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DETERMINATION OF BENZOYLECGONINE AND COCAINE IN BIOLOGIC FLUIDS BY AUTOMATED GAS CHROMATOGRAPHY

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SUMMARY

A method is described for extraction of the cocaine metabolite benzoylecgonine, conversion to the butyl ester derivative and gas chromatographic analysis using packed or capillary columns. Using a capillary column, cocaine and benzoylecgonine may be determined simultaneously. The extraction scheme has been designed to facilitate processing of large numbers of samples generated in pharmacokinetic studies. Structural analogues, *m*-toluylecgonine and *m*-toluylecgonine methyl ester, are used as internal standards. Concentrations as low as 10 ng/ml in 1-ml samples of plasma or urine are readily determined. Between-run coefficients of variation were 1.01% for cocaine and 4.18% for benzoylecgonine for concentrations of 75 and 350 ng/ml, respectively.

INTRODUCTION

Cocaine is rapidly metabolized by humans, the elimination half-life in plasma being less than 2 h in most subjects [1, 2]. The two major urinary metabolites reported are benzoylecgonine and ecgonine methyl ester [3–7], although relatively little quantitative data on the metabolites exist. Only a small fraction of ingested cocaine is excreted unchanged [8]. Benzoylecgonine, which results from hydrolysis of the methyl ester group of cocaine, is found in relatively high concentrations in blood and urine of cocaine users. Measurement of benzoylecgonine is of interest because of its potential pharmacologic activity [9] and as a marker for cocaine use in forensic and toxicologic cases [8, 10, 11].

In determining the metabolic profile of cocaine in humans, we required a method for determination of benzoylecgonine in plasma. Gas chromatographic (GC) methods using nitrogen–phosphorus detection [12], electron-capture detection [3, 13] or mass spectrometry (MS) with selected-ion monitoring [11, 14]

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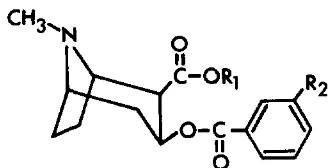

Fig. 1. Structures of cocaine, benzoylecgonine and internal standards. Cocaine, $R_1=CH_3$, $R_2=H$; benzoylecgonine, $R_1=H$, $R_2=H$, *m*-toluylecgonine methyl ester, $R_1=R_2=CH_3$; *m*-toluylecgonine, $R_1=H$, $R_2=CH_3$.

are the most suitable methods for determining benzoylecgonine in plasma. Immunoassay [8, 15, 16] and high-performance liquid chromatographic (HPLC) methods [17, 18] for benzoylecgonine determination have also been reported, but problems with specificity or sensitivity make these methods less attactive than GC methods for determining low plasma concentrations.

This paper describes a new method for benzoylecgonine determination using GC following conversion to the butyl ester derivative. A structural analogue, *m*-toluylecgonine, is used as an internal standard (Fig. 1). Using a capillary column, cocaine and benzoylecgonine may be determined simultaneously. Good precision and limits of quantitation in the low nanogram per milliliter range are obtained for 1-ml plasma samples.

EXPERIMENTAL

Chemicals and reagents

Cocaine hydrochloride was from Mallinckrodt (St. Louis, MO, U.S.A.). Benzoylecgonine tetrahydrate was purchased from Alltech Assoc., Applied Science Labs., or was synthesized in our laboratory. *m*-Toluylecgonine methyl ester (cocaine internal standard) was synthesized as previously described [19] and *m*-toluylecgonine (benzoylecgonine internal standard) was synthesized as described below. Potassium carbonate (anhydrous) was Baker analyzed reagent; sodium chloride was ACS reagent grade and phosphoric acid (85%) was HPLC grade (Fisher). Isoamyl alcohol and chloroform were ACS reagent grade (Fisher); *tert.*-amyl alcohol was Baker analyzed reagent; toluene, isopropyl alcohol and *n*-butyl acetate were glass-distilled HPLC grade from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Each new bottle of solvent was checked by GC for interfering substances. Oxalyl chloride, 98%, was from Aldrich (Milwaukee, WI, U.S.A.). 1-Butanol was reagent grade and kept dry by storing in a glass-stoppered reagent bottle over anhydrous potassium carbonate. The carbonate buffer was prepared by saturating a 1.0 *M* solution of potassium carbonate with sodium chloride, and then adjusting the pH to 8.5 with phosphoric acid.

Instrumentation

GC analyses were performed using a Hewlett-Packard Model 5711A instrument equipped with nitrogen–phosphorus detectors, a 7672 automatic sampler and split–splitless capillary inlet system. Injections were made in the splitless

mode for capillary column analyses, and on-column for packed column analyses. Chromatograms were recorded on a Hewlett-Packard 3390A plotter–integrator. Extractions were carried out with a multi-tube vortex mixer (Kraft Apparatus). A narrow-bore, 12 m×0.20 mm I.D., fused-silica capillary column coated with cross-linked methylsilicone (0.33 μm film thickness) attached to a fused-silica injection port liner was used for the simultaneous analyses of cocaine and benzoylecgonine. For benzoylecgonine determination on a packed column, a 6-m glass column packed with 3% SP2100 DB on Supelcoport (100–120 mesh) (Supelco, Bellefonte, PA, U.S.A.) was used and the oven run isothermally at 250°C. Carrier gas (helium) flow-rates were 1 ml/min for capillary column analyses and 30 ml/min for packed column analyses; the detector make-up gas (helium) flow-rate was 30 ml/min; the detector hydrogen and air flow-rates were 5 and 50 ml/min, respectively. Injection port and detector temperatures were 250 and 300°C, respectively. For benzoylecgonine and cocaine determination on a capillary column, the oven temperature was programmed from 120 to 250°C at 32°/min, and held at 250°C for 4 min. The total run time was 8 min per sample. The automatic sampler parameters were as follows: number of sample pumps, 3; number of sample prewashes, 3; number of solvent postwashes, 4; injection volume, 1 μl. A toluene–isoamyl alcohol (90:10) postwash reduced ghosting of cocaine and of benzoylecgonine to non-detectable levels.

Extraction procedure

All glassware was washed and EDTA-treated as previously described [19]. Aliquots (1 ml) of plasma and 500 ng of *m*-toluylecgonine internal standard (and *m*-toluylecgonine methyl ester internal standard as well if cocaine was to be assayed) in 100 μl of 0.005 *M* sulfuric acid were pipetted into 16×100 mm screw-top glass culture tubes and briefly vortexed. A plasma control was run with every ten samples. Samples were alkalinized with sodium chloride-saturated potassium carbonate buffer, pH 8.5 (0.5 ml, 1 *M*). A 5-ml volume of chloroform–isopropyl alcohol (50:50) was added, the tubes were fitted with PTFE caps and vortex-mixed for 4 min. The tubes were centrifuged at 0°C for 25 min at 800 *g* to break up emulsions. The organic layers (lower) were pipetted into clean 13×100 mm culture tubes and the extracts were evaporated to dryness with a gentle current of air or nitrogen, heating in a warm water-bath (20 min at 60°C). The tubes were removed and allowed to cool. Oxalyl chloride (100 μl) was added to each tube, the samples were vortexed and let stand 10 min. Then 1 ml of 1-butanol was added to each tube. (This step should be performed in a fume hood, since there is a vigorous reaction with evolution of hydrochloric acid). The tubes were capped and heated in a boiling water-bath (90–95°C for 20 min). The samples were cooled, then made alkaline with potassium carbonate (1 ml, 2 *M*). A 2-ml volume of toluene–*tert.*-amyl alcohol (90:10) was added, the tubes were vortex-mixed and centrifuged, then placed in a dry ice–acetone bath to freeze the aqueous layers. The toluene layers were poured into clean tubes containing sulfuric acid (0.5 ml, 0.5 *M*) which were then vortex-mixed, centrifuged and frozen as above. The organic phase was removed and discarded; the acidic aqueous layer was washed with 2 ml of toluene–*tert.* amyl alcohol (90:10). The extraction could be inter-

rupted at this point, if necessary, and the samples stored frozen ($-10°C$). Potassium carbonate (1 ml, 2 *M*) and *n*-butyl acetate (500 μl) were added, the tubes vortex-mixed, centrifuged and frozen. The butyl acetate extracts were poured into EDTA-treated autosampler vials and left uncapped for GC analysis. Baseline separation of cocaine, benzoylecgonine and internal standards was obtained on both packed and capillary columns (see Figs. 3–5). The assay for urine samples was identical, with the exception that 1 ml of a 2 *M* potassium carbonate buffer, 3 ml of chloroform–isopropyl alcohol (50:50) and larger amounts of internal standard (1000 ng) were added.

Calibration procedure

Standard curves, prepared from drug-free plasma samples to which cocaine, benzoylecgonine and internal standards were added and extracted as described above, were linear over the range 0–1000 ng/ml for cocaine and 0–10 000 ng/ml for benzoylecgonine. Standard curves prepared from aqueous standards were found to be identical to those prepared from spiked plasma. Quantitation was based on peak-height ratios of cocaine or benzoylecgonine to internal standard. A standard in the middle of the expected concentration range was re-injected and its concentration was used to calibrate the computing integrator (i.e., a two-point standard curve including the origin) using the internal standard method. A standard curve was constructed with each set of samples analyzed. For plasma cocaine, the standards were 0, 10, 20, 50, 100, 200, 500 and 1000 ng/ml. The equation for a typical cocaine standard curve, determined by linear regression, was $y=0.00349x+0.033919$; $r=0.99917$. For plasma benzoylecgonine, the standards were 0, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/ml. An equation for a typical benzoylecgonine standard curve was $y=0.00465x-0.033916$; $r=0.99959$.

Synthesis of m-toluylecgonine perchlorate

A suspension of ecgonine hydrochloride [20] (0.22 g, 1 mmol) in 20 ml toluene containing *m*-toluic anhydride (0.35 g, 1.4 mmol) and 4-dimethylaminopyridine (0.36 g, 3 mmol) was heated under reflux for 1 h. The mixture was cooled, and enough methanol (ca. 5 ml) was added to bring the solids into solution. This was chromatographed on a silica column (10×1.5 cm) eluting with ethyl acetate–methanol–concentrated ammonia (50:50:2) and collecting fractions of ca. 3 ml. The first eight fractions contained unreacted anhydride and 4-dimethylaminopyridine as shown by thin-layer chromatography (TLC) and were discarded. Fractions 9–18 contained the product and were evaporated using a rotary evaporator to give 50 mg of a light yellow oil. This was taken up in 1 ml isopropyl alcohol, to which two drops of 60% perchloric acid were added. Stirring with a glass rod led to the formation of white solids, which were collected by filtration, washed successively with 1 ml isopropyl alcohol, 1 ml isopropyl alcohol–methyl *tert.*-butyl ether (50:50) and 1 ml of methyl *tert.*-butyl ether, and then air-dried. There was obtained 42 mg of white powder, m.p. (decomposes) 248–250°C. Calculated for $C_{17}H_{22}NO_8Cl$: C, 50.56; H, 5.49; N, 3.47. Found: C, 50.70; H, 5.78; N, 3.31.

RESULTS AND DISCUSSION

A major challenge in benzoylecgonine determination is its extraction from the biological matrix. Being an amphoteric compound, it exists as a zwitterion at neutral pH, is positively charged under acidic conditions and is negatively charged under basic conditions. Consequently, rather polar extracting solvents, such as mixtures of chloroform and ethanol or isopropyl alcohol [3, 14] must be employed. This leads to co-extraction of many endogenous substances which may interfere with the analysis.

Due to its polarity, benzoylecgonine is not volatile enough to be analyzed directly by methods which require vaporization, such as GC or GC–MS. Consequently, a variety of methods have been devised for converting benzoylecgonine to ester derivatives that have sufficient volatility to be analyzed by GC. These include pre-column [14] and on-column alkylation [21] with acetals of dimethylformamide, on-column alkylation with tetraalkyl ammonium salts [22] and esterification with ethanol–sulfuric acid [11], pentafluorobenzyl bromide–pyridine [3] or butyl iodide–trimethylphenyl ammonium hydroxide [23]. Reduction of the carboxyl group to an alcohol followed by derivatization with electron capture-sensitive reagents has also been employed to improve chromatographic properties and sensitivity [13].

Oxalyl chloride is a mild reagent for conversion of carboxylic acids to the corresponding acid chlorides [24]. Since acid chlorides react readily with alcohols to form esters, we anticipated that treatment of benzoylecgonine with oxalyl chloride followed by an alcohol would be a convenient procedure for preparing ester derivatives. Indeed, addition of oxalyl chloride to benzoylecgonine followed by gentle heating with 1-butanol resulted in facile formation of the butyl ester. The reaction proceeds smoothly on nanogram quantities, and the ester derivative can be taken through acid–base partitioning steps to clean up extracts derived from biologic samples.

In any chromatographic method it is desirable to utilize a structural analogue of the analyte as an internal standard, but this is especially so with procedures which require derivatization. If conversion to the derivative is incomplete, a close structural analogue will be converted to the analogous derivative in the same yield as the analyte, and the ratio of analyte to internal standard will remain constant over the course of the analysis. Consequently, we synthesized a benzoylecgonine analogue, *m*-toluylecgonine (Fig. 1), for use as an internal standard. This compound differs from benzoylecgonine only by addition of a methyl group on the benzene ring, which is remote from the carboxyl group and unlikely to affect the rate of derivatization. It should be pointed out that in previous methods for benzoylecgonine determination, unrelated compounds or esters of benzoylecgonine were used as internal standards.

An extraction procedure was developed which is suitable for both plasma and urine specimens (Fig. 2). Benzoylecgonine and internal standard are extracted using a mixture of chloroform–isopropyl alcohol (50:50). Extraction efficiency for cocaine and benzoylecgonine averaged 75% (range 67–80%) and 70% (range 68–74%), respectively, for plasma concentrations of 100–2000 ng/ml. The extract

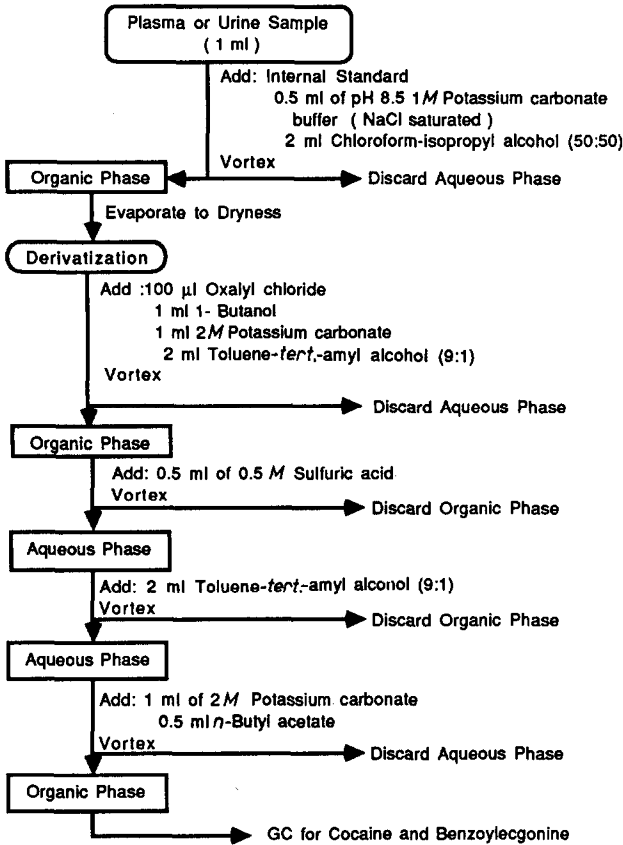

Fig. 2. Flow diagram of the extraction procedure.

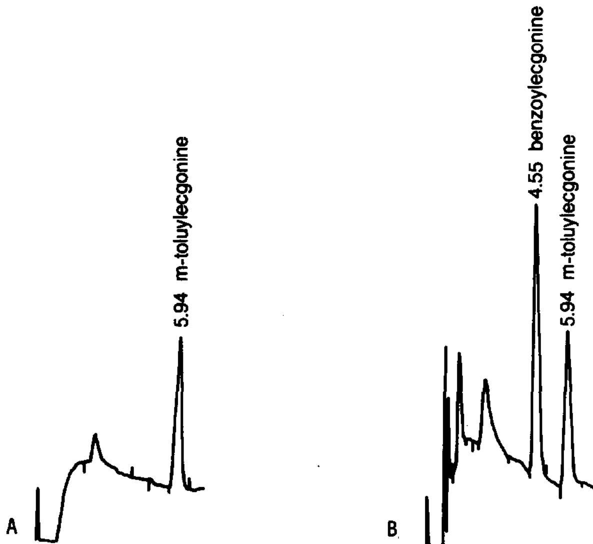

Fig. 3. Packed column gas chromatograms of extracts of human plasma. (A) Extract of drug-free plasma. (B) Extract of plasma from subject following intravenous cocaine, containing 505 ng/ml benzoylecgonine.

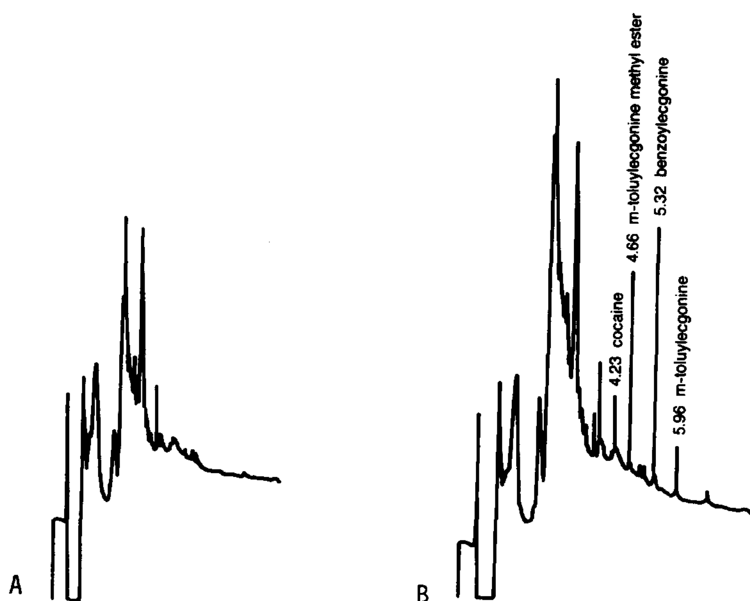

Fig. 4. Capillary column gas chromatogram of extracts of human plasma. (A) Extract of drug-free plasma. (B) Extract of plasma from subject following intravenous cocaine, containing 75 ng/ml cocaine and 899 ng/ml benzoylecgonine. Chart-speed 0.5 cm/min.

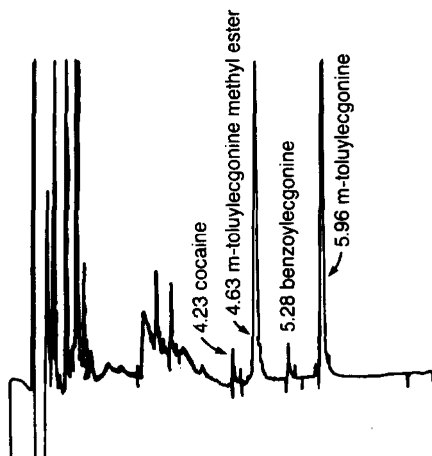

Fig. 5. Capillary column chromatogram of extract of plasma containing 25 ng/ml cocaine and 50 ng/ml benzoylecgonine. Chart-speed 1.0 cm/min.

is evaporated to dryness, and the residue is treated with oxalyl chloride followed by 1-butanol. This results in formation of the butyl ester derivatives, which are lipophilic and readily purified by acid–base solvent partitioning steps. The final extracts are analyzed by GC using either packed (Fig. 3) or capillary (Figs. 4 and 5) columns.

Due to the mildness of the derivatization procedure, cocaine present in the extracts remains intact. Control experiments have shown that negligible benzoyl-

TABLE I

BETWEEN-RUN PRECISION FOR SPIKED PLASMA SAMPLES

Compound	Concentration spiked (ng/ml)	*n*	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
Packed column				
Cocaine*	75	10	73.9 ± 6.64	1.41
Benzoylecgonine	350	10	335 ± 14.5	4.60
Capillary column				
Cocaine	75	10	74.2 ± 4.20	1.08
Benzoylecgonine	350	10	336 ± 11.5	4.18

*Analyzed by the method described in ref. 19.

TABLE II

WITHIN-RUN PRECISION FOR CAPILLARY COLUMN DETERMINATIONS: PERCENTAGE DEVIATION FOR DUPLICATE ANALYSES

Compound	Concentration range (ng/ml)	*n*	Mean concentration (ng/ml)	Mean percentage deviation*
Cocaine	11–50	10	22	1.22
	50–100	8	70	1.61
	100–200	10	165	1.15
	200–500	10	390	0.92
Benzoylecgonine	96–200	6	146	1.38
	200–500	6	383	1.82
	500–2000	10	1240	1.04
	2000–6000	10	3480	1.12

*Calculated as in Table II of ref. 19.

TABLE III

WITHIN-RUN PRECISION AND ANALYTICAL RECOVERY FOR SPIKED PLASMA SAMPLES WITH CAPILLARY COLUMN DETERMINATION

Compound	Concentration added (ng/ml)	*n*	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Recovery (%)
Cocaine	25	8	25.2 ± 1.3	5.0	101
Benzoylecgonine	50	8	49.4 ± 2.4	4.9	99

ecgonine is generated when samples containing cocaine only are carried through the extraction and derivatization procedures. Consequently, benzoylecgonine and cocaine may be determined simultaneously. A capillary column is advantageous for the simultaneous determination, since co-extracted endogenous substances

TABLE IV

WITHIN-RUN PRECISION FOR PACKED COLUMN DETERMINATIONS: PERCENTAGE DEVIATION FOR DUPLICATE ANALYSES

Compound	Concentration range (ng/ml)	*n*	Mean concentration (ng/ml)	Mean percentage deviation★
Cocaine★★	11-50	8	24	2.30
	50-100	8	71	1.91
	100-200	16	169	1.5
	200-500	14	306	1.9
Benzoylecgonine	70-200	6	147	1.67
	200-400	6	248	1.08
	400-1000	10	619	2.6

★Calculated as in Table II of ref. 19.
★★Analyses carried out as described in ref. 19.

interfere with the determination of low concentrations of cocaine if packed columns are used. We utilize a separate internal standard for cocaine, *m*-toluylecgonine methyl ester, as described in a previous publication [19].

The method has good precision and accuracy (Tables I–IV). Using 1-ml plasma or urine samples, concentrations as low as 10 ng/ml are readily determined. As little as 2 ng/ml cocaine and 5 ng/ml benzoylecgonine can be detected. Standard curves were linear over the range of concentrations expected in plasma of cocaine users: from 10 ng/ml to greater than 2000 ng/ml for cocaine and from 10 ng/ml to 10 000 ng/ml for benzoylecgonine. A gas chromatograph with an automatic sampler and computing integrator allows up to 100 samples to be analyzed in one 24-h period.

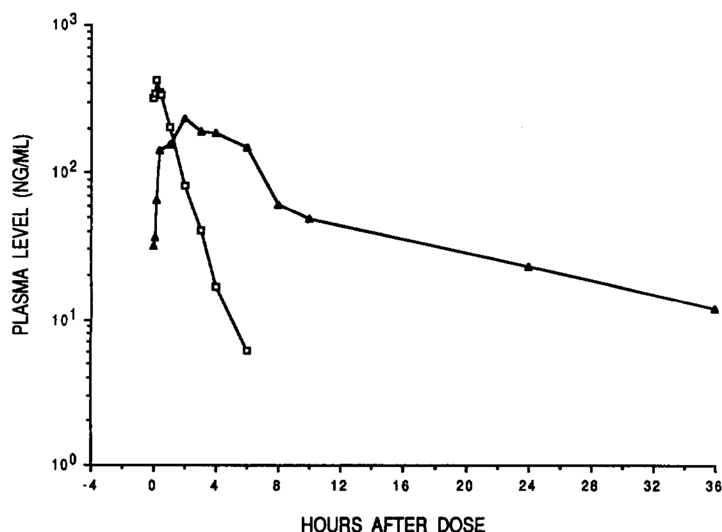

Fig. 6. Benzoylecgonine (△) and cocaine (□) concentrations in plasma following intravenous administration of 0.6 mg/kg cocaine to a human subject.

The method has been in use for over two years in studies on the pharmacokinetics of benzoylecgonine in humans. Well over 1000 plasma and urine samples have been analyzed. Data from a representative subject were used to construct a semilogarithmic plot of concentration versus time shown in Fig. 6. Benzoylecgonine is eliminated much more slowly than cocaine, and using our method it may be readily determined in plasma for 24 h or more following a modest dose of cocaine.

In conclusion, a method for benzoylecgonine determination has been developed which is sensitive, has good precision and is convenient for analysis of large numbers of samples generated in pharmacokinetic studies. It is the first method to use a structural analogue of benzoylecgonine as an internal standard, which we feel is a significant improvement over previously reported methods.

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