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ANALYSIS OF BENZOYLECGONINE IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Benzoylecgonine, a polar metabolite of cocaine, was extracted and purified from urine using a reversed-phase high-performance liquid chromatographic column. The portion of the eluent corresponding to elution of the drug was collected, derivatized, and quantified using gas chromatography–mass spectrometry with selected ion monitoring. Using this procedure, analysis of 1 ng/ml of benzoylecgonine in urine can be achieved.

INTRODUCTION

It is presently very difficult to detect and quantify cocaine in body fluids of the drug abuser. This is due primarily to the rapid and extensive metabolism of cocaine in the body to the metabolites benzoylecgonine and ecgonine. Only about 1% of the parent drug is excreted in the urine[1]. Benzoylecgonine is excreted in higher concentration than ecgonine[2] and has been the metabolite of choice for drug monitoring. Benzoylecgonine has been analyzed by thin-layer chromatography[3,4], enzyme multiplied immunoassay[3], and gas chromatography with flame ionization[5,6], electron capture[7], and nitrogen detection[8]. A review has been published by Bastos and Hoffman[9].

A major problem in the analysis of benzoylecgonine is the difficulty of extracting the metabolite from biological media. Since the molecule contains both amine and carboxyl functions, it is amphoteric and therefore highly lipophobic. Solvent extraction from aqueous media requires exhaustive continuous extraction[10], or a salting out procedure[3], or liquid–solid extraction of previously lyophilized material[11]. These methods are cumbersome, inefficient, and relatively unselective.

We have developed a high-performance liquid chromatographic (HPLC) procedure which is capable of extracting and sufficiently purifying benzoylecgonine from urine to enable its analysis by gas chromatography–mass spectrometry (GC–MS) without further cleanup. This development has led to a simple analytical procedure for the analysis of benzoylecgonine in urine which shows promise of wide applicability to other drugs and their metabolites. By using the combination of HPLC and GC–MS with selected ion monitoring and and isotope-labeled variant as

an internal standard, analysis of benzoylecgonine has been achieved at concentrations as low as 1 ng/ml of urine.

EXPERIMENTAL

A reversed-phase HPLC column (μBondapak C_{18}, Waters Assoc., Milford, Mass., U.S.A.) was used to simultaneously extract and purify benzoylecgonine from urine. A 5-cm pre-column packed with pellicular reversed-phase particles was used to protect the analytical column. A solvent program* was generated by a Varian 8500 liquid chromatograph and the column eluent was monitored at 254 nm (DuPont, Model 837). One hundred ng of pentadeuterated benzoylecgonine were added to each of the 1-ml urine samples as internal standard. The 1-ml samples were then introduced onto the column via a large-volume loop injector (Rheodyne, Model 7105). The mobile phase flow-rate was maintained at 100 ml/h resulting in an initial column pressure drop of 1500 p.s.i. at room temperature. Distilled-in-glass methanol and distilled water were used as mobile phase solvents. The portion of the effluent (approx. 4 ml)

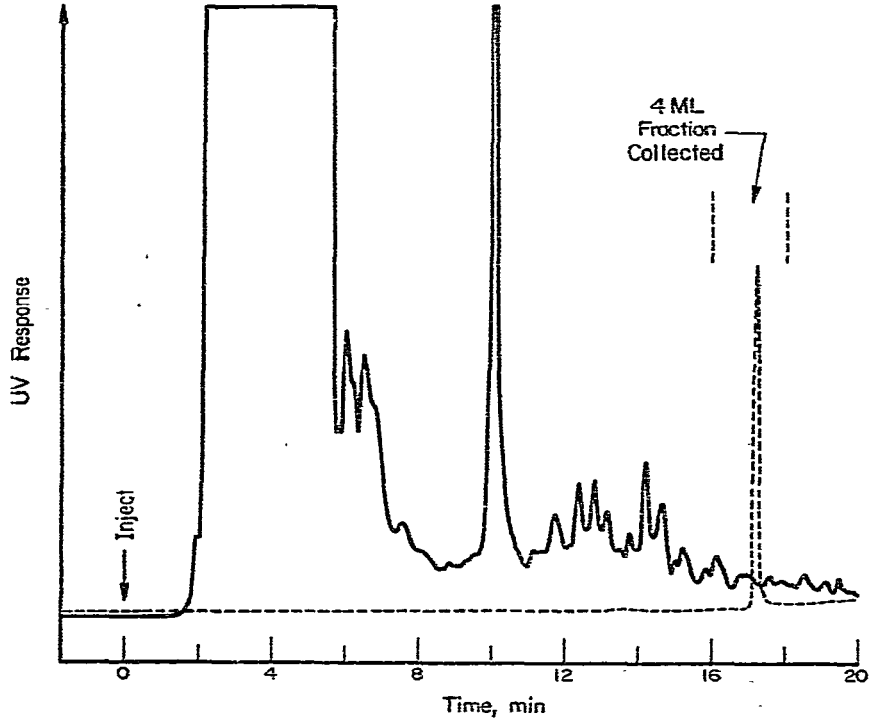

Fig. 1. Chromatograms of 1 ml of urine (———) and 3 μg of benzoylecgonine (– – –). Column, μBondapak C_{18}; solvent, 5 min isocratic, 5% methanol in water; 10 min gradient, 5% methanol/min; 5 min gradient, 10% methanol/min; flow-rate: 100 ml/h; UV attenuation: urine, absorbance 0.128; benzoylecgonine, absorbance 0.08.

* Solvent program: 5 min isocratic, 5% methanol in water; 10 min gradient, 5% methanol/min; 5 min gradient, 10% methanol/min; reset to initial conditions.

corresponding to where benzoylecgonine eluted (the window is as shown in Fig. 1) was collected. The effluent was evaporated at 40° under a gentle stream of nitrogen and then trimethylsilylated with 25 μl of bis(trimethylsilyl)trifluoroacetamide (Pierce, Rockford, Ill., U.S.A.). A 2- to 4-μl volume of the trimethylsilylated solution was injected onto a 6 ft. × 2 mm column packed with 3% OV-1. The column temperature was kept at 220°. Methane which was used as the carrier gas entered directly into the ion source. Ammonia (0.1 torr) was also bled into the ion source so that a mixture of methane and ammonia (8:1) was the chemical ionization reagent gas. The source pressure was approximately 1 torr. The protonated-molecule ions of trimethylsilyl benzoylecgonine (*m/e* 362) and the internal standard (*m/e* 366) were monitored by a Finnigan 3200 mass spectrometer (Fig. 2).

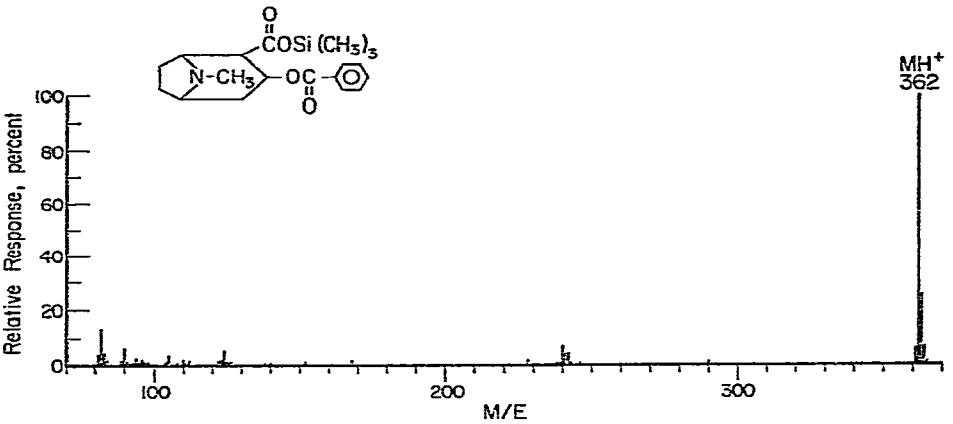

Fig. 2. The methane-ammonia chemical ionization mass spectrum of trimethylsilyl benzoylecgonine.

The ion source voltages were adjusted to give maximum sample ion intensities under methane chemical ionization conditions while unit resolution and symmetrically shaped peaks were maintained. Typical operating conditions were: ionization energy, 200 eV; ion repeller, 0 V; lens, −20 V; filament emission, 1 mA; ion source temperature, 130 to 160°; and electron multiplier gain, 5×10^4 to 7×10^5.

The pentadeuterated benzoylecgonine was obtained from National Institute on Drug Abuse. The mass spectrum of the deuterated compound shows that it is not isotopically pure. The most intense ion at the molecular ion region is for the d_4-variant (*m/e* 366); therefore, this ion was monitored as the internal standard.

RESULTS AND DISCUSSION

Reversed-phase liquid chromatography is a useful technique for extracting and purifying benzoylecgonine from urine prior to analysis by GC–MS. Although the metabolite benzoylecgonine is a polar compound, it is retained considerably longer on a reversed-phase column than most polar urinary constituents and therefore it can be effectively purified (Fig. 1). This has been verified by collecting the portion of the eluent (t_R = 16–18 min) corresponding to where benzoylecgonine elutes, silylating,

and analyzing for trimethylsilyl benzoylecgonine by GC–MS with selected ion monitoring. Fig. 3 shows the GC peak corresponding to 1 ng of benzoylecgonine collected from the HPLC column. Hardly any interferences from endogeneous compounds were observed while monitoring this mass number (*m/e* 362).

Before incorporating this HPLC procedure into an analysis method for benzoylecgonine, three important studies were performed: the reproducibility in HPLC retention, the lifetime of the reversed-phase column, and the quantification of benzoylecgonine by GC–MS.

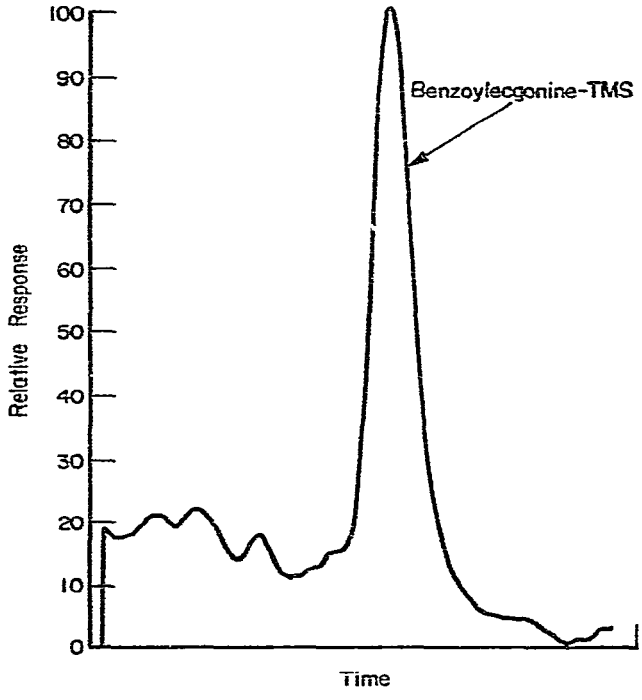

Fig. 3. Analysis of 1 ng of benzoylecgonine in 1 ml of urine by GC–MS with selected ion monitoring (*m/e* 362.4). Benzoylecgonine was extracted and purified by the method illustrated in Fig. 1.

Since the quantities of benzoylecgonine being analyzed are below the detection limit of the ultraviolet (UV) photometer, a time window corresponding to the elution of the drug is collected. It is therefore extremely important that the retention time of the drug remains constant. We monitor the reproducibility in retention by injecting 3 μg of standard benzoylecgonine before and after injection of urine samples. The relative standard deviation was 0.64% over 12 measurements in 20 days (retention time, 1011.33 $\pm$ 6.46 sec). The reproducibility in retention of later eluting peaks in gradient elution is more dependent on the reproducibility of the solvent gradient than on the initial solvent conditions. Column re-equilibration is therefore shortened (10 min). This allows a fast sample throughput time (30 min) while maintaining a reproducible retention time.

An additional concern was the ability of the reversed-phase column to maintain

its performance (*i.e.*, efficiency, permeability, and retention reproducibility) after injections of large quantities of biological fluids. To date over fifty 1-ml urine samples have been injected with essentially no change in column performance. Also, there has not yet been a need to replace the pre-column. Column performance is continually being measured to determine its useful lifetime.

Once the HPLC conditions had been determined, a GC–MS quantification procedure specific for benzoylecgonine was developed using selected ion monitoring. A 4-ml volume of water–methanol (1:1) (to approximate the collected eluent) was spiked with 100 ng of deuterated benzoylecgonine and 1, 3, 6, 10, and 50 ng of benzoylecgonine. These solutions were evaporated at 40° with a gentle stream of nitrogen, derivatized and analyzed by GC–MS (see Experimental). Fig. 4 shows the standard curve in which the correlation coefficient obtained was 1.000.

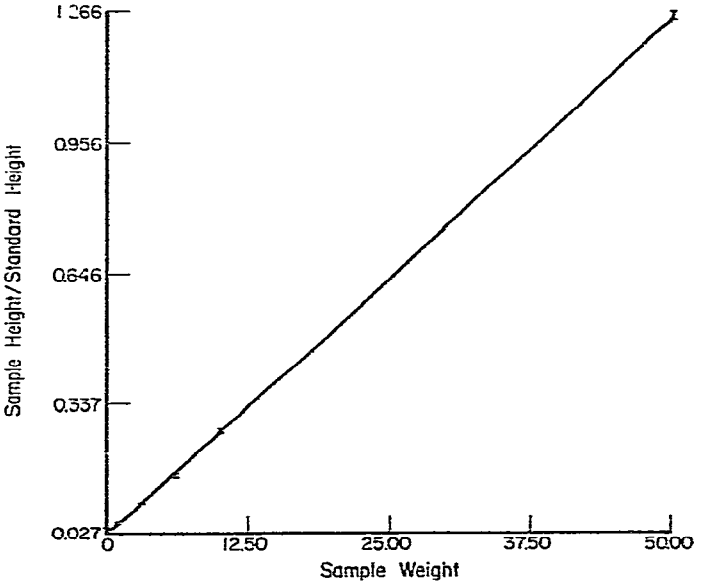

Fig. 4. Standard curve for the range 0–50 ng of benzoylecgonine using 100 ng of internal standard.

GC–MS with selected ion monitoring constitutes a highly specific and sensitive method for the analysis of many drugs and related compounds[12]. Nevertheless, the analysis of some drugs and their metabolites, especially the more polar ones, has been hampered due to poor extraction and cleanup techniques. Often, similar drugs require two entirely different extraction and purification steps to allow their analysis by GC–MS.

There are a number of advantages in using HPLC for the extraction and purification of drugs and their metabolites from body fluids prior to GC–MS analysis. Using benzoylecgonine as an example, we have demonstrated that a polar metabolite, previously very difficult to concentrate in biological fluids, can be easily extracted and purified using HPLC. Twitchett and Moffat[13] have listed retentions of a large

number of drugs, on reversed-phase columns, all of which behave similar to benzoylecgonine.

This approach, therefore, shows promise as a general method for the extraction and cleanup of drugs from body fluids. Additional advantages are: extraction selectivity can be enhanced for polar, ionic drugs by controlling the pH of the mobile phase[14]; several drugs can be extracted and purified in a single run; and the extraction and purification procedure can now be automated. The combination of HPLC and GC–MS with selected ion monitoring allows very sensitive drug analysis with minimum sample preparation.

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