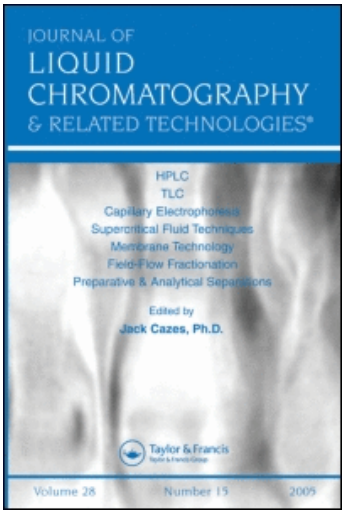

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High Performance Liquid Chromatographic Determination of Cocaine and Its Main Metabolites In Biological Samples: A Review

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To cite this Article Clauwaert, K. , Lambert, W. and De Leenheer, A.(1995) 'High Performance Liquid Chromatographic Determination of Cocaine and Its Main Metabolites In Biological Samples: A Review', Journal of Liquid Chromatography & Related Technologies, 18: 11, 2097 — 2114
To link to this Article: DOI: 10.1080/10826079508010257
URL: http://dx.doi.org/10.1080/10826079508010257

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(11), 2097-2114 (1995)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF COCAINE AND ITS MAIN METABOLITES IN BIOLOGICAL SAMPLES: A REVIEW

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ABSTRACT

The HPLC determinations of cocaine and its main metabolites are critically reviewed with special attention to the matrices analysed. A comparison of the different extraction methods is given and the chromatography, internal standardization and detection modes are discussed.

INTRODUCTION

Cocaine, the major alkaloid of Erythroxylum coca, has a long history of human use and abuse. More than four thousand years ago the coca leaves were already chewed. In the beginning of our century cocaine was an ingredient of tonics and soda's. Nowadays cocaine is almost exclusively associated with abuse, it is snorted, injected or smoked as "freebase" or "crack" (1,2). The instantaneous and overwhelming effect of such applications explain the popularity of cocaine among drug users. The extreme danger and high mortality rate attract attention of the phy-

sicians and analysts. This results in an increasing amount of scientific effort with respect to the neuronal activity of cocaine, to the effects on the human organism and of course to the various ways and techniques for analysis of this compound.

Cocaine is a local anaesthetic and vasoconstrictor : it blocks the sodium transport across the membranes which forms the basis of its medical application in otorhinolaryngeal surgery (1,2).

The effects of cocaine on the central nervous system are well known. It blocks the reuptake of norepinephrine, dopamine and serotonin, mono-amines which are implicated in memory function. The high synaptic concentrations of mono-amines and especially of dopamine result in physiological and psychotropic effects such as an increased sense of alertness, well-being and euphoria. Subsequent mono-amine depletion in the presynaps results in a crash with depression and physical discomfort. This cycle is responsible for the reinforcing properties of cocaine : drug-users prevent the crash by taking new and higher doses (1,2). Much effort is put into the search for cocaine antagonist but at present no such compound is available and today cocaine intoxication can only be treated symptomatically (3).

METABOLISM

Cocaine is rapidly and almost completely metabolized and deactivated. Benzoylecgonine is one of the main degradation products formed by either an hepatic carboxyesterase or spontaneous hydrolysis (4), (Fig.1). Another important metabolite is ecgonine methyl ester, which is formed by hepatic cholinesterase. Although they have no pharmacological activity, both metabolites are of great toxicological and analytical importance : due to their long half-life in biological matrices (four to six times longer than cocaine) they remain much longer detectable than the parent compound, cocaine. Unfortunately, their hydrophylic nature renders them difficult to extract.

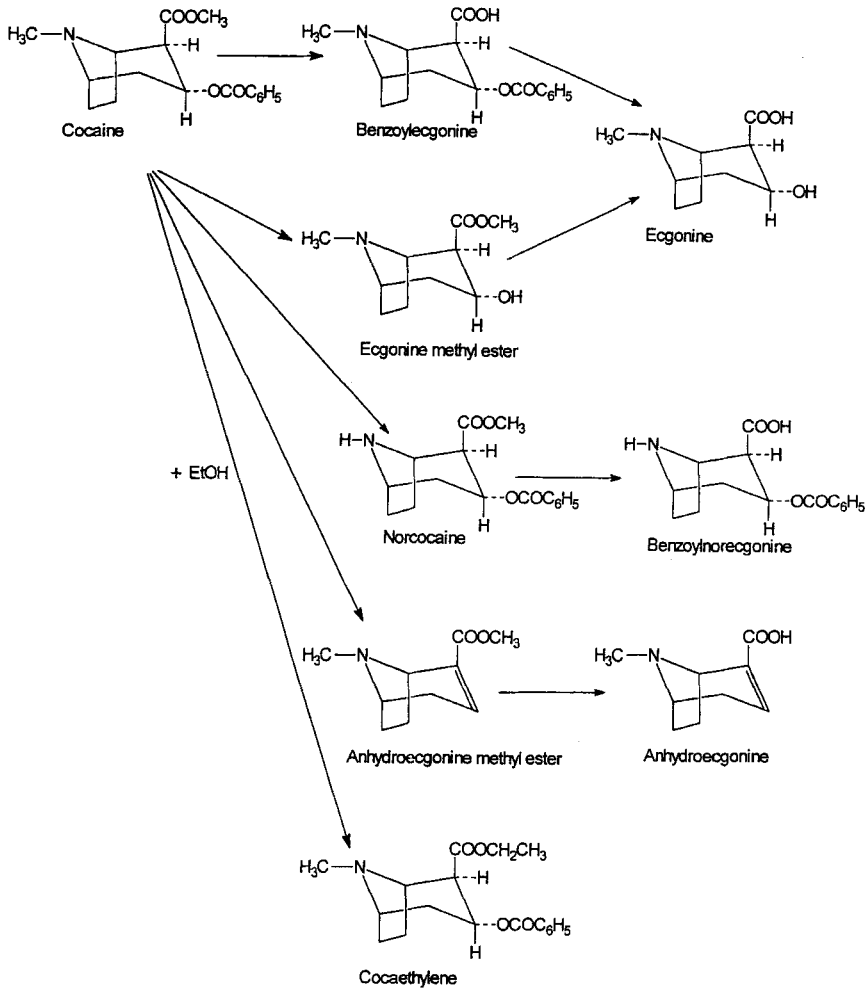

Figure 1 : Main biotransformation pathways.

Benzoylecgonine as well as ecgonine methyl ester are then further hydrolysed to the inactive ecgonine.

N-demethylation of cocaine to norcocaine has been identified as a minor metabolic pathway in humans. Norcocaine is one of the few cocaine metabolites found to have in vivo pharmacological activity in animals and to block amine re-uptake in vitro. Norcocaine appears to be unimportant as a determinant of cocaine's behavorial effects in humans. However, metabolites of norcocaine such as N-hydroxy norcocaine and norcocaine nitroxide have been suggested as mediators of cocaine's hepatotoxicity in animals (1). Norcocaine can be hydrolysed to benzoylnorecgonine, a metabolite causing seizures in rats and accumulating in the guinea pig foetus following maternal cocaine administration. Large concentrations of benzoylnorecgonine are reported in the urine of pregnant cocaine users, suggesting a similar accumulation in the human foetus (5,6).

The anhydroecgonine methylester, a pyrolytic product of cocaine, is formed as a result of thermal degradation when cocaine free base is smoked and can be found together with anhydroecgonine in the urine of crack smokers (7).

The prevalence of alcoholism in cocaine abusers is about twice that seen in heroin abusers. The cocaine addicts state that ethanol improves and prolongs their euphoria and reduces the crash that occurs upon cessation of cocaine use. Cocaethylene, a pharmacologically significant analog of cocaine, formed by transesterification of cocaine with ethanol when the two are present together, might be responsible for this. Its formation is catalysed by the hepatic carboxyesterase which is also responsible for the formation of benzoylecgonine. Although the psychomotor stimulant effects produced by cocaethylene and cocaine are similar, cocaethylene is less potent than cocaine in production of such effects. At the same time the lethal dose of cocacethylene is much less than that of cocaine in mice. These findings suggest that there may be increased risk of morbidity and mortality as users titrate their cocaine intake based on the weaker stimulant effects of cocaethylene, during which time additional cocaine may be converted to the more toxic cocaethylene (8,9).

Arylhydroxy and arylmethoxy metabolites of cocaine have also been identified as minor metabolites in urine of cocaine users. Meta-hydroxybenzoylecgonine is a metabolite that is also present in meconium and the fact that it is not a routine analyte in commercial meconium testing laboratories is in some cases responsible for misdiagnosis (10) (Fig.2). Interestingly, ethylester homologs of these compounds have also been detected in urine of individuals using cocaine and ethanol concurrently (11-13).

MATRICES OF INTEREST

Urine and blood are the most popular biological fluids for drug research. As cocaine is rapidly hydrolysed to benzoylecgonine many studies are performed on its stability in these two matrices. It was proved that in refrigerated blood samples and in alkaline buffers the cocaine concentration decreases as a function of time when no precautions are taken. Degradation can be prevented by lowering both pH and temperature (14-18). In blood, cocaine is also hydrolysed to ecgonine methylester when the enzymes responsible for this are not deactivated by an anticholinesterase agent (16,17).

Several other matrices are also examined by HPLC. In liver (19), high concentrations of cocaine and its metabolites are found. In brain (20), cocaine levels are higher than in blood and cocaine is stable in frozen brain tissue up to three months post-mortem. Vitreous humour (21,22) has also been examined for the presence of cocaine : it is a fairly simple matrix and is not influenced by any post-mortem alteration. The lack of post-mortem metabolic activity in this matrix suggests that drug levels found in vitreous fluid may give a quite accurate indication of body drug concentrations at the time of death.

The analysis of meconium (7,23), the first passed stool of newborns, is an increasingly prevalent method for detection of substances which were present in the mother uterus. Meconium begins to form between ten to twelve weeks of gestation and continues to be formed throughout intrauterine life. It may

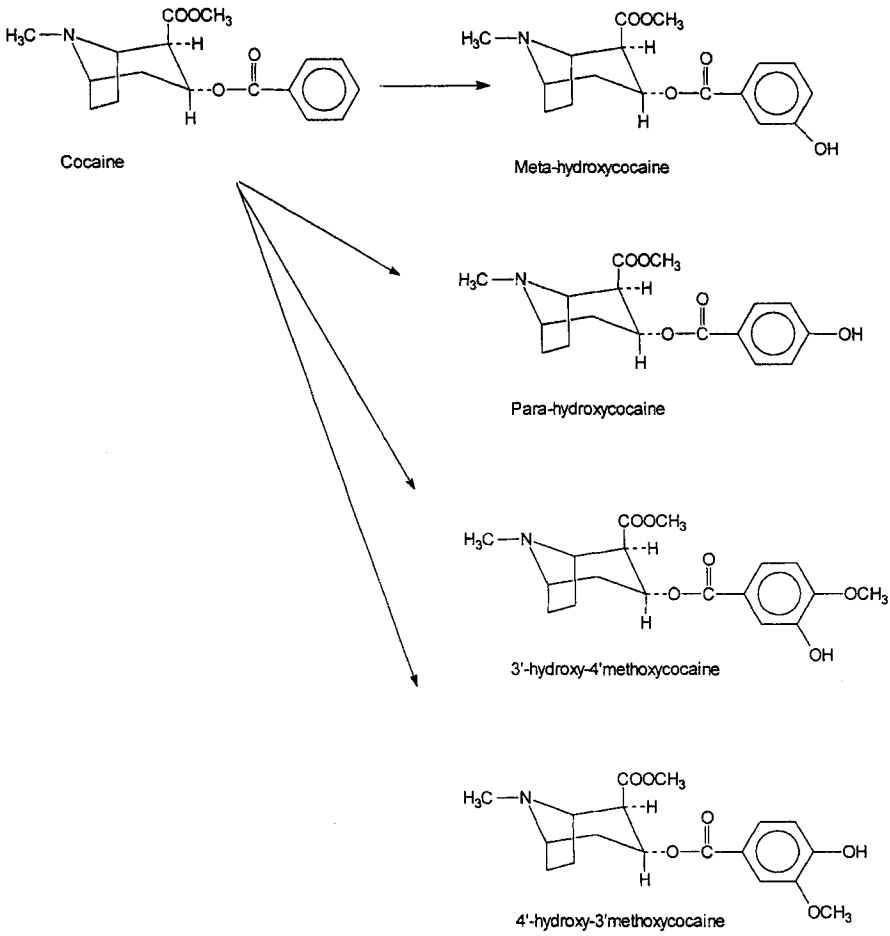

Figure 2 : Minor metabolites of cocaine.

serve as a reservoir for substances taken by the mother, and therefore it is a record of fetal drug exposure for up to the last twenty weeks of gestation. The concentrations of cocaine in meconium are relatively high. Amniotic fluid (24) is also examined as an alternative to meconium, because it is present during the entire pregnancy.

Hair (25-27) affords also an interesting matrix for HPLC analysis of cocaine. Drugs pass from the body fluids into the hair and remain bound in this matrix. Hence, hair analysis may provide information regarding the past use of drugs. Taking growth into consideration, hair can be divided into single strands for estimating the timespan at which the drug was taken.

Saliva (28,29) has already been analysed for cocaine with GC and concentrations have been found exceeding those in plasma. In this way HPLC analysis of saliva can become important in the cocaine abuse investigation.

EXTRACTION

A suitable sample preparation is an important prerequisite for applying liquid chromatography to biosamples. The isolation is usually performed by liquid-liquid extraction at a pH at which the analyte is non-ionized, or by Solid Phase Extraction.

a. Liquid-liquid extraction

A first group of liquid-liquid extraction procedures was developed for the extraction of cocaine and of the active metabolites such as norcocaine and cocaethylene. The coextraction of those three compounds is due to their similar lipophilicity.

The samples are brought to an alkaline pH with a sodium carbonate buffer (30-34), a borate buffer (35,36), or sodium hydroxide (37), and then extracted with hexane-iso amylalcohol (98:2, by vol) (30,32,33), hexane-iso-amylalcohol (99:1, by vol) (37), diethylether (31,35), hexane (34) or chloroform (36). In some cases an additional aqueous back-extraction step is performed in hydrochloric acid (30,32,33,37), tetramethylammonium hydrogen sulphate (35) or acetic acid (31). The aqueous acidic fraction can be directly injected onto the HPLC (30,32,33,35,37) or submitted to a second alkaline extraction comparable to the first one ; the organic phase is then evaporated to dryness and injected after redissolution in the mobile phase (31).

TABLE 1

Survey of the Determination of the Compounds Extracted with a Liquid-liquid Method.

Ref.	Matrix[a]	Analyte[b]	Col[c]	Eluent[d]	Mode[e]	Intern.St.[f]	Det[g](nm)
6	Pl,TH	C,BE	C18	H_2O-CH_3CN-CH_3OH+HAc	I	Lidocaine	235
18	L	C,BE,CE,NC	C18	PB-CH_3CN	I	Cocaine PE	235
29	Pl	C,CE	C8	PB-CH_3CN-CH_3OH	I	Cocaine PE	235
30	Pl	C	C18	PB-CH_3OH	I	Tetracaine	232
31	Pl	C,CE	C6	PB-CH_3CN+HS	I	Cocaine PE	235
32	Pl	C,CE	C8	PB-CH_3CN+HS	I	Cocaine PE	235
33	Pl	C,CE	PCN	PB-CH_3CN-CH_3OH	I	Protryptiline	214-230
34	Pl	C,NC	C18	TMAHS-CH_3CN	I	None	230
35	Pl	C	PH	PB-CH_3CN-NaCl	I	Desipramine	210
36	L,S,U	C,BE	PCN	PB-CH_3OH+TEA	I	Benzoctamine	233
37	Pl,U	C,BE	C17	AAB-CH_3CN-CH_3OH	I	Bupivacaine	230
38	U	C,BE	C18	PB-CH_3CN	I	Cocaine EE	200-235
41	S	C,BE,NC,NB,E	C18	SAB-CH_3CN	I	Lidocaine	230
42	Pl	C,BE,EM	Pol	PB-CH_3CN	G	Cocaine EE	235-E.D.
50	S	C,CE,NC	C18	PB-CH_3CN	I	Mazindol	230
51	U	BE	C18	PB-CH_3OH	I	None	Fl.

Abbrevations used :

a : Pl = Plasma ; TH = Tissue homogenate ; L = Liver ; S = Serum ; U = Urine
b : C : Cocaine ; BE = Benzoylecgonine ; CE = Cocaethylene ; NC = Norcocaine
c : PCN = Polycyano ; Pol = polymeric ; PH = Phenyl
d : PB = Phosphate Buffer ; HS = Hexane Sulfonic Acid ; TMAHS = Tetramethyl ammonium hydrogen sulphate ; TEA = Triethanolamine ; SAB-AAB = Sodium, Ammonium Acetate Buffer,
e : I = Isocratic System, G = Gradient elution
f : EE = Ethylester ; PE = Propylester
g : E.D. = Electrochemical Detection

The extraction of lipophilic compounds such as cocaine, norcocaine and cocaethylene, can be performed with a simple liquid-liquid extraction, with a high extraction efficiency and results in pure extracts. These determinations can be satisfactory for some clinical and pharmacokinetic tests but are insufficient for judicial testing or to establish the cause of death. Failure to detect the parent drug or the active metabolite in blood or urine does not exclude fairly recent use due to the short elimination life of those compounds.

An evaluation of the different liquid-liquid extractions has confirmed that benzoylecgonine, containing both an amine and a carboxylic acid group, possesses high hydrophobicity but can be extracted simultaneously with cocaine from neutral or basic media with chloroform or dichloromethane. The extraction efficiency is further enhanced by addition of alcohols such as isopropanol or ethanol ; on the other hand, the extraction with diethylether, diethylether-benzene or butylchloride is not effective for benzoylecgonine. The addition of alcohols, however, increases the recovery at the expense of the purity of the extracts of biological samples which negatively affects the detection limits (38).

The samples are buffered at an alkaline pH with a sodium carbonate buffer, ammonium hydroxide (41,42), a phosphate (18) or borate buffer followed by extraction with chloroform-isopropanol (3:2, by vol) (6,39), (95:5, by vol) (43) or (9:1, by vol) (42), chloroform-ethanol (80:20, by vol) (40) or (82.5:17.5, by vol) (44), dichloromethane-isopropanol (90:10, by vol) (19) or dichloromethane-ethanol (50:50, by vol) (41). This alkaline extraction can be preceded by an acidic extraction in which the organic phase is discarded (40) or followed by an acid aqueous extraction (39). None of these methods seems to be outstanding. The chromatograms shown are not convincing and due to the unspecific wavelength that is mostly used (around 230 nm) problems in UV-detection may arise with post-mortem matrices.

Miller and DeVane (45) describe the extraction with acetonitrile after alkalinization of the plasma. After centrifugation the upper organic layer is evaporated, the

residue is redissolved in 0.1% hydrochloric acid and injected. The miscibility of acetonitrile and water will certainly result in very long evaporation times.

The main advantage of the liquid-liquid extraction is its linearity over a wide range of concentrations : the same extraction can be applied to clinical and experimental samples where the concentrations are low and also to post-mortem samples where the concentrations for cocaine and benzoylecgonine are often very high.

b. Solid phase extraction

Solid phase extractions have become popular as a result of high recoveries, pure extracts and ease of automation. Solid phase extractions of cocaine and metabolites can be divided into three groups according to the nature of the sorbent.

A first group is based on a non-polar sorbent, such as ethyl- (46), or octadecylphases (47,48). After conditioning of the column with methanol and an alkaline buffer, samples are applied. Subsequently, columns are washed and the compounds are eluted with a mixture of chloroform and methanol. Due to the strongly different polarity of cocaine and e.g. benzylecgonine a wide range of contaminants which will interfere in the chromatographic step will also be eluted. Probably, this is the reason why several authors do not include a chromatogram.

Strong cation exchange columns are used (23,47,49) to extract positively charged basic compounds. After conditioning with methanol and an acidic buffer, the sample is diluted with the acidic buffer so that the amino-functions of the compounds are positively charged. After application the compounds of interest are eluted with a mixture of an alkaline buffer or ammonium hydroxide and methanol.

Recently mixed-mode sorbents became quite popular (7,20,21, 24,38,50). These resins combine hydrophobic and cation-exchange properties and have the potential to retain analytes covering a wide range of polarity. The mixed-mode isolation of benzoylecgonine is clearly described by Mills et al. (52) and recoveries are from 60% (blended resin) to 95% (in a copolymerized resin).

TABLE 2

Survey of the Determination of the Compounds Extracted with a SPE method.

Ref.	Matrix[a]	Analyte[b]	Col[c]	Eluent[d]	Mode[e]	Intern.St.[f]	Det[g](nm)
16	Meconium	C,BE,NC,NBE	C18	PB-CH_3CN+TBAH	I	Lidocaine	233
19	BT	C,BE,CE,NC	C18	PB-CH_3CN+BA	I	Bupivacaine	230-255-275
20	VH	C,BE	C18	PB-CH_3OH	I	Tetracaine	235-275
22	Meconium	C,BE	C18	PB-CH_3CN	I	Non	230-255-275
24	H	C	Pol	PB-CH_3OH+THF	I	Tetracaine	DAD-Fl
36	L,S,U	C,BE	PCN	PB-CH_3CN+TEA	I	Benzoctamin	DAD
40	Pl,U,AF	C,BE,NC,NBE	C18	PB-CH_3CN+TBAH	I	Lidocaine	233
43	Pl	C,BE,CE	C18	PB-CH_3CN+BA	I	None	230-255-275
44	S	C,BE,NC,NBE	PCN+S	PB-CH_3CN	I	Tolazoline	228
45	U	BE	C18	PB-CH_3CN+DS	I	None	233
46	U	C,BE,NC,EME,E	Pol	AAB-CH_3CN	G	None	MS
47	U	C,BE,CE,EME	C18	PB-CH_3CN	G	Methadone, Cocaine HE	Fl

Abbreviations used

a : Pl = Plasma ; BT = Brain tissue ; L = Liver ; S = Serum ; U = Urine ; VH : Vitreous humour ; H = Hair ; AF = Amniotic fluid

b : C = Cocaine ; BE : Benzoylecgonine ; CE = Cocaethylene ; NC = Norcocaine ; NBE = Norbenzoylecgonine ; EME = Ecgonine methylester ; E = Ecgonine

c : PCN = Polycyano ; Pol = polymeric ; S = Silica

d : PB = Phosphate Buffer ; TBAH = Tetrabutyl ammonium hydroxide ; BA = Butylamine ; THF = Tetrahydrofuran ; TEA = Triethanolamine ; DS = Dodecylsulphate ; AAB = Ammonium Acetate Buffer ;

e : I = Isocratic System ; G = Gradient Elution

f : HE = Hexylester

g : DAD = Diode array detection ; Fl = Fluorescentie ; MS = mass spectrometry.

The sample is diluted with a weak acidic phosphate buffer and columns are conditioned successively with methanol, water and with the phosphate buffer ; the columns are then washed with water, hydrochloric acid and methanol and elution is performed with a mixture of dichloromethane-isopropanol-ammoniumhydroxide (78:20:2, by vol).

Sample preparations used for GC can be subdivided in the same categories, but we only focus on these extraction procedures that were directly applied to HPLC.

CHROMATOGRAPHY

Most methods use non-polar phases chemically bonded to silica, octadecyl being the funtional group that is used predominantly. The eluent applied to this stationary phase is a mixture of methanol and/or acetonitrile and a phosphate buffer pH 2.0-7.0 (7,19,21,23,31,36,40,42,43,46,48,50). End-capped columns (19,50) or a modifier such as tetrabutylammoniumhydroxide (7,43), tetrabutylammonium phosphate (36), tetrabutylammoniumbromide (42), diethylamine (23), dodecylsulphate (48) and butylamine (20,46) are used. The phosphate buffer is sometimes replaced by an acetate buffer (39,44). Evans and Morarity (5) elute the octadecyl column with water-acetonitrile and methanol containing 1 % acetic acid and 0.3 M methylenediamine-tetraacetate. Bouis et al. (35) use a 0.1 % tetramethylammoniumhydrogeniumsulphate in water mixed with acetonitrile.

Jatlow and Nadim (32) chromatographed the samples on an hexylsilica with a mixture of acetonitrile and phosphate buffer at pH 3, containing the sodium salt of hexane sulphonic acid as an ion pairing reagent. The same mixture is used by Bailey (29,32) on an octylsilica column while Logan and Stafford (22) omit the ion pairing reagent. Hackett and co-workers (37) chromatograph on a phenyl phase, the solvent being acetonitrile in an aqueous solution of 0.01 % phosphoric acid and 0.01 % sodium chloride.

Balíková and Večerková (38) use a nitrile phase which is a medium-polar phase bonded to silica, whereas their eluent is a phosphate buffer-methanol-acetonitrile mixture. Puopolo and co-workers (34) use the same phase but elute with a phosphate buffer-methanol-triethylamine mixture.

Lampert and Stewart (47) describe a more complex HPLC assay of cocaine and his metabolites in serum. The compounds are chromatographed on tandem-cyanopropylsilica columns used in the reversed-phase mode. The mobile phase consists of an acetonitrile-phosphate buffer mixture.

Miller and DeVane (45) elute ecgonine methylester from a polymeric styrene divinylbenzene column with a mixture of acetonitrile-phosphate buffer pH 8.8. The same column is used by Tagliaro et al. (25,26) who use a mobile phase of methanol-tetrahydrofuran and a phosphate buffer pH 3. Nishikawa and co-workers (49) use a chromatographic separation for liquid chromatography / atmospheric pressure chemical ionisation / mass spectrometry, on a column with a hydrophillic polymer used for aqueous steric exclusion chromatography and a gradient elution with ammoniumacetate and acetonitrile.

INTERNAL STANDARDS

There is a substantial diversity in the internal standards used in the published procedures. The same compound, sometimes strongly deviating regarding to pKa value and lipophilicity, is used for the internal standardization of different substances. Most of the internal standards differ completely in structure from cocaine or its metabolites e.g. bupivacaine (20,39), lidocaine (5,6,43), protriptyline (34), tolazoline (47), tetracaine (21,25,31), methadone (50), benzoctamine (38) and desipramine (37). Lidocaine is a known adulterant of illegal street cocaine and thus not very useful for the determination of cocaine in those samples.

Cocaine propylester (19,30,32,33) or hexylbenzoylecgonine (50) are chemically more closely related to cocaethylene and

cocaine but they are completely inadequate to compensate for losses during the extraction of benzoylecgonine.

Cocaethylene is used as an internal standard by Miller and DeVane (45) and Jatlow et al. (40). However, cocaethylene is formed in the body during concomitant use of cocaine and ethanol. Therefore its use as an internal standard should be restricted to certain well-controlled clinical cases where co-ingestion of the two compounds is excluded.

In a great number of methods (23,26,35,45,48,49) no internal standard is used. This, however, is not acceptable in a quantitative analysis of compounds in biological matrices.

DETECTION METHODS

Although cocaine and most of its metabolites show UV-absorption at 200-230 nm, this unspecific wavelength is only suitable for detection after efficient prepurification as several matrix constituents are also absorbing at this wavelength. A second disadvantage of UV-detection is the inability to detect ecgonine methylester and ecgonine since they lack a chromophore group and thus UV absorbancy. The UV-detection is mostly performed around 230 nm (6,19,21,23,30-36,38-40,43-45,47,48). Higher wavelengths result in a decreased sensitivity fn cocaine but suffer less from interferences.

Balíková and Vecerková (38) and Puopolo and co-workers (34) apply a diode-array detector which specificity is much better than that of a single wavelength detector. Two independent parameters determine the final specificity : the retention time of the compounds and the UV spectrum.

Tagliaro and co-workers (25,26) use the weak native fluorescence of cocaine (related to its phenyl ring) to develop a sensitive and specific HPLC-procedure for the determination of cocaine and benzoylecgonine in hair samples. The excitation and emission wavelengths are set at 230 and 315 nm, respectively.

Attempts have been made to detect ecgonine methylester as well as the other metabolites of cocaine together with cocaine

by HPLC. Miller and DeVane (45) use serial UV and electrochemical detection. Under the conditions described benzoylecgonine and cocaine are detected by UV absorbancy while ecgonine methylester and cocaine are detected electrochemically.

Roy et al. (50) proposed an HPLC method with fluorescence detection . This method was aimed specifically to the determination of the non-UV absorbing metabolite ecgonine methylester and requires a complex system of on-line post-column ion-pair extraction and derivatization, in which benzoylecgonine and norcocaine are alkylated and ecgonine methylester is silylated.

Nakashima et al. (42) developed a method with 3, bromo-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone (Br-DMEQ), a highly fluorerescent labeling reagent for the determination of benzoylecgonine and ecgonine. The fluorescence of these derivatives is monitored at 455 nm with excitation at 370 nm.

Recently, liquid chromatography/mass spectrometry (LC-MS) has gained widespread recognition as an analytical tool since it permits the separation and ionization of polar, non-volatile or thermally labile compounds without derivatization. In addition, LC-MS provides structural information. For the determination of cocaine and its metabolites in biological fluids it widens our possibilities since low UV sensitive compounds such as ecgonine methylester and ecgonine can easily be detected. Nishikawa and co-workers (49) published the analysis of cocaine and its metabolites by liquid chromatography/atmospheric pressure chemical ionisation mass spectrometry.

CONCLUSION

A survey of the different extractions, chromatographic conditions, internal standards and methods of detection is given. A lot of work has been already done on the liquid chromatographic determination of cocaine and its metabolites in biological samples. Yet, additional research is still necessary to find a good internal standard for the determination of benzoylecgonine ; other matrices than the classic ones should be examined and the detection of ecgonine and ecgonine methylester appears another important objective for future developments.

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Received: January 10, 1995
Accepted: January 25, 1995