Considering this discussion and the results, it is possible to formulate a liquid dosage form with limited stability containing sorbitol, alcohol (10–20% v/v), and preservatives (methylparaben and propylparaben). The pH should be adjusted to at least 8.5 with sodium hydroxide in water. The shelflife of the dosage form may be extended for more than 1 year by adding an overage of about 5% of furosemide.

The data were not treated mathematically because the pH values were not constant (Table III) because of the hydrolysis of sugar. The commercial vehicle also contained high concentration of sugar. No attempt was made to buffer the dosage forms heavily to prevent pH changes. If a biologically safe buffer can be used to maintain the pH value at around 8.5, it would certainly stabilize the dosage form further.

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Quantitation of Cocaine and Its Principal Metabolite, Benzoylecgonine, by GLC–Mass Spectrometry Using Stable Isotope Labeled Analogs as Internal Standards

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Abstract □ A quantitative GLC–mass spectrometric assay was developed for the determination of cocaine and its principal metabolite, benzoylecgonine, in human urine. The assay utilizes selective ion focusing to monitor in a GLC effluent the molecular ions of cocaine and benzoylecgonine generated by electron-impact ionization. Cocaine-d_3 and benzoylecgonine-d_3 were the internal standards. The assay can measure 2 ng of cocaine/ml and 5 ng of benzoylecgonine/ml with about 5% precision. The curves relating the amounts of cocaine and benzoylecgonine added *versus* the amounts found over a large range of cocaine and benzoylecgonine concentrations were straight lines with nearly zero intercepts and slopes of 0.98 ± 0.01 and 0.97 ± 0.01, respectively. The method was used for the analysis of urinary cocaine and benzoylecgonine in cocaine addicts. Assay specificity was confirmed by complete identity of the mass spectra of cocaine and benzoylecgonine with those of authentic materials.

Keyphrases □ Cocaine—GLC–mass spectrometric analysis in human urine □ Benzoylecgonine—GLC–mass spectrometric analysis in human urine □ GLC–mass spectrometry—analyses, cocaine and benzoylecgonine in human urine □ Narcotics—cocaine and benzoylecgonine, GLC–mass spectrometric analyses in human urine

Cocaine abuse (1–5) has prompted considerable interest in methods for the detection and quantitation of the parent drug and its metabolites in biological fluids and tissues. TLC and the enzyme multiplied immunoassay technique (EMIT) are the most frequently employed screening methods for the detection of cocaine in human urine. However, these methods are inherently only semiquantitative at best. Although cocaine may be determined by GLC, the amount of unchanged cocaine excreted in human urine (2, 6) is generally below the method's limits of detection (7, 8).

BACKGROUND

A rather sensitive GLC procedure (9) for cocaine is based on the reduction of cocaine with lithium aluminum hydride, derivatization of the reduced product with pentafluoropropionic anhydride, and subsequent detection by electron capture. This method is sensitive but unspecific, and it is not applicable to cocaine in biological specimens. Besides cocaine, benzoylecgonine, ecgonine, and possibly other metabolites (10) of cocaine are converted by the procedure (9) to the same derivative. Therefore, the GLC-measured cocaine concentration cannot be the true concentration of cocaine alone.

Recently, a GLC procedure for cocaine and benzoylecgonine in the urine of cocaine users was reported (11). The method involves methylation of urinary benzoylecgonine to cocaine by treatment with diazomethane, thereby measuring combined benzoylecgonine and cocaine. Original cocaine was determined in a separate extract without the methylation step. Thus, benzoylecgonine could be determined by difference. Cocaine is metabolized in the human body rather rapidly (12), benzoylecgonine being the major metabolite (13, 14). Consequently, most urine samples of cocaine users contain trace amounts of unchanged drug and large concentrations of benzoylecgonine. Furthermore, cocaine and benzoylecgonine have rather widely different and variable extraction efficiencies, and one ends up dealing with differences of rather large and very small numbers. As a result, this assay is of limited usefulness.

This paper describes a GLC–mass spectrometric assay of cocaine and benzoylecgonine in human urine. Selective ion monitoring, the technique built on combined GLC–mass spectrometry with selective focusing on suitable fragments of the molecular ion (mass fragmentography) or the molecular ion itself, is a well-established technique, used widely in pharmacology (15, 16). This technique was used to develop a sensitive and specific assay for cocaine and benzoylecgonine in human urine with site-specific deuterium-labeled cocaine and benzoylecgonine as internal standards.

EXPERIMENTAL

Materials—Analytical grade cocaine hydrochloride[1], benzoylecgonine[2], norcocaine hydrochloride[3], methyl iodide-d_3[4], and *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine[5] were used without further purification. All solvents were analytical grade[6]. Silanized tubes[7] (10 ml) with screw caps[8] were used for urine extraction; final solvent evaporation was performed in 5-ml glass-stoppered centrifuge tubes[9]. Pasteur pipets with hand-

[1] Mallinckrodt Chemical Works, New York, N.Y.
[2] Techman Inc., Park Forest South, Ill.
[3] Norcocaine hydrochloride was a gift from Dr. Sally, Chemistry Department, University of Wisconsin.
[4] International Chemical & Nuclear Corp., Irvine, Calif.
[5] Pfaltz & Bauer, Flushing, N.Y.
[6] Fisher Scientific Co., Pittsburgh, Pa.
[7] Kimble, Owens-Illinois, Toledo, Ohio.
[8] Lined with Teflon (DuPont).
[9] Pyrex 8084.

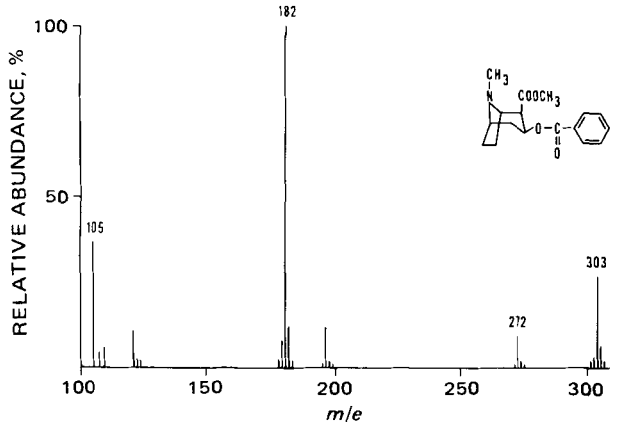


Figure 1—*Electron ionization (70 ev) mass spectrum of cocaine.*

drawn constricted tips were utilized for all solution transfers. Urine samples of cocaine addicts[10] were processed as soon as obtained.

Cocaine-d_3 (*N*-methyl-d_3) was synthesized by treatment of norcocaine with methyl iodide-d_3 using an established procedure for the alkylation of secondary amines (17). Benzoylecgonine-d_3 (*N*-methyl-d_3) was prepared from the labeled cocaine using a known procedure (18) for the synthesis of unlabeled benzoylecgonine from cocaine. Both labeled compounds showed satisfactory mass spectral (electron-impact ionization) characteristics. A selective ion detection analysis of cocaine-d_3 showed the presence of an ion equivalent to 97 ± 0.15% (n = 5) cocaine-d_3. A similar analysis of benzoylecgonine-d_3 showed the presence of an ion equivalent to 96 ± 0.16% (n = 5) benzoylecgonine-d_3.

Extraction of Cocaine and Benzoylecgonine from Urine—*Cocaine*—To 3 ml of urine was added an appropriate amount of cocaine-d_3 (typically 46.7 ng/ml) as an internal standard. The urine was adjusted to pH 9 with 1 *N* NH_4OH and extracted twice with 5 ml of chloroform. The organic fractions were combined, 1 ml of 0.1 *N* HCl was added, and the solution was shaken for 15 min. The organic layer was discarded, and the aqueous phase was adjusted to pH 9 with 1 *N* NH_4OH and extracted twice with 4 ml of chloroform. The organic fractions were combined and dried, and the solvent was evaporated at 40° under a gentle stream of nitrogen. Recovery of cocaine added to control human urine was studied at the 10-ng/ml level.

Benzoylecgonine—To 1 ml of urine was added an appropriate amount of benzoylecgonine-d_3 (typically 120 ng/ml) as an internal standard. The urine was adjusted to pH 9 with 0.1 *N* NH_4OH and extracted twice with 5 ml of chloroform–2-propanol (4:1 v/v). The organic fraction was dried with sodium sulfate and filtered, and the solvent was removed at 60° under a gentle stream of nitrogen. Recovery of benzoylecgonine added to control human urine was studied at the 50-ng/ml level.

Formation of Derivatives—Cocaine is thermally stable, has enough vapor pressure, and can be gas chromatographed as such. Consequently, the dried urinary extract was reconstituted in 50 μl of benzene, and approximately 1 μl was injected into the GLC–mass spectrometer for mass fragmentographic assay.

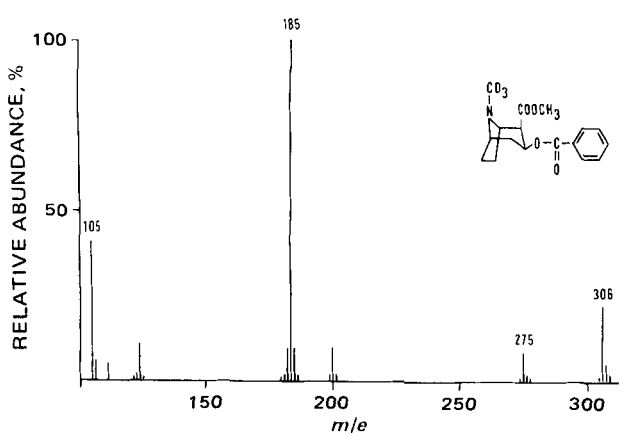


Figure 2—*Electron ionization (70 ev) mass spectrum of cocaine-*d_3.

[10] Aliquots (20 ml) of urine samples of cocaine addicts from metropolitan New York City were obtained from the New York State Narcotic Addiction Control Commission, Testing and Research Laboratory, Brooklyn, N.Y.

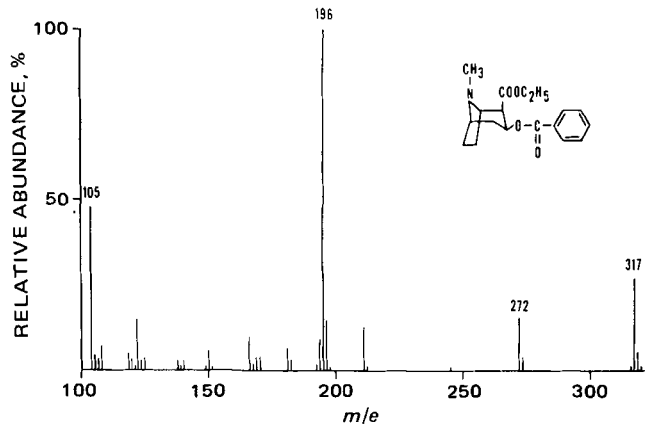


Figure 3—*Electron ionization (70 ev) mass spectrum of benzoylecgonine ethyl ester.*

Benzoylecgonine in the urinary extract was converted to the corresponding ethyl ester by treatment with diazoethane (19). In a typical experiment, potassium hydroxide (1 g) was dissolved in 2 ml of water and 5 ml of ether was added. The biphasic solution was stored in the freezer at −20° for 1 hr. Then *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine (~2 mg) was added, and the reaction mixture was placed in the freezer. In a few minutes, the ether phase turned pale yellow.

Benzoylecgonine from the urinary extract was taken up in ethanol (200 μl) and cooled to −20°, and 250 μl of pale-yellow ether solution containing diazoethane was added. The solution was stored in the freezer for 15 min. The material was removed from the freezer and warmed to room temperature, and the solvents were removed under a gentle stream of nitrogen at 40°. The residue was taken up in 5 ml of chloroform, 1 ml of 0.1 *N* HCl was added, and the mixture was shaken for 15 min and centrifuged.

The organic phase was discarded, the aqueous layer was adjusted to pH 9 with 0.1 *N* NH_4OH, 5 ml of benzene was added, and the solution was shaken for 15 min and centrifuged. The benzene solution was dried with sodium sulfate and filtered, benzene was removed at 40° under a stream of nitrogen, and the residue was reconstituted in 50 μl of benzene. An aliquot (1 μl) of this solution was injected into the GLC–mass spectrometer for the mass fragmentographic assay.

Instrumentation—The magnetic sector, single-focusing mass spectrometer[11] was interfaced with a gas chromatograph and equipped with a multiple ion detector/peak matcher accessory (MID/PM) (15, 16). GLC was performed on a 1.8-m glass column (2 mm i.d.) silanized with 5% dimethyldichlorosilane in toluene and packed with 1.5% OV-1 on 100–200-mesh Gas Chrom Q. The column was conditioned for 24 hr at 280° with a flow rate of 20 ml of helium/min. The column temperature was 205°, the flash heater was at at 230°, the separator was at 235°, and the ion source was at 250°.

The accelerating voltage was 3.5 kv in the scan mode and 3.0 kv in the

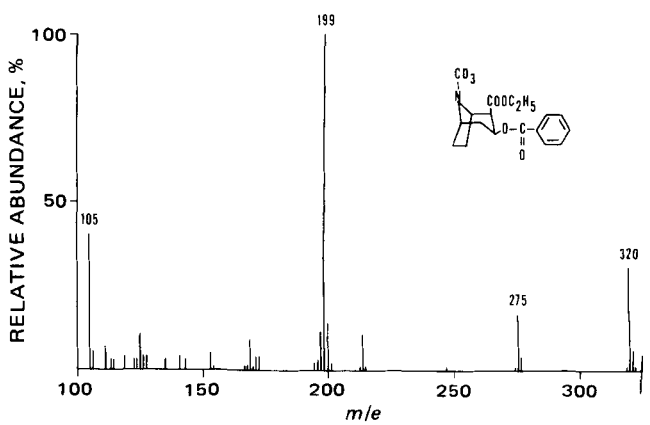
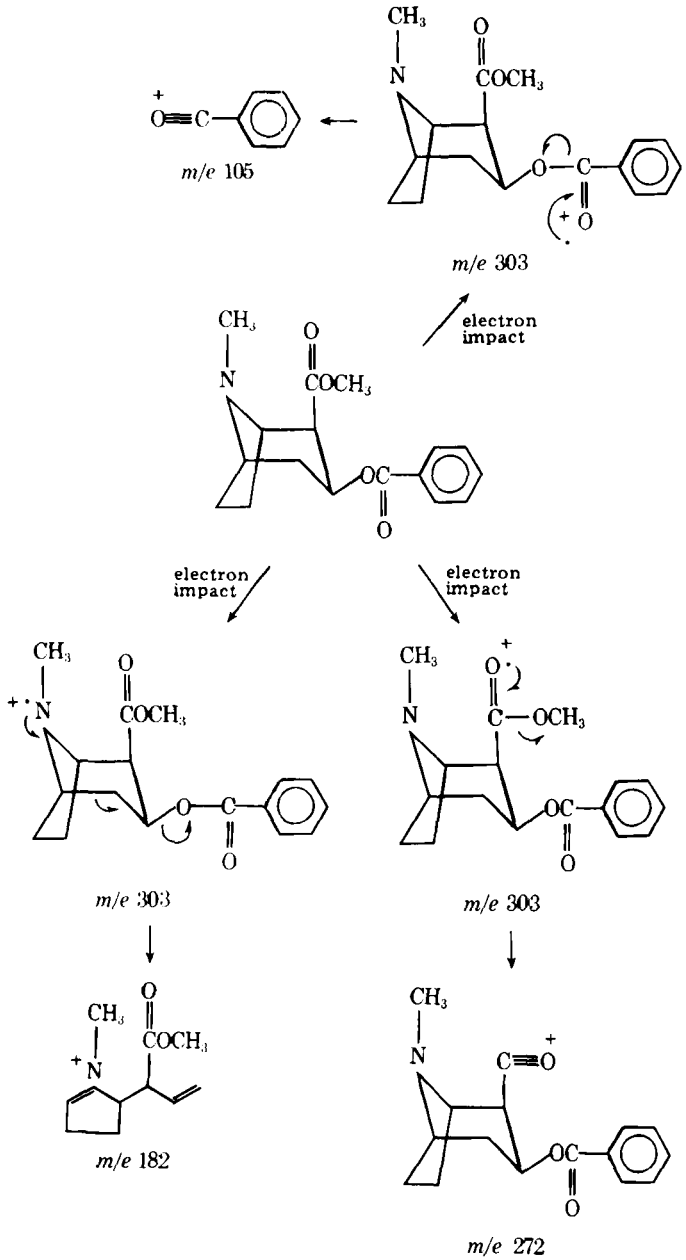


Figure 4—*Electron ionization (70 ev) mass spectrum of benzoylecgonine ethyl ester-*d_3.

[11] LKB 9000, LKB, Stockholm, Sweden.



Scheme I

multiple ion detector mode, the ionization potential was 70 ev, and the trap current was set at 60 μamp. The magnetic field was kept constant by focusing the background ion (column bleed) at *m/e* 281, and the additional voltages were 245, 213, 102, and 73 v for measuring ion intensities at *m/e* 303, 306, 317, and 320, respectively. The retention times of cocaine and benzoylecgonine ethyl ester were 2.5 and 3.5 min, respectively.

RESULTS AND DISCUSSION

The mass spectrum of cocaine (Fig. 1) showed a molecular ion at *m/e* 303, a base peak at *m/e* 182 ($M - C_6H_5CO_2$), and another intense peak at *m/e* 105 (benzoyl cation). The fragmentation pattern depicted in Scheme I was readily discernible. The molecular ion formed either by loss of a lone-pair electron from a nitrogen atom or a lone-pair electron from an oxygen atom underwent energetically favorable fragmentation (20–22) to give the observed ions.

The mass spectrum of cocaine-d_3 (Fig. 2) showed an M^+ at *m/e* 306 and was similar to that of cocaine. Most ions were shifted to a higher mass by 3 amu, except the benzoyl cation, *m/e* 105, which was a common fragment ion from both cocaine and cocaine-d_3. The mass spectrum of benzoylecgonine ethyl ester (Fig. 3) showed an M^+ at *m/e* 317, and the fragmentation pattern was rather similar to that of cocaine. The spectrum of benzoylecgonine ethyl ester-d_3 (Fig. 4) was similar to that of the unlabeled isomer, the appropriate ions being shifted to a higher mass by 3 amu.

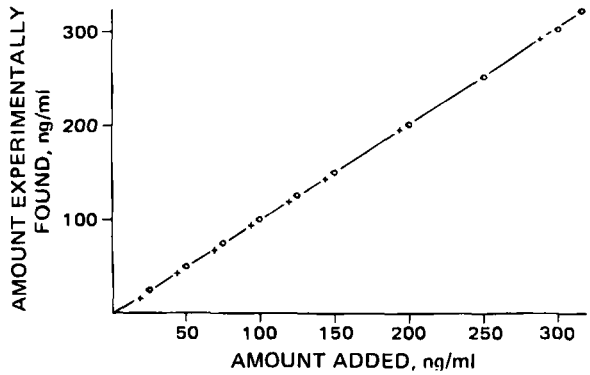


Figure 5—*Curve relating amounts of cocaine (0–255 ng) (+) and benzoylecgonine (0–321 ng) (O) added to control urine* versus *the amounts found using the method described. For these samples, 46.7 ng of cocaine-*d_3*/ml and 121 ng of benzoylecgonine-*d_3*/ml were added as internal standards.*

Control human urine samples subjected to the described procedure for cocaine and benzoylecgonine analysis showed no significant background ions at *m/e* 303, 306, 317, and 320. Known amounts of cocaine and benzoylecgonine, along with their isotopic analogs in fixed amounts, were added to control urine and processed as described. Cocaine and benzoylecgonine were quantitated from the ratios of ion intensities at *m/e* 303/306 and 317/320, respectively. Analysis of cocaine data (Fig. 5) gave a slope of 0.98 ± 0.01 and an intercept of 0.18 ± 0.2 ng. Similarly, benzoylecgonine data (Fig. 5) gave a slope of 0.97 ± 0.01 and an intercept of 0.3 ± 0.2 ng. These data affirm a simple linear relationship between the appropriate ion intensity ratios and concentrations of cocaine and benzoylecgonine and exclude any isotope exchange or any significant kinetic isotope effect in the fragmentation process.

Five samples containing 10 ng of cocaine/ml and 50 ng of benzoylecgonine/ml were analyzed using 7.5 ng of cocaine-d_3/ml and 19.5 ng of benzoylecgonine-d_3/ml as internal standards. The results for these samples were 9.7 ± 0.3 ng/ml for cocaine and 49.0 ± 3.0 ng/ml for benzoylecgonine. These samples were assayed in duplicate; in this set, exactly the same amounts were taken as before but the internal standards were added after the extraction. The recoveries for these samples, based on comparison of the ion intensity ratios of the two sets, were 80 ± 9% for cocaine (*m/e* 303/306) and 65 ± 12% for benzoylecgonine (*m/e* 317/320). The wide range of recoveries observed is expected in trace analysis and is attributed to variable glassware and GLC column adsorption.

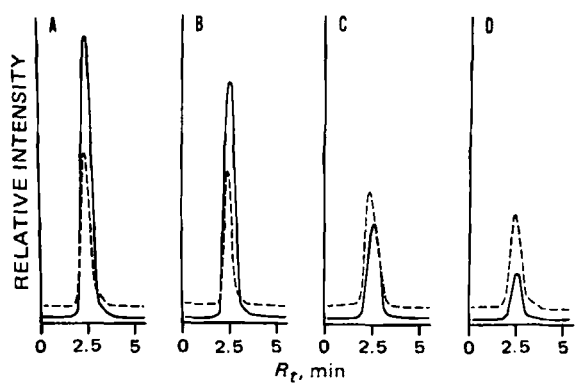


Figure 6—*Selective ion chromatograms for cocaine* (m/e *303*) (—) *along with cocaine-*d_3 (m/e *306*) (- - -). *A: Urine obtained from Subject 1. The internal standard cocaine-*d_3 (m/e *306*) *concentration was 46.7 ng/ml. The cocaine concentration was found to be 91 ng/ml. B: Urine obtained from Subject 2. The internal standard cocaine-*d_3 (m/e *306*) *concentration was 46.7 ng/ml, and the cocaine concentration was found to be 75 ng/ml. C: Urine obtained from Subject 3. The internal standard cocaine-*d_3 (m/e *306*) *concentration was 46.7 ng/ml, and the cocaine concentration was found to be 42 ng/ml. D: Urine obtained from Subject 4. The internal standard cocaine-*d_3 (m/e *306*) *concentration was 5.1 ng/ml, and the cocaine concentration was found to be 2.2 ng/ml.*

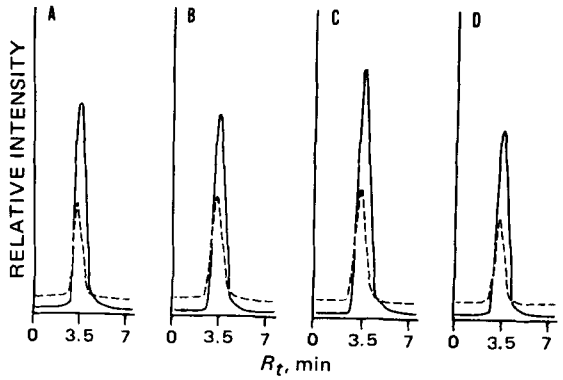


Figure 7—*Selective ion chromatograms for benzoylecgonine ethyl ester* (m/e *317*) (—) *along with benzoylecgonine ethyl ester-*d_3 (m/e *320*) (- - -). *In each sample, 750 ng of deuterated analog/ml was added. A: Urine obtained from Subject 1. Benzoylecgonine* (m/e *317*) *was found to be 1.60 μg/ml. B: Urine obtained from Subject 2. Benzoylecgonine* (m/e *317*) *was found to be 1.50 μg/ml. C: Urine obtained from Subject 3. Benzoylecgonine* (m/e *317*) *was found to be 1.80 μg/ml. D: Urine obtained from Subject 4. Benzoylecgonine* (m/e *317*) *was found to be 1.80 μg/ml.*

Several urine samples of cocaine addicts were assayed for cocaine and benzoylecgonine. Figures 6 and 7 illustrate the mass chromatograms at *m/e* 303–306 (cocaine–cocaine-d_3) and 317–320 (benzoylecgonine–benzoylecgonine-d_3), respectively. The urinary analyses of cocaine and benzoylecgonine of some cocaine addicts are presented in Table I.

The assay for cocaine and its principal metabolite presented here is sensitive, specific, and applicable to other body fluids and tissues. The sensitivity of the assay, being a function of extraction efficiencies, GLC column conditions, and the ion source, cannot be quoted in absolute terms. With near zero leak current in the ion source, clean and freshly silanized GLC columns and glassware, and better than 50% recoveries, an assay sensitivity of approximately 2 ng of cocaine/ml and 5 ng of benzoylecgonine/ml is possible.

The results from the control human urine show good specificity of the assay; however, the observed specificity from control human urine cannot be extrapolated to the urinary analysis of cocaine addicts. Recently, several illicit cocaine samples were found to contain significant amounts of lidocaine, procaine, heroin, and aspirin (23). These materials, if present in the urine, might interfere in current screening methods for cocaine analysis. None of these mentioned drugs shows significant response at *m/e* 303–320 on electron-impact ionization at the retention time of materials of interest (24); therefore, these contaminants can be safely ignored.

The mass chromatogram obtained from biological extracts (Figs. 6 and 7) showed clean and symmetrical peaks; furthermore, these biological extracts were pure enough so that the entire mass spectra of cocaine and

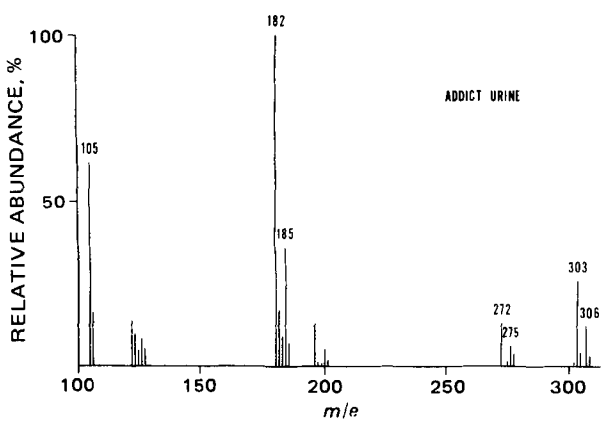


Figure 8—*Mass spectrum of cocaine along with the added internal standard cocaine-*d_3 *from the urine extract of Subject 1.*

Table I—Analyses of Cocaine and Benzoylecgonine in Urine of Cocaine Addicts [a]

Subject	Cocaine Found, ng/ml	Benzoylecgonine Found, μg/ml
1	91 ± 1	1.60 ± 0.02
2	76 ± 0.5	1.50 ± 0.02
3	42 ± 0.4	1.80 ± 0.03
4	2 ± 0.21	1.80 ± 0.022

[a] Analysis of duplicate runs, using 3 ml of urine for cocaine determination and 1 ml of urine for benzoylecgonine determination.

benzoylecgonine could be recorded. The mass spectrum of urinary cocaine with added cocaine-d_3 (Fig. 8) showed expected doublets at *m/e* 303–306 and 182–185. Also of some significance was a singlet of increased relative ion intensity at *m/e* 105, a common fragment ion from the two isomeric species. The mass spectrum of urinary benzoylecgonine was also identical to that of the authentic material.

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