50—100	100—200	200—400
Number of samples	16	11	8	11	8
Mean concentration (ng/ml)	12.0	37.3	65.4	143	304
Mean percent deviation*	6.3	7.5	5.8	5.0	2.9

*Calculated as in Table II.

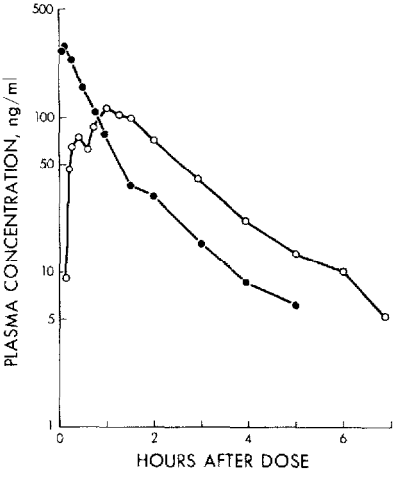


Fig. 4. Plasma cocaine concentrations in human subjects following intravenous administration of 0.4 mg/kg (•, I.H.), and intranasal administration of 2 mg/kg (○, I.P.).

Pharmacokinetic studies

The assay has been in use for over a year in studies of the pharmacokinetics of cocaine in humans. Data from two representative subjects was used to construct semilogarithmic plots of concentration versus time shown in Fig. 4. One subject (I.H.) had received a 0.4 mg/kg intravenous bolus, the other (I.P.) a 2 mg/kg intranasal dose. Readily measurable quantities of cocaine were present at 6 h, about four half-lives in these subjects.

CONCLUSION

A sensitive and precise method for the GC determination of cocaine in plasma has been developed. The operational simplicity of the procedure and use of an automatic sampler make the method well suited for the analysis of large numbers of samples generated in pharmacokinetic studies.

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REFERENCES

1 R.L. Foltz, A.F. Fentiman and R.B. Foltz, NIDA Research Monograph No. 32, U.S. Government Printing Office, Washington, DC, August 1980, p. 90.
2 P.I. Jatlow and D.N. Bailey, Clin. Chem., 21 (1975) 946.
3 B.H. Dvorchik, S.H. Miller and W.P. Graham, J. Chromatogr., 135 (1977) 141.
4 J.I. Javaid, H. Dekirmenjian, E.G. Brunngraber and J.M. Davis, J. Chromatogr., 110 (1975) 141.
5 D.M. Chinn, D.J. Crouch, M.A. Peat, B.S. Finkle and T.A. Jennison, J. Anal. Toxicol., 4 (1980) 37.
6 P.I. Jatlow, C. Van Dyke, P. Barash and R. Byck, J. Chromatogr., 152 (1978) 115.
7 M.A. Evans and J. Morarity, J. Anal. Toxicol., 4 (1980) 19.
8 A.N. Masoud and D.M. Krupski, J. Anal. Toxicol., 4 (1980) 305.
9 S.M. Fletcher and V.S. Hancock, J. Chromatogr., 206 (1981) 193.
10 V.D. Gupta, Int. J. Pharmaceut., 10 (1982) 249.
11 D.J. Stewart, T. Inaba, M. Lucassen and W. Kalow, Clin. Pharmacol. Ther., 25 (1979) 464.
12 D.J. Stewart, T. Inaba, B.K. Tang and W. Kalow, Life Sci., 20 (1977) 1557.
13 H.-L. Schmidt and G. Werner, Justus Liebigs Ann. Chem., 653 (1962) 184.
14 P. Jacob, III, M. Wilson and C. Savanapridi, unpublished results.