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Determination of cocaine and its metabolites in human urine by gas chromatography/mass spectrometry after simultaneous use of cocaine and ethanol

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Abstract: Cocaethylene is an active metabolite produced when cocaine is consumed jointly with ethanol. The development of analytical techniques for determining cocaethylene and other cocaine metabolites is highly relevant for pharmacokinetic and toxicology studies of the cocaine and alcohol interaction in humans. The gas chromagraphy/mass spectrometry (GC/MS) method here reported is based on a single solid-phase extraction together with deuterated internal standards previously added to urine, followed by derivatization with pentafluoropropionic anhydride (hydroxyl and amine functions) and 1,1,1,3,3,3 hexafluor-2-propanol (carboxylic acid function) and injection into a capillary GC system coupled to a mass spectrometric detector in the selected ion monitoring acquisition mode. A sensitivity of 1–2 ng ml^{-1} for the quantitative analysis of cocaine and its main metabolites (ecgonine methyl ester, benzoylecgonine, cocaethylene and norcocaine) was achieved. In addition, some other minor metabolites were easily extracted and detected.

Keywords: *Cocaine and alcohol interaction; cocaethylene; norcocaethylene; capillary gas chromatography/mass spectrometry; urinary metabolic profile.*

Introduction

There is an increasing interest in the pharmacological and toxicological interactions of cocaine and ethanol since recent surveys have shown the concurrent use of both drugs by a relevant proportion of the population [1, 2]. In forensic studies, cocaine and ethanol are increasingly found in biological samples from fatally-injured drivers [3, 4], and an increased cardiovascular toxicity due to their association has been postulated [5]. It has been shown that the cardiovascular effects of the combination of cocaine and ethanol are greater than those elicited by cocaine alone [6, 7].

An ethyl analogue of cocaine–cocaethylene (benzoylecgonine ethyl ester) — has been detected in blood and urine samples from subjects intoxicated with alcohol and cocaine [8] and from healthy volunteers receiving doses of cocaine and alcohol compatible with social consumption [7, 9]. Cocaethylene has shown cocaine-like properties in receptor binding studies and seems to have similar biological activity in dopaminergic neurones [10, 11].

In man, cocaine is mainly metabolized to ecgonine methyl ester by plasma and liver cholinesterases, spontaneously hydrolysed in plasma to benzoylecgonine, and transformed to norcocaine by liver isoenzymes of the cytochrome P_{450} system [12, 13]. Cocaethylene seems to be produced by hepatic transesterification from cocaine in the presence of alcohol [11, 14]. Cocaethylene arylhydroxy- and arylhydroxy-methoxy metabolites have also been identified in urine samples from individuals consuming cocaine and alcohol simultaneously [15], similar to the pathways recently described for cocaine itself [16]. The identification of cocaethylene and its metabolites in urine and other specimens may be of relevance as biological markers of the concurrent consumption of both drugs, and their pharmacokinetic profile may provide new insights on the time-course of cocaine and alcohol interaction. In fact, cocaethylene and norcocaine have been

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proposed as markers of cocaine ingestion in hair of drug abusers to overcome hair contamination by "environmental" cocaine [17].

Although various analytical methods for the simultaneous detection of cocaine and its metabolites have been described [18–21], the coextraction of these compounds is very difficult due to differences in their physicochemical properties. Liquid–liquid extraction techniques yield low recoveries for polar metabolites, such as benzoylecgonine [18]. Solid-phase extraction techniques improve the recovery of benzoylecgonine [22] and allow the coextraction of ecgonine methyl ester [19, 23–25]. GC coupled to MS seems to be the procedure that yields adequate specificity and sensitivity for these type of studies [19, 22–27]. However, analysis of some of the more polar cocaine metabolites by GC requires the use of derivatization techniques. Sometimes use of two independent processes of derivatization [18] or alternatively, the combination of derivatization techniques with the detection of some underivatized compounds have been described [23].

The present work describes an analytical method based on a single clean-up step by solid-phase extraction [23] and a single derivatization procedure using 1,1,1,3,3,3-hexafluor-2-propanol and pentafluoropropionic anhydride [16], for the identification of cocaine, cocaethylene and other minor metabolites in urine samples from simultaneous cocaine and ethanol users.

Materials and Methods

Chemicals and reagents

Cocaine, ecgonine methyl ester, benzoylecgonine, cocaethylene, and their corresponding deuterated analogues were provided by Radian Corporation (Austin, TX, USA). Norcocaine was provided by Research Triangle Institute (Durham, NC, USA). Methanol, chloroform, ethyl acetate, hydrochloric acid and potassium dihydrogen phosphate were reagent grade (Merck, Darmstadt, Germany). Isopropyl alcohol and ammonium hydroxide, 25% reagent grade, were supplied by Scharlau (Barcelona, Spain). 1,1,1,3,3,3 hexafluor-2-propanol (spectroscopy grade) was supplied by Merck (Darmstadt, Germany). Pentafluoropropionic anhydride was provided by Supelco (Bellefonte, USA). Bond Elut Certify™ columns were provided by Analytichem International (Harbor City, USA) and the Visiprep™ vacuum manifold to operate with columns in a semiautomatic mode for solid–liquid extraction was provided by Supelco (Bellefonte, USA). Deionized water was obtained in our laboratory with a MILLI Q System (Millipore, Mulheim, France).

Experimental standard solutions

Standards were supplied as solutions of cocaine (in N,N-dimethylformamide), ecgonine methyl ester (in methanol), benzoylecgonine (in methanol), cocaethylene (in acetonitrile), norcocaine (in methanol), at a concentration of 1 mg ml^{-1} (S1). Working solutions of 100 μg ml^{-1} (S2) were prepared by diluting 1 ml of the above solutions to 10 ml with methanol. Solutions of 10 μg ml^{-1} (S3) were prepared by diluting 1 ml of S2 to 10 m with methanol. Solutions of N-methyl-D_3-labelled cocaine (in acetonitrile), ecgonine methyl ester (in methanol), benzoylecgonine (in methanol) and cocaethylene (in acetonitrile) which were used as internal standards, were supplied at a concentration of 100 μg ml^{-1}. Solutions were checked by ultraviolet spectrophotometry and stored at −20°C.

Apparatus

A Hewlett–Packard 5890A model gas chromatograph fitted with a HP 7673A autosampler was coupled to a HP 5970 mass selective detector (Hewlett–Packard, Palo Alto, CA, USA). The separation was carried out using a cross-linked capillary column (Hewlett-Packard) 25 m long × 0.2 mm internal diameter, 5% phenyl-methyl silicone gum (0.33 μm film thickness). The injector in splitless mode (valve activation time of 30 s after injection) and the interface were operated at temperatures of 280 and 290°C, respectively. The oven temperature was programmed with three consecutive rates: first rate, from 100 to 200°C at 20°C min^{-1}; second rate, from 200 to 240°C at 3°C min^{-1}; and third rate from 240 to 280°C at 20°C min^{-1}. Helium was used as carrier gas at a flow rate of 0.65 ml min^{-1} (measured at 180°C). The mass spectrometer was operated by electron impact ionization (EI, 70 eV) and in the selected ion monitoring (SIM) acquisition mode. Two ions (molecular ion and base peak) were selected for those compounds routinely quantitated. In the case of other minor metabolites monitored, those

Table 1
Monitored ions and relative retention times of each of the analysed compound

Compound	Derivatives	*m/z*	Relative ret. time
Ecgonidine	COO-HFIP	288	0.24
Ecgonidine methyl ester		152	0.33
Ecgonine methyl ester	O-PFP	182	0.33
		345*	
Ecgonine methyl ester-D_3	O-PFP	185	0.34
		348*	
Ecgonidine ethyl ester†		166	0.35
Ecgonine ethyl ester	O-PFP	359	0.36
Norecgonidine methyl ester	N-PFP	313	0.37
Norecgonine methyl ester	N,O-bis-PFP	477	0.37
Norecgonidine ethyl ester†	N-PFP	327	0.39
Norecgonine ethyl ester	N,O-bis-PFP	491	0.39
Benzoylecgonine	COO-HFIP	318	0.73
		439*	
Benzoylecgonine-D_3	COO-HFIP	321	0.73
		442*	
Cocaine		182	1.00
		303*	
Cocaine-D_3		185	1.00
		306*	
Cocaethylene		196*	1.07
		317	
Cocaethylene-D_3		199*	1.07
		320	
Norcocaine	N-PFP	313*	1.09
		435	
Norcocaethylene	N-PFP	327	1.15

* Ions used for quantitation.
† Tentative structures.

ions giving the cleanest ion trace were selected. Table 1 summarizes the selected ions.

Experimental procedure

Urine samples used for this study were obtained from a clinical trial, where nine male volunteers received either cocaine alone or in conjunction with alcohol [7] (DGFPS authorization 87/334). Urine was collected after the following time periods following cocaine administration: 0, 0–4, 4–8 and 8–24 h. Samples were acidified to about pH 3 with hydrochloric acid (6N) immediately after collection to prevent degradation of cocaine and/or metabolites [28]. Aliquots of 2.5 ml of these acidified urine samples were transferred to 15 ml silanized tubes, and 5 μl (500 ng) of each of the deuterium-labelled standards and 1 ml of phosphate buffer (pH 7.0;0.1 M) were added. The mixture was vortexed, centrifuged at 3000 rpm for 5 min and poured into Bond Elut Certify™ columns and gently sucked through. Columns had been previously inserted into a vacuum manifold, conditioned by washing once with 2 ml of methanol and 2 ml of phosphate buffer (pH 7.0;0.1 M) and prevented from running dry. After applying the samples, the columns were successively washed with 3 ml of deionized water, 3 ml of hydrochloric acid (0.1 M) and 9 ml of methanol. Elution of the analytes was performed with 2 ml of a mixture of a chloroform–isopropyl alcohol (80:20, v/v) containing 2% of ammonium hydroxide. The eluates were collected and evaporated to dryness under a gentle nitrogen stream at room temperature and kept in a desiccator under vacuum for 1 h before derivatization of the residues. For the derivatization procedure, 70 μl of pentafluoropropionic anhydride and 30 μl of 1,1,1,3,3,3 hexafluor-2-propanol were added to the dried residue and vortexed for 10 s. The tubes were heated for 10 min at 70°C and, after cooling, taken to dryness under a stream of nitrogen. The residue was redissolved in 50 μl of ethyl acetate and 1 μl was injected into the CG/MS system.

Calibration procedure

Standard curves were prepared with blank human urine over the concentration range 100–2000 ng ml^{-1} for cocaine, 250–4000 for ecgonine methyl ester, 500–4000 ng ml^{-1} for

benzoylecgonine and 25–500 ng ml^{-1} for both cocaethylene and norcocaine.

Known amounts of cocaine equivalent to 100, 250, 500, 1000 and 2000 ng ml^{-1}; ecgonine methyl ester equivalent to 250, 500, 1000, 2000 and 4000 ng ml^{-1}; benzoylecgonine equivalent to 500, 750, 1000, 2000 and 4000 ng ml^{-1}; cocaethylene and norcocaine equivalent to 25, 50, 100, 250 and 500 ng ml^{-1} were taken to dryness before adding the acidified blank urine. Peak height ratios (x) between cocaine, ecgonine methyl ester, benzoylecgonine, cocaethylene and norcocaine analytes and their corresponding deuterated standard analogues (ecgonine methyl ester-D_3 was used as internal standard for norcocaine) vs the corresponding concentrations (y) were subjected to least-squares regression analysis.

Results

Good linearity was obtained over the ranges studied ($r = 0.9998$, intercept = −0.0910, slope = 0.0056 for cocaine; $r = 0.9990$, intercept = 0.2960, slope = 0.0027 for ecgonine methyl ester; $r = 0.9998$, intercept = 0.0095, slope = 0.0054 for benzoylecgonine; $r = 0.9997$, intercept = −0.0216, slope = 0.0057 for cocaethylene; and $r = 0.9990$, intercept = −0.0510, slope = 0.0067 for norcocaine). The limits of detection (four times the signal to noise ratio) were 1 ng ml^{-1} for benzoylecgonine, ecgonine methyl ester and norcocaine, and 2 ng ml^{-1} for cocaine and cocaethylene.

Absolute recoveries ($n = 4$) were as follows: 87.5 ± 2% for cocaine, 41.5 ± 3% for ecgonine methyl ester, 83.5 ± 5% for benzoylecgonine, 93.0 ± 7% for cocaethylene, and 98.0 ± 2% for norcocaine over their corresponding concentration ranges. Within and between-day coefficients of variation of low and high control samples are shown in Table 2.

Some other minor metabolites of cocaine were extracted and detected in the urine samples of subjects receiving cocaine and alcohol (see Fig. 1), and among them, two cocaethylene related substances: norcocaethylene and norecgonine ethyl ester, were detected (full mass spectra) and their chemical structures and fragmentation pattern elucidated from their mass-spectra as compared with their methyl ester analogues (see Figs 2 and 3).

Discussion

The solid–liquid extraction of urine allowed identification and quantitation of cocaine and its main active (cocaethylene and norcocaine) and inactive (ecgonine methyl ester and benzoylecgonine) metabolites in a single step. In addition, clean urinary extracts were obtained as a result of the selectivity of the technique. This is an important factor for the high sensitivity achieved, which is particularly important for the detection of norcocaine. The relatively selective extraction of the sample increased the sensitivity and the specificity of the analytical technique, based on specific derivatization and analysis of the final extracts by GC/MS operated in SIM acquisition mode. The use of deuterated-labelled compounds as internal standards also provided good results for linearity, accuracy, and precision. In the

Table 2
Within-day and between-day coefficient of variation of spiked urine

	Low control			High control			
Compound	Spiked (ng ml^{-1})	Measured (ng ml^{-1})	RSD %	Spiked (ng ml^{-1})	Measured (ng ml^{-1})	RSD %	n
Within-day							
Cocaine	150	164.4	4.3	1500	1522.3	3.4	9
Ecgonine methylester	300	300.8	1.6	3000	3307.7	10.8	6
Benzoylecgonine	300	294.0	4.9	3000	2943.0	3.2	9
Cocaethylene	30	31.8	5.4	400	422.3	4.3	8
Norcocaine	30	34.1	8.6	400	415.6	7.0	5
Between-day							
Cocaine	150	170.3	4.7	1500	1505.0	5.5	4
Ecgonine methylester	300	310.4	2.6	3000	2977.5	11.9	4
Benzoylecgonine	300	291.3	4.5	3000	2995.9	2.6	4
Cocaethylene	30	32.5	5.8	400	438.6	3.6	4
Norcocaine	30	30.2	19.0	400	407.9	9.3	4

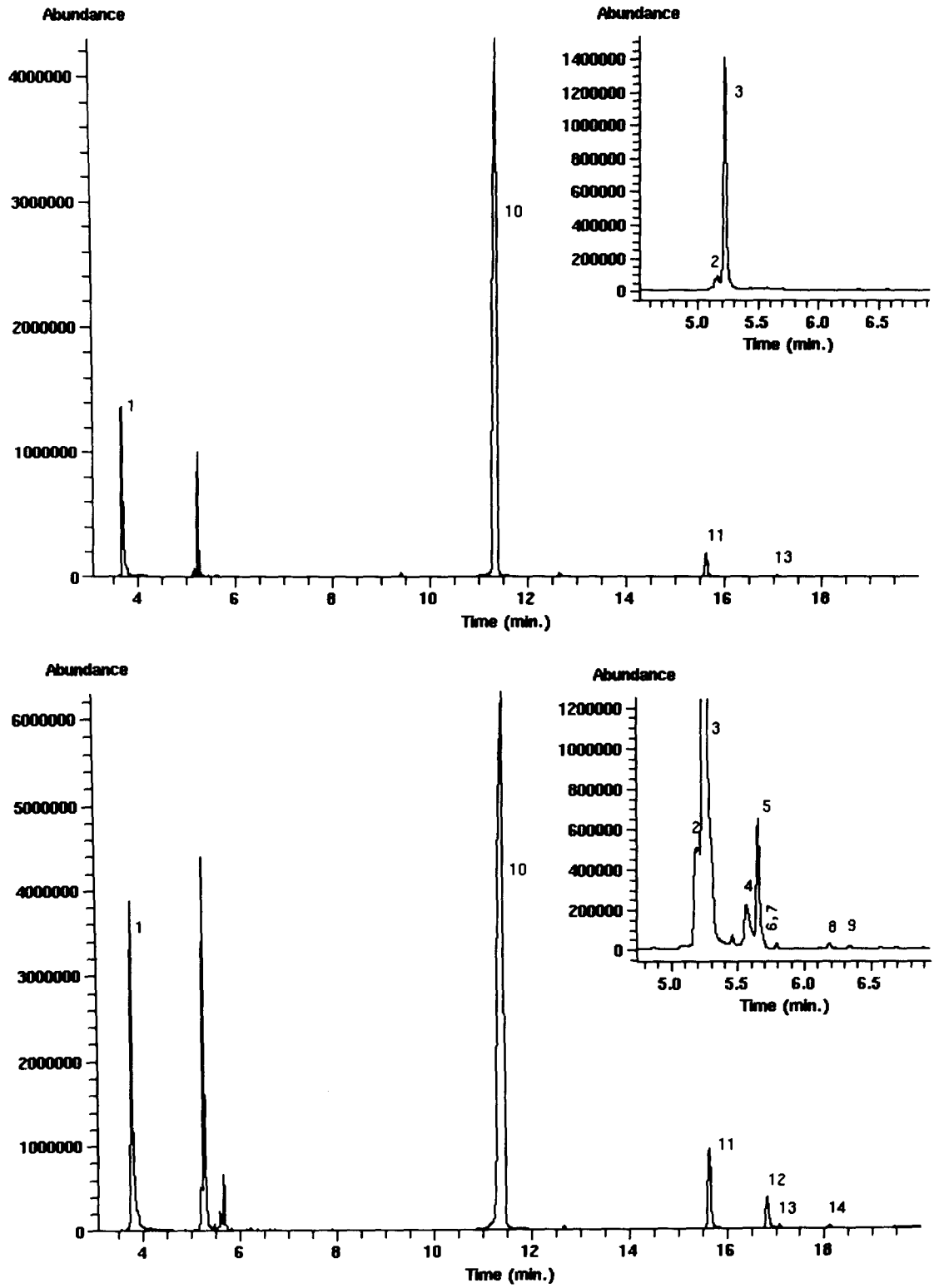

Figure 1
Total ion current profile (SIM acquisition mode) from the GC/MS analysis of a derivatized urine extract from a healthy volunteer who consumed cocaine alone (a), and cocaine and alcohol (b). (1) COO-HFIP-ecgonidine; (2) ecgonidine methyl ester; (3) O-PFP-ecgonine methyl ester; (4) tentative ecgonidine ethyl ester; (5) O-PFP-ecgonine ethyl ester; (6) N-PFP-norecgonidine methyl ester; (7) N,O-bis-PFP-norecgonine methyl ester; (8) tentative N-PFP-norecgonidine ethyl ester; (9) N,O-bis-PFP-norecgonine ethyl ester; (10) COO-HFIP-benzoylecgonine; (11) cocaine; (12) cocaethylene; (13) N-PFP-norcocaine; (14) N-PFP-norcocaethylene. HFIP = Hexafluoroisopropionyl derivative. PFP = Pentafluoropropionyl derivative.

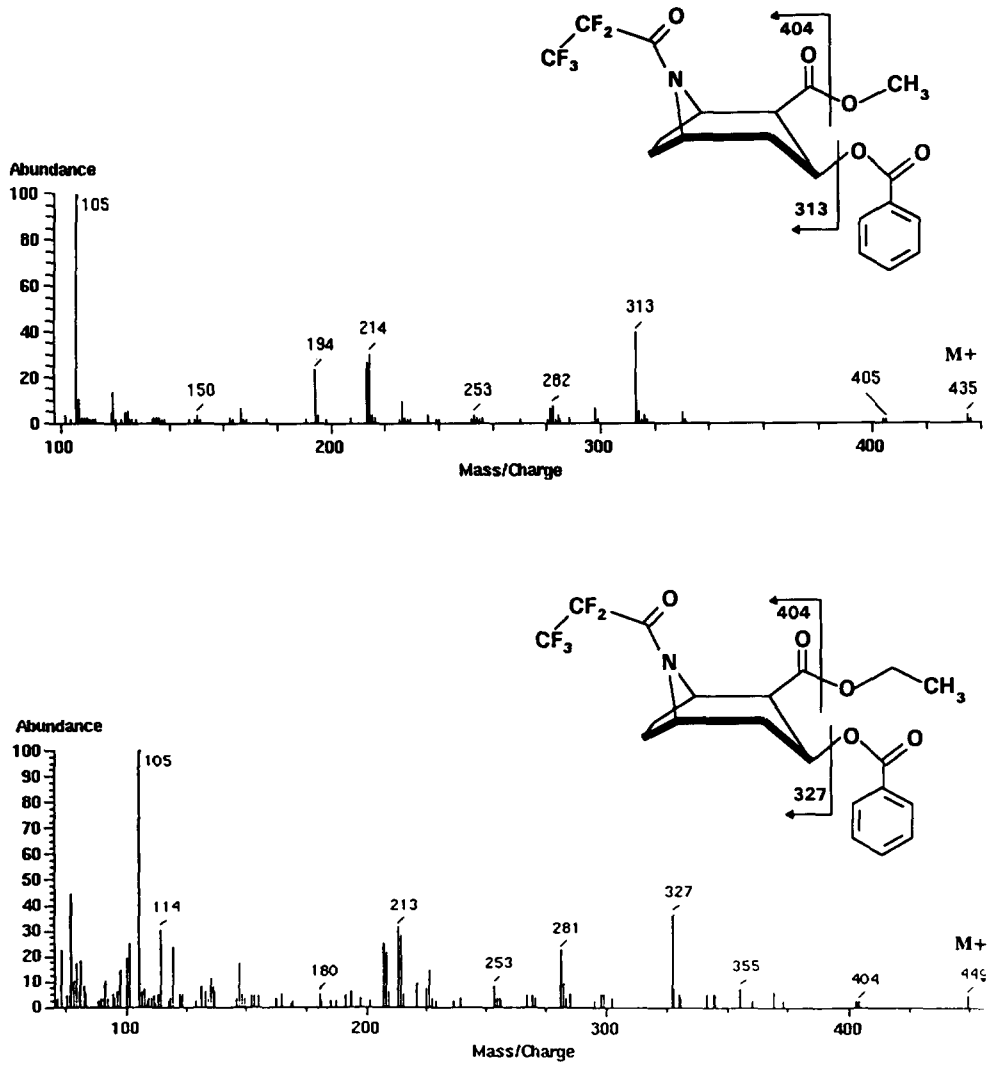

Figure 2
Mass spectrum and chemical structure of some metabolites identified in human urine after simultaneous intake of alcohol and cocaine: (a) N-PFP-norcocaine, (b) N-PFP-norcocaethylene.

case of norcocaine, where a deuterated analogue was not available, ecgonine methyl ester-D_3 was used as internal standard and acceptable results, similar to those obtained with more automated analytical methods recently described for the analysis of cocaine and its metabolites [29] were obtained. As expected, ecgonine was not extracted with the present extraction procedure. Previous studies have suggested that a stronger acidic cation exchange resin may be used for the analysis of this particular compound [27].

A number of cocaethylene related substances have been identified in urine of simultaneous users of cocaine and alcohol. The mass spectra of PFP/HFIP derivatives of some of them have been published previously: norcocaethylene and ecgonine ethyl ester [30, 31]. Another substance was identified: the derivative of norecgonine ethyl ester (see Fig. 3). This substance is the ethyl analogue of the derivative of norecgonine methyl ester [16]. The present analytical method has the potential of being able to monitor cocaine and cocaethylene, their metabolites and also some other minor compounds of interest in toxicological studies.

Conclusions

The present analytical technique allows the simultaneous quantitation of cocaine and its main metabolites (ecgonine methyl ester, benzoylecgonine, cocaethylene and nor-

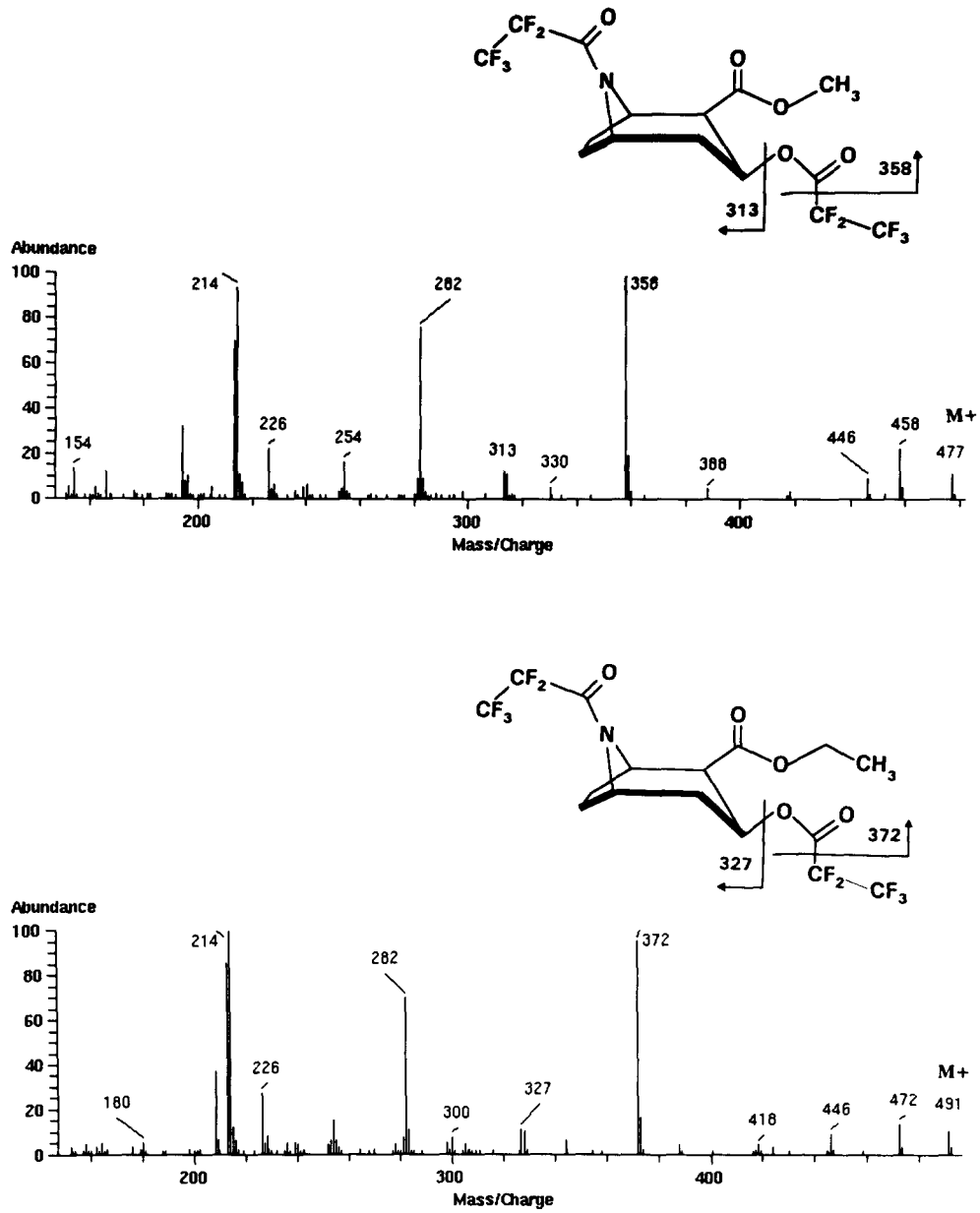

Figure 3
Mass spectrum and chemical structure of some metabolites identified in human urine after simultaneous intake of alcohol and cocaine: (a) N,O-bis-PFP-norecgonine methyl ester (b) N,O-bis-PFP-norecgonine ethyl ester.

cocaine) in urine as well as the detection of norcocaethylene, ecgonine ethyl ester and other minor metabolites. The analytical technique is a relevant contribution to the study of the urinary excretion profile of cocaine metabolites because only one extraction procedure is required, a single method of derivatization is used, and good sensitivity is achieved as a result of the low background of the biological extract.

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