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Simultaneous Determination of Cocaine and Benzoyl Ecgonine in Urine by Gas Chromatography with On-Column Alkylation

Cocaine, as a pharmacologically active alkaloid from the leaves of the *Erythroxylon coca* bush, is the most powerful naturally occurring stimulant known. The history of its use as a stimulant by the Indians of Peru and Bolivia goes back as far as 1200 years. In the past 116 years the pharmacological and addictive properties of cocaine have been studied. More recently, reports have shown cocaine abuse among 10 to 24.1% of narcotic addicts for infrequent periods dating back to 1968 [*1*], and seizures of illicit cocaine are frequently reported in the news. Yet in spite of its extensive use the user is not readily identified, nor has cocaine's role in fatal poisoning been fully examined. Cocaine's potential for abuse is well known, but exhaustive epidemiological and toxicological data are, on the whole, unavailable. This lack of data may be due to the technological inability to assay for free cocaine by normal analytical methods because of its extensive metabolism to benzoyl ecgonine [*2–6*]. The obvious point of attack, then, in the problem of cocaine abuse detection would be the perfection of a reliable analysis for benzoyl ecgonine.

Benzoyl ecgonine (demethylated cocaine) is a nitrogenous compound containing a carboxylic functional group. Normal extraction methods do not remove this compound efficiently from aqueous solutions because of its high water solubility. This phenomenon, combined with its relative insensitivity to many visualizing agents and the inability of many solvent systems to move it from the origin, makes thin-layer chromatographic (TLC) identification somewhat difficult, although feasible [*7*].

Recent developments in immunochemical methods such as enzyme multiplied immunoassay technique (EMIT), hemagglutination inhibition (HI), and radioimmunoassay (RIA) facilitate the detection of benzoyl ecgonine. A good gas-liquid chromatographic (GLC) method for confirmation and quantitation, however, is desirable.

The highly polar nature of benzoyl ecgonine precludes gas chromatographic analysis in its underivatized form [*8*]. There have been several analytical methods developed in the past few years for the derivatization of benzoyl ecgonine for both GLC and TLC analysis [*6,9–13*]. Among these are alkylation with acidified alcohols, acylation to various halogenated derivatives, and silylation. These methods all necessitate lengthy derivatization techniques which may include chemical reduction, refluxing, neutralization, back extractions, and washes. Koontz et al [*14*] reported a GLC method for the analysis of benzoyl ecgonine using dimethylformamide dimethyl acetal to methylate it back to cocaine.

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Their procedure, however, required preparative TLC and a lengthy derivatization procedure, did not incorporate an internal standard, and was not quantitative. Wallace et al [*15*] recently reported a relatively rapid and simple GLC method for the simultaneous determination of cocaine and benzoyl ecgonine in urine by comparison of the quantitation of cocaine both before and after a methylation procedure. Recovery of benzoyl ecgonine, however, was only 65% by this method. In addition, the internal standard is not added until after the initial extraction procedure and the single derivatization allows little flexibility in the event of interfering peaks [*15*].

We report a procedure for the simultaneous and separate quantification of cocaine and its primary metabolite, benzoyl ecgonine, from a urine matrix. Our procedure uses on-column alkylation incorporating the homologous series of dimethylformamide dialkyl acetals which produce quantitative cocaine derivatives of particular utility when other drugs or their metabolites are present in the urine. Quantification is aided by the incorporation of isopropylated benzoyl ecgonine as an internal standard which is similar in structure to both cocaine and benzoyl ecgonine.

Instrumentation

A Varian Aerograph Model 2700 equipped with dual flame ionization detectors was used. The columns were silane-treated glass, either 6 ft (1.8 m) by 2 mm inside diameter packed with 3.8% UC W-98 2 on Gas Chrom Q 80/100 mesh or 3 ft (0.9 m) by 2 mm inside diameter packed with 3% OV-17 on Chromosorb WHP 80/100 mesh. The oven temperature was 200°C; the detector temperature, 300°C; and the injection port temperature, 300°C. It is very important that the injection port temperature be maintained at 300°C. The carrier gas was nitrogen at a flow rate of 35 ml/min. The range was 10^{-11} A/mV, and the attenuation, ×32. A Hewlett-Packard Model 3380B recorder-integrator was used for automatic data reduction of peak areas.

Experimental Procedure

Reagents

All reagents are analytical grade unless otherwise indicated and include dimethylformamide dialkyl acetals, sodium bicarbonate and potassium carbonate (2:1 w/w) as the solid bisalt buffer, and dimethylformamide (DMF). The dimethylformamide dialkyl acetals used are as follows:

(1) N,N dimethylformamide dimethyl acetal (Aldrich Chemical Co.),
(2) N,N dimethylformamide diethyl acetal (Aldrich Chemical Co.),
(3) N,N dimethylformamide dipropyl acetal (DMF-di-*n*-propyl acetal) (Aldrich Chemical Co.),
(4) N,N dimethylformamide diisopropyl acetal (Aldrich Chemical Co.),
(5) N,N dimethylformamide dibutyl acetal (DMF-di-*n*-butyl acetal) (Aldrich Chemical Co.),
(6) N,N dimethylformamide ditertbutyl acetal (DMF-di-*t*-butyl acetal) (Pierce Chemical Co.), and
(7) N,N dimethylformamide dicyclohexyl acetal (Aldrich Chemical Co.).

The extraction solvent is spectrograde chloroform:isopropanol (95:5 v/v), and the standard reference solution, calculated as the free base, is 1 μg/ml cocaine (Applied Science Laboratories) and benzoyl ecgonine (Applied Science Laboratories) in dimethylformamide.

The internal standard is constructed as follows: benzoyl ecgonine (10 mg) is placed in a conical, 12-ml tube and dissolved in a minimum amount (1 to 2 ml) of dimethylfor-

mamide. A small boiling chip and 0.5 ml of dimethylformamide diisopropyl acetal is added, and the solution is refluxed for 10 min over a low flame. The entire solution is then evaporated to dryness. Removal of the boiling chip is unnecessary. The residue is reconstituted by the addition of 10 ml isopropyl alcohol with a volumetric pipet. This results in a 1 mg/ml solution of isopropylated benzoyl ecgonine. The purity of the isopropylated benzoyl ecgonine is checked by removing a 50-μl aliquot and evaporating it to dryness, adding 25 μl DMF and 25 μl DMF-di-*n*-propyl acetal and vortexing. If any unreacted benzoyl ecgonine is in the solution, injection into the gas chromatograph will give a peak for *n*-propylated benzoyl ecgonine.

One millitre of the isopropylated benzoyl ecgonine is diluted with water to 5 ml, yielding a standard with concentration of 200 μg/ml.

Sample Extraction

A 0.1-ml aliquot of the internal standard solution (200 μg/ml isopropylated benzoyl ecgonine in water) is added to 10 ml urine in a 50-ml, round-bottom centrifuge tube. A single 10-ml aliquot of the reference solution (1 μg/ml cocaine and benzoyl ecgonine in water) is extracted in parallel with the specimen as a control. A sufficient amount of the bisalt buffer is added to saturate the urine (about 5 g) and mixed by vortex. Thirty millilitres of the extraction solvent is added, and the sample is shaken for 10 min. Separation of phases is accomplished by centrifugation at 1500 rpm for 3 to 5 min. The aqueous layer is then aspirated and discarded, and the organic layer filtered through Whatman #2 filter paper into a 40-ml conical centrifuge tube and evaporated to dryness in a 60°C water bath under air or nitrogen.

Gas-Liquid Chromatography Preparation

Prior to injection of the underivatized sample, the column is "scrubbed" by injecting 2 μl of dimethylformamide dimethyl acetal under normal operating conditions. All syringes used in this procedure should be thoroughly rinsed with dimethylformamide and care should be taken to exclude alcohol from interfering in the analysis since intermediate reactions occur with the dimethylformamide dialkyl acetals and alcohols in the esterification of benzoyl ecgonine. Care should also be taken to exclude water, since the dimethylformamide acetals are hygroscopic.

The residue is reconstituted with 50 μl of dimethylformamide, vortex-mixed, and 1 μl is injected into the gas chromatograph to determine (1) the complexity of the resulting chromatogram, that is, the retention times of interfering compounds, if any; (2) which derivative of benzoyl ecgonine will be subject to the least interference; and (3) if cocaine itself is present in the extract. After injection of an underivatized sample, the column should be scrubbed by the injection of 2 μl of dimethylformamide dimethyl acetal to react any free benzoyl ecgonine adsorbed on the column.

Derivatization

Ten microlitres of the dimethylformamide-dissolved residue is transferred to a 5-ml conical centrifuge tube to which 10 μl of the appropriate dialkyl acetal is added and vortex-mixed. An aliquot of 2 to 4 μl is injected on the gas chromatograph. Completion of the derivatization reaction is accomplished on-column. We found the injector port temperature of 300°C to be a critical condition for efficient derivatization. A separate 10-μl aliquot of the residue may be reacted in a similar manner with a different DMF-dialkyl acetal to form an additional confirming derivative.

Extraction efficiency and GLC response factors for quantification as well as the re-

tention times of the corresponding derivatives may be obtained by reacting parallel aliquots of the DMF-reconstituted residue of the reference solution.

Calculation

The following formula is used for quantification

$$C_x = C_s(A_x/R_x)(R_s/A_s)$$

where

C_x = concentration of cocaine or benzoyl ecgonine in the sample,
C_s = concentration of the internal standard (2 μg/ml),
A_x = peak area of cocaine or benzoyl ecgonine in the specimen,
R_x = peak area of the internal standard in the specimen,
R_s = peak area of the internal standard in the reference solution, and
A_s = peak area of cocaine or benzoyl ecgonine in the reference solution.

Results

Quantification of cocaine and benzoyl ecgonine is achieved through the use of an internal standard (isopropylated benzoyl ecgonine) and the parallel treatment of a reference solution of known concentration for the two drugs. The use of an appropriate internal standard can automatically compensate for extraction losses, differential GLC response, and so forth. The success of this technique for quantification is illustrated by the consistent accuracy of cocaine and benzoyl ecgonine determinations as shown in Tables 1 and 2.

TABLE 1—*Cocaine recovery from spiked urine.*

Amount of Cocaine Added, μg/ml	Average Cocaine Recovered, μg/ml[a]	Standard Deviation
10	10.14	0.16
5	4.94	0.30
2	2.00	0.14
1	1.09	0.05
0.5	0.50	0.01

[a] Average of triplicate analyses.

TABLE 2—*Benzoyl ecgonine[a] recovery from spiked urine.*

Amount of Benzoyl Ecgonine Added, μg/ml	Average Benzoyl Ecgonine Recovered, μg/ml[b]	Standard Deviation
10	11.57	0.78
5	5.26	0.33
2	2.01	0.03
1	1.04	0.06
0.5	0.55	0.05

[a] As *n*-propyl ester.
[b] Average of triplicate analyses.

These tables list the results of triplicate quantitative determinations of urines spiked with specified concentrations of cocaine or benzoyl ecgonine. Average quantitations for all concentrations were within ± 16% of the amount added and 90% were within ± 10%.

Figures 1 and 2 show the linearity of detector response to concentrations of the two

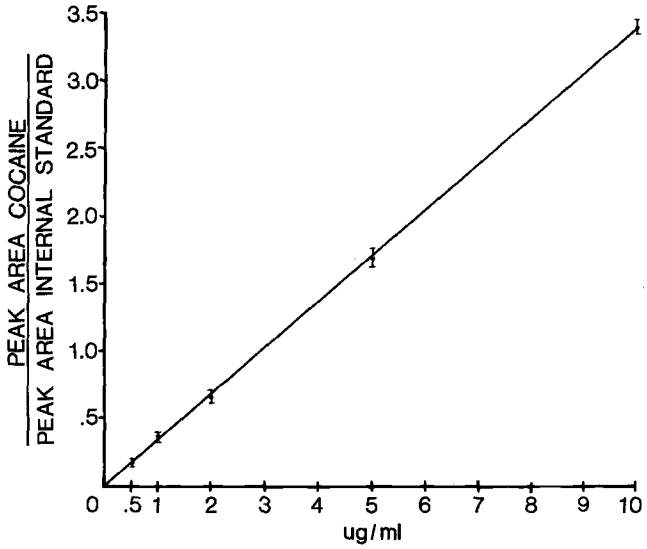

FIG. 1—*Cocaine standard curve.*

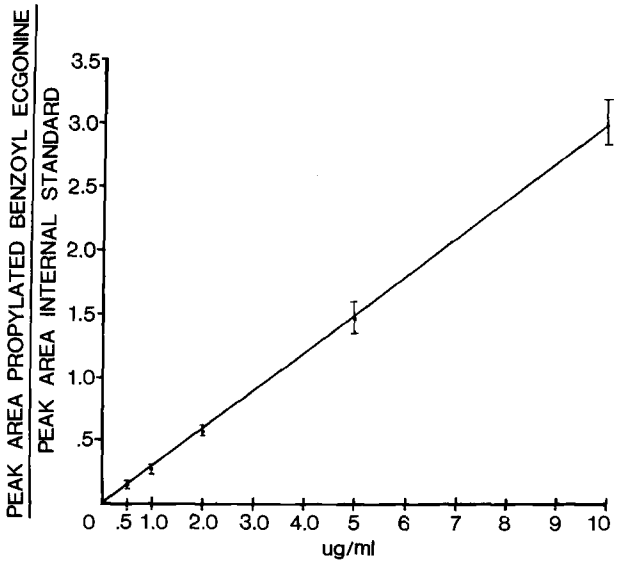

FIG. 2—*Benzoyl ecgonine (as the* n*-propyl ester) standard curve.*

compounds ranging from 0.5 to 10 μg/ml. Day to day precision could not be established because of the instability of cocaine in biological media; however, quadruplicate determinations of freshly spiked samples (0.5 to 2.0 μg/ml) of cocaine and benzoyl ecgonine processed similarly were found to yield similar linear results.

Figure 3 illustrates the separation of a nonquantitative blend of various esters of benzoyl ecgonine achieved on the 3% OV-17 column. Figure 4 (*left*) represents a urine specimen spiked to 1.0 μg/ml with both compounds and derivatized as described on the 3% OV-17 column, and Fig. 4 (*right*) shows the chromatogram for another urine spiked to 0.5 μg/ml and injected on the same column.

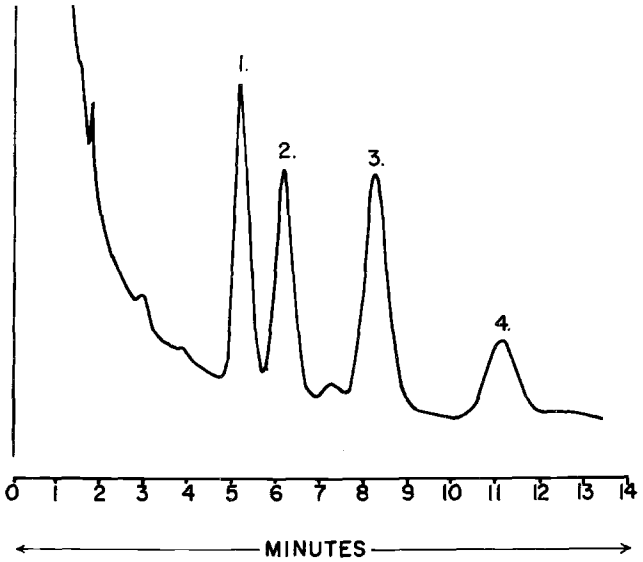

FIG. 3—*Separation of esters of benzoyl ecgonine on 3 ft (0.9 m) by 2 mm inside diameter, 3% OV-17 on Chromosorb WHP at 200°C (nitrogen, 20 ml/min):* (1) *methyl ester (cocaine);* (2) *ethyl ester;* (3) n-*propyl ester; and* (4) n-*butyl ester.*

Discussion

Gas liquid chromatography with flame ionization detection was employed for the confirmation of cocaine/benzoyl ecgonine samples screened positive by RIA, EMIT, or HI. Cocaine itself is easily chromatographed. The structure of benzoyl ecgonine, however, necessitates the formation of a derivative for GLC analysis. In preliminary experiments to develop a sufficiently rapid derivatization method, we tried silylation and methylation of benzoyl ecgonine. Various silyl donors and methods of silylation were investigated but were found to be extremely unreliable in our hands. We experienced excellent results using trimethylanililium hydroxide (Meth-Elute®, Pierce Chemical Co.) to convert benzoyl ecgonine back to cocaine, but this procedure lacked the simultaneous and separate determination of free cocaine which we desired as an additional form of identification. We surveyed other methods of analysis which either (1) lacked the sensitivity required; (2) lacked the differentiation of cocaine and benzoyl ecgonine; (3) did not incorporate an internal standard; (4) required special instrumentation; (5) required long derivatization techniques; or (6) most commonly, were too laborious for routine confirmation.

The method we have developed for the esterification of benzoyl ecgonine is both simple and rapid. The group of reagents selected for the alkylation of benzoyl ecgonine to its various esters gives the analyst the flexibility to form the derivatives which will be most suited for the particular analytical problem, since multiple drug abuse is not uncommon and the appearance of other peaks may complicate the identification of benzoyl ecgonine. The structure of the dimethylformamide dialkyl acetals is given in Fig. 5, and the reaction

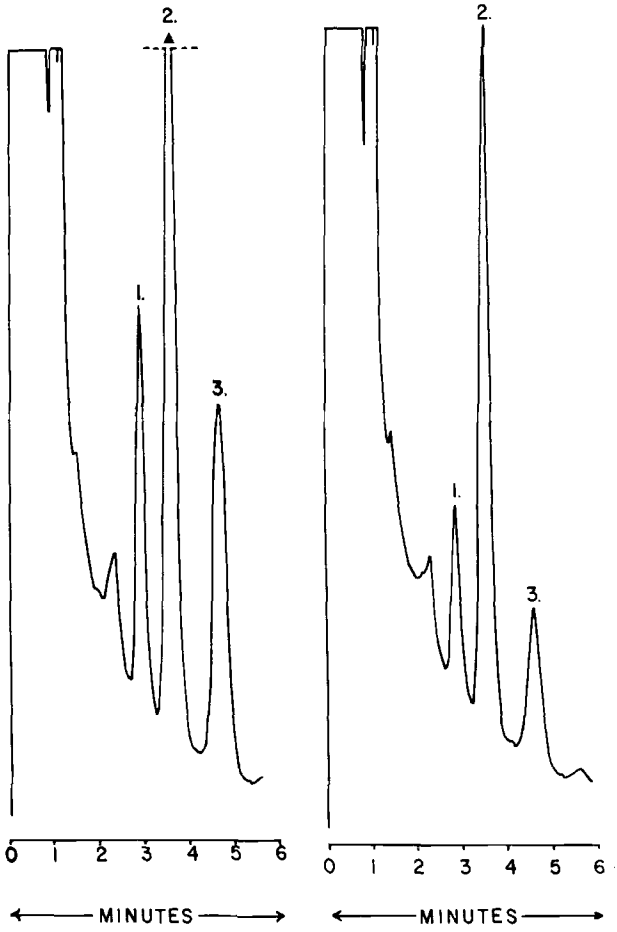

FIG. 4—(left) *Urine spiked with 1.0 μg/ml cocaine and benzoyl ecgonine:* (1) *cocaine;* (2) *internal standard (isopropyl ester of benzoyl ecgonine, 2 μg/ml); and* (3) n-*propyl ester of benzoyl ecgonine. Injection: 3 μl: column: 3 ft (0.9 m) by 2 mm inside diameter, 3% OV-17 on Chromosorb WHP at 200°C.* (right) *Urine spiked with 0.5 μg/ml cocaine and benzoyl ecgonine:* (1) *cocaine;* (2) *internal standard (isopropyl ester of benzoyl ecgonine, 2 μg/ml); and* (3) n-*propyl ester of benzoyl ecgonine. Injection: 3 μl: column: 3 ft (0.9 m) by 2 mm inside diameter, 3% OV-17 on Chromosorb WHP at 200°C.*

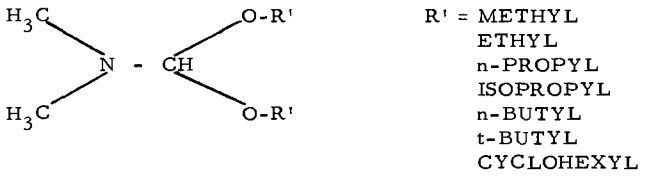

FIG. 5—*DMF-dialkyl acetals.*

of this series of reagents with carboxyl groups with special reference to the esterification of benzoyl ecgonine is given in Fig. 6 [*16*]. Note that the by-products of the reaction are relatively inert and therefore not harmful to the column. These derivatives were confirmed by gas chromatographic-mass spectral analysis.[2]

[2] D. Pearce and R. Cravey, unpublished data, 1975.

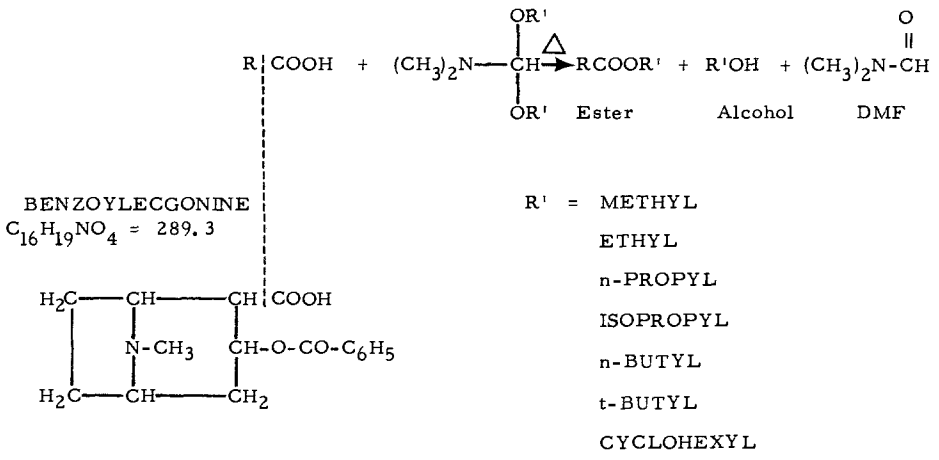

FIG. 6—*Esterification of benzoyl ecgonine with DMF-dialkyl acetals.*

On-column methylation through the use of dimethylformamide dimethyl acetal with an injection port heated to 300°C gives a minimum yield of 95%. Further investigation of on-column alkylation with the other dimethylformamide dialkyl acetals gave similar results and yielded quantitative derivatives which were, in all but one instance (the ethyl ester of benzoyl ecgonine), well separated without unusual chromatographic conditions from cocaine and the internal standard on two commonly used columns, 3.8% UC W-98 2 and 3% OV-17. Table 3 gives the relative retention times of the possible esters of benzoyl ecgonine with the DMF-dialkyl acetals.

TABLE 3—*Relative retention times of benzoyl ecgonine esters formed by DMF-dialkyl acetals.*[a]

Ester	Retention Time, s	Relative Retention Time
Methyl (cocaine)	147	1.00
Ethyl	181	1.27
Isopropyl	192	1.31
n-propyl	239	1.63
t-butyl	246	1.67
n-butyl	329	2.24
cyclohexyl	849	5.78

[a] Column: 3 ft (0.9 m) by 2 mm inside diameter, 3% OV-17 on Chrom WHP at 200°C.

The residue resulting from the extraction process must be dry and alcohol-free. Our experience shows that even residual alcohol left in syringes from other analyses will react with benzoyl ecgonine and any of the DMF-dialkyl acetals. This contamination produces esters by alkylation with alkyl groups donated by DMF-dialkyl acetals and the alcohol. For example, the process of mixing methanol in the presence of DMF-dipropyl acetal and benzoyl ecgonine produces mainly propylated benzoyl ecgonine and a variable amount of methylated benzoyl ecgonine. Therefore, the use of DMF is recommended for syringe cleaning and for reconstituting the residue.

Quantitative yields of these derivatives are predicated on the temperature of reaction. Kinetic studies of the derivative formation indicate that quantitative yields are not pro-

duced even when heated at 150°C for 1 h in a dry bath. We use on-column alkylation as a method of reaction. However, if TLC analysis is desirable, heating by an open flame may be substituted. The minimum time required for quantitative yields from our experience is the time necessary to just boil the mixture for a few seconds. The derivative thus formed is stable in the DMF-dialkyl acetals for several hours. If the excess reagent is removed by complete evaporation immediately following chromatography, the residues are stable indefinitely. A sample treated in such a manner may also be applied to a thin-layer plate, if desired. We have chromatographed old residues left in the laboratory atmosphere after three weeks and have found no degradation.

On-column esterification will not convert cocaine to any of the other esters of benzoyl ecgonine when reacted with the other DMF-dialkyl acetals; similarly, the various esters of benzoyl ecgonine will not interconvert once they have been formed.

The derivatization procedure having been established, we investigated the extraction efficiency. Salting out with sodium chloride gave better than 92% recoveries of both compounds but yielded an extremely dirty extract with an unacceptable level of background noise. The bisalt method yielded recoveries of 70 ± 5% for both cocaine and benzoyl ecgonine and a much cleaner residue. The efficiencies obtained were acceptable in view of the sample size used, the single extraction employed, and the use of an internal standard.

The isopropyl derivative of benzoyl ecgonine was chosen as the internal standard for this procedure for these reasons: (1) retention time between cocaine and propylated benzoyl ecgonine; (2) structural similarity to cocaine and benzoyl ecgonine; (3) easy synthesis; and (4) elution from the column free of interfering substances. However, should an interfering substance obscure the internal standard, one of the other esters may be used for this purpose, provided a standard curve is prepared.

From our experience the two most useful derivatives for the confirmation of benzoyl ecgonine have been the *n*-propyl and *n*-butyl. Both these derivatives are easily formed from the DMF-dialkyl acetals and are well resolved from other common drugs and their metabolites on these columns. As can be seen in Table 3, the ethyl derivative is difficult to separate from the internal standard on these columns and, hence, is of limited value. However, a more polar column or altered chromatographic conditions may help in the separation of ethylated benzoyl ecgonine and the isopropyl ester, thereby increasing its usefulness. When cocaine is absent, confirmation of benzoyl ecgonine may be accomplished by methylating benzoyl ecgonine back to cocaine.

Although *t*-butylated benzoyl ecgonine and the *n*-propyl ester co-chromatograph, this phenomenon should not preclude the use of dimethylformamide di-*t*-butyl acetal as an alternative to *n*-propylation. With the five derivatives (methyl, ethyl, *n*-propyl, *n*-butyl, and *t*-butyl) normally employed, we have not needed the cyclohexyl ester in routine analysis.

Final selection of the most suitable derivative rests upon the analyst. Consideration should be given to (1) the lower limits of detectability desired; (2) the presence or absence of interfering drugs or their metabolites extractable by this procedure; (3) whether single or duplicate derivatives are desirable; and (4) whether single or dual column chromatography is employed. We have adopted as the criterion for proof of cocaine use the method of dual derivatives on a single, moderately polar column (3% OV-17).

Summary

A gas chromatographic procedure has been developed for the simultaneous determination of cocaine and benzoyl ecgonine in urine specimens. The two drugs are extracted by isopropanol/chloroform from urine samples saturated with a bisalt buffer. The organic extract is evaporated to dryness, and an aliquot of the residue is injected onto the gas

chromatograph to determine the presence of cocaine and the location of any extraneous peaks. Benzoyl ecgonine is then analyzed as its particular alkyl ester subject to the least interference from extraneous peaks as observed in an initial underivatized injection. The reconstituted residue is co-injected with the appropriate dimethylformamide dialkyl acetal for on-column alkylation. The use of two columns or more than one benzoyl ecgonine alkyl ester gives positive identification, and the use of isopropyl benzoyl ecgonine as an internal standard allows accurate quantification.

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