CHROMBIO. 5118

Determination of cocaine and norcocaine in plasma and cell cultures using high-performance liquid chromatography

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SUMMARY

A new simple high-performance liquid chromatographic (HPLC) method was developed for the determination of cocaine and norcocaine. Cocaine and norcocaine in biological samples were buffered to pH 9.0, extracted with diethyl ether and reextracted in a 0.1% aqueous solution of tetramethylammonium hydrogen sulfate (TMAHS) with a theoretical yield of extraction of 100%. The HPLC elution of cocaine and norcocaine was performed using a Spherisorb RP-18, 100 mm × 4.6 mm I.D., 5 μm particle size column with a mobile phase containing acetonitrile–0.1% TMAHS aqueous solution (60·40). The compounds were entirely separated, and a reliable limit of quantitation was set at 20 ng/ml when extracted from 0.5 ml of plasma No interference with 26 other drugs was found. Cocaine and norcocaine stability studies showed that their half-lives in human plasma incubated at 37°C were 50.8 and 43.2 min, respectively. In contrast, plasma from dogs or rats exhibited only weak or no enzymatic esterase activity towards cocaine and norcocaine resulting in less rapid degradation. Hydrolysis could be efficiently inhibited with sodium fluoride and prevented by storage of the sample at −20°C. The highly sensitive assay also allowed the assessment of the oxidative metabolism pathway of cocaine to norcocaine in primary rat hepatocyte cultures

INTRODUCTION

Cocaine (benzoylmethylecgonine) is a potent central nervous system (CNS) stimulant which induces dramatic psychological dependence. Studies in humans and in vitro showed that hydrolysis of the ester linkages is the major route of cocaine metabolism [1,2] and that a quantitatively minor oxidative pathway metabolises cocaine to norcocaine (N-desmethylcocaine) followed by further metabolites [3,4]. This biotransformation, catalysed by the hepatic microsomal fraction [5,6], is implicated in cocaine-induced hepatotoxicity in animal models [7–9]. Although norcocaine has also been found in man after cocaine administration [10,11], its implication in cocaine toxicity still remains unknown.

Among the large number of analytical procedures for cocaine determination using radioimmunoassay (RIA), gas chromatography (GC) and high-performance liquid chromatography (HPLC) [11–19], only a few refer to the determination of norcocaine. In particular, the HPLC method described by Evans and Morarity [13] allowed the simultaneous determination of benzoylecgonine, cocaine and norcocaine. However, due to the lack of sensitivity and the incomplete separation of the two latter compounds, the method is not suitable to assess cocaine metabolism in milligram quantities of biological material such as cell cultures. Therefore, this paper describes a new, simple HPLC method for a reproducible, specific and highly sensitive determination of cocaine and norcocaine.

EXPERIMENTAL

Chemicals

Cocaine was provided by Siegfried (Zofingen, Switzerland). Norcocaine was synthesised according to Borne et al. [20]. The structural identification of norcocaine was confirmed using nuclear magnetic resonance analysis and its purity, as determined by HPLC analysis, was greater than 99%. Benzoylecgonine was prepared from cocaine by refluxing in water during 13 h. The excess of cocaine was then extracted with diethyl ether, and the water, containing benzoylecgonine, was evaporated. The purity of the product was greater than 98%, as assessed by HPLC. Sterile water was obtained by deionisation and filtration through a Milli-Q and Nanopure system (Millipore, Molsheim, France). Acetonitrile, HPLC grade, was provided by Rathburn (Walkerburn, U.K.). Tetramethylammonium hydrogen sulphate (TMAHS) and dimethylsulphoxide were obtained from Fluka (Buchs, Switzerland). Analytical-grade diethyl ether, purchased from Merck (Darmstadt, F.R.G.), was freshly distilled on potassium hydroxide before use.

Chromatographic system and conditions

The liquid chromatograph consisted of a pump (Gilson Model 303 with a manometric module 802 C, Synmedic, Switzerland), an autosampler (Perkin Elmer ICS 100, Überlingen, F.R.G.), a UV detector (ABI Analytic Kratos Division Spectroflow 783, Paul Bucher, Basel, Switzerland) and an integrator (Perkin Elmer LCI 100). The column used was a Brownlee Labs. 100 mm × 4.6 mm I.D. Spherisorb RP-18, 5 μm particle size. The mobile phase consisted of acetonitrile–0.1% aqueous TMAHS (60:40, v/v) and the flow-rate was maintained at 1 ml/min. Depending on the column series, the acetonitrile concentration in the mobile phase was adjusted to keep the retention time of cocaine and norcocaine constant during the study. The UV detector was set at 230 nm with 0.02 a.u.f.s. The quantitation of the eluted compounds was based on peak heights.

Stock solutions

Norcocaine base and cocaine base, 10 mg of each, were dissolved together in 10 ml of acetonitrile. This stock solution was then stored at 4°C until use. The stability of this solution was periodically determined as follows. An aliquot of the stock solution was diluted to 1 μg/ml. Samples containing 100 ng of cocaine and norcocaine were evaporated to dryness, and the residues were dissolved in 150 μl of mobile phase. A 120-μl aliquot was injected onto the column.

Effect of pH on the extraction yield of cocaine and norcocaine

A 100-μl volume each of 1 μg/ml cocaine and norcocaine in acetonitrile was evaporated to dryness under a stream of nitrogen at 40°C. The residues were then dissolved in 1 ml of buffer solution at the indicated pH and extracted using 4 ml of freshly distilled diethyl ether. After centrifugation at 1000 g for 5 min, the maximal volume of the organic phase was transferred into another glass tube and evaporated to dryness. The residue was reconstituted in 150 μl of mobile phase, and 120 μl were injected onto the column. The peak heights were compared with those obtained after injection of the standard which was prepared without extraction, after dissolution of the residues in 150 μl of mobile phase and injection of 120 μl.

Standard curves and extraction procedure

After evaporation to dryness of the acetonitrile, cocaine and norcocaine were redissolved in either 0.5 ml of blank plasma or 2 ml of hepatocyte homogenate to give final concentrations between 20 and 2000 ng/ml. Each concentration was prepared in duplicate. Following vigorous agitation, 0.5 ml of 0.1 M borate buffer, pH 9.0, was added to each tube. The drugs were then extracted with 4 ml of diethyl ether for 10 min, and the tubes were centrifuged at 1000 g for 5 min. A 3.5-ml volume of the organic phase was then reextracted with 150 μl of 0.1% aqueous TMAHS for 10 min. After centrifugation, the diethyl ether was

aspirated off, and 120 μl of the acidic phase were injected onto the column. The concentrations of cocaine and norcocaine in unknown samples were determined by extrapolation of the linear regression line of the peak-height ratios versus the concentrations of calibration standards.

Stability of cocaine and norcocaine in human plasma

A 100-ng amount of cocaine and norcocaine was dissolved in either 0.5 ml of untreated human plasma, or in human plasma inactivated at 60°C for 10 min, or in human plasma containing 1 mg/ml sodium fluoride. To assess the stability of cocaine and norcocaine during storage, the samples were stored for one and seven days at 4°C and −20°C and analysed as described above. To determine the hydrolysis and metabolism of the compounds, plasma samples were incubated at 37°C. The reaction was stopped by rapid cooling and freezing of the glass tubes in dry ice–acetone. The analysis was performed within two days.

Metabolism of cocaine in primary rat hepatocyte cultures

The isolation and subsequent culture of rat hepatocytes were performed as described earlier [21]. The hepatocyte cultures ($1.5 \cdot 10^6$ cells per 60-mm dish) were incubated in 3 ml of Williams' medium E, with or without 10^{-4} M cocaine hydrochloride, and supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), insulin (10^{-7} M), dexamethasone (10^{-7} M) and 1.6% dimethylsulphoxide to reduce the loss of cytochrome P-450 [22]. After various times of incubation, the culture media were removed. The cell monolayers were washed three times with 3 ml of cold saline and immediately frozen at −20°C for a maximum of two days. The cells were then scraped off with a rubber policeman into pure water and ultrasonicated (10 s, 10 W). Cocaine and norcocaine were extracted and determined as described above. Protein concentration was determined according to Bradford [23].

RESULTS AND DISCUSSION

In the present study a simple procedure was developed to determine both cocaine and its N-demethylated metabolite, norcocaine, in plasma or in cultured cells. The method combines a two-step liquid–liquid extraction for an efficient clean-up procedure of the crude material, followed by the separation of the compounds using HPLC. Since cocaine in plasma or in water is rapidly degraded, optimisation of the storage conditions of biological samples containing these drugs appears to be a critical point. Finally, the sensitivity of the method allowed assessment of the capacities of primary rat hepatocyte cultures to metabolise cocaine to norcocaine.

As shown in Fig. 1A, cocaine and norcocaine eluted at 7.16 and 5.82 min, respectively. The limit of quantitation for both compounds was found to be 20 ng/ml based on measurements not exceeding 5% variation using 0.5-ml ali-
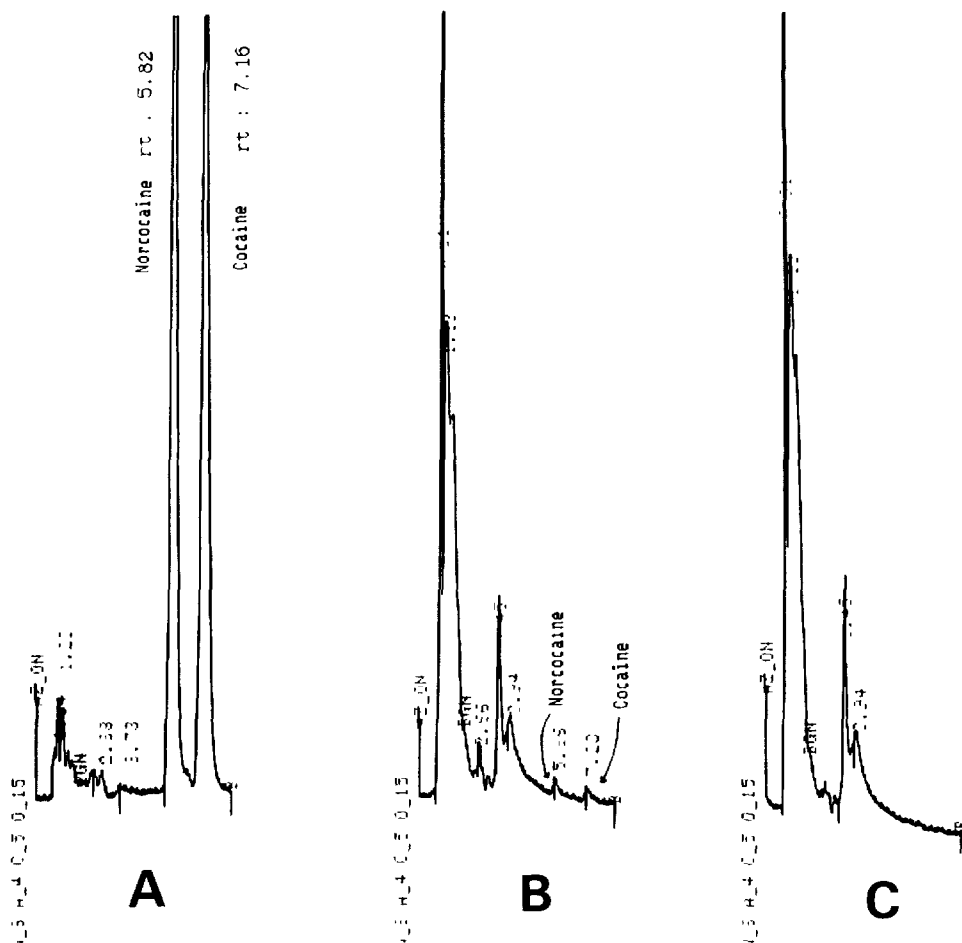
Fig. 1. Chromatograms of (A) a standard solution containing 80 ng each of cocaine and norcocaine in an injected mobile phase volume of 120 μl, (B) 1 ng each of cocaine and norcocaine extracted from 0.5 ml of dog plasma and (C) blank dog plasma.

quots. However, cocaine and norcocaine could still be detected following extraction, at a concentration of 2 ng/ml, based on a signal-to-noise ratio of 2 (Fig. 1B).

Cocaine and norcocaine degradation was low in acetonitrile. A 6 and 8% decrease in cocaine and norcocaine concentration, respectively, was measured after one month of storage at 4°C, whereas no degradation occurred after one day (Table I). The acetonitrile stock solutions containing 1 mg/ml cocaine and norcocaine could therefore be used for at least one month.

As for most of the alkaloids, the nitrogen atom of the cocaine molecule can be protonated to give a water-soluble salt, whereas the alkaline form of cocaine

TABLE I

STABILITY OF COCAINE AND NORCOCAINE IN ACETONITRILE

Sample	Percentage remaining[a]	
	Norcocaine	Cocaine
One-day-old solution	100 ± 2	100 ± 1
One-month-old solution at $4\,^{\circ}$C	92 ± 2	94 ± 3

[a]The results represent the means $\pm$ S.D. of three determinations and were expressed as percentage remaining substance in acetonitrile stock solutions.
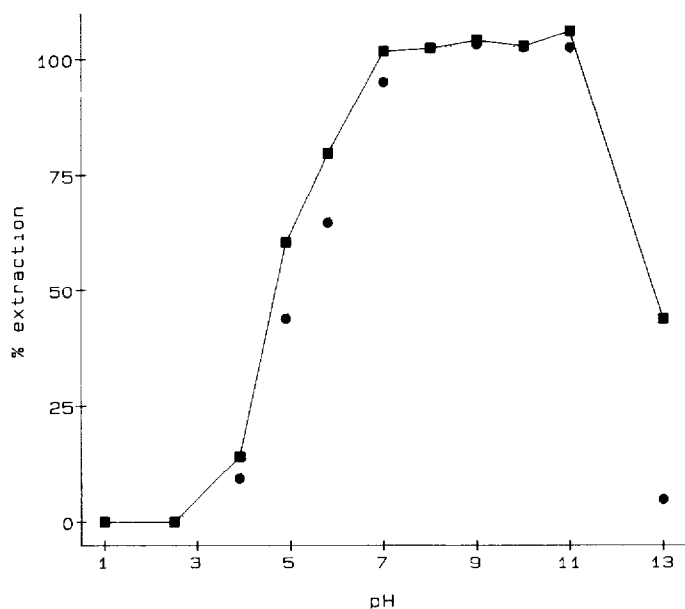

Fig. 2. Effect of pH on the extraction yield of cocaine (■) and norcocaine (●).

is very soluble in organic solvents such as diethyl ether. As shown in Fig. 2, the yield of extraction in diethyl ether is 100% between pH 7.0 and 11.0 for both compounds. At higher pH, a decrease in the yield of extraction is observed which is explained by the hydrolysis of the molecules as demonstrated elsewhere [14, 24]. Therefore, we arbitrarily decided to perform our standard extraction procedure at pH 9.0.

The evaluation of the external standard curve of cocaine and norcocaine covering a broad concentration range of cocaine showed that the concentration–peak height response factor (RF) was constant between 20 ng/ml and 2 μg/ml cocaine and norcocaine. Below 20 ng/ml the height surelevated the con-
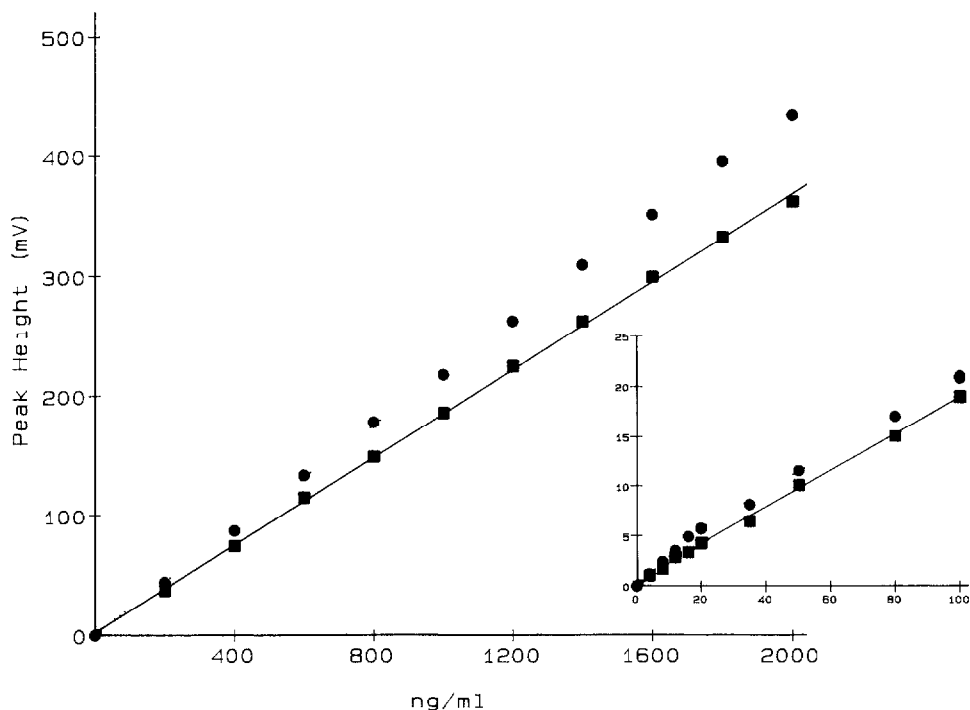
Fig. 3. External standard of cocaine (■) and norcocaine (●) extracted from dog plasma.

centration present in the plasma, whereas the RF increased with concentrations higher than 2 μg/ml (data not shown). The line fitting of this concentration range (Fig. 3) gave a correlation coefficient of 0.999. The extraction efficiency for cocaine and norcocaine from 0.5 ml of plasma containing 100 ng was 100%.

The method developed was highly reproducible for both compounds. The results summarised in Table II show that, with decreasing concentrations, the relative standard deviations varied between 0.9 and 8.3% for cocaine and were even smaller for norcocaine.

Cocaine and norcocaine were rapidly hydrolysed in human plasma at 37°C (Fig. 4A and B). Their half-lives were 50.8 and 43.2 min, respectively. The degradation of these drugs results from two separate processes: firstly, hydrolysis of the methyl ester linkage, giving benzoylmethylecgonine which occurs spontaneously in water, is increased with raising the pH or the temperature [24]. A second hydrolysis catalysed by cholinesterases cleaves the benzoyl ester linkage to produce ecgonine methyl ester [1]. When human plasma was heated for 10 min at 60°C to inactivate the thermolabile cholinesterase activity, the half-life of cocaine and norcocaine was greatly enhanced to 348 and 287 min, respectively (Fig. 4). Incubation of the drugs in human plasma in the

TABLE II

WITHIN-DAY REPRODUCIBILITY OF COCAINE AND NORCOCAINE ANALYSIS AFTER
THEIR EXTRACTION FROM 0.5 ml PLASMA

Concentration (ng/ml)	Peak height[a] (mV)	
	Norcocaine	Cocaine
10	3.03 ± 0.24	2.17 ± 0.18
20	5.12 ± 0.22	4.02 ± 0.16
100	24.06 ± 0.98	20.17 ± 0.34
1000	223.68 ± 2.27	189.54 ± 1.79

[a]The values represent means $\pm$ S.D. of five analyses per concentration.
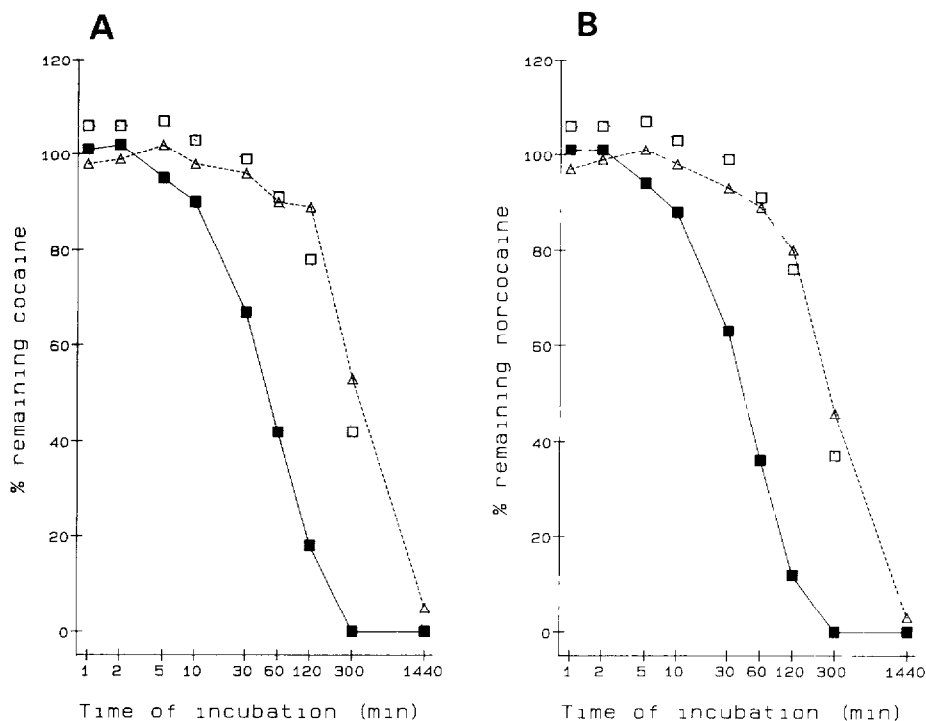

Fig. 4. Hydrolysis of cocaine (A) and norcocaine (B) in human plasma. The results were expressed
as a percentage of the substances determined without incubation. The values represent the means
of three analyses per time point. The S.D. (not shown) were always lower then 5%. (■) Untreated
plasma; (□) plasma containing 1 mg/ml sodium fluoride; (△) heat-inactivated plasma.

presence of 1 mg/ml sodium fluoride increased their half-lives in a similar
manner as heating the plasma. These results are in accordance with a direct
measurement of cholinesterase activity using a Sigma kit (Sigma diagnostics

TABLE III

REMAINING COCAINE AND NORCOCAINE CONCENTRATIONS IN PLASMA FROM VARIOUS SPECIES AFTER 1 h INCUBATION AT 37°C

Sodium fluoride (1 mg/ml)	Percentage remaining[a]							
	Dog		Rat		Human		Control plasma	
	Nor	Coc	Nor	Coc	Nor	Coc	Nor	Coc
−	76, 80	72, 72	87, 89	81, 83	33, 38	40, 44	28, 28	25, 29
+	83, 87	82, 84	88, 89	84, 88	88, 93	90, 92	80, 81	80, 83

[a]Cocaine (Coc) and norcocaine (Nor) (100 ng each) were dissolved in 0.5 ml plasma from various species. The results represent two determinations and were expressed as percentage remaining substance after incubation versus extraction from water without incubation.

TABLE IV

STABILITY OF COCAINE AND NORCOCAINE IN HUMAN PLASMA SAMPLES

Storage (days)	Incubation conditions[a]	Percentage remaining[b]	
		Norcocaine	Cocaine
1	4°C	5 ± 1	13 ± 1
	4°C, +NaF	92 ± 1	95 ± 1
	−20°C	91 ± 1	95 ± 1
	−20°C, +NaF	108 ± 2	107 ± 2
7	4°C	0	0
	4°C, +NaF	22 ± 3	43 ± 2
	−20°C	92 ± 3	97 ± 3
	−20°C, +NaF	110 ± 3	110 ± 3

[a]Sodium fluoride at a concentration of 1 mg/ml of plasma
[b]Fraction of remaining substance after incubation as compared to the 100-ng standard without incubation. The values represent means ± S.D. of triplicate determinations.

cholinesterase, procedure No. 420) (data not shown) and with previous findings [25, 26].

Upon incubation of cocaine in plasma from various species including man, marked differences were observed. For example, in the lyophilised control plasma Validate® (Organon Teknika, Eppelheim, F.R.G.) cocaine and norcocaine were most rapidly hydrolysed, leaving only 27 and 28% of cocaine and norcocaine, respectively, after 1 h of incubation at 37°C (Table III). The thawed human plasma hydrolysed cocaine by 58%, and 28% cocaine hydrolysis was obtained with thawed dog plasma. The least effective species in hydrolysing

cocaine was the rat; in fresh plasma only 18% total hydrolysis occurred of which only 4% could be inhibited using sodium fluoride (Table III).

The instability of cocaine and norcocaine in plasma can therefore distort the results and give false negative data since sample collection and analysis are rarely done within a day. Therefore, some usual storage conditions were examined. As shown in Table IV, the drugs were unstable at 4 °C during seven days, even in the presence of sodium fluoride. Only 43 and 22% of the initial

TABLE V

DRUGS TESTED FOR POSSIBLE INTERFERENCE WITH COCAINE

Substance	Relative ϵ^a	RRT^b
Atropine sulfate	0.05	0.44
Amitriptyline	0.87	2.55
Barbital sodium	0 04	0.14
Benzocaine	0.31	0.20
Benzoylecgonine	0.65	0.34
Butacaine	0.34	1.12
Caffeine	0.35	0.13
Chlorpromazine hydrochloride	0.99	3.03
Clomipramine hydrochloride	0.60	3.17
Clonazepam	1.29	0.20
Cocaine hydrochloride^c	1.00	1.00
Dibucaine hydrochloride	1.92	2.42
Dexamphetamine sulfate	—^{d}	—^{e}
Flunitrazepam	1.27	0.24
Imipramine	0.36	2.19
Lidocaine	0.26	0 70
Medazepam	0.75	2.19
Meprobamate	—^{d}	—^{e}
Methaqualone	2.19	0 28
Morphine hydrochloride	0.63	0.27
Norcocaine	1.08	0.77
Pentobarbital sodium	0.05	0.19
Phenobarbital sodium	0.23	0.15
Procaine hydrochloride	0.44	0.46
Quinine sulphate	4.41	1.43
Stovaine	1.01	1.23
Tetracaine hydrochloride	0.50	1.15
Tropacocaine hydrochloride	0.82	0.84

^{a}Molar absorbance at 230 nm relative to cocaine hydrochloride.
^{b}Retention time of the compounds, relative to cocaine, after elution under the same HPLC conditions (see Experimental).
^{c}Reference substance with $\epsilon = 13354$ mol^{-1} l cm^{-1} and $t_R = 7.87$ min.
^{d}No absorption at 230 nm.
^{e}Could not be determined at 230 nm.

concentrations of cocaine and norcocaine, respectively, were recovered in the plasma. At $-20°C$, practically all of the initially spiked cocaine and norcocaine were recovered in the samples.

A series of 26 compounds were tested for possible interference with cocaine or norcocaine detection (Table V). Some of the drugs were structurally related to cocaine, e.g. tropacocaine and benzoylecgonine, whereas others were common substitutes of cocaine found in illicit samples of cocaine [26], e.g. most of the local anesthetics like benzocaine, lidocaine, procaine or stovaine but also CNS stimulants such as caffeine, quinine and dexamphetamine. We have also examined substances often associated with cocaine in multi-drug abusers, e.g. barbiturates, benzodiazepines and various other psychotropics.

The calculated molar absorbance at 230 nm using a concentration of 10 $\mu g/$ ml of the compounds in the mobile phase showed that only a few of them did not absorb or absorbed weakly at this wavelength, e.g. most of the barbiturates, meprobamate, atropine and dexamphetamine (Table V). The relative retention time of the others indicated that most of the substances tested in this study did not interfere with either cocaine or norcocaine determination. Although tropacocaine and butacaine were eluted close to cocaine and norco-
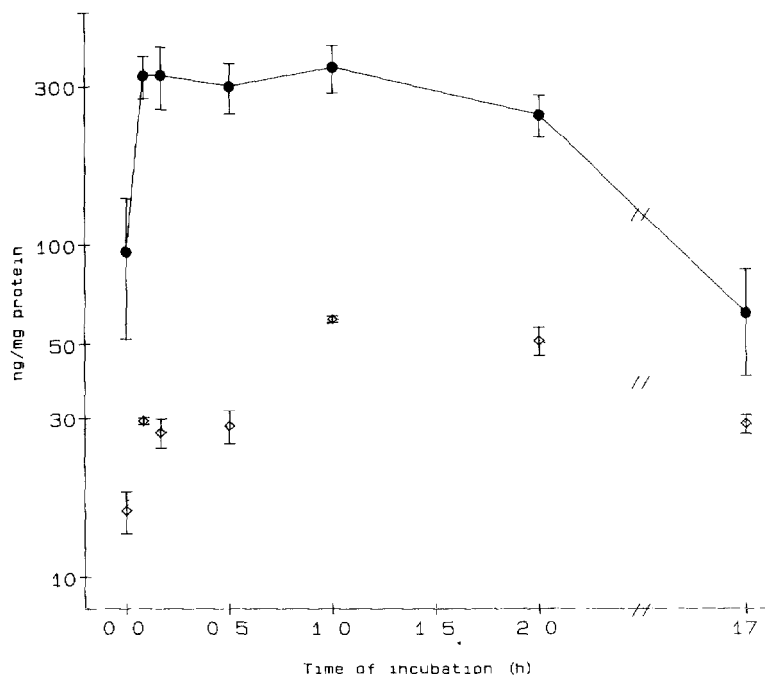

Fig. 5. Cocaine (●) and norcocaine (◇) concentrations in rat hepatocyte cultures after incubation with 100 μM cocaine.

caine, respectively (Table V), the resolution of the adjacent peaks was greater than 90% (data not shown).

The method described in this paper was originally developed to assess cocaine metabolism in cell cultures. Some results are shown in Fig. 5. Cocaine was rapidly taken up into rat hepatocytes, and with 10^{-4} M extracellular cocaine an intracellular steady-state concentration was reached after 5 min of incubation (Fig. 5). The intracellular cocaine concentration amounted to approximately 300 ng/mg of protein and was stable for 2 h, followed by a decrease which resulted from both metabolic and non-enzymatic cocaine degradation. Using this sensitive HPLC method, the oxidative metabolism of cocaine could be identified in rat hepatocyte cultures as evidenced by norcocaine production in these cells. The intracellular norcocaine concentration was stable for 1 h at 50 ng/mg of protein and decreased thereafter, concomitant with cocaine decrease (Fig. 5).

In conclusion, this sensitive HPLC method for the simultaneous determination of cocaine and norcocaine may not only find its application in forensic medicine, but also allows the assessment of the oxidative metabolic pathway of cocaine to norcocaine in cell cultures.

ACKNOWLEDGEMENTS

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