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QUANTITATIVE DETERMINATION OF COCAINE AND ITS METABOLITES BENZOYLECGONINE AND ECGONINE BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and reliable method was developed to determine quantitatively cocaine and its metabolites benzoylecgonine and ecgonine. The method involved the formation of fluoro derivatives which were separated on 3 % and 5 % OV-1 columns and detected in picomole quantities using an electron capture detector. Ecgonine and benzoylecgonine were derivatized with a mixture of hexafluoroisopropanol–heptafluorobutyric anhydride (1:2). Cocaine was first reduced by LiAlH$_4$ and then acylated by pentafluoropropionic anhydride. Benzoylecgonine, but not ecgonine, could also be determined by reduction and subsequent acylation. This provided the basis for the determination of cocaine, benzoylecgonine and ecgonine from the same sample. Cocaine could be determined in urine and plasma by this method.

INTRODUCTION

It has been suggested that cocaine is extensively metabolized[1–3]. However, little is known about its absorption, distribution and excretion. It is thought to be metabolized through the hydrolysis of two ester bonds, first to benzoylecgonine and finally to ecgonine. In recent years the emphasis has been on the development of methodology for the detection of cocaine in drug abuse programs for screening urines and, to date, there is no method available to determine cocaine and its metabolites together from the same sample. A number of thin-layer chromatography (TLC) and gas–liquid chromatography (GLC) methods are available for the detection of cocaine or benzoylecgonine[4–10]. However, most of these methods are nonquantitative and are not very sensitive. An enzyme multiplied immunoassay technique[11] (EMIT) has been developed for the detection of urinary benzoylecgonine. Although the method is rapid and generally reliable it does not determine unchanged cocaine and does not differentiate between benzoylecgonine and ecgonine. Also, the method is not applicable to plasma because of high protein concentration and lysozyme activity in plasma. Because of the suspected low amounts of ecgonine there is no report for its determination, although recently Bastos *et al.*[9] have described a TLC method for the detection of ecgonine.

This communication describes a sensitive and reliable GLC method for the quantitative determination of cocaine, benzoylecgonine, and ecgonine and has the potential of being useful in studying the metabolism, distribution and excretion of cocaine.

EXPERIMENTAL

Materials and methods

All chemicals used were reagent grade. Cocaine hydrochloride and nanograde cyclohexane were obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). Benzoylecgonine (Lot No. 81-24-D) and ecgonine (Lot No. 70-220-D) were purchased from Technam (Park Forest South, Ill., U.S.A.). LiAlH$_4$, hexafluoroisopropanol, heptafluorobutyric anhydride and pentafluoropropionic anhydride were from Chemical Procurement Labs. (College Point, N.Y., U.S.A.). All samples were run on a Packard Model 824 gas chromatograph equipped with a ^{3}H-electron capture detector. The derivatives were separated on a coiled column (6 ft. × 2 mm I.D.) packed with 3 % or 5 % OV-1 on 80–100 mesh GHP (Supelco, Bellefonte, Pa., U.S.A.). Nitrogen was used as carrier and purge gas.

Procedures

Determination of cocaine. Cocaine was determined by the method of Blake et al.[10] with a slight modification in chromatographic conditions. Briefly, cocaine hydrochloride solution (0.1–2.0 μg in methanol) was taken in Kimax culture tubes (125 × 6 mm). The methanol was evaporated under nitrogen and 2 ml of cyclohexane added to the tube. After mixing, 50 μl of saturated LiAlH$_4$ solution in ether were added. The solution was mixed and left at room temperature for 10 min. Then 50 μl of water were added to destroy the excess of LiAlH$_4$. The solution was mixed vigorously and 50 μl of pentafluoropropionic anhydride were added. The mixture was mixed thoroughly after 3 min and left at room temperature for another 2 min. Then 6 ml of saturated sodium borate solution were added and the solution was mixed for 4 min on a shaker. The tubes were centrifuged, if necessary, to separate the organic and aqueous phases and 1 μl of the cyclohexane phase was injected. The chromatographic conditions for separation are summarized in Table I.

TABLE I

CHROMATOGRAPHIC CONDITIONS AND RETENTION TIMES FOR COCAINE, ECGONINE AND BENZOYLECGONINE DERIVATIVES

	Reduced cocaine and benzoylecgonine	Ecgonine	Benzoylecgonine
Column temperature*	110°	130°	190°
Injection port temperature	160°	160°	200°
Detector temperature	160°	160°	200°
Gas flow-rate (ml/min)	20	30	50
Range	1 × 10^{-9}	1 × 10^{-9}	1 × 10^{-9}
Retention time (min)**	4.2	12.5	15.6

* The column was operated at isothermal temperatures.
** These retention times are for the 5 % OV-1 column. For the 3 % OV-1 column the retention time for cocaine, ecgonine and benzoylecgonine under the above conditions is 1.8 min, 6.0 min and 5.8 min, respectively.

Determination of ecgonine. Ecgonine solution (0.05–1.0 μg in methanol) was taken in 1.5 ml capacity Miniaktor tubes (Applied Science Labs., State College, Pa., U.S.A.). After the methanol was evaporated under nitrogen, 100 μl of the derivatizing mixture hexafluoroisopropanol–heptafluorobutyric anhydride (1:2, v/v) were added and the tubes were heated for 30 min at 75°. The excess of the reagents was then removed by evaporation under nitrogen until there was no odor of heptafluorobutyric anhydride (3–5 min). Then 1 ml of cyclohexane was added and 1 μl of the sample was injected. The operation conditions for chromatography are given in Table I.

Determination of benzoylecgonine. Benzoylecgonine solution (1–15 μg in methanol) was derivatized exactly as described for ecgonine and determined under the conditions given in Table I.

Benzoylecgonine was also determined after reduction and acylation as in the cocaine determination.

RESULTS AND DISCUSSION

The use of acyl derivatives in GLC has been extensively investigated and is commonly used for the determination of compounds containing hydroxyl groups[12–19]. Although cocaine (benzoyl methyl ecgonine) does not have any free functional groups, recently Blake *et al.*[10] were able to reduce cocaine to 2-hydroxymethyl tropine. The reduced product was then O-acylated by treating with pentafluoropropionic anhydride or heptafluorobutyric anhydride and the derivative was detected by an electron capture detector with greatly increased sensitivity. We have extended this method to determine cocaine quantitatively. Fig. 1B shows a typical chromatogram of reduced cocaine acylated with pentafluoropropionic anhydride. Under the conditions given in Table I a single symmetrical peak was obtained in which the peak height was proportional to the concentration of the sample injected. Fig. 1A is a reagent blank. The arrow points to the position where reduced cocaine was eluted. Fig. 4C shows the linearity of the plot of peak heights with increasing concentrations of cocaine. Since the detector response depends on the specific sensitivity setting, the linearity range should be checked with each instrument. Under the conditions described here 50–750 pg of the injected cocaine derivative gave a linear response. It was found that the best reproducible results were obtained at concentrations lower than 0.5 μg/ml. Irrespective of the original concentration, the sample could be diluted to desired concentration with cyclohexane. The derivatives were stable for more than 12 h. Since cocaine is a benzoylmethyl ester of ecgonine one should be able to reduce both ecgonine and benzoylecgonine to 2-hydroxymethyl tropine. Reduction and subsequent acylation of benzoylecgonine under the conditions used for cocaine resulted in a single symmetrical peak identical to that obtained from cocaine (Fig. 1C). Although the derivative obtained from benzoylecgonine was not identified by mass spectroscopic analysis in these studies, we assume from the gas chromatographic behavior that it was identical to pentafluoropropyl 1,2-difluoro-2-hydroxymethyl tropine obtained from cocaine and identified by mass spectroscopic analysis[10]. This assumption was further supported by the fact that cocaine and benzoylecgonine gave a similar response after reduction and acylation when separated on 3% and 5% OV-1 columns. Also, when equimoles of cocaine and benzoylecgonine were reduced and derivatized
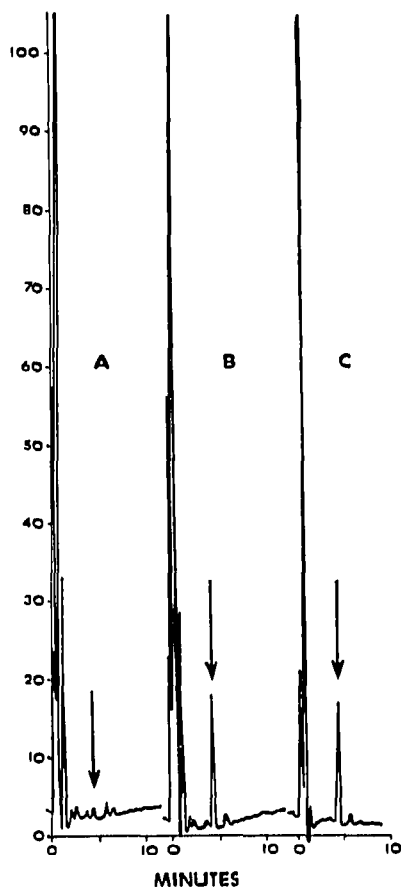
Fig. 1. Typical chromatograms of (A) reduced and acylated reagent blank, (B) reduced and acylated cocaine, (C) reduced and acylated benzoylecgonine. The derivatives were separated on a 5% OV-1 column under the conditions described in Table I.

the response was additive. Ecgonine, however, was not reduced under these conditions. The reason for this is not clear.

For the determination of ecgonine and benzoylecgonine, a variety of derivatization conditions were tried. Optimal derivatization for both was achieved by heating with a mixture of hexafluoroisopropanol–heptafluorobutyric anhydride (1:2, v/v). Fig. 2B shows a single peak obtained from ecgonine and Fig. 3B shows a typical chromatogram of benzoylecgonine. The two were separated under different chromatographic conditions (Table I). The presence of one did not interfere with the analysis of the other. The linearity range for ecgonine and benzoylecgonine under the conditions described here is shown in Figs. 4A and 4B, respectively. Ecgonine gave a linear response from 50–500 pg whereas benzoylecgonine was linear from 1–10 ng. The sensitivity of the method can be increased by increasing the sensitivity of the instrument or by decreasing the final volume of the sample.
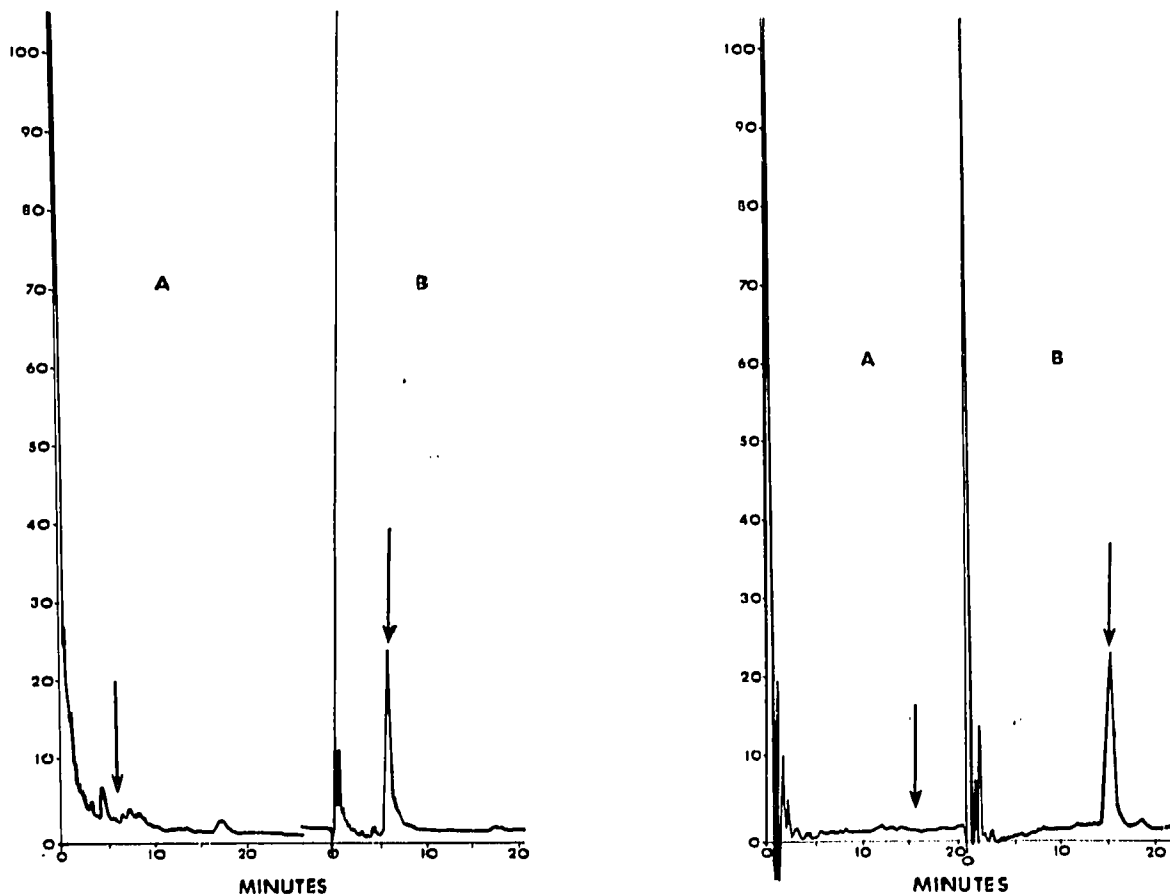
Fig. 2. Typical chromatogram of (A) derivatizing reagent blank (hexafluoroisopropanol–hepta-fluorobutyric anhydride, 1:2), the position of the arrow indicates where ecgonine was eluted; (B) derivatized ecgonine. Column used was 3% OV-1. Chromatographic conditions are given in Table I.

Fig. 3. Typical chromatogram of (A) derivatizing reagent blank, (B) derivatized benzoylecgonine. Column used was 5% OV-1. Chromatographic conditions are given in Table I.

Simultaneous determination of cocaine, ecgonine and benzoylecgonine

The method described here was used to determine cocaine, ecgonine and ben-zoylecgonine in a given sample simultaneously. A methanol solution containing known amounts of cocaine, ecgonine and benzoylecgonine was prepared. An aliquot was taken to determine ecgonine and benzoylecgonine. Since benzoylecgonine has only one free carboxyl group with benzoyl ester at the hydroxyl position, it gave a less volatile fluoro derivative than ecgonine, in which carboxyl as well as hydroxyl groups were acylated. Ecgonine and benzoylecgonine derivatives were separated on 3% and 5% OV-1 columns under different conditions (Table I). At an isothermal temperature of 130°, ecgonine had a retention time of 6.0 min (3% OV-1) whereas benzoylecgonine was eluted after 40 min as a very broad peak. However, at 190° benzoylecgonine gave a narrow, symmetrical peak with a retention time of 5.8 min whereas ecgonine was eluted with the solvent front. Cocaine and benzoylecgonine were determined in
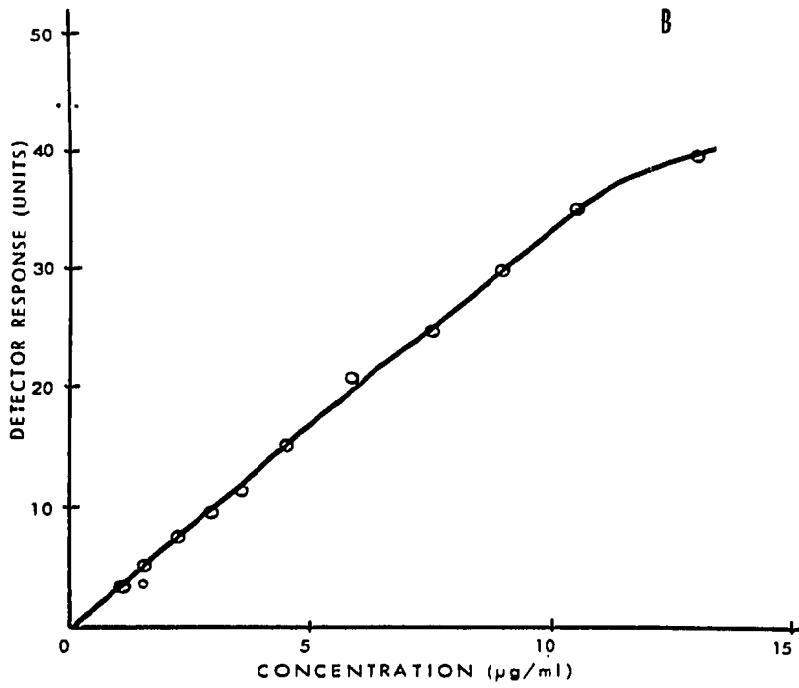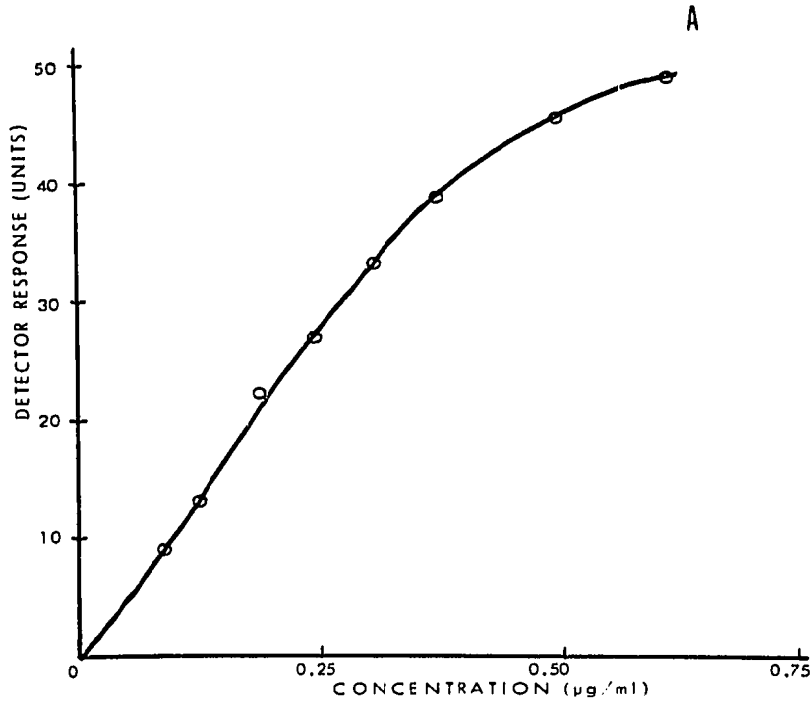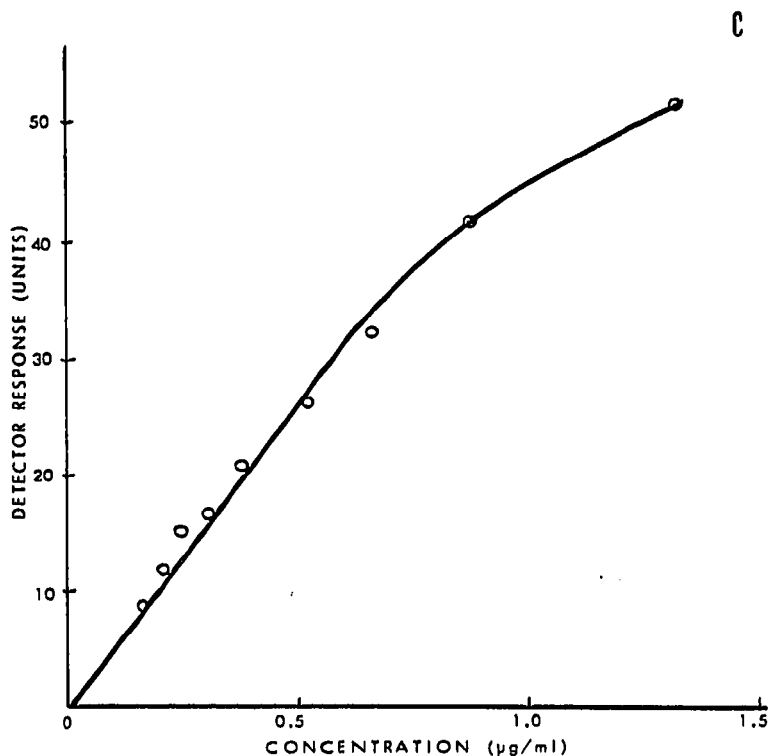
Fig. 4.

C

Fig. 4. Standard curves for (A) derivatized ecgonine, (B) benzoylecgonine, (C) reduced and acylated cocaine.

another aliquot for the sample after reduction and acylation. Knowing the amount of benzoylecgonine the amount of cocaine was calculated. These results are given in Table II. The method appears to be reliable and reproducible.

Extraction of cocaine from urine and plasma

To check whether the method could be extended to biological materials, cocaine was added to normal human urine (0.2 μg/ml urine). 5 ml of the urine were extracted with 2 ml of cyclohexane as described by Blake *et al.*[10] Cocaine was determined after

TABLE II

SIMULTANEOUS DETERMINATION OF COCAINE, ECGONINE AND BENZOYLEC-GONINE IN THE SAME SAMPLE

	Concentration (μg/ml)	
	Calculated	Determined*
Cocaine	0.25	0.21
Ecgonine	0.1	0.12
Benzoylecgonine	3.0	2.9

* These values represent average of three determinations.
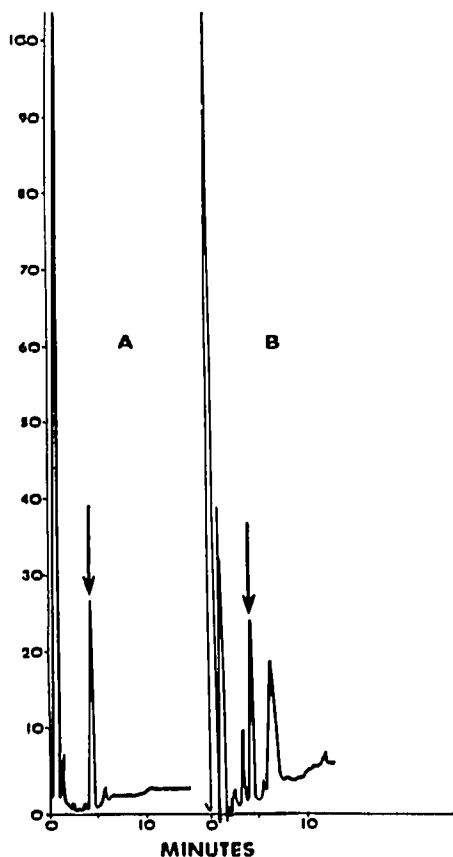
Fig. 5. Chromatograms of (A) cocaine added to normal human urine (0.2 μg/ml) and determined after extraction with cyclohexane as described in the text, (B) cocaine from rat plasma determined after extraction with cyclohexane as described in the text.

reduction. Fig. 5A shows that cocaine gave a symmetrical peak in urine without any interfering peaks. Under these conditions ecgonine and benzoylecgonine were not extracted.

In another experiment a rat was injected with cocaine hydrochloride solution in physiological saline (20 mg per kg body weight). The rat was sacrificed after 15 min and 5 ml of blood were collected in a beaker containing 0.5 ml of 0.15 M EDTA. Blood was centrifuged for 10 min at 1000 g and 0.1 ml of the plasma were diluted to 5 ml with water and extracted with 2 ml of cyclohexane as above. Fig. 5B shows the cocaine peak obtained after reduction and acylation.

These results indicate that the method described here would be useful in the determination of cocaine and its metabolites in biological samples.

ACKNOWLEDGEMENT

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