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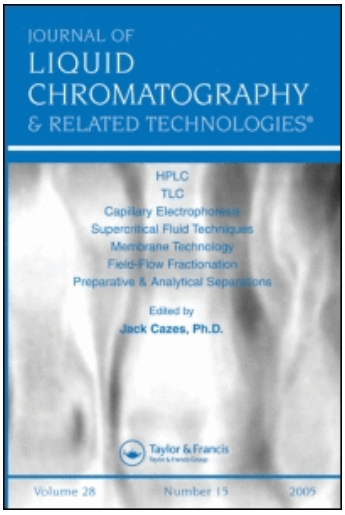

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Liquid Chromatographic Analysis of Cocaine, Benzoylecgonine, Local Anaesthetic Agents and Some of Their Metabolites in Biological Fluids

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LIQUID CHROMATOGRAPHIC ANALYSIS OF COCAINE, BENZOYLECGONINE, LOCAL ANAESTHETIC AGENTS AND SOME OF THEIR METABOLITES IN BIOLOGICAL FLUIDS

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ABSTRACT

Cocaine and benzoylecgonine, other local anaesthetic agents (amides, esters) and some of their metabolites were determined in biological samples, after liquid-liquid or solid-liquid extraction, by high performance liquid chromatography, using a reversed-phase column, an appropriate internal standard, three mobile phases, with two flow rates, UV detection at 230 nm and 280 nm, and scanning UV spectra with photodiode array detector. Fluorimetric detection (ex. : 280 nm, em : 350 nm) was also available for characterizing the ester anaesthetic compounds. The method is selective and suitable for clinical pharmacology purposes as well as for forensic toxicology.

INTRODUCTION

Since cocaine was classed as narcotic, a wide variety of synthetic local anaesthetic agents, esters and amides (Fig. 1) have been proposed as alternative compounds (1,2). Furthermore, these drugs are frequently used as illicit cocaine adulteration (3,4) . Most of them are not psychoactive, but develop the same local anaesthetic effect as cocaine. Many of the suspected cocain samples were found to

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contain only local anaesthetic in combination with other stimulant substances (caffeine, ephedrine,...). The widespread abuse of cocaine has prompted many studies for analyzing these compounds, understanding of their pharmacokinetic and pharmacodynamic effects. Numerous metabolites of cocaine (benzoylecgonine, ecgonine, norcocaine, ecgonine methylester, norecgonine methylester, ecgonidine, ecgonidine methylester, norecgonidine methylester, m-hydroxycocaine, p-hydroxy-cocaïne and m-hydroxybenzoylecgonine), have been identified in humans (5-7). Only benzoylecgonine is found in relatively high concentrations in blood and urine of cocaine users (8). Its measurement is of interest as a marker for cocaine abuse in forensic toxicology (9-12).

Synthetic local anaesthetic agents are prescribed for local or regional anaesthesia. Among these drugs, lidocaine is often used as antiarythmic agent. In some clinical situations, local anaesthetics may develop toxic reactions, increasing especially their neuro - or cardio-toxicity (13-15). Their placental transfer and their foetal toxicity during obstetric anaesthesia have been reported (16,17). Thus the determination of these agents and their metabolites is useful for clinical pharmacology as well as for forensic toxicology.

Several methods have been proposed for the analysis of cocaine and/or other local anaesthetics io biological media using gas chromatography (10,18-35), liquid chromatography (12, 36-40) and immuno-assays (34, 35, 41-49). The present study was undertaken to develop a HPLC procedure for the determination of cocaine, benzoylecgonine, other local anaesthetics and some of their metabolites in biological samples.

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MATERIAL AND METHODS

Reagents and glassware

All reagents were of analytical grade. Ammonium acetate (Normapur), sodium carbonate (Normapur), sodium bicarbonate (Normapur) were from Prolabo (Paris, France). Chloroforme (RS : Reagente Speciale), methylene chloride (RS), diethylether (RS), methanol (RS, for HPLC), acetonitrile (RS, for HPLC), isopropanol (RPE : Reagente Puro Erba), hydrochloric acid (RPE) and sulphuric acid (RPE) were from Carlo Erba (Milano, Italy). 3 ml Extrelut® pre-packed column were from Merck (Nogent sur Marne, France). All glassware was washed with a 3 % "RBS 25" biodegradable alkaline solution from Serflam (Marseille, France), which contains a mixture of anionic and non-ionic detergents, and then rinsed successively with distilled water, acetone and extraction solvent.

Drugs and internal standard

Cocaine, benzoylecgonine and other local anaesthetics and some of their metabolites (Fig. 1) were kindly supplied by the respective manufacturers.

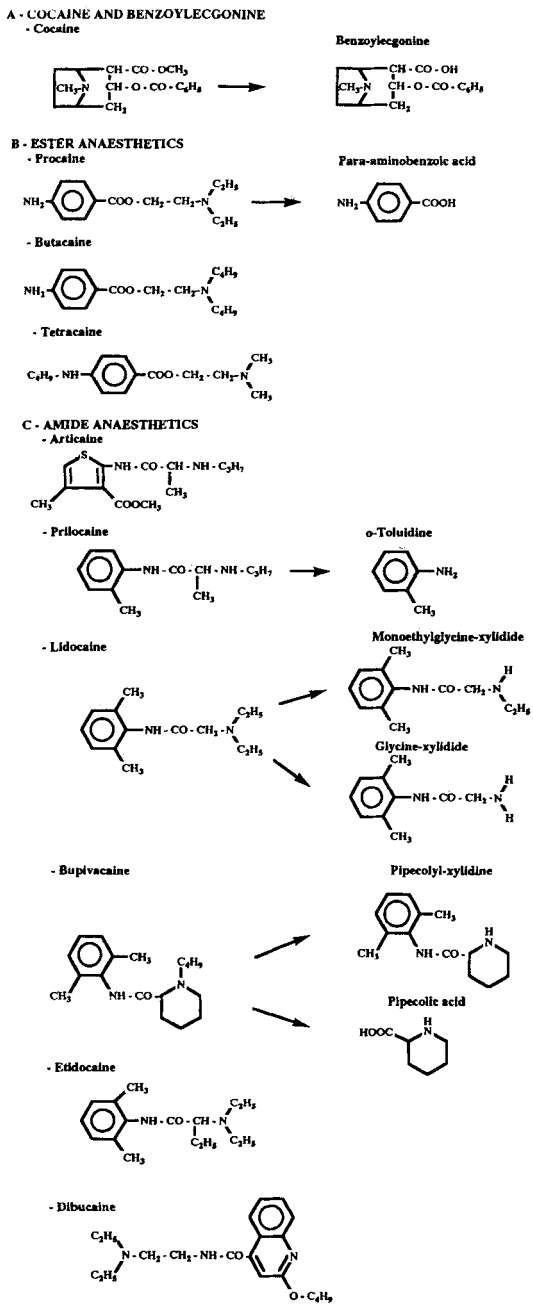

Fig 1 : Tested compounds and formula

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Stock solutions were made in methanol at a concentration of 1 $\mu g\ \mu l^{-1}$ and stored at -20°C. They are stable for at less one month. They were diluted to 100 ng μl^{-1}, 10 ng μl^{-1} and 1 ng μl^{-1} in the same solvent before use.

The internal standard was chosen according to the analyzed compounds (Table 1).

Apparatus and chromatographic parameters

The chromatographic analysis was performed on a component "Waters" system consisting of a M590 pump (A) and a M510 pump (B), a WISP 712 automatic sample injection module, a μ Bondapak C18 column (30 cm x 3.9 mm I.D. ; particle size 10 μm, ambient temperature) connected to a μ Bondapak C18 T.M. guard pak column (5 mm x 6 mm I.D. ; two filters, one at either end, held the packing in place and provided a 2 μm filtering capability), a UV-VIS M990 photodiode-array detector (which permits scanning chromatographic and spectral data) coupled with a Kontron SF25 fluorimetric detector and a 840 control station.

Two mobile phases were used. Each one was a mixture of two liquids distributed by the (A) pump : 0.1 M ammonium acetate, and the (B) pump : pure acetonitrile or mixture of acetonitrile and methanol (50:50,v/v) :

- Phase n° 1 : 0.1 M ammonium acetate/acetonitrile (50:50, v/v) with a flow rate of 1.5 ml min^{-1} was used for the analysis of amide and ester local anaesthetics.
- Phase n° 2 : 0.1 M ammonium acetate/acetonitrile-methanol mixture (40:60,v/v) with a flow rate of 1 ml min^{-1} was used for the analysis of cocaine and benzoylecgonine.

The UV detection wavelength was monitored at 230 nm for cocaine and benzoylecgonine and amide anaesthetic compounds (except for articaine), and at 280 nm for the ester compounds and articaine. In addition the fluorimetric detection (ex. 280 nm, em : 350 nm) was used for characterizing the ester compounds. The UV spectrum of each compound corresponding to a peak was established between 200 and 350 nm.

Extraction procedure

. Local anaesthetic drugs (amide or ester) : solid-liquid extraction

To 2 ml of the sample (whole blood, plasma, urine) to be analyzed, 1 ml of sodium carbonate saturated solution and a known amount of the appropriate internal standard (Table 1) were added. The mixture was passed onto a 3 ml Extrelut® cartridge. Elution was then carried out with 15 ml methylene chloride. The eluate was collected in a 20 ml evaporation glass tube and evaporated to dryness under a stream of nitrogen in a 40°C water bath. For the ester analysis, the residue was dissolved in 100 μl of mobile phase n° 1 and 40 μl of this solution were injected into the chromatograph. For the amide analysis the residue was dissolved in100 μl of 0.01M hydrochloric acid. The acid solution obtained was

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TABLE 1 : Choice of Internal Standard

Compound to be analyzed	Internal standard	Amount to be used
Cocaine and Benzoylecgonine	Bupivacaine	1 μg (10 μl sol. 0.1 μg μl^{-1})
Procaine Paraaminobenzoic Acid Tetracaine	Butacaine	0.5 μg (50 μl sol. 0.01 μg μl^{-1})
Butacaine	Tetracaine	2 μg (20 μl sol. 0.1 μg μl^{-1})
Lidocaine Monoethylglycine-xylidide* Glycine-xylidide* Bupivacaine Pipecolyl-xylidine* Dibucaine Prilocaine o-Toluidine	Etidocaine	1 μg (10 μl sol. 0.1 μg μl^{-1})
Articaine	Butacaine	1 μg (10 μl sol. 0.1 μg μl^{-1})
Etidocaine	Dibucaine	2 μg (20 μl sol. 0.1 μg μl^{-1})

* Prilocaine can be used as internal standard for these compounds (see "selectivity" chapter).

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washed by vortex-mixing with 3 ml of diethylether for 20s on a whirlimixer and centrifuged for 5 min at 2800 g. The ether layer was discarded. 40 μl of the acid solution were injected into the chromatograph.

. *Cocaine and benzoylecgonine : liquid-liquid extraction*

Benzoylecgonine extraction requires a solvent containing a high proportion of an alcohol : for instance chloroform/isopropanol (3/2). But, when this solvent is passed on the Extrelut® cartridge, the aqueous phase is also eliminated from the

column. For this reason a liquid-liquid extraction of benzoylecgonine and cocaine from alkaline medium was developed and followed by an acidic back extraction.

2 ml of the sample (whole blood, plasma, urine) to be analyzed were collected into a 20 ml glass centrifuge tube. After introducing 10 μl of bupivacaine (internal standard) solution at 0.1 μg μl^{-1} and 1 ml of the mixture of sodium carbonate and bicarbonate saturated solutions (50:50,v/v) the liquid was shaken for 10 min with 8 ml of chloroform/isopropanol (3/2, v/v). After centrifugation, the organic layer was transfered into a second tube containing 2 ml of 0.1M sulphuric acid and the mixture was shaken for 10 min. The mixture was then centrifuged and the organic phase was discarded. 2 ml of sodium carbonate and bicarbonate saturated solution and 10 ml chloroforme/isopropanol (3/2) were added to the aqueous phase. The resulting mixture was shaken for 10 min. Following centrifugation, the organic phase was removed to a clean glass tube and evaporated to dryness under a stream of nitrogen in a 40°C water-bath. The residue was dissolved in 100 μl of mobile phase n° 2 and 40 μl of this solution were injected into the chromatograph.

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Calculation

The ratio between the peak areas of the analyzed drugs and that of the appropriate internal standad was calculated and plotted against the concentrations of the tested drugs after analysis of blank samples spiked respectively with increasing concentrations of each drug (50, 100, 200, 500 and 1000 ng ml^{-1}) and a constant amount of the appropriate internal standard. Within these concentration ranges the relations were linear for all compounds. The coefficients of correlation were showed in table 2.

RESULTS AND DISCUSSION

Table 3 shows the retention time, the absorption characteristics in ultraviolet and the obtained responses by fluorimetric detection of the analyzed compounds.

Fig. 2 shows the chromatograms and the spectra obtained after solid-liquid extraction of anaesthetic drugs from a blank plasma before (A) and after spiking with a mixture of the ester-drugs (B) and amide drugs (C). Fig. 3 is concerning a plasma of a patient treated with therapeutic doses of lidocaine and bupivacaine. Fig. 4 shows the chromatograms and the spectra obtained after liquid-liquid extraction of cocaine and benzoylecgonine from whole blood (blank, spiked blank and necropsic samples) and from urine (blank, spiked blank and necropsic samples).

Recovery

The percentage extraction of each drug at 50 and 500 ng ml^{-1} concentrations was calculated by comparison of the peak area ratios from the reference sample and from the assay. For the assay the tested drug was added before the extraction

TABLE 2 : Coefficients of Variation (CV % : highest values), Correlation coefficients (Cr) and Extraction Percentage (%) issued from Reproducibility, Linearity and Recovery Studies on Whole Blood (WB), Plasma (P) and Urine (U)

Tested Compounds		Reproducibility (CV %)		Linearity (Cr)	Recovery (%) Mean ± SD, n = 6	
		Intraday (n = 7)	Day to day (n = 8)	(50-1000 ng ml^{-1})	50 ng ml	500 ng ml
COCAINE	WB	12	12.8	0.99	82 ± 6	84 ± 5
	P	11.2	13.8	0.99	82 ± 5	82 ± 4
	U	10.4	13	0.99	83 ± 6	84 ± 4
BENZOYL-ECGONINE	WB	12	12.5	0.98	80 ± 8	81 ± 7
	P	12	13.5	0.99	81 ± 8	82 ± 8
	U	10.6	13.6	0.99	82 ± 7	83 ± 6
ESTERS						
Procaine	WB	16.2	17.2	0.96	74 ± 8	74 ± 5
	P	12.5	17	0.97	74 ± 7	75 ± 5
	U	12.6	17	0.96	75 ± 6	76 ± 6
Paraamino-benzoic Acid	WB	12.5	12	0.98	65 ± 5	66 ± 4
	P	10.5	12.2	0.98	66 ± 5	67 ± 5
	U	11.8	11	0.98	68 ± 4	68 ± 3
Butacaine	WB	14.5	18	0.96	70 ± 7	71 ± 5
	P	12	12.5	0.96	71 ± 5	72 ± 4
	U	9	12.5	0.96	71 ± 3	71 ± 2
Tetracaine	WB	14.3	14	0.96	70 ± 6	72 ± 4
	P	12	13.5	0.97	70 ± 2	71 ± 3
	U	11.5	13	0.97	72 ± 4	72 ± 5
AMIDES						
Articaine	WB	10.8	8.7	0.99	86 ± 4	85 ± 4
	P	11	9.6	0.99	86 ± 3	87 ± 6
	U	9.7	9.5	0.99	87 ± 7	88 ± 6
Prilocaine	WB	10.5	11	0.99	86 ± 7	87 ± 4
	P	12.4	9.7	0.99	86 ± 4	87 ± 4
	U	9.6	10.2	0.99	88 ± 4	88 ± 5
o-Toluidine	WB	12.4	12.7	0.98	85 ± 10	86 ± 8
	P	11	10.5	0.99	87 ± 9	87 ± 7
	U	10.5	7.8	0.99	88 ± 5	89 ± 8
Lidocaine	WB	8.6	11.8	0.99	88 ± 7	89 ± 4
	P	10.7	10.6	0.99	86 ± 4	87 ± 7
	U	10.8	11	0.99	88 ± 4	88 ± 5
Monoethyl-glycine-xylidide	WB	11.5	10.9	0.98	82 ± 4	83 ± 5
	P	10.5	11.8	0.99	82 ± 6	83 ± 4
	U	9.8	10.2	0.99	84 ± 5	84 ± 5
Glycine-xylidide	WB	11.5	11	0.98	80 ± 8	81 ± 6
	P	11	10.8	0.98	82 ± 7	81 ± 7
	U	10.2	10.5	0.98	82 ± 4	82 ± 5
Bupivacaine	WB	10.5	9.8	0.99	88 ± 7	87 ± 4
	P	10.5	10.2	0.99	90 ± 6	88 ± 6
	U	9.3	10.6	0.99	89 ± 6	89 ± 5
Pipecolyl-xylidine	WB	9.5	9.6	0.99	89 ± 8	86 ± 6
	P	8.5	9	0.99	88 ± 7	87 ± 6
	U	7.3	8.2	0.99	90 ± 7	89 ± 5
Etidocaine	WB	11	9.8	0.99	87 ± 8	88 ± 6
	P	10.4	10.5	0.99	86 ± 6	86 ± 5
	U	9.8	8.8	0.99	89 ± 4	88 ± 4
Dibucaine	WB	10.5	11.2	0.99	80 ± 8	81 ± 4
	P	10.8	10.6	0.99	80 ± 7	82 ± 5
	U	8.8	9.7	0.99	81 ± 6	81 ± 7

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TABLE 3 : Retention Time (related to Cocaine), UV Absorption Characteristics and Fluorescence Response of Tested Compounds

Compounds	Retention time related to cocaine		UV absorption characteristics (between 200-350 nm)				Fluorescence response (ex. : 280 nm, em. : 350 nm)
	mobile phase n° 1	mobile phase n° 2	maximum wavelength		minimum wavelength		
COCAINE	1	1	233*	275	260	295	-
BENZOYL-ECGONINE	0.37	0.55	232*	274	260	295	-
ESTERS							
Procaine	0.74	0.65	220	295*	240	340	++
Paraaminobenzoic acid	0.55	0.50	215	290*	240	340	++
Butacaine	1.69	1.18	220	290*	240	340	++
Tetracaine	2.61	2.17	225	310*	250	345	+
AMIDES							
Articaine	0.77	0.68	210	275*	230	330	-
Prilocaine	0.80	0.84	208*	235*	220	300	-
o-Toluidine	0.63	0.59	208	234*284	260	330	-
Lidocaine	1.09	1.20	210*	265	255		-
Monoethylglycine-xylidide	0.69	0.72	210*	265	255		-
Glycine-xylidide	0.53	0.59	210*	265	255		-
Bupivacaine	1.77	1.64	210*	265	256		-
Pipecolyl-xylidine	0.69	0.71	210*	265	256		-
Etidocaine	2.15	2.21	210*	265	256		-
Dibucaine	2.47	2.88	210*	225*325	260		-
STIMULANTS							
Caffeine	0.34	0.36	215*	275*	240	300	-
Amphetamine	0.40	0.44	210*	260	230		-
Ephedrine	0.45	0.46	215*	257	280		-
Adrenaline	0.47	0.48	214	256*	225	290	
OPIATES							
Morphine	0.61	0.79	215*	285	262		++
O_6 monoacetyl-morphine	0.80	0.90	215*	285	262		±
Heroine	1.33	1.34	215*	285	262		-
Ethylmorphine	1.07	1.30	215*	285	262		++
Codeine	0.72	0.98	215*	285	262		++
Acetylcodeine	1.4	1.54	215*	285	262		±

* : strong absorption ; ++ : very good response ; + : good response ; ± : medium response ; - : no response.

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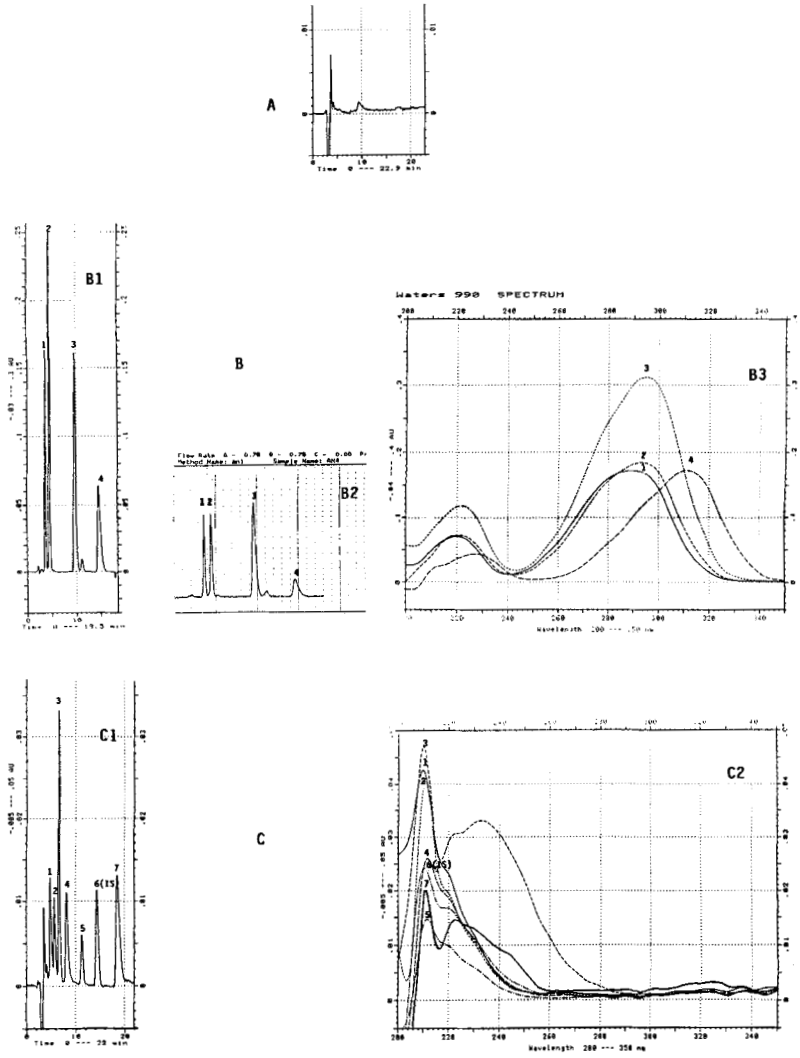

Fig 2 : Chromatograms and Spectra of blank plasma (2 ml) after solid-liquid extraction of anaesthetic drugs (mobile phase n° 1) :

A - Standard of blank plasma

B - Blank plasma spiked with ester anaesthetic compounds : (1) para-aminobenzoic acid : 1 μg ml^{-1}, (2) procaine : 1 μg ml^{-1}, (3) butacaine (IS) : 2 μg added, (4) tetracaine : 1 μg ml^{-1}

B1 - UV detection (280 nm)

B2 - Fluorescence detection (ex 280 nm, em 350 nm)

B3 - Corresponding spectra

C - Blank plasma spiked with amide anaesthetic compounds : (1) glycine-xylidine : 500 ng ml^{-1}, (2) monoethylglycine-xylidide : 500 ng ml^{-1}, (3) prilocaine : 500 ng ml^{-1}, (4) lidocaine : 500 ng ml^{-1}, (5) bupivacaine : 500 ng ml^{-1}, (6) etidocaine (IS) : 1 μg added, (7) dibucaine : 1 μg ml^{-1}

C1 - UV detection (230 nm)

C2 - Corresponding spectra (dibucaine spectrum over written).

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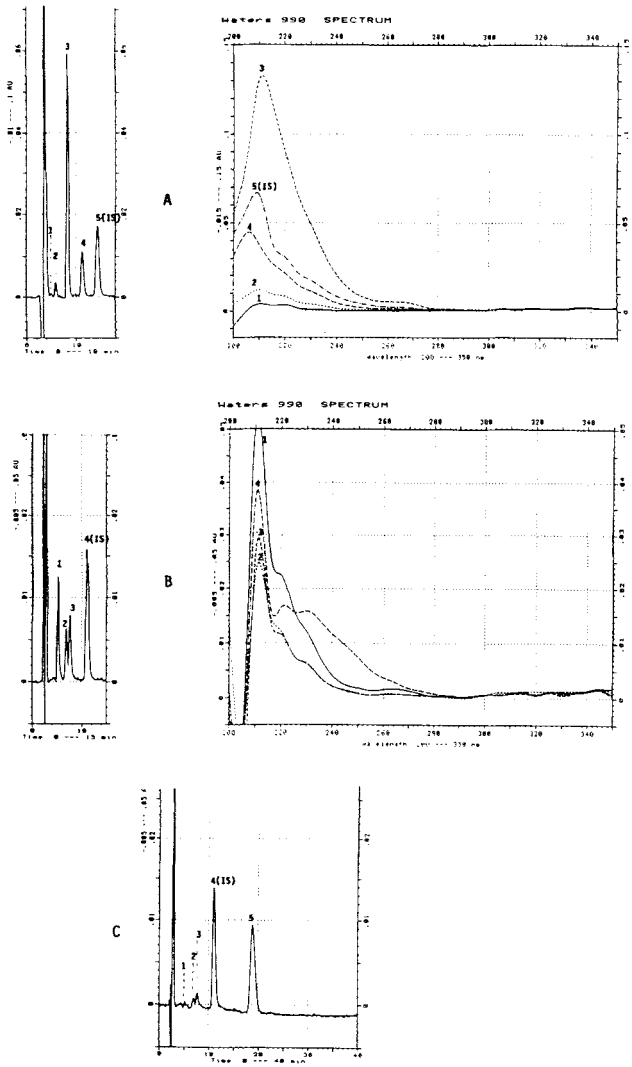

Fig 3 : Chromatograms and spectra of plasma (2 ml) from a patient treated with lidocaine and bupivacaine :

A - Measurement of lidocaine and bupivacaine (mobile phase n° 1) : (1) glycine-xylidide : trace, (2) monoethylglycine-xylidide + pipecolyl-xylidine (no mesurable), (3) lidocaine : 1900 ng ml^{-1}, (4) bupivacaine : 500 ng ml^{-1}, (5) etidocaine (IS) : 1 μg added

B - Measurement of glycine-xylidide, monoethylglycine-xylidide and pipecolyl-xylidine (mobile phase n° 3) on blank plasma spiked with (1) glycine-xylidide : 500 ng ml^{-1}, (2) pipecolyl-xylidine : 500 ng ml^{-1}, (3) monoethylglycine-xylidide : 500 ng ml^{-1}, (4) prilocaine (IS) : 1 μg added.

C - Measurement of glycine-xylidide, monoethylglycine-xylidide and pipecolyl-xylidine (mobile phase n° 3) on plasma (2 ml) from the patient (A) : (1) glycine-xylidide : 20 ng ml^{-1}, (2) pipecolyl-xylidine : 68 ng ml^{-1}, (3) monoethylglycine-xylidide : 108 ng ml^{-1}, (4) prilocaine (IS) : 1 μg added, (5) lidocaine (measured before, see fig. 3.A.), bupivacaine : no response during 40 min. No significant UV spectra.

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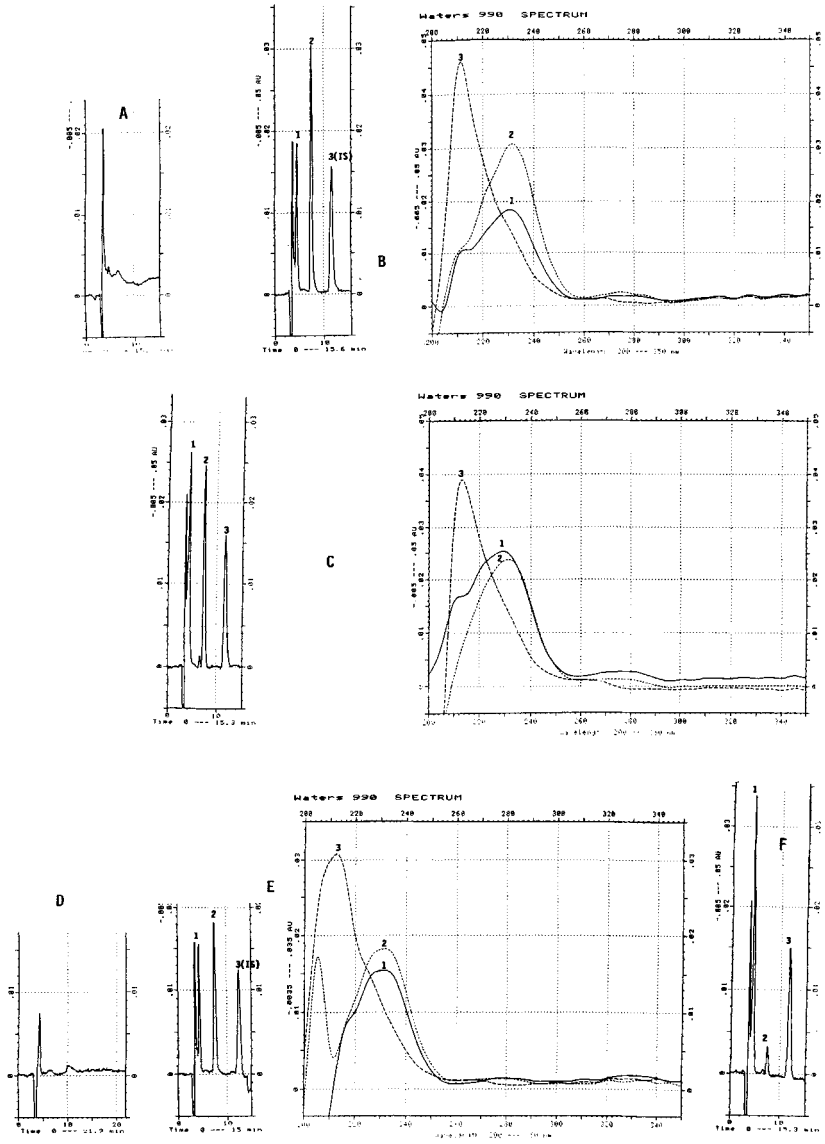

Fig. 4 : Chromatograms and spectra of cocaine and benzoylecgonine after liquid-liquid extraction with acid back-extraction (mobile phase n° 2).

A - Blank whole blood (1 ml)

B - Blank whole blood (1 ml) spiked with (1) benzoylecgonine : 500 ng ml^{-1}, (2) cocaine : 1000 ng ml^{-1}, (3) bupivacaine (IS) : 1 μg added

C - Necropsic whole blood (1 ml) : (1) benzoylecgonine : 740 ng ml^{-1}, (2) cocaine : 840 ng ml^{-1}, (3) bupivacaine (IS) : 1 μg added

D - Standard of blank urine (1 ml)

E - Blank urine spiked with (1) benzoylecgonine : 600 ng ml^{-1}, (2) cocaine : 800 ng ml^{-1} (3) bupivacaine (IS) : 1 μg added

F - Necropsic urine (1 ml) : (1) benzoylecgonine : 980 ng ml^{-1}, (2) cocaine : 116 ng ml^{-1}, (3) bupivacaine (IS) : 1 μg added.

procedure and the appropriate internal standard into the last organic phase (only for recovery studies). For the reference sample, drug and internal standard were added together into the last organic phase. The recoveries reach about 80-90 % for all tested substances (Table 2) except for ester compounds for which the recoveries were between 65-75 %. Paraaminobenzoic acid was extractable from both acidic and alkaline medium ; it is preferably extractable from acidic medium. Pipecolic acid (minor metabolite of bupivacaine) was not extracted with the described procedure.

Reproducibility

The reproducibility was tested on a pool of blank sample spiked with 50, 100 and 500 ng ml^{-1} of each drug. Table 2 shows the highest coefficients of variation obtained. The within day coefficients of variation were less than 12 % for cocaine and benzoylecgonine, less than 16.2 % for the ester drugs and less than 12.4 % for amide drugs. The day to day coefficients of variation on a period of two weeks were less than 13.8 % for cocaine and benzoylecgonine, less than 18 % for the ester drugs and less than 12.7 % for the amide drugs. The coefficients of variation were high for the esters, revealing the instability of these compounds in aqueous medium during storage or extraction procedure.

Sensitivity

The noticed limits of determination were 20 ng ml^{-1} for cocaine, benzoylecgonine, lidocaine, monoethylglycine-xylidide, glycine-xylidide, pipecolyl-xylidine, prilocaine, articaine, o-toluidine, procaine, paraaminobenzoic acid and butacaine, and 40 ng ml^{-1} for bupivacaine, etidocaine, dibucaine and tetracaine.

Selectivity

The selectivity of the method is good. The retention times (related to cocaine) of the tested drugs, their responses to UV and fluorescence detection and their spectral characteristics are presented in table 3. The fluorimetric detection allowed to distinguish the ester drugs and certain opiates from cocaine, benzoylecgonine and the amide drugs.

As denoted from fig 2B lidocaine and bupivacaine, often associated in anaesthetic procedure [Bromage method (50, 51)] are well separated. However their metabolites monoethyl-xylidide and pipecolyl-xylidine are not separated by the described procedure. A mobile phase (n° 3) containing 0.1 M ammonium acetate and acetonitrile (80:20, v/v) at a flow rate of 1.5 ml min^{-1} and prilocaine as internal standard (1 μg added) allows the determination of these two compounds (Fig. 3 B and 3 C). Thus, when lidocaine and bupivacaine are given in the same treatment, two assays must be carried out on the same sample.

Other interferences were observed : between cocaine and lidocaine, between benzoylecgonine, caffeine and amphetamine, between procaine and codeine or

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O_6 monoacetylmorphine. They can be removed by acting on the nature of the mobile phase (n° 2 instead of n° 1) and if necessary on the detection wavelength.

CONCLUSION

The proposed procedure can be applied for diagnosis and explanation of some toxic reactions caused by cocaine and/or its adulterations by synthetic agents among victims, for studying placental transfers or therapeutic controls on local anaesthetic drugs, as well as for forensic toxicology.

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