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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.

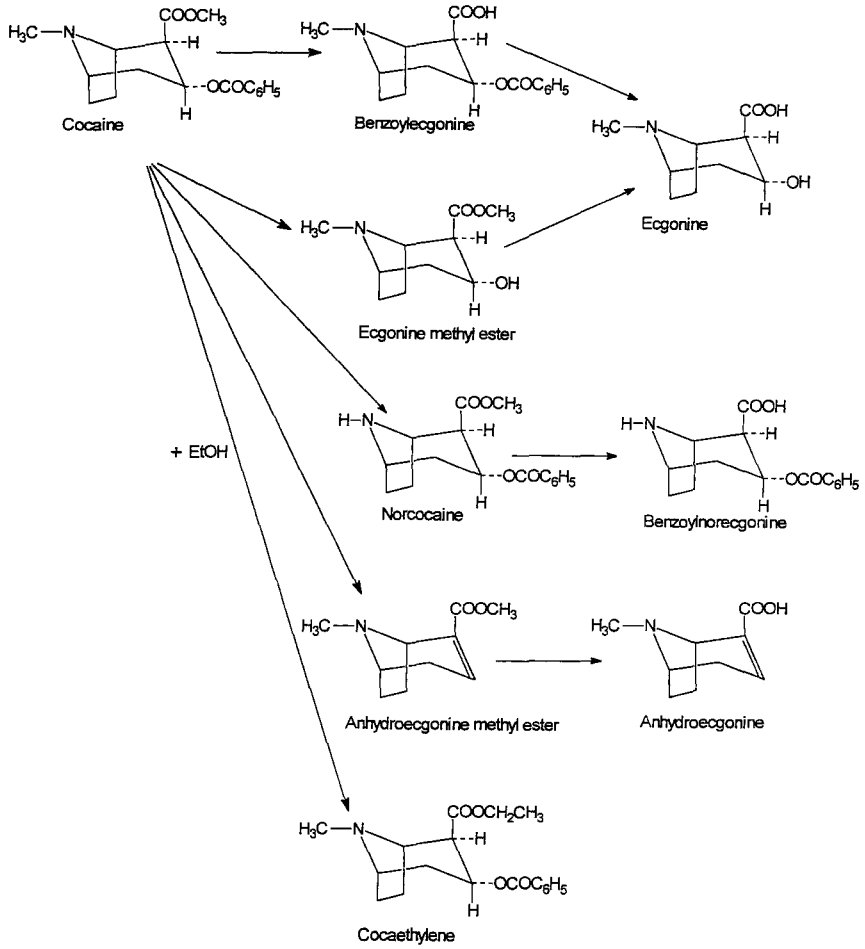

Figure 1 : Main biotransformation pathways.

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

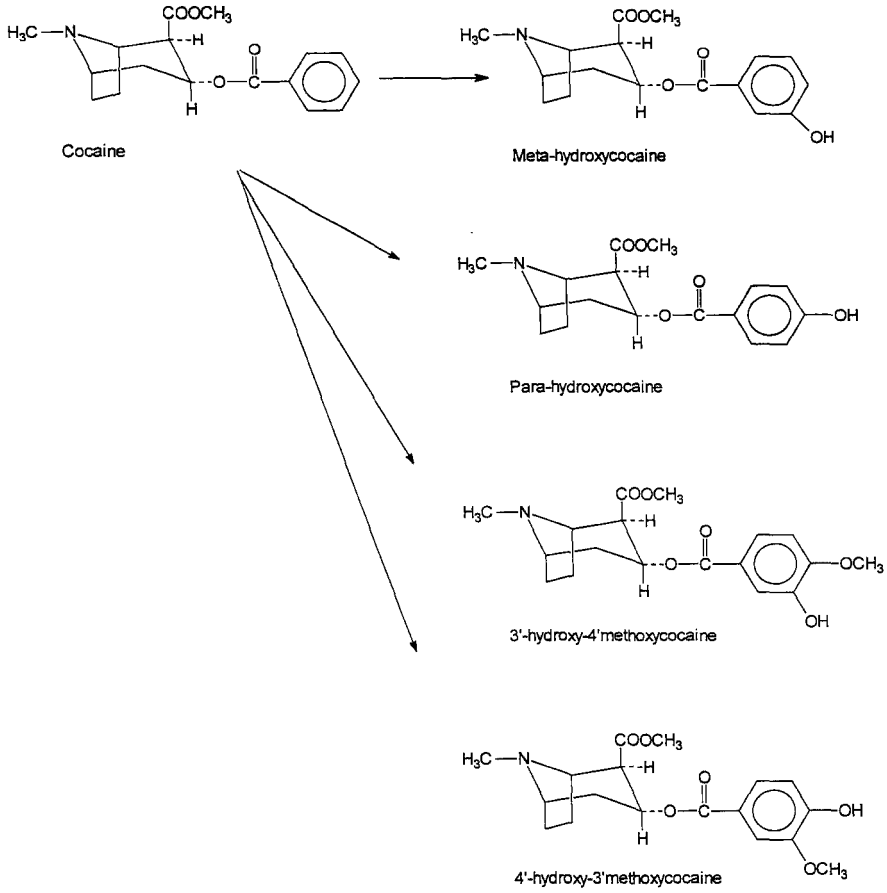

Figure 2 : Minor metabolites of cocaine.

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 fluoreresecent 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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SIMULTANEOUS QUANTITATION OF DIQUAT AND ITS TWO METABOLITES IN SERUM AND URINE BY ION-PAIRED HPLC

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ABSTRACT

We describe here the simultaneous quantitation of diquat (DQ) and its two metabolites (DQ-monopyridone and -dipyridone) in serum and urine. Serum or urine was mixed vigorously with ten times volume of trichloroacetic acid and centrifuged at 12,000 rpm for 5 min. A 20 μl of the upper layer was injected into the HPLC. The major operating conditions of the HPLC were as follows: Column; Zorbax ^{R} C8 (24cm x 4.6mm i.d.), Mobile phase; 5 % acetonitrile(v/v) containing ortho-phosphoric acid (0.2 M), diethylamine(0.1 M), and sodium octansulfonate(7.5 mM) and flow rate; 1.0 ml/min and UV detection by combined two UV detectors: 310 nm for DQ and 365 nm for DQ-monopyridone and DQ-dipyridone. Linear calibration curves for DQ, DQ-monopyridone and DQ-dipyridone were in the range of 0.1 - 10 μg/ml. In the serum and urine dosed intravenously DQ and its two metabolites were also detected.

INTRODUCTION

Herbicides containing diquat (DQ) only and combined with DQ and praquat (PQ) are widely used in Japan. An average of 200-400 deaths per year in Japan were registered during the past five years due to these herbicide poisoning(1).

Pharmacokinetic of DQ is summarized as a slight absorption after oral ingestion and rapid elimination into the urine with unchanges(2). *In vitro* studies using rats liver homoginate, however, we have identified two metabolites of DQ, DQ-monopyridone and DQ-dipyridone, as shown in Figure 1(3).

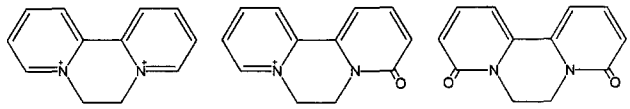
There has been reported DQ and PQ determination in urine and serum using HPLC(4-7), but not reported these metabolites.

We describe here simultaneous determination of DQ and DQ-monopyridone and DQ-dipyridone in serum and urine by HPLC after deprotenization by trichloroacetic acid.

MATERIALS AND METHODS

Reagents

We purchased methyl viologen as PQ(Sigma), $DQBr_2$ (Sigma), organic solvents (HPLC grade, Wako). DQ-monopyridone and DQ-dipyridone were isolated and purified after incubation with rat liver homoginates with $DQBr_2$ according to Fuke et al(3).



Diquat (DQ) **DQ-monopyridone** **DQ-dipyridone**

FIGURE 1. Chemical Structures of Diquat(DQ), DQ-monopyridone and DQ-dipyridone.

Sample preparation for HPLC analysis

Serum or urine samples (0.2-1.0 ml) was deprotenized vigorously with ten times volume of 10 % trichloroacetic acid solution and centrifuged at 12,000 rpm for five minutes. A 20 μl of the upper clean layer was injected into the HPLC.

Operating conditions of HPLC

The major operating conditions of the HPLC were as follows: Column; Zorbax ^{R} C8 (24 cm x 4.6 mm i.d.), Mobile phase; 5 % acetonitrile(v/v) containing ortho-phosphoric acid (0.2 M), diethylamine(0.1 M), and sodium octansulfonate(7.5 mM) and flow rate; 1.0 ml/min and UV detection by combined two UV detectors: 310 nm for DQ and 365 nm for DQ-monopyridone and DQ -dipyridone.

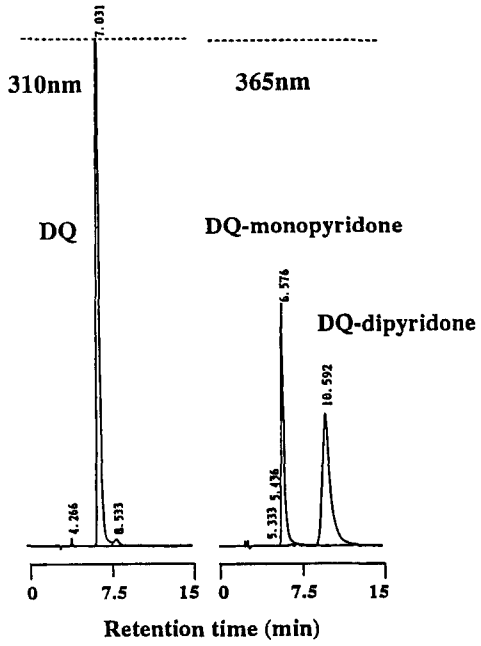


FIGURE 2. HPLC Chromatograms of Diquat(DQ), DQ-monopyridone and DQ-dipyridone in Rat Serum after Deprotenization by Trichloroacetic Acid Solution.

RESULTS AND DISCUSSION

Figure 2 shows typical HPLC chromatograms of blank serum spiked with DQ (10 μg/ml), DQ-monopyridone (5 μg/ml) and DQ-dipyridone (5 μg/ml). The retention time of DQ peak was 6.8 min at 310 nm, but not detected at 365 nm. The retention times of DQ-monopyridone and DQ-dipyridone peaks were 3.3 and 10.6 min at 365 nm, but both peaks were not detected at 310 nm.

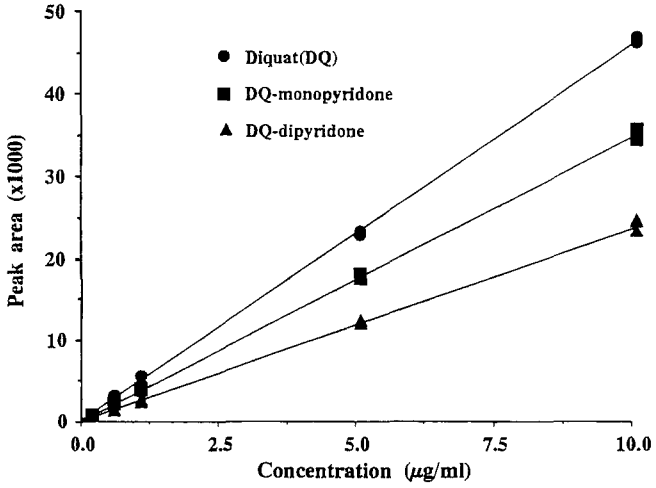


FIGURE 3. Calibration Curves of Diquat(DQ), DQ-monopyridone and DQ-dipyridone in Spiked with Rat Serum after Deprotenization by Trichloroacetic Acid Solution.

These peaks were clearly separated. The retention time of PQ peak at 10 μg/ml was 9.0 min at 310 nm (chromatogram not shown), but its peak was not detected at 365 nm in the analysis of 10μg/ml. PQ solution. No interference by PQ was observed for the quantitation of DQ itself and its two metabolites in this method. Similar chromatograms were also obtained in the analysis of urinary samples.

Calibration curves were produced using absolute peak area of DQ, DQ-monopyridone and DQ-dipyridone in the HPLC chromatograms versus concentrations in serum or urine samples. Figure 3 shows the calibration curves of DQ, DQ-monopyridone

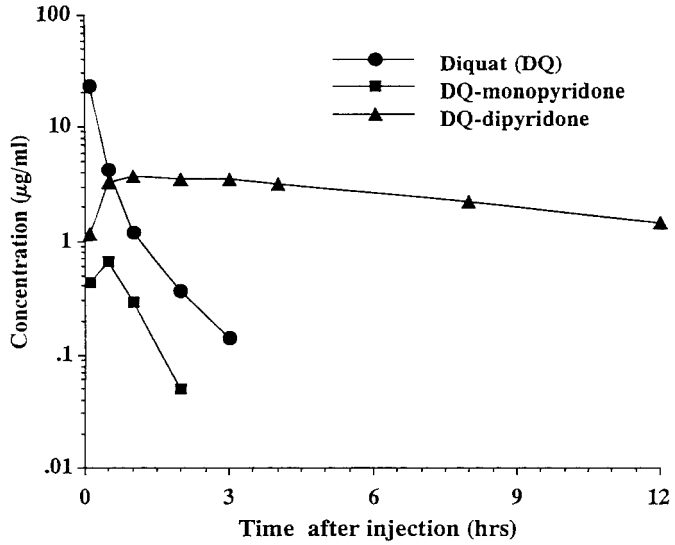


FIGURE 4. Time Course Changes of Diquat(DQ), DQ-monopyridone and DQ-dipyridone in Serum of a Rat Dosed Intravenously(10 mg/kg).

and DQ-dipyridone. Each linear calibration curve of DQ, DQ-monopyridone and DQ-dipyridone was in the range of 0.1 - 10 μg/ml, respectively. Each detection limit of Pq-monopyridone, DQ-monopyridone and DQ-dipyridone was 0.05 μg/ml, respectively. The precision of the method was examined using six replicate analyses of spiked serum and urine at 1.0 μg/ml. The value of the within-day coefficient of variation of the method and the day-to-day were less than 6%.

We applied this method to a rat intravenously injected DQ (10 mg/kg). Figure 4 shows time course changes of DQ, DQ-

monopyridone and DQ-dipyridone concentrations in serum. In urine collected for 24 hours, DQ, DQ-monopyridone and DQ-dipyridone were also detected. These results suggest that DQ is metabolized *in vivo* to DQ-monopyridone and DQ-dipyridone as our previous *in vitro* report(3).

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HPLC QUANTITATION OF FENITROTHION AND 3-METHYL-4-NITROPHENOL IN BIOLOGICAL FLUIDS AFTER PREPACKED CARTRIDGE EXTRACTION AND ITS APPLICATION TO A POISONING CASE

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ABSTRACT

We describe here the simultaneous determination of fenitrothion and its metabolite, 3-methyl-4-nitrophenol (MNP), in biological fluids by HPLC after SepPakC18 cartridge extraction and its application to a human poisoning case. One ml of serum or urine mixed with an equal volume of 0.1 N HCl was applied to the SepPakC18 cartridge, which was preactivated using methanol (5 ml), hexane/isopropanol (9/1 v/v, 10 ml), methanol (5 ml) and distilled water (5 ml). After washing by 0.1N HCl(5 ml), the eluate with 5 ml of hexane/isopropanol (9/1 v/v) was concentrated, and the residue was dissolved in 100 μl of methanol containing methaqualone (internal standard, 30 μg/ml). A 20 μl of the aliquot

was injected into the HPLC. Recovery yields of fenitrothion and MNP from serum and urine ranged from 78.5-93.3%. Linear calibration curves for fenitrothion and MNP were in the range 0.1 - 10 μg/ml. In a human poisoning case at 18.5 hrs after ingestion of Sumithion, concentrations of fenitrothion and MNP in serum were 11.8 and 3.9 μg/ml and those in urine were less than 0.01 μg/ml and 2.8 μg/ml, respectively.

INTRODUCTION

Fenitrothion (Figure 1), an organophosphate insecticide, is widely used in the world. In Japan, an average of 54 deaths per year were registered during the past six years due to fenitrothion poisoning(1).

There has been reported the determination of fenitrothion or its major metabolite, 3-methyl-4-nitrophenol (MNP)(2), using gas chromatography equipped with a flame ionization detector, a flame photometric detector or a mass spectrograph and HPLC(3-6), but reports of their simultaneous determination are few.

We report here the simultaneous determination of fenitrothion and MNP in serum and urine by HPLC after SepPakC18 cartridge extraction and its application to human poisoning cases.

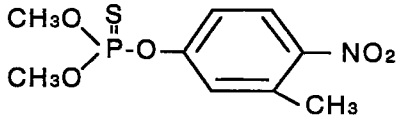

FIGURE 1. Chemical Structure of Fenitrothion.

MATERIALS AND METHODS

Reagents

Fenitrothion (Sumitomo Kagaku), MNP (Aldrich), SepPakC18 cartridge (Waters), methanol (HPLC grade) and the other reagents (Wako Purechemical) were used as purchased. Serum and urine samples were collected from healthy volunteers.

Extraction procedure using a SepPak C18 cartridge

One ml of serum or urine mixed with an equal volume of 0.1 N HCl was applied to the SepPakC18 cartridge, which was preactivated using methanol (5 ml), hexane/isopropanol (9/1 v/v, 10 ml), methanol (5 ml) and distilled water (5 ml). After washing with 0.1N HCl (5 ml), the eluate with 5 ml of hexane/isopropanol (9/1 v/v) was concentrated, and the residue was dissolved in 100 μl of methanol containing methaqualone (internal standard, 30 μg/ml). A 20 μl aliquot was then injected into the HPLC.

Operating conditions of HPLC

The major operating conditions of the HPLC were as follows: Pump; Waters M-600, Column; μBondapakC18 (10 cm x 8 mm i.d.), Detector; Waters-M490, programmed at 310 nm from start to 7 min and at 270 nm after 7 min, Mobile phase; methanol/water (6/4 v/v), Flow rate; 2.0 ml/min.

A poisoning case history

A male (42 year-old-age) ingested Sumithion for suicidal purpose. He was found by his wife 3 hrs after ingestion and gastric lavage subsequently performed. He was then transmitted to our hospital 18.5 hrs after ingestion and given intensive medical treatment such as administration of atropine sulfate (At) and pralidoxime iodide (PAM), direct hemoperfusion (DHP), forced diuresis by dosing of Lasix (furosemide), etc.(7). He left the hospital 65.5 days after ingestion.

RESULTS AND DISCUSSION

Figure 2 shows typical HPLC chromatograms of blank serum (or urine) and of blank serum (or urine) spiked with fenitrothion (1.0 μg/ml) and MNP (1.0 μg/ml). There were no interference peaks for the determination of fenitrothion and MNP in the chromatograms obtained from blank serum (or urine) after SepPackC18 extraction. Each symmetrical peak of MNP, methaqualone (IS) and fenitrothion, which was detected at the retention times of 3.3, 5.2 and 8.4 min, respectively, was clearly separated. No peaks of At, PAM and furosemide were also detected in the chromatogram (data not shown).

Calibration curves were produced using the ratio of peak area of fenitrothion and MNP to that of IS in the HPLC

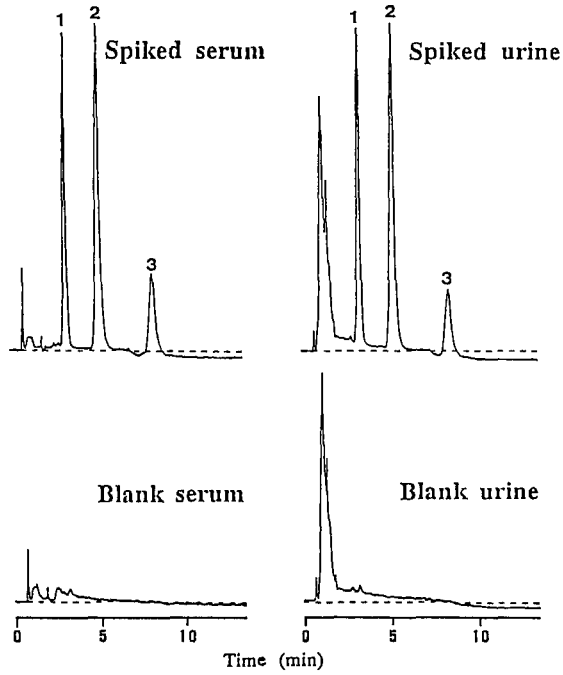

FIGURE 2. HPLC Chromatograms of Serum and Urine Spiked with Fenitrothion (1.0 μg/ml) and 3-Methyl-4-nitrophenol (1.0 μg/ml) and of Blank Serum and Urine after SepPakC18 Extraction. Peaks; 1:3-Methyl-4-nitrophenol 2: Methaqualone (IS) 3: Fenitrothion

chromatograms versus concentration. Figure 3 shows the calibration curves of fenitrothion and MNP. Each calibration curve for fenitrothion and MNP was linear in the range of 0.1 - 10 μg/ml, respectively. The detection limit of fenitrothion was 0.05 μg/ml and that of MNP was 0.01 μg/ml. These detection limits were lower than that obtained by gas chromatography equipped with a

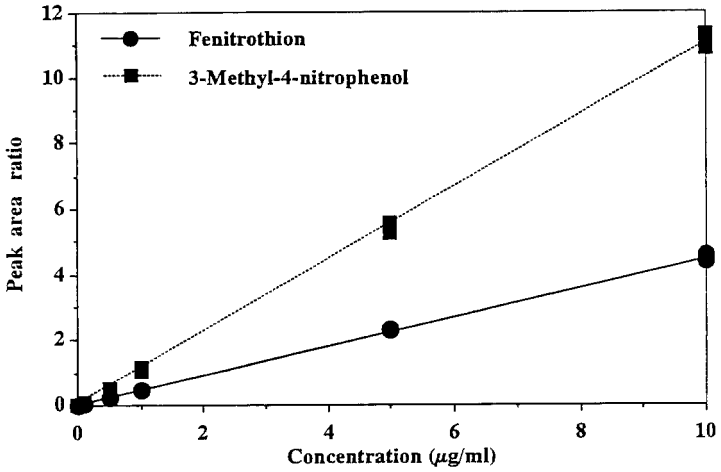

FIGURE 3. Calibration Curves of Fenitrothion and 3-Methyl-4-nitrophenol.

flame photometric detector (4), but sufficiently sensitive for poisoning cases.

Recovery yields of fenitrothion and MNP in serum and urine by SepPakC18 extraction are shown in Table 1. High recovery yields of both fenitrothion and MNP in the range 78.5-93.3% (n=5) were obtained. This extraction procedure is complete within 10 min for the serum and urine samples. The precision of the method was examined using six replicate analyses of the spiked serum and urine at 1.0 μg/ml. The value of the within-day coefficient of variation of the method and the day-to-day were less than 6%. Our quantitation method is relatively rapid and sufficiently sensitive to be useful for poisoning cases due to fenitrothion.

TABLE 1. Recovery Yields of Fenitrothion and 3-Methyl-4-nitrophenol by SepPakC18 Cartridge Extraction.

Sample	Fenitrothion	3-Methyl-4-nitrophenol
Serum (n=5)	**92.1±3.0** (%)	**90.0±2.9** (%)
Urine (n=5)	**78.5±4.3** (%)	**88.2±0.7** (%)

Serum and urine spiked with fenitrothion and 3-methyl-4-nitrophenol (1.0 μg/ml) were used.

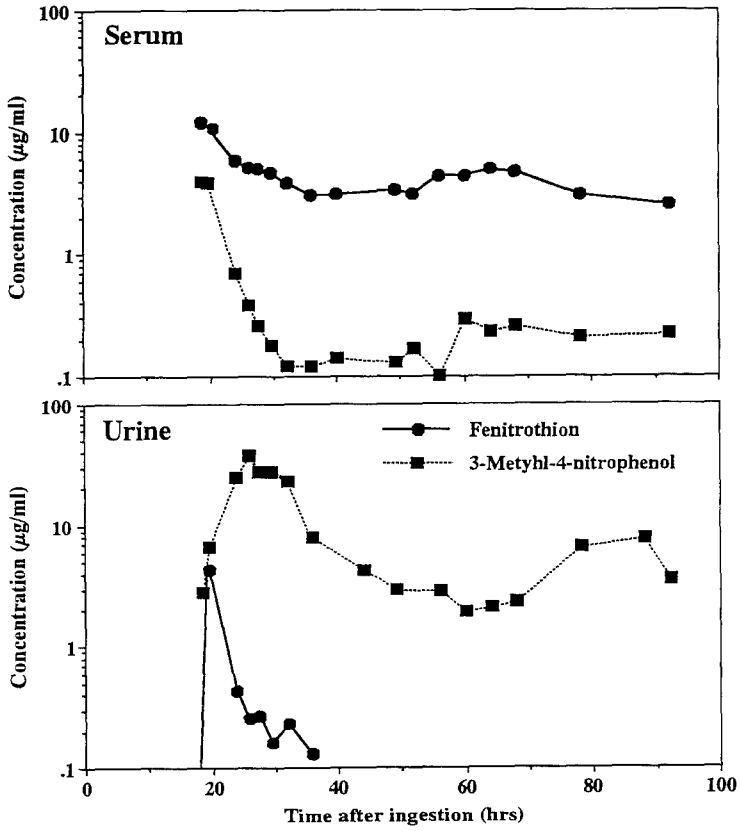

FIGURE 4. Time Course Changes of Fenitrothion and 3-Methyl-4-nitrophenol in Serum and Urine of a Patient Ingested Sumithion.

We applied this method to a poisoning case as shown in Figure 4. Fenitrothion and MNP concentrations in serum at 18.5 hrs after ingestion were 11.8 and 3.9 μg/ml, and those in urine were less than 0.01 μg/ml and 2.8 μg/ml, respectively. In a death case previously reported by Yoshida et al.(8), 16 μg/ml of fenitrothion in the serum was detected. Fenitrothion concentration in the serum detected at the admission was a lethal concentration, but he survived due to our intensive medical treatments. In this case, a decrease in fenitrothion and MNP in serum was rapidly achieved by the emergency treatments such as administration of PAM, forced diuresis and DHP. Fenitrothion in urine also rapidly decreased like that observed in serum, but MNP in urine significantly increased during the first 10 hrs after admission. Activity of acetylcholinesterase inhibited by organophosphate is rapidly reactivated by the administration of PAM(9) the reactivated acetylcholinesterase induces the rapid esterification of organophosphate. Therefore, this significant increase of MNP in urine might be as the results of the rapid esterification of fenitrothion, which produces MNP, by the reactiveted acetylcholinesterase.

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SEPARATION AND DETERMINATION OF (l)-EPHEDRINE AND (d)-PSEUDOEPHEDRINE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

The separation and determination of (l)-ephedrine (l-Ep) and (d)-pseudoephedrine (d-Ps) in Plasma by high-performance liquid chromatography (HPLC) is described. The newly developed method is based on precolumn derivatization with 5-dimethylaminonaphthalene - 1 - sulfonyl chloride (DNSCl) in 0.03% triethylamine in acetonitrile at 50 C° . The diastereomers formed were separated on a reversed phase column by HPLC with fluorescence detection employing 0.6 % phosphate buffer (pH 6.5)-methanol (3:8,v/v) as mobile phase. Clean-up of the ephedrine stereoisomers in plasma was efficiently attained by the combined use of a Sep-pak C_{18} cartridge and an ion-exchange gel, Carboxymethyl Sephadex LH-20 (CM-LH-20). The detection limit of each ephedrine stereoisomer was 100 pg at a signal-to-noise ratio of 3:1. The plasma-level profile of l-Ep and d-Ps in a guinea pig was investigated by the newly developed method. The determination of l-Ep and d-Ps in a volunteer's plasma after oral administration of Xiao Qing-long Heji [an oriental pharmaceutical preparation clinically provided now in China] was also performed.

INTRODUCTION

High-performance liquid chromatography (HPLC) is presently being extensively utilized for the separation and determination of isomeric compounds in biological fluids. The application of HPLC for optical resolution of isomers has been developed in two ways: One is the use of a chiral stationary phase or a chiral mobile phase; the other is the use of a chiral reagent for derivatization followed by the separation of the diastereomers using conventional column and mobile phase. Irrespective of the inevitable disadvantages regarding simplicity , the derivatization method is more favorable for the determination of isomeric compounds in biological specimens with respect to both sensitivity and versatility. (l)-Ephedrine and (d)-pseudoephedrine are known to have the same chemical structure but with different pharmacological activities (1, 2). A reliable method for separation and determination of those stereoisomers in biological fluids is therefore required . This paper reported a highly resolving and sensitive methods for separation and determination of those stereoisomers in plasma by a reversed phase HPLC method through a newly developed precolumn dansyl derivatization reaction combined with the use of a Sep-pak C18 cartridge and an ion-exchange gel, CM-LH-20, for clean-up of the biological samples.

MATERIALS

Optically pure (l)-ephedrine hydrochloride , (d)-pseudoephedrine hydrochloride, (l)-norephedrine hydrochloride and (d)-norpseudoephedrine hydrochloride were characterized and supplied by Professor Da-dun Cheng (Nanjing Pharmaceutical Institute ,China). Xiao Qing-long Heji (901101) was provided by Pharmaceutical Institute of Liaozhong (Liaoning , China). Dansyl chloride was purchased from Fluka co.,ltd (Switzerland) . The Sep-pak C18 cartridge (Millipore co., USA) was kindly donated by Professor Junichi Goto (Tohoku University, Japan) and washed successively with ethanol , 5% aqueous bovine serum albumin solution and then water prior to use. Carboxymethyl Sephadex LH-20 (CM-LH-20) (0.95 mequiv. /g) was prepared in our laboratory according to the known method. All other chemicals employed were of analytical reagent grade. Solvents were purified by distillation prior to use. All glassware used was silanized with trimethylchlorosilane.

METHODS

Instruments

The apparatus used for this work was a LC-10AD solvent delivery system (Shimadzu, Kyoto) equipped with a RF-535 fluorescence spectrophotometer (excitation wavelength 316 nm; emission wavelength 486 nm). A Shimpack CLC-ODS column (5 μm, 15 cm x 6.0 mm ID) and a Shimpack G-ODS Guard column (5 μm, 1 cm x 4.0 mm ID) (Shimadzu, Kyoto) were used at ambient temperature.

Procedure for separation and determination of (l)-ephedrine and (d)-pseudoephedrine in plasma

To a plasma specimen (100-500 μl) was added (l)-norephedrine (100 ng) as an internal standard (I.S.). The mixture was diluted with a 0.5 M phosphate buffer solution (pH 7.0, 2 ml) and then passed through a Sep-pak C_{18} cartridge. After successive washing with water (5 ml) and 20% ethanol (3 ml), (l)-ephedrine and (d)-pseudoephedrine were eluted with ethanol (8 ml). The eluate was evaporated down and redissolved in acetonitrile (100μl) and mixed with DNSCl (600 μg) in 0.03% triethylamine in acetonitrile (100 μl). The resulting solution was heated at 50 C° for 20 min and then evaporated down under N_2 The residue obtained was redissolved in 90% ethanol (1 ml) and applied to a column (18 mm x 6 mm ID) of CM-LH-20 (80 mg). Elution was carried out at a flow rate of 0.1 ml/min. After washing with 90% ethanol to remove neutral and acidic compounds, the diastereomers formed were eluted with 0.05 M methylamine in 90% ethanol (6 ml). The dried eluate was redissolved in the mobile phase (50-100 μl) and an aliquot of the solution was injected into the HPLC system.

Administration of (l)-ephedrine and (d)-pseudoephedrine to a guinea pig

(l)-Ephedrine hydrochloride (835 μg/kg) and (d)-pseudoephedrine hydrochloride (855 μg/kg) dissolved in saline was provided i.v. to a male guinea pig weighing 0.92 kg. The blood was withdrawn at 0.5, 1, 2, 4, 6 and 8 hr after injection and centrifuged at 1500 g for 20 min to separate the plasma.

Administration of Xiao Qing-long Heji to a healthy volunteer

20 ml of Xiao Qing-long Heji (a traditional Chinese pharmaceutical preparation) was orally administered to a healthy male volunteer (0.16 ml/kg). The blood sample was withdrawn at 2 hr after administration and centrifuged at 1500 g for 20 min to separate the plasma.

RESULTS

Determination of (l)-ephedrine and (d)-pseudoephedrine in plasma

Following the established standard procedure, (l)-norephedrine was added to the plasma specimens as I.S. and the ephedrine stereoisomers were extracted with a Sep-pak C_{18} cartridge then treated with DNSCl in the presence of triethylamine in acetonitrile. After purified on a Sephadex LH-20 column, the diastereomers were subjected to HPLC on a Shimpack CLC-ODS column using a mixture of 0.6% phosphate buffer (pH 6.5)-methanol (3:8,v/v) as mobile phase, monitored by fluorescence detection (excitation wavelength 316 nm; emission wavelength 486 nm). A calibration graph was constructed by plotting the ratio of the peak area of (l)-ephedrine and (d)-pseudoephedrine to that of (l)-norephedrine against the amount of those stereoisomers, a linear response to each stereoisomer being 1-800 ng/ml. After providing i.v. (l)-ephedrine and (d)-pseudoephedrine to a guinea pig, the blood specimens were collected at 0.5, 1, 2, 4, 6 and 8 hr, the plasma level of l-Ep and d-Ps was investigated. It is evident from the data on Fig 1 that the pharmacokinetic patterns of (l)-ephedrine and (d)-pseudoephedrine in a guinea pig are closely similar, in spite of the metabolic rate of d-Ps being a little quicker than that of l-Ep. This method was further applied to the determination of (l)-ephedrine and (d)-pseudoephedrine in a volunteer's plasma obtained at 2 hr after oral administration of 20 ml of Xiao Qing-long Heji (a traditional Chinese pharmaceutical preparation). A clear chromatogram was obtained (Fig 2), following the standard procedure. The peaks of (l)-ephedrine and (d)-pseudoephedrine on the chromatogram represent 163 and 73 ng/ml, respectively.

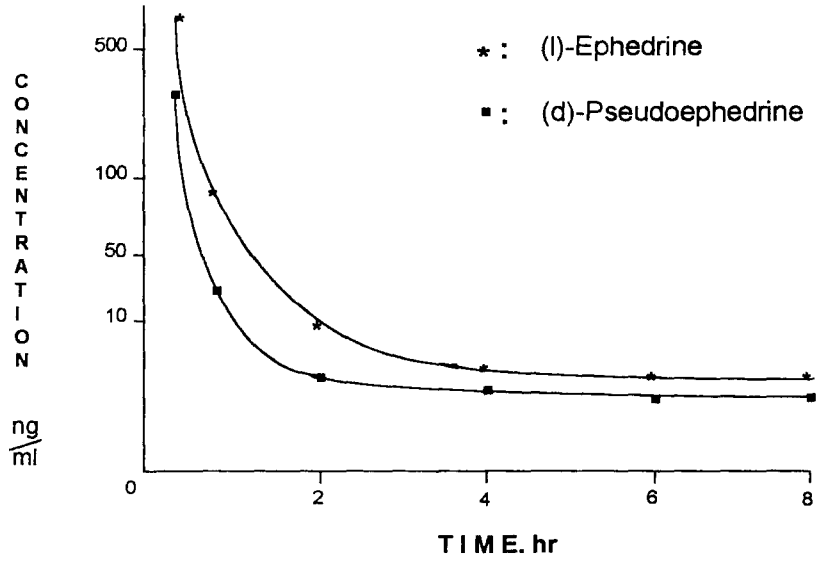


Figure 1. Plasma level of (l)-ephedrine and (d)-pseudoephedrine in a guinea pig after providing i.v. l-Ep and d-Ps at 835μg/kg and 855 μg/kg , individually.

DISCUSSION

Derivatization of (l)-ephedrine , (d)-pseudoephedrine , (l)-norephedrine and (d)-norpseudoephedrine with DNSCl to form the diastereomers

A number of methods for analysis of ephedrine stereoisomers have been reported in recent years, including nuclear magnetic resonance (NMR)(3), high-performance liquid chromatography (HPLC)(4), enzyme immunoassay (EIA)(5) and other methods. Most of these methods, however, seem to be unsuitable for quantitative determination of ephedrine stereoisomers in plasma, owing to either poor resolution or low sensitivity. To provide a novel diastereomeric methods for separation and determination of ephedrine stereoisomers in plasma with satisfactory resolution and sensitivity was therefore attempted. An easily available reagent 5-dimethylaminonaphthalene-1-sulfonyl chloride (DNSCl) was used as the derivatization

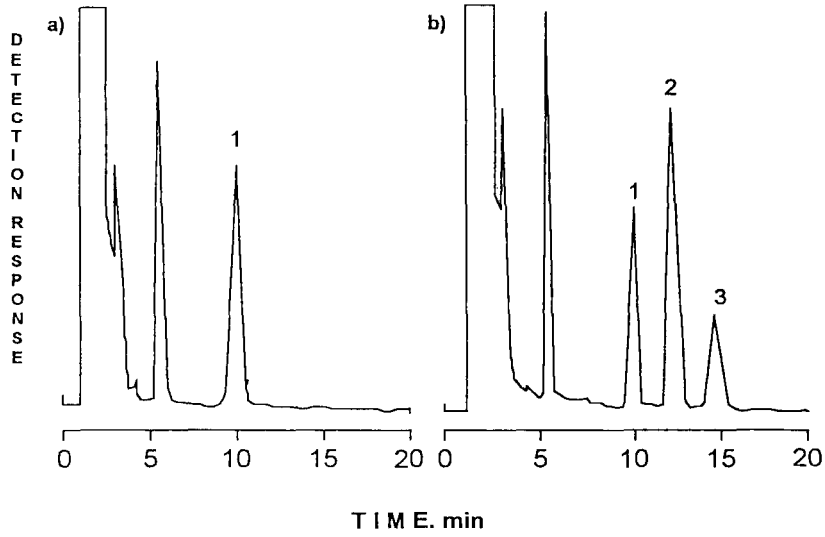
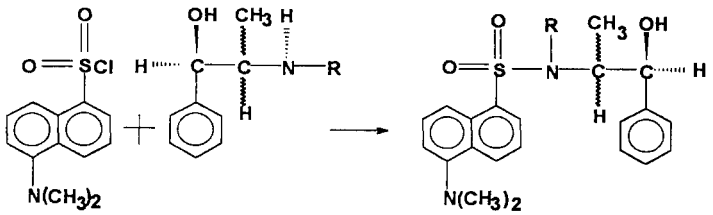


Figure 2. Chromatograms of (l)-ephedrine and (d)-pseudoephedrine in plasma Conditions: column, Shimpack CLC-OD column (5 μm, 15 cm x 6.0 mm i.d.) guarded with a Shimpack G-ODS column (5μm, 1cm x4.0 mm i.d.) mobile phase, 0.6% phosphate buffer (pH 6.5)-methanol / 3:8; flow rate,1.3ml/min; a): blank sample spiked with l-NE as I.S. b): plasma sample obtained from a volunteer 2 hr after oral administration of Xiao Qing-long Heji (0.16 ml/kg); peaks: 1=l-NE (I.S.), 2=l-Ep, 3=d-Ps .



R = CH_3 : (l) - Ephedrine (l - Ep) ; (d) - Pseudoephedrine (d - Ps)

= H : (l) - Norephedrine (l - NE); (d)-Norpseudoephedrine(d - NP)

Figure 3. Derivatization of ephedrine stereoisomers with 5-dimethylaminonaphthalene-1-sulfonyl chloride (DNSCl).

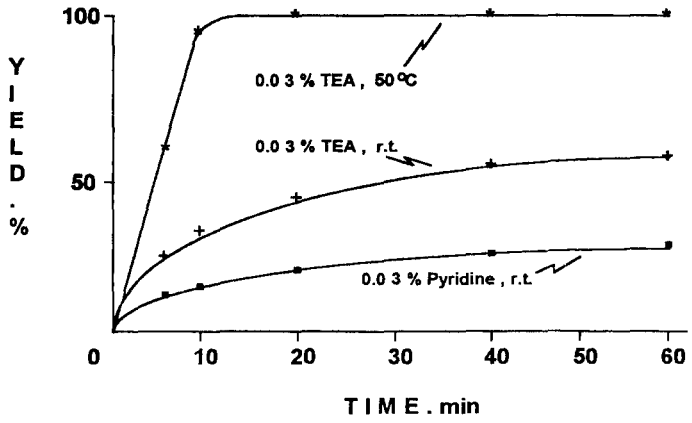


Figure 4. Time course for the derivatization of ephedrine stereoisomers with 5-dimethylaminonaphthalene-1-sulfonyl chloride(DNSCl)

reagent to react with two pairs of ephedrine stereoisomers (Fig 3) and the suitable derivatization conditions were investigated. DNSCl reagent (600 μg) was added to a solution of ephedrine stereoisomers (each 300 ng) in various concentrations of triethylamine or pyridine, in acetonitrile (100 μl) at different temperatures. An aliquot of the solution was applied to HPLC and the yields of fluorescent products were estimated by comparing the peak areas with those of synthetic standard samples. As shown in Fig 4, 0.03% triethylamine in acetonitrile at 50 C° is the most desirable pattern, since the reaction rate increased along with the incubation time up to 15 min, resulting in a quantitative formation of diastereomeric compounds. Based on these results, two pairs of ephedrine stereoisomers (l-Ep/d-Ps and l-NE/d-NP) were treated with 5-dimethylaminonaphthalene-1-sulfonyl chloride in 0.03% triethylamine in acetonitrile at 50 °C for 20 min.

Separation of diastereomeric ephedrines

Various combinations of organic solvents were examined for a suitable mobile phase on a Shimpack CLC-ODS column. The 0.6 % phosphate buffer and

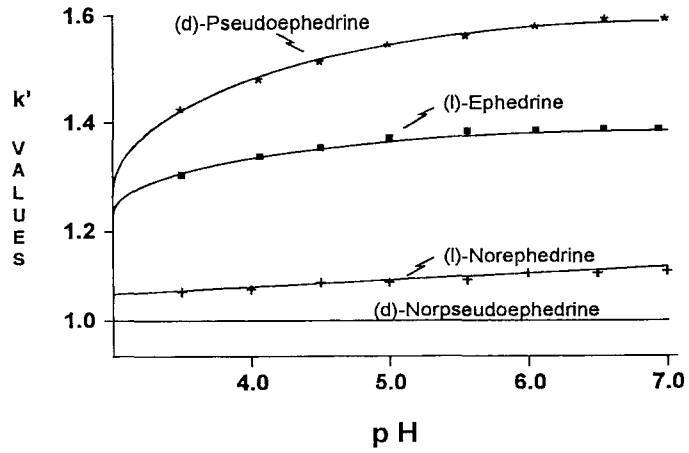


Figure 5. Effects of pH of mobile phase on k'values of (l)-ephedrine, (d)-pseudoephedrine and (l)-norephedrine relative to (d)-norpseudoephedrine.

methanol system appeared to be promising , since no significant leading and / or tailing peak was observed on chromatograms. Accordingly, the effect of pH of the mobile phase on the capacity ratio (k') was investigated with the 0.6% phosphate buffer-methanol (3:8,v/v) system. As shown in Fig 5, the k' values were influenced by pH of the mobile phase. The k' values of l-Ep, d-Ps and l-NE relative to d-NP increased along with the increasing of pH up to 7.0, beyond which the k' values decreased. On the base of above results, pH 6.5 was chosen as a preferred condition for the HPLC. Chromatographic separation of diastereomeric ephedrines was monitored by fluorescence detection (excitation wavelength 316 nm; emission wavelength 486 nm) with satisfactory resolution (Tab 1), the limit of detection being 100 pg (at signal-to-noise ratio = 3). The interesting finding is that among the two pairs of stereoisomers, (l)-ephedrine was eluted earlier than (d)-pseudoephedrine , in contrast, (l)-norephedrine was eluted later than (d)-norpseudoephedrine (Fig 6), which is in good accordance with the elution order of the underivatized forms under the same chromatographic system. This suggests that there may exist some identical characteristics on configuration both for the earlier eluted

Tab.1 HPLC Separation of Diastereomers Derived from Ephedrine Stereoisomers with Dansyl Chloride

	d-NP	*l-NE*	*d-Ps*	*l-Ep*
K'	7.6	9.2	13.1	16.7
α	1.18		1.24	
R	*2.13*		*2.27*	

Conditions : column, a Shimpack CLS-ODS column and a Shimpack G-ODS Guard column ; mobile phase ,0.6% phosphate buffer (pH 6.5)-methanol (3:8); flow rate, 1.3 ml/ min

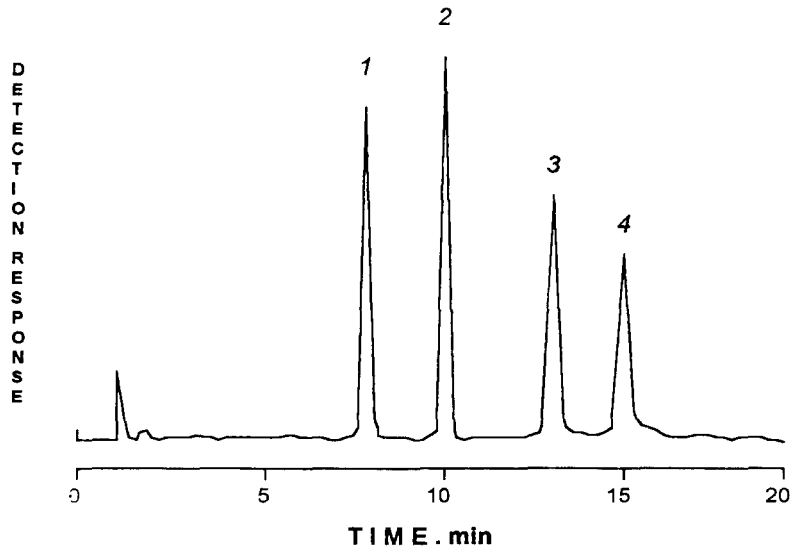


Figure 6. Chromatogram of diastereomeric ephedrines derived from ephedrine stereoisomers with DNSCl Conditions:column,Shimpack CLC-ODS column (5μm,15 cm x 6.0 mm i.d.)guarded with a Shimpack G-ODS column(5μm,1cm x 4.0mm i.d.);mobile phase,0.6% phosphate buffer (pH 6.5)-methanol/3:8; flow rate,1.3 ml/min peaks:1=d-NP, 2=l-NE, 3=l-Ep, 4=d-Ps.

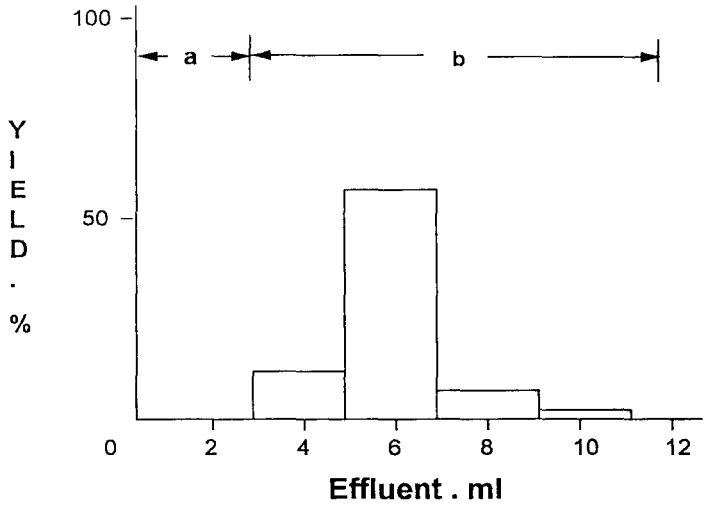


Figure 7. Elution pattern of ephedrine stereoisomers on a Sep-pak C_{18} cartridge Eluent: a) 20% ethanol, b) ethanol.

Tab.2 Recovery of (l)-Ephedrine and (d)-Norpseudoephedrine Added to Human Plasma

Isomer	Amount (ng/ml) Added	Found	Recovery ± S.D.(%)*
(l)-Ephedrine	30.1	26.0	86.3 ± 9.1
	298.3	270.6	90.7 ± 6.2
(d)-Norpseudo-ephedrine	28.7	25.6	89.2 ± 8.4
	290.6	271.4	93.4 ± 5.9

* n=8

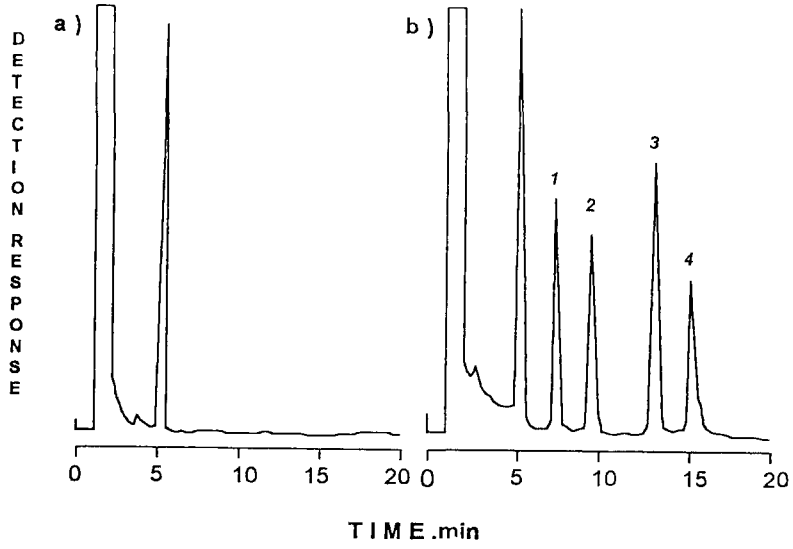


Figure 8. Separation of (l)-ephedrine/(d)-pseudoephedrine and (l)-norephedrne/ (d)-norpseudoephedrine in plasma Conditions: column, Shimpack CLC-ODS column (5 μm, 15 cm x 6.0 mm i.d.) guarded with a Shimpack G-ODS column(5 μm, 1cm x 4.0mm i.d.); mobile phase, 0.6% phosphate buffer (pH 6.5)-methanol/3:8; flow rate, 1.3 ml/min; a): blank b): plasma sample; peaks: 1=d-NP, 2=l-NE, 3=l-Ep, 4=d-Ps.

out parts l-Ep and d-NP and/or for the later eluted out parts l-NE and d-Ps, which is hoped to be useful for further study on those stereoisomers.

Clean-up of (l)-ephedrine / (d)-pseudoephedrine and (l)-norephedrine / (d)-norpseudoephedrine in plasma

In previous study, a Sep-pak C_{18} cartridge was reported for extraction of enantiomeric propranolols, having the similar functional groups as ephedrine stereoisomers, in plasma successfully (6). In this paper, we tried to use this cartridge combined with the use of an ion-exchange gel, Carboxymethyl Sephadex LH-20 (CM-LH-20), for efficient clean-up of the ephedrine stereoisomers in plasma. Ephedrine stereoisomers

in phosphate buffer (0.5 M, pH 7.0) was applied to the cartridge. After washing with water and 20 % ethanol to remove co-existing inorganic salts and other polar substances, the elute obtained with ethanol was separated and analyzed by HPLC. As illustrated in Fig 7 , the ephedrine stereoisomers were recovered at a rate of more than 90 % in an initial 8 ml of the effluent. After derivatization with DNSCl , the reaction products were dissolved in 90 % ethanol and applied to a column of CM-LH-20. The neutral and acidic interfering compounds were almost entirely removed by eluting with 90 % ethanol and the desired basic diastereomers were quantitatively recovered with 0.05 M methylamine in 90% ethanol (Tab 2). An excellent chromatogram for these stereoisomers was thus obtained following this procedure (Fig 8).

The newly developed diastereomeric HPLC method by using an easily available derivatization reagent DNSCl together with a novel clean-up procedure proved to be effective for the separation and determination of secondary , or primary, amide type of ephedrine stereoisomers in plasma. The separation and determination of ternary amide type of ephedrine stereoisomers like methylephedrine in plasma were undergoing in this laboratory and the results will be published elsewhere.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF KEBUZONE AND ITS METABOLITES IN THE SAMPLES OF ERYTHROCYTES, PLASMA, AND WHOLE BLOOD

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ABSTRACT

Within the framework of behaviour study of non-steroidal anti-inflammatory drugs of different structural types kebuzone was analysed as a representative of the 3,5-pyrazolidinedione group. High-performance liquid chromatographic method has been developed to determine kebuzone and its three metabolites in erythrocytes, plasma and whole blood samples. Isolation of kebuzone from these samples has been optimized with respect to transformation of kebuzone. Samples of erythrocytes, plasma and whole blood were deproteinated, but before this adjustment it was necessary to carry out haemolysis of erythrocytes. The supernatant was chromatographed on a glass column of SGX C-18 (150mm x 3.3mm I.D.) using methanol and water with final apparent pH 2.7 as the mobile phase pumped at a flow-rate of 0.5 ml/min and ultraviolet detection at 247 nm. The method employs an internal standard resulting in good accuracy and precision. The method has been successfully applied to a 2-h pharmacokinetic study of kebuzone in rabbits.

INTRODUCTION

Kebuzone [4-(3-oxobutyl)-1,2-diphenyl-3,5-pyrazolidinedione] is a non-steroidal anti-inflammatory drug successfully used in the treatment of rheumatic diseases. Kebuzone has also a prominent antiuratic effect [1,2]. Several methods have been developed for the determination of kebuzone [1-16]. Various analytical techniques have been used for the assay, such as spectrophotometry [3-6], coulometric method [7], paper chromatography(PC) [1], thin-layer chromatography(TLC) [2,8,9], gas chromatography(GC) [10,11] and high-performance liquid chromatography(HPLC) [12-16]. HPLC determination of 88 drug (including kebuzone) with potential toxicity was described by Eigendorf [12-14]. Dependence of retention characteristics of kebuzone and other 3,5-pyrazolidinediones on eluent pH in reversed-phase HPLC has been published [15, 16]. Determination of kebuzone in biological material has been performed using GC[10, 11], PC [1] and TLC [2,8,9]. PC and TLC methods [1,2,8,9] were used to observe kebuzone and its metabolites (Fig. 1). In paper [2] analytical characteristics of metabolites after its separation by TLC, isolation, clean-up and analysis of spectral methods were compared with the characteristics of synthetically prepared metabolites.

The aim of the present study was to develop a procedure to isolate kebuzone and its metabolites from three different biological matrices - whole blood, erythrocytes and plasma. Optimizations of these biological sample preparations and the analytical procedures were intended to assay kebuzone in the above-mentioned biological matrices using unified HPLC conditions. This method has been applied to the determination of kebuzone in samples arising from a pharmacokinetic study investigating drug transport in the organism of some representative non-steroidal anti-inflammatory drugs (kebuzone represents the 3,5-pyrazolidinedione group).

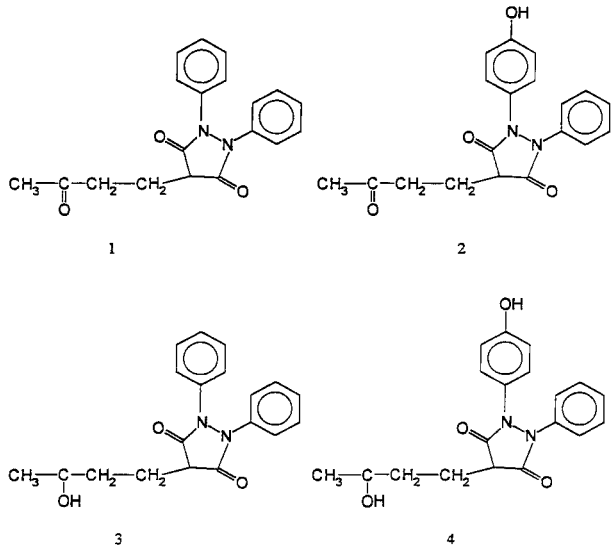

Fig. 1 Structual formulae of kebuzone and its metabolites. 1 = kebuzone, 2 = *p*-hydroxykebuzone, 3 = *γ*-hydroxykebuzone, 4 = *p*-hydroxy-*γ*-hydroxykebuzone

MATERIALS AND METHODS

Reagents and chemicals

Kebuzone, Ketazon Spofa inj. and methaqualone (internal standard, IS) were supplied by Léčiva (Prague, Czech Republic). The metabolites *p*-hydroxykebuzone(p), *γ*-hydroxykebuzone(γ) and *p*-hydroxy-*γ*-hydroxykebuzone(di) were gifts from the Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic). Propyl gallate and ascorbic acid were obtained from Lachema (Brno, Czech Republic) and Farmakon (Olomouc, Czech Republic), respectively. Methanol, acetone, methylene chloride and hydrochloric acid (35%) were also

obtained from Lachema (Brno, Czech Republic) and perchloric acid was from Merck (Darmstadt, Germany).

Kebuzone and IS were PhBs 4 grade, methanol was HPLC grade, and all other chemicals were analytical-reagent grade. Water was doubly distilled.

Chromatographic system

The HPLC system consisted of a Model 8500 Varian pump, Varichrom UV-VIS detector (both: Varian, Palo Alto, CA, USA) and a SP 4100 integrator (Spectra Physics, Santa Clara, CA, USA). Analytical samples were introduced onto the column using a model LCI 30 injection valve (Laboratory Instruments, Prague, Czech Republic) with a 20 μl (or 10 μl) loop. The analytical glass column contained Separon SGX C-18 (150x3.3 mm I.D., 5 μm)(Tessek, Prague, Czech Republic). The mobile phase was a mixture of methanol - water (50:50, v/v) with a final apparent pH of 2.7 adjusted with 5% perchloric acid solution. The flow rate was set at 0.5 ml/min. The UV absorbance of the column effluent was monitored at 247 nm.

Standard solution

Working standards for kebuzone were prepared by dilutions of 25 mg/ml (in methanol) of the stock standard to aliquot concentrations.The stock solutions of kebuzone metabolites (2 mg/ml) and the internal standard (1mg/ml) were prepared in methanol.

Biological sample

Sample of whole blood, erythrocytes and plasma were obtained from the Department of Pathological Physiology, Faculty of Medicine, Charles University

(Hradec Králové, Czech Republic). Here also three rabbits were treated intravenously with the Ketazon Spofa inj. preparation in a 50 mg/kg dose, and the blood samples were withdrawn in the pharmacokinetics study. Samples were withdrawn 3, 6, 15, 30, 60 and 120 min after drug administration.

The withdrawn heparinized rabbit blood was placed in a 10-ml test tube. A portion of each sample of the withdrawn blood was left for analysis (as the sample of whole blood), and for the other portion of the blood, the erythrocytes were isolated from the plasma by centrifugation at 1500 *g* for 10 min. The samples of whole blood, erythrocytes and plasma were immediately frozen.

Sample preparation

Erythrocytes and whole blood. A 0.5 ml volume of sample was pipetted into a 10-ml glass-stoppered centrifuge test tube, 5 μl of the internal standard solution was added and the sample was haemolyzed by adding 0.9 ml of water. The sample was shaken for 5 min, placed in an ultrasonic bath for 5 min, and left at room temperature for 5 min. To the sample 2 ml of acetone was added and the sample was shaken for 5 min, centrifuged at 1930 *g* for 5 min, the supernatant was separated and thickened to a volume of 0.5 ml under a gentle stream of nitrogen. A 20 μl aliquot of the thickened supernatant was injected onto the HPLC column.

Plasma. A 0.5 ml volume of plasma sample was pipetted into a 10-ml glass-stoppered centrifuge test tube, 5 μl of the internal standard solution and, after shaking, 0.75 ml of acetone were added. Then the procedure continued in the same manner as described for the whole blood and erythrocytes samples.

Preparation of the standard curves

Calibration standards were prepared by adding 10 μl of the appropriate working standard and 5 μl of the internal standard to 0.5 ml of whole blood, 0.5 ml of

erythrocytes and 0.5 ml of plasma. Five calibration concentrations of kebuzone were used for the standard curve in each matrix - 2, 10, 20, 30 and 40 μg/ml of isolated erythrocytes, 100, 200, 300, 400 and 500 μg/ml of plasma and 50, 100, 150, 200 and 300 μg/ml of whole blood. Sample preparation was carried out as described above.

Concentrations of kebuzone were calculated from the linear regression equation of the calibration curve constructed by plotting the peak area ratio (y) of kebuzone and the internal standard versus the concentration of kebuzone (x).

RESULTS AND DISCUSSION

In the elaboration of HPLC method for the analysis of kebuzone there was a need to allow a "relatively easy" transformation of kebuzone. Kebuzone in aqueous and methanolic solution[17,18] gives in to very easy and rapid transformation. Series of the factors (e.g. light, warmth, change in pH, etc.) [19,20] produce transformation owing to oxidation and hydrolytic processes. There occur [9,18,21] either transformation on the pyrazolidinedione ring (e.g. it can be oxidized to 4-hydroxyderivate of kebuzone) and/or the decomposition of the pyrazolidinedione ring (hydrazobenzen, azobenzen are formed). In the present paper undesirable transformation was observed, which is analogous to the difficulties described in papers [9,22,23].

Isolation technique

The original aim of our work was to develop a liquid-liquid extraction procedure to isolate kebuzone from three different biological matrices - whole

blood, isolated erythrocytes and plasma. The samples were adjusted as follows: To 0.5 ml of whole blood (or erythrocytes) in a glass-stoppered centrifuge test tube, 50 μg standard solution of kebuzone and 5 μl of the internal standard solution were added and the sample was haemolysed . After haemolysis the sample was acidified with hydrochloric acid (5 mol/l), and after shaking kebuzone was extracted to 6 ml methylene chloride for 5 min. Methylene chloride was separated and evaporated to dryness under a gentle stream of nitrogen. The dry residue was reconstituted with methanol and 10 μl was injected to the HPLC column. The same procedure, but without haemolysis, was used when isolating kebuzone from the plasma sample. Fig. 2 shows that in chromatogram A there is an undesirable peak Y (t_R 5.6 min) in addition to the peak of kebuzone(K) and peaks of blood residues. Peak Y was recorded on the chromatogram in a modification of this extraction process (alteration of acid and its strength, use of another extraction solvent, *e.g.* chloroform, etc).

To stabilize kebuzone, an antioxidant was added to the samples of the biological matrix. Of the antioxidants, ascorbic acid and propyl gallate were effective.

Ascorbic acid (100 μl 1% methanol solution added to 0.5 ml volume of each biological sample) prevented transformation of kebuzone during the extraction step in all three biological samples. But after solution of dry residues of the samples in methanol, slow transformation of kebuzone gradually. Occurred on the HPLC chromatogram (Fig 2., chromatogram B) a small peak Y developed 15 min after solution of the residues, which gradually advanced on repeated injection.

Only propyl gallate (PG) (100 μl 0.5% methanol solution added to a 0.5 ml volume of each biological sample) stopped the transformation of kebuzone. After solution of dry residues samples in methanol the kebuzone peak was standing still after 24 hours. A disadvantage of the use of propyl gallate as the stabilizing agent

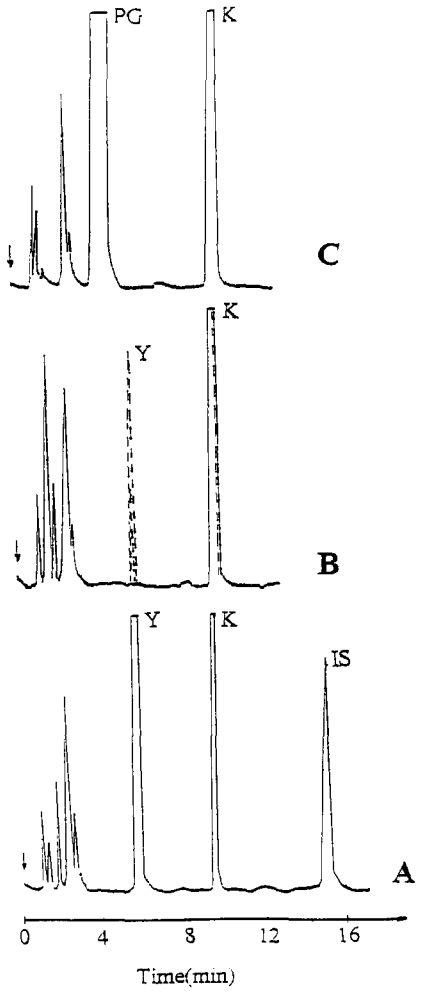

Fig.2 Effect of stabilizing agents on kebuzone(K) during its extraction into methylene chloride. A - chromatogram without the addition of the stabilizing agent (Y - undesirable peak), B - chromatogram with the addition of ascorbic acid solution (Y - undesirable peak), C - chromatogram with the addition of propyl gallate (PG) solution

was its very good recovery (85.3%) into methylene chloride. See the bulky peak (PG) in chromatogram C in Fig 2. An added smaller amount of propyl gallate to the sample was not effective on examination.

This extraction process (with an addition of propyl gallate solution to the sample before its extraction) is useful for the isolation and examination of HPLC analysis of kebuzone from the samples of the whole blood, isolated erythrocytes and plasma. It is true that then the peak of propyl gallate can interfere with the peaks of kebuzone metabolites. There is no doubt that it is disadvantageous in the pharmacokinetic study of kebuzone. Therefore an attempt was made to use deproteination of samples instead of extraction into an organic solvent.

Methanol, perchloric acid (30%), ammonium sulfate, acetonitrile and acetone were tested as protein precipitants. Acetone proved to be the best of the protein precipitants tested for the determination of kebuzone in the whole blood sample, isolated erythrocytes sample and plasma sample because the undesirable peak Y didnot appear in the chromatogram even after 24 hours. When using acetone to deproteinate the biological matrix, samples must be evaporated under a gentle stream of nitrogen until all acetone is removed from the separated supernatant, because acetone absorbs ultraviolet light up to 330 nm.

In order to increase the efficacy of the isolation procedure, it was necessary to carry out the haemolysis of erythrocytes in the samples of whole blood and isolated erythrocytes. The recovery values are listed in Table 1.

Chromatography

Under chromatographic conditions described in the Experimental part, kebuzone, *p*-hydroxy-*γ*-hydroxykebuzone, *p*-hydroxykebuzone, *γ*-hydroxykebuzone and methaqualone (I.S.) were completely separated with retention times of 9.2, 3.9,

TABLE 1

RECOVERY OF KEBUZONE IN RABBIT ERYTHROCYTES, PLASMA AND WHOLE BLOOD

Biological material	Added conc. (μg/ml)	Recovery (n=5) mean ± S.D. (%)
Erythrocytes	2.0	67.7 ± 2.9
	10.0	68.8 ± 2.7
	40.0	70.0 ± 3.1
Plasma	150.0	93.1 ± 2.1
	300.0	94.9 ± 2.2
	450.0	95.0 ± 2.1
Whole blood	50.0	79.0 ± 2.4
	150.0	79.5 ± 2.2
	250.0	80.2 ± 2.3

4.4, 8.8 and 15.0 min, respectively. No interfering peaks of the retention times of analysed compouds were seen in blank samples of whole blood, isolated erythrocytes a plasma. Fig. 3 and 4 show the representative chromatograms of blank (A), control (B) and dosed (C) rabbit whole blood and erythrocyte samples, respectively.

Quantitative determination of kebuzone was performed. Metabolites of kebuzone were not quantified, because in a 2-hour pharmacokinetic study they were not detected. The calibration curves of kebuzone displayed good linearity

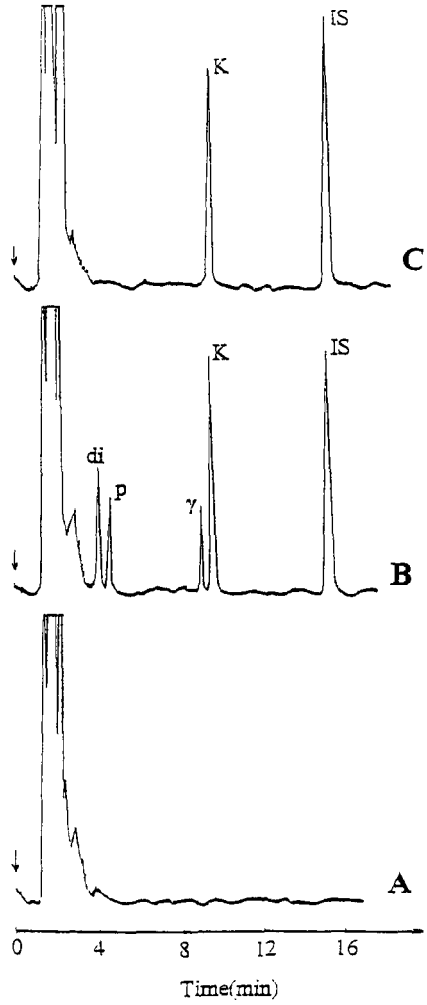

Fig.3 Chromatograms for kebuzone(K), its metabolites / *p*-hydroxy-*γ*-hydroxy-kebuzone(di), *p*-hydroxykebuzone(p) and *γ*-hydroxykebuzone(γ) / and the internal standard (IS) in rabbit erythrocytes. A-blank samples, B-control samples spiked with standard solution of kebuzone (10 μg/ml) and its metabolites (and IS), C-30-min sample from a rabbit given a single dose of 50 mg/kg kebuzone.

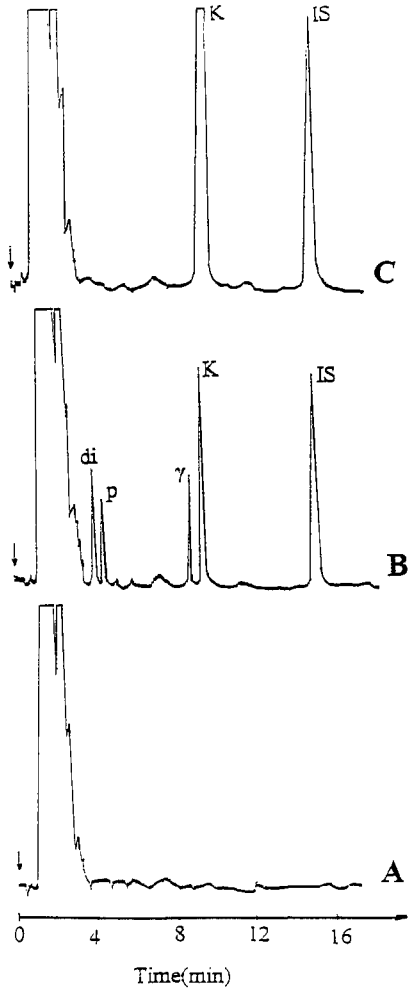

Fig.4 Chromatograms for kebuzone(K), its metabolites / *p*-hydroxy-*γ*-hydroxykebuzone(di), *p*-hydroxykebuzone(p) and *γ*-hydroxykebuzone(γ) / and the internal standard (IS) in rabbit whole blood. A-blank samples, B-control samples spiked with standard solution of kebuzone (10 μg/ml) and its metabolites (and IS), C-30-min sample from a rabbit given a single dose of 50 mg/kg kebuzone.

TABLE 2

REGRESSION EQUATIONS AND CORRELATION COEFFICIENTS OF KEBUZONE IN ERYTHROCYTES, PLASMA AND WHOLE BLOOD

Biological material	Regression equation $y = ax + b$	Correlation coefficient r
Erythrocytes	$y = 0.0794x + 0.0060$	0.9998
Plasma	$y = 0.0794x + 1.0000$	0.9985
Whole blood	$y = 0.0682x + 0.1373$	0.9992

over the range examined in each biological matrix. Their regression equation and correlation coefficients are given in Table 2.

Both within-day and day-to-day precision and accuracy of standard curves are examined. Within-day precision was calculated from the analysis of six samples in each matrix of four concentrations of kebuzone. Day-to-day reproducibility was investigated during a four-week period. Measured concentrations and coefficients of variation (C.V.) are presented in Table 3; the C.V. values were all less than 5%.

The detection limit for kebuzone was 3 μg/ml in whole blood and plasma and 10 μg/ml in erythrocytes. The limit of quantification was 10 μg/ml in whole blood and plasma and 22 μg/ml in erythrocytes. The sensitivity of this HPLC assay of kebuzone depends on the adjustment of the sample of protein precipitation. In papers [12-16] in HPLC analysis of kebuzone only some retention characteristics (retention times, capacity factors) are stated, but none of these paper introduces the validation of the kebuzone HPLC assay (or its HPLC determination in biological samples) as the present paper.

TABLE 3

ASSAY PRECISION AND ACCURACY OF THE DETERMINATION OF KEBUZONE IN ERYTHROCYTES, PLASMA AND WHOLE BLOOD

Conc. added (μg/ml)	Within-day (n=6)		Day-to-day (n=12)	
	Concentration found (mean ± S.D)(μg/ml)	C.V. (%)	Concentration found (mean ± S.D)(μg/ml)	C.V. (%)
Erythrocytes				
4.0	4.2 ± 0.1	3.3	4.3 ± 0.2	4.2
16.0	15.8 ± 0.4	2.4	16.4 ± 0.5	3.0
24.0	24.6 ± 0.4	1.2	24.8 ± 0.6	2.3
36.0	36.5 ± 0.7	1.8	37.0 ± 0.8	2.2
Plasma				
150.0	154.5 ± 2.2	1.4	153.1 ± 2.1	1.4
250.0	252.0 ± 2.6	1.0	251.5 ± 2.9	1.1
350.0	354.2 ± 3.2	0.9	355.0 ± 3.7	1.1
450.0	447.3 ± 4.1	0.9	449.1 ± 5.2	1.1
Whole blood				
60.0	61.8 ± 1.1	1.8	62.4 ± 1.4	2.2
120.0	122.3 ± 2.0	1.6	123.2 ± 2.5	2.1
180.0	183.4 ± 2.7	1.5	181.9 ± 2.8	1.6
240.0	244.0 ± 4.1	1.7	244.8 ± 4.0	1.6

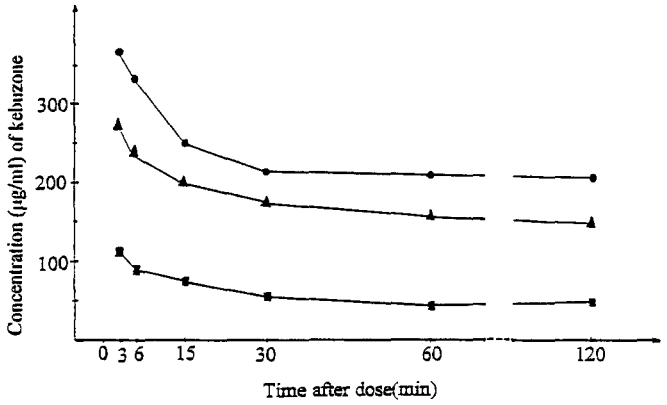

Fig.5 Concentration of kebuzone in erythrocytes (■), plasma (●) and whole blood (▲) of rabbit following intravenous administration of Ketazon inj.

Pharmacokinetic study

In order to investigate drug levels in the transport of some representative non-steroidal anti-inflammatory drugs in the organism, representatives of different structural types were selected. Kebuzone, a representative of 3,5-pyrazolidine-diones, was determined similarly as ibuprofen [24] and acetylsalicylic acid[25], representatives of profens and salicylates, respectively.

In a 2-hour pharmacokinetic study the samples of whole blood, isolated erythrocytes and plasma were analysed and the amounts of kebuzone determined. The observed blood, erythrocytes and plasma levels of kebuzone (Fig.5) with a simultaneous examination of the distribution of kebuzone between erythrocytes and plasma for three laboratory rabbits are presented. Fig.6 compares the concentration obtained by the sum of the concentrations found in erythrocytes and plasma (related to 1 ml of withdrawn blood with regard to the haematocrit value) and the

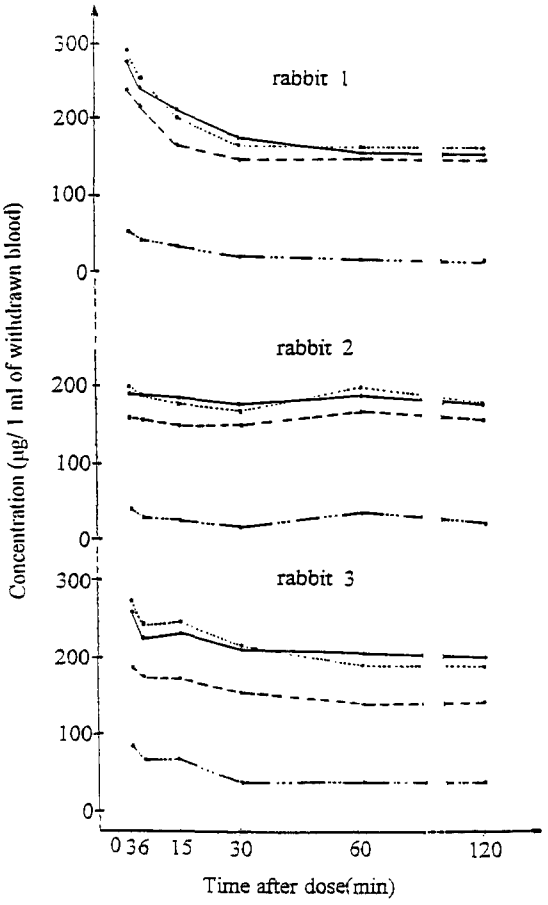

Fig.6 Distribution of kebuzone between erythrocytes and plasma in 1 ml of withdrawn blood during 2h after intravenous administration. (— ··· —) erythrocytes; (– – – –) plasma; (. . . . . .) sum of the levels; (———) level found in the analysis of the whole blood sample.

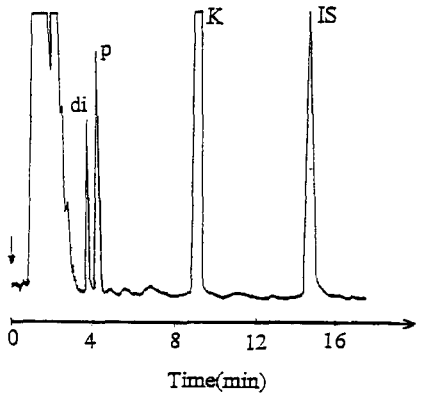

Fig.7 Chromatogram for kebuzone(K), its metabolites / *p*-hydroxy-γ-hydroxy-kebuzone(di) and *p*-hydroxykebuzone(p) / and the IS in rabbit whole blood 24-h sample from a rabbit given a single dose of 50 mg/kg.

concentration found in whole blood. The comparison of kebuzone levels in plasma, erythrocytes and whole blood shows that directly after administration kebuzone is already evenly distributed between erythrocytes and plasma and its levels almost do not change in both matrices. 20% of released kebuzone is demonstrated in erythrocytes and 80% in plasma . The metabolites of kebuzone were demonstrated in no biological matrix in the course of a 2-hour pharmacokinetic study. Althrough for the purposes of our cooperation with the Faculty of Medicine the determination of the levels of kebuzone during two hours in this biological matrix was sufficient, the samples of blood were still withdrawn 10 and 24 hour after drug administration. It was found that *p*-hydroxy-γ-hydro-xykebuzone and *p*-hydroxykebuzone were detected in samples of whole blood (Fig. 7) and plasma, but not in erythrocytes samples. γ-hydroxykebuzone was not detected in the samples of any biological matrix withdrawn after 24 hour.

Conclusion

In the present paper, a method using HPLC for the analysis of kebuzone and its metabolites in the samples of erythrocytes, plasma and whole blood was developed. HPLC analysis of kebuzone and its metabolites has not been described in the literature yet. If we want to determine only kebuzone (without its metabolites), it can be used for the adjustment of blood, erythrocytes and plasma sample extraction with methylene chloride after adding propyl gallate solution (as the stabilizing agent). In the simultaneous determination of kebuzone and its metabolites it was suitable to perform, before HPLC analysis, precipitation of proteins in the mentioned samples and inject the supernatant to the column. This HPLC method makes it possible to obtain a more detailed picture of the distribution of kebuzone in blood between erythrocytes and plasma and to contribute to a more objective view of the results of the analysis as dependent on the isolated biological matrix. It can be generally stated that whole blood provided more objective information about drug levels than plasma, though it is a more complicated biological matrix. Besides determining kebuzone levels in blood and distribution of kebuzone between erythrocytes and plasma, other physiological or pathophysiological parameters were investigated in laboratory rabbits by our collaborator from the Faculty of Medicine.

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SOLID PHASE EXTRACTION (SPE) OF BLOOD UREA COMPARED WITH LIQUID-LIQUID EXTRACTION REGARDING ARTIFACT FORMATION

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ABSTRACT

Solid phase extraction (SPE) of blood urea is described. Differential analysis of free type and bound type blood urea analysis for diagnosis is important because free type urea is more essential causing symptom of uremia. The differential analysis is attained by ultrafiltration by centrifugation using a cellulose membrane with cut-off molecular weight of 10,000 daltons from native and acidified blood. Compared with SPE, liquid-liquid extraction is more problematic due to the formation of artifacts during extraction and condensation by evaporation. Artifacts were observed in solvent extraction as a result of reactions between the extraction solvent and the target compounds.

INTRODUCTION

When a known compound is to be extracted by a solvent, the property of solubility and polarity of a target compound must be considered. When an unknown compound is to be extracted, systematic studies are prepared to extract the compound with different solvents and analyze the biological as well as physiological activities of each extraction fraction to examine in what fractions physiologically-active compounds were extracted. Thereafter, further separation using a proper method such as chromatography will be considered. This is a normal procedure when separating an unknown compound.

It is a so-called bioassay to examine in what fraction the physiologically-active compounds were extracted. Based on the author's experiences, the author intends to stress that in addition to the solvent properties there is another factor to be considered when selecting an appropriate solvent for extraction due to the artifact formation during solvent extraction.

As another subject, the author wishes to present blood urea analysis by using solid phase extraction (SPE) because of no previous studies of its superiority to liquid-liquid extraction procedure. There has been many papers describing chromatograpy of an immobilized enzyme, post column reaction or column switching, which required complicated procedure, set-up systems and troublesome technique[1,2]. This is because the separation of blood urea from other blood components is so difficult, therefore researchers designed complicated techniques. However, the complicated procedures are not easily applicable to routine clinical analysis. In this paper the author will present the simple technique of blood urea analysis using SPE instead of the complicated chromatographic separation.

MATERIALS AND METHOD

Toxic compounds in polyurethane (PU) were studied and the PUs used are identical to these reported previously [3].

HPLC analysis method for blood urea is as follows: Mitsubishi Kasei MCI^{R} CK 08S strong cation exchange column (SO_3H type, 4.6X 150 mm) was used. Column capacity and particle size were as follows: more than 1.9 meq/ml and 11-14 um. The material is SDB polymer base (degree of crosslinking of DVB is 8%). Other information on MCI Gel CK/CPK series for strong cation exchange columns are available from Mitsubushi Kasei Co.Ltd. The eluent is 1.5 mM HCl aqueous solution and urea is detected by ultraviolet at the wavelength of 210 nm. Flow rate is 2 ml/min. Retention time of blood urea peak is 24 min. HPLC apparatus is SP-8750 from Spectra-Physics.

Liquid-liquid extraction using methanol or acetone is identical to that reported previously[3].

SPE of blood urea is as follows: Blood is acidified at pH 3 with HCl or used as is. These were applied for ultrafiltration by centrifugation at 4.000 rpm using a Centricon^{R} (cut-off molecular weight 10,000 daltons) supplied by Amicon and the centrifugated

solution was acidified to pH3 with HCl. These were applied to the strong cation exchange column (H type) of Bond Elut^{R} SCX (500 mg of resin weight and 0.6 ml of void volume) supplied by Analytichem (Harbor City, CA, USA). The column was conditioned with 2 ml of methanol followed by 2ml of 0.1M HCl aqueous solution, which is a normal manner previously described[4]. One ml of blood was applied to the conditioned SCX column at an application flow rate of 0.3 ml/min and washed with 2 ml of water. The retained urea on SCX column was eluted with 2 ml of 1M HCl aqueous solution at the flow rate of 0.3 ml/min. Conditioning and elution in SPE were carried out using a Model AP-115 AN vacuum pump supplied by Iwaki (Tokyo, Japan)[4].

RESULTS AND DISCUSSION

I Artifact formation during extraction

While studying the extraction of toxic compounds and the physiological activity of the extracts (such as carcinogenic, mutagenic, cytotoxic, teratogenic compounds), the author found that the physiological and biological activities and recovery rate of an extract vary depending on the extraction solvents. These phenomena may not seem particularly surprising but it should be stressed here that these are not always caused by the difference in chemical as well as physical properties and polarities of extraction solvents.

For example, a medical device such as an artificial dialyzer was extracted with methanol or acetone, and each extract is tested for mutagenicity Ames test with or without S9 Mix of metabolic activity enzyme (Table I)[5]. Acetone indicated a greater quantity of extracted substances than methanol, but the substances extracted with acetone indicated a lower mutagenicity (Table I). Therefore, the researcher may speculate that mutagenic compounds are more favorably extracted with methanol rather than acetone. Cunningham et al[6] stated that the solvent, especially methanol, reacts with the target compound such as amine compounds and produced physiologically more active and more toxic compounds, which resulted in an artifact formation. If the researcher overlooks this kind of artifact formation, he may have an incorrect decision and misunderstanding for his results. The results reported by Cunningham et al are

TABLE I Mutagenicity test of irradiated and non-irradiated polyurethane extracts

Solvent	Extracted Amount (g)	Number of Mutant at 5000 μg/plate in the Absence of S9Mix*	
		non-irradiation	10Mrad
MeOH	4.97	173	1014
Acetone	8.10	134	249

*Salmonella typhimurium TA 100 was used.
Extraction was from 46 g of each polyurethane.

briefly outlined below followed by the methods for solving or preventing the artifact formation problems, some of which were based on the author's own experience.

During extraction with methanol or a vacuum evaporation for condensation process Cunningham et al observe that some aromatic amine compounds undergo a Mannich reaction with formaldehyde from methanol[6]. During these reaction, an artifact of dimer or oligomer compounds was produced. These artifact compounds indicated a greater mutagenicity more than 5 times than the original target compound[6]. The author also confirmed the formation of similar oligomer artifacts when extracting aromatic amine compound with methanol from polyurethane. Aromatic amine compound in this case is 4,4'-methylenedianiline (MDA) from PU and the dimer or oligomer linked at R-NHCH2NH-R was produced. Formation of such oligomer artifacts is prevented by replacing methanol with ethanol.

Furthermore, the liquid-liquid extraction process is replaced by SPE in order to prevent artifact formation and increase recovery rate of target compound[3]. This is because the former requires vacuum evaporation/concentration and allows prolonged contact of the target compound with the extraction solvent. Additionally, it is a well-known fact that compounds were lost without successfully trapping during a vacuum

evaporation/concentration process, thus reducing the recovery rate and causing thermal decomposition[3].

Cunningham et al also observe the formation of acetylated artifact when using ethylacetate as an extraction solvent. This is because the compounds with hydroxyl or amino functional group will be acetylated during extraction and reduce the recovery rate of the target compounds with hydroxyl and/or amino functional groups. These acetylated compounds also indicated a greater toxicity, therefore they warn that the results may lead to the misunderstanding that compounds appearing to be strongly mutagenic are collected[6].

II Blood urea analysis using SPE combined with ion exchange chromatography

Blood urea analysis using HPLC has already been reported. However, most of them are complicated set-up system such as using an immobilized enzyme column prior or posterior to the analytical column, post-column calorimetry or florescence reaction method, pre-column calorimetry or florescence reaction method or column switching method[1,2]. They required complicated technique, thus they are not favorably applicable to the routine clinical laboratory.

SPE for blood urea has not been reported so far, therefore the author intended to prevent blood components with SPE in place of chromatographic separation in addition to the differential analysis of endogenous ammonia and urea. Some part of blood urea existed in combination with blood protein, therefore ultrafiltration using the centrifugation at 4,000 rpm is thought to be most appropriate for differential analysis of free type from protein-bound type of blood urea.

By acidifying the blood, urea is totally isolated from blood protein due to denaturation of blood protein, therefore the differential analysis of free type and free plus bound type is attainable. The analysis of free type of blood urea is more essential for the purpose of diagnosis of patient's status of disease. Current clinical test is carried out using a selective ammonia electrode. By this method, the differential analysis of endogenous ammonia and urea is not attainable, thus the selective analysis of endogenous urea analysis is required.

For SPE procedure of blood urea, the centrifuged solution is applied to Bond Elut^{R} SCX column and eluted with 1 M HCl as mentioned in the experimental section.

For HPLC analysis, urea elution will vary depending on the concentration of HCl aqueous solution used for the eluent as well as the retention time and separation of blood urea from blood components. As the results, 1.5 mM HCl aqueous solution for the eluent and 2 ml/min flow rate were found to be appropriate in the current experiment. This result is dependent on the difference of blood or other factors such as the amount of components in blood. Thus the reader can select between 1-2 mM HCl for the eluent at the flow rate of 2-3 ml/min. The author did not believe current result in this paper was most appropriate and required modification depending on sample blood. The selection of columns for blood urea analysis was studied and compared the current and conventional ion exchange column with the ion chromatography column with less ion exchange capacity. The former was found to be superior to the latter in terms of urea separation from blood components[7].

Blood urea peak was not interfered with blood components in my experiment as shown in Figure I. In order to confirm urea peak was not overlapped and a single peak, photodiodearray detection with multiple wavelength for three dimension chromatogram was used. This result indicated urea peak was single and pure.

By using the combination of SPE and HPLC methods, complicated HPLC analytical procedure is not required. Routine and differential analysis (free and bound type) is easily attainable. Blood urea is free from blood components using SPE in place of chromatographic separation as shown in Figure I. The average recovery rate of blood urea following this method of SPE is 98% (n=10), which is satisfactory.

Current SPE procedure was carried out manually, but the author thinks the combination of autoinjector, automated ultrafiltration using centrifugation, the automated SPE, and HPLC in series will lead to the development of a laboratory clinical test device that will be more convenient than the current clinical test method using an ammonia-selective electrode. The main reason is due to the capability of differential analysis of free and total urea amount in blood in addition to more precious, selective and sensitive than current method using the ammonia-selective electrode. In terms of automation, for example, when combining ASTED^{R} (Automated Sequential Trace Enrichment of Dialysates) and ASPEC^{R} (Automatic Sample Preparation with Extraction Columns) from Emuesu Co. Ltd were used combined with autoinjector and HPLC, automated equipment for differential analysis of free and bound type blood urea will be attainable for clinical laboratory routine analysis.

Moreover, the necessity of differential analysis is not always limited to the determination of blood urea. Blood guanidines, calcium in milk as an example are also

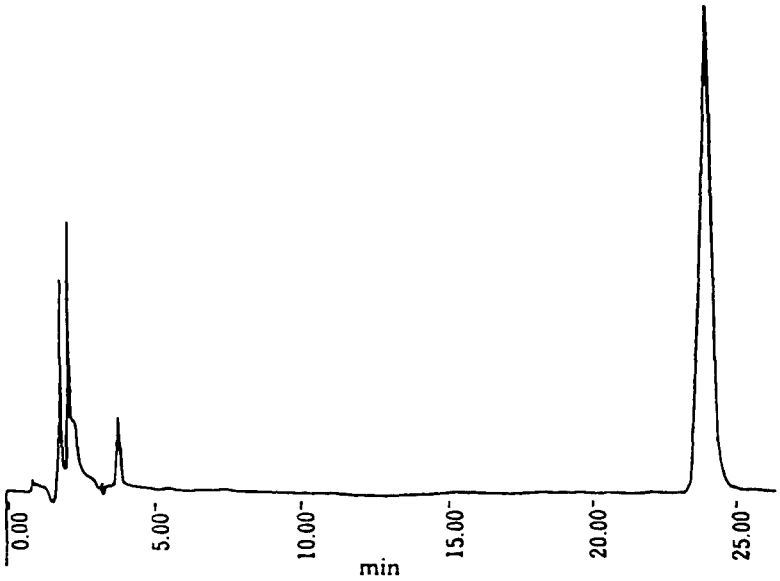

FIGURE 1 HPLC chromatogram of blood urea after SPE treatment by SCX column

The peak eluted at around 24 min is blood urea.

required. Therefore, the current method as well as an automated procedure may be applicable for the determination of other components in clinical laboratory as well as other industries.

CONCLUSION

Analytical chemists are not well informed on artifact formation during solvent extraction by reacting the extraction solvent with the target compound, which may lead to incorrect decision and misunderstanding for their results. In order to avoid these, the author stresses the necessity of taking into consideration the factors other than solvent properties and polarities when selecting appropriate extraction solvents and the necessity replacing liquid-liquid extraction with SPE.

SPE is superior to liquid-liquid extraction. By using SPE, routine and differential analysis (free and bound type) of blood urea is easily attainable and blood urea is free from blood components by using SPE.

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CHARGE-TRANSFER CHROMATOGRAPHY USED TO STUDY THE INTERACTION OF CHLORHEXIDINE WITH PROTEINS AND AMINO ACIDS

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ABSTRACT

The interaction of the antibacterial agent chlorhexidine (1,1-hexamethylene-bis-/5-(4-chlorophenyl)-bisguanidine/) with human albumin and pepsin and with amino acids was studied with reversed-phase charge transfer chromatography in the presence of various monovalent cations. It was established that the mobility of chlorhexidine increases in the presence of ions in the lower concentration range (0.001 - 0.1 M) then increases at higher concentrations. The ion radii significantly influences the effect. Stepwise regression analysis proved that the chlorhexidine binds both to pepsin and human albumin, the binding is stronger with human albumin. The ions modify the character of chlorhexidine-human albumin binding indicating the hydrophilic character of the interaction. Except Asp and

Glu the other amino acids exhibited weak interactions with chlorhexidine, no significant difference was found between the interactive strength of L- and D-forms of Asp and Glu. Our data make probable that hydrophilic binding of chlorhexidine to the acidic side chains in proteins may be involved in its mode of action.

INTRODUCTION

Many studies have been devoted to the exploration of the effect of chlorhexidine (1,1-hexamethylene-bis-/5-(4-chlorophenyl)-bis-guanidine/) on the reduction of gingivitis (1-6) and plaque (7-12) formation. Chlorhexidine effectively reduces oral microflora (13-23) and the incidence of oral mucosal complications after bone marrow transplantation (24), inactivates human immunodeficiency virus (25), inhibits the incorporation in DNA of thymidine, decreases lactate dehydrogenase content in human buccal epithelial cells in culture (26) and desorbs adsorbed salivary constituents (27). However, chlorhexidine causes burns of the lips, mouth and tongue (28) and anaphylactic symptoms (29). Chlorhexidine interacts with surfactants (30), the addition of Triton-X-100 (31) or sodium lauryl sulphate (32) decreased its effect.

The mode of action of chlorhexidine has not been elucidated in detail. Chlorhexidine is thought to interact with acidic lipid components to cause changes in the membrane permeability (33), however, it changes only slightly the phase transition temperature of phosphatidylcholine and phosphatidylglycerol (34).

Charge-transfer reversed-phase thin-layer chromatography has been frequently used to study interactions between different organic molecules of low molecular

weight. The theory and practice of the determination of relative interactive strength by this method has also been described (35).

The objectives of our work were to study the interaction of chlorhexidine with some proteins and amino acids and to elucidate the effect of ions on the strength of interaction.

MATERIALS AND METHODS

Chlorhexidine diacetate (Serva Chemical Co.. USA) was of analytical purity. Human albumin and pepsin were the products of Human Vaccine Works (Budapest, Hungary) and Chemical Works of Gedeon Richter Ltd. (Budapest, Hungary), respectively. Amino acids were of analytical purity and of L-conformation, the D-forms were included in the experiments only in the case of Asp and Glu. To study the interaction of chlorhexidine with the proteins cellulose powder for TLC (Merck, Darmstadt, Germany) was mixed with the proteins in 9:1, 19:1 and 99:1 w/w ratios. Layers of 20 x 20 cm (0.25 mm thickness) were prepared from the mixed slurries and after drying their UV spectra was determined with a Model CS-930 Dual Wavelength TLC Scanner (Shimadzu, Kyoto, Japan). The UV spectra of chlorhexidine adsorbed on cellulose layer was determined in separate experiment. As the chlorhexidine was easily detectable even at the highest protein:cellulose ratio (1:9 w/w), this mixed sorbent was used in the experiments to study the interaction between chlorhexidine and the proteins. The use of unimpregnated cellulose layer as reversed-phase sorbent was motivated by the theoretical considerations stating that any layer may

behave as a reversed-phase one when the stationary phase is less polar than the mobile phase (36). The validity of the hypothesis outlined above was proved to be true for unimpregnated cellulose (37). Chlorhexidine diacetate was dissolved in distilled water at the concentration of 2 mg/ml, 5 μl of this solution was spotted onto the plates. Distilled water was used as eluent containing LiCl, NaCl and KCl in the concentration range of 0.001 - 1 mM. At each salt concentration unmixed cellulose layers served as control. After development the plates were dried at 105°C and the maximum of the chlorhexidine spot was determined with the same TLC scanner at 270 nm. The R_M values characterizing the lipophilicity were calculated in each case.

As it was assumed that the protein and salt concentration and the interaction between proteins and salts may influence simultaneously the R_M value of chlorhexidine we used stepwise resression analysis to elucidate this problem (38). As the exact type of correlation (linear, quadratic or logarithmic) between the independent and dependent variables was not previously established we used the following independent variables: x_1 = human albumin content of the sorbent (%); x_2 = pepsin content of the sorbent (%); x_3 = cation radii; $x_4 = (\log x_6)^2$; $x_5 = (x_1.x_7)^2$; $x_6 = (x_7)^2$; x_7 = salt concentration in the eluent (M); $x_8 = x_1.x_7$; $x_9 = x_2.x_7$; $x_{10} = (x_2.x_7)^2$; $x_{11} = \log x_7$. The number of accepted variables was not limited, the significance level for each independent variables was set to 95%.

To study the interaction of chlorhexidine with amino acids DC-Alufolien cellulose plates (Merck, Darmstadt, Germany) were used without any pretreatment.

Chlorhexidine was spotted onto the plates as described above. For the screening test distilled water was used as the eluent containing the amino acids at 10 mM concentration. The detection of the spot maximum was carried out by the TLC scanner as described above. As Asp and Glu showed the highest effect their interaction with chlorhexidine was studied more in detail, the concentrations of Asp and Glu varying between 0 - 10 and 0 - 100 mM depending on their solubilitv in water. To elucidate the role of stereospecificity the experiments with Asp and Glu were carried out with the L- and D-forms too. As the correlation between the Asp and Glu concentration and the R_M value of chlorhexidine seemed to be markedly nonlinear, logarithmic correlations were calculated between the R_M value and amino acid concentration separately for the L- and D-forms of Asp and Glu.

RESULTS AND DISCUSSION

The UV spectra of chlorhexidine and human albumin adsorbed on cellulose are shown in Fig.1. The chlorhexidine has an UV maximum at 270 nm. The human albumin (and also pepsin) has a relatively low absorbance at this wavelength, therefore the adsorbed proteins interfere to a negligible extent with the UV detection of chlorhexidine.

The lipophilicity of chlorhexidine decreasad rapidly with the growing concentration of salt in the eluent (Fig.2) then - having a minimum - increased again at higher salt concentrations. However, this increasing phase was slower than the decreasing phase. The effect was similar for each salt but depended also

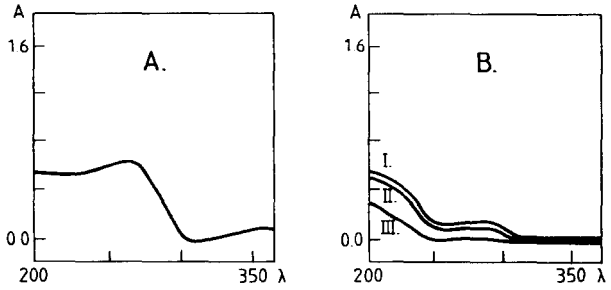

FIGURE 1. UV spectra of chlorhexidine (A) and human albumin (B) adsorbed on cellulose. I. human albumin: cellulose 1.9 w/w; II. human albumin:cellulose 1:19 w/w; III. human albumin:cellulose 1:99 w/w

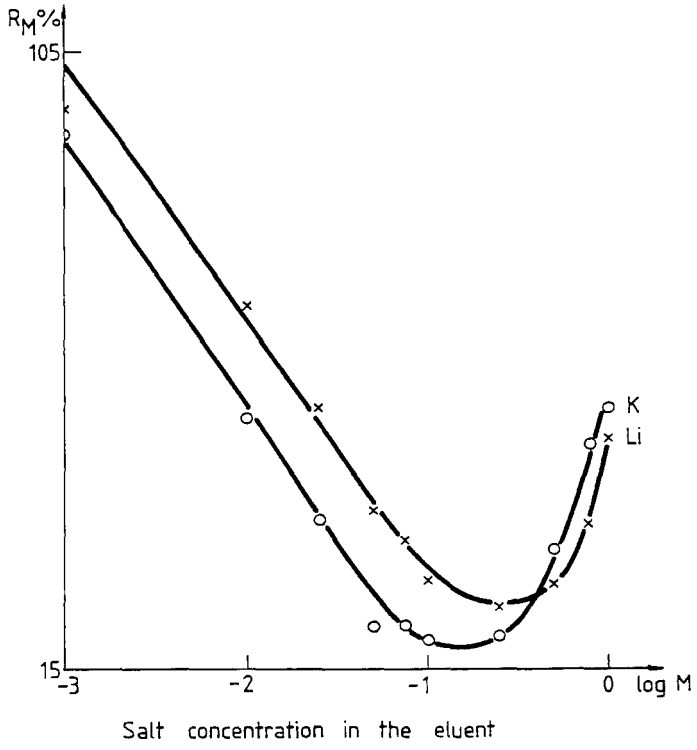

FIGURE 2. Effect of various salts on the R_M value of chlorhexidine.

on the type of salt. Our data make obvious that the salt in the eluent influences the R_M value of chlorhexidine in minimally two different ways. We assume that the polar groups of chlorhexidine interact with the polar adsorptive site of cellulose. The ions present in the eluent also bind to the polar adsorption centers of cellulose. This coadsorption is of competitive character, the higher the ion strength the higher is the ratio of adsorptive site occupied by the ions in the eluent. The lessening number of binding sites results in continuous decrease of chlorhexidine retention. Similar masking effects of eluent additives was also observed in the reversed-phase chromatography of peptides (39). After the probable saturation of free adsorptive sites of cellulose by the ions in the eluent the situation changes markedly. The fact that higher salt concentrations increase again the R_M values of chlorhexidine may be due to the dissociation suppressing effect of salts. The undissociated chlorhexidine molecule shows a higher reversed-phase retention than the dissociated one. Similar anomalous effect of salts on the reversed-phase retention behavior of heterocyclic quaternary ammonium salts has been previously reported (40).

The proteins markedly decreased the retention of chlorhexidine at higher salt concentration (Fig.3) indicating that the proteins really interact with chlorhexidine.

The results of stepwise regression analysis are compiled in Table 1.

The equation fits well to the experimental data the significance level being over 99.9% (compare calculated and tabulated F values). Six independent variables

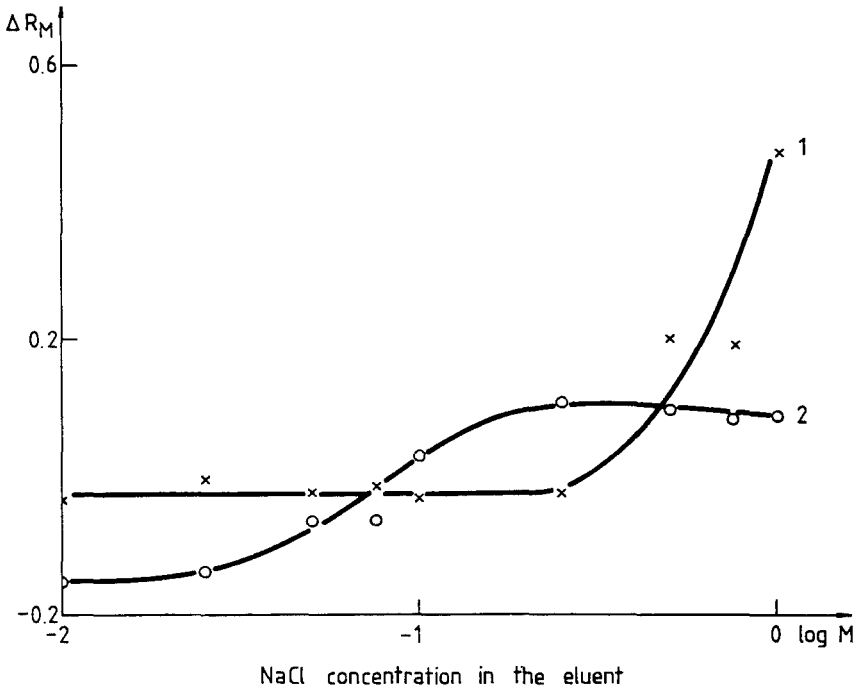

FIGURE 3. Effect of human albumin (1) and pepsin (2) on the lipophilicity of chlorhexidine.

of the eleven included ones account for about 74% of the total variance (see r^2 value). It can be concluded from the slope (b) values that both human albumin and pepsin significantly increase the retention of chlorhexidine that proves the binding of chlorhexidine to these proteins. The ions with greater ion radii decrease more strongly the retention of chlorhexidine that is not only the ion concentration but also the character of the cation influences the lipophilicity

TABLE 1.
Effect of Salts and Proteins on the Lipophilicity (R_M) of Chlorhexidine. Results of Stepwise Regression Analysis.

$$R_M = a + b_1.x_1 + b_2.x_2 + b_3.x_3 + b_4.(\log x_7)^2 + b_5.x_1.x_7 + b_6.(x_7)^2$$

$n = 74$ $a = 0.52$ $F_{calc.} = 32.59$ $F_{99.9\%} = 4.37$ $r^2 = 0.7420$

Number of independent variable	$b.10^2$	$s_b.10^3$	Path coefficient %
1	2.10	5.16	12.4
2	1.27	5.26	6.0
3	-21.97	10.73	5.0
4	10.55	8.50	33.5
5	-0.46	1.28	11.9
6	74.72	75.07	30.9

x_1 = human serum albumin content of the sorbent (%)
x_2 = pepsin content of the sorbent (%)
x_3 = cation radii
x_7 = salt concentration in the eluent (M)

of chlorhexidine. The exact type of correlation between the salt concentration in the eluent and the R_M value of chlorhexidine is not clear. Our data suggest that it can be quadratic or log quadratic. This finding supports our previous assumption that the influence of salts is composed of more than one physicochemical processes which are yet not clearly understood. The interaction between human albumin and salt concentration decreases the R_M value of chlorhexidine proving again the hydrophilic character of protein-chlorhexidine interaction. The path coefficients show the relative impact of the independent variables on the R_M value. The salt concentration exerts the highest effect on the retention followed by the pro-

TABLE 2.
Effect of Amino Acids on the Lipophilicity of Chlorhexidine.

Amino acid	Lipophilicity (R_M value) Mean	Standard deviation	Coefficient of variation%
Control	1.35	0.03	2.57
Ala	1.29	0.03	2.41
Arg	1.03	0.04	3.88
Asn	1.14	0.02	1.91
Asp	0.28	0.03	11.81
Cys	1.22	0.02	1.41
Glu	0.33	0.03	5.47
Gly	1.25	0.02	1.64
Ile	1.15	0.08	7.35
Leu	1.12	0.01	0.52
nor-Leu	1.09	0.10	9.30
Lys	1.09	0.05	4.18
Orn	1.04	0.02	2.24
Pro	1.36	0.02	1.54
Ser	1.34	0.03	2.57
Trp	1.40	0.05	3.57
Tyr	1.30	0.02	1.21
Val	1.29	0.04	3.10

tein concentrations. The impact of the ion radii is of secondary importance.

The results of the screening test for amino acid-chlorhexidine interaction are compiled in Table 2. Many amino acids decrease the lipophilicity of chlorhexidine. This phenomena is probably due to the fact that the more hydrophilic amino acids interact with the chlorhexidine reducing its lipophilicity. The highest effect was observed with the dicarboxylic amino acids Asp and Glu indicating that the interaction is of hydrophilic character.

We assume that the quaternary amino groups of chlorhexidine form hydrogen bonds with the carboxyl

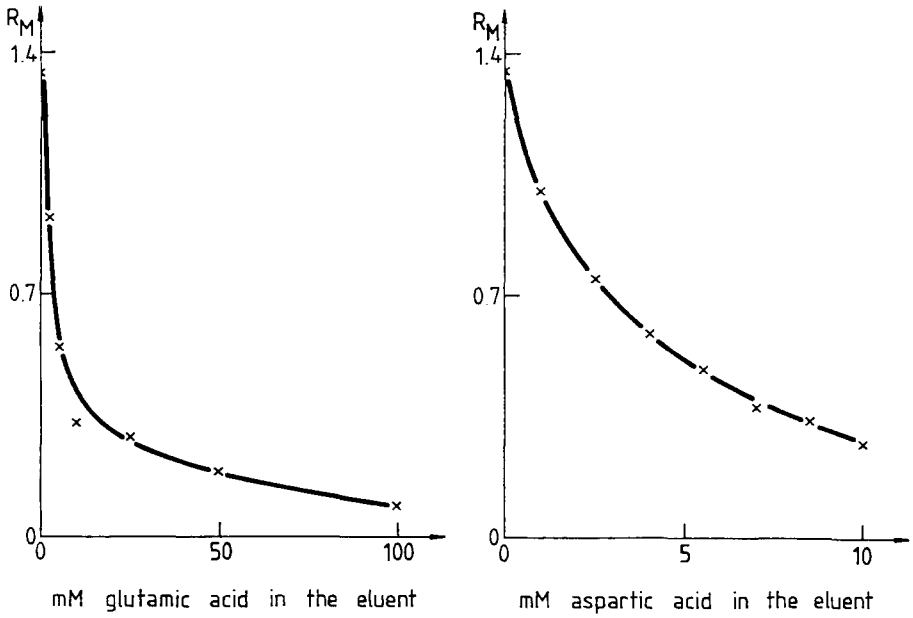

FIGURE 4. Effect of dicarboxylic amino acids on the R_M value of chlorhexidine.

groups of the amino acids resulting in charge transfer complexes of unknown stoichiometry. Unfortunately, charge transfer chromatography does not give information about the composition of the complexes. The correlation between the concentration of dibasic amino acids in the eluent and the R_M value of chlorhexidine is markedly nonlinear (Fig.4). As in the majority of cases the correlation between the two complexing molecules is significantly linear (41,42), this finding can be tentatively explained by the assumption that the complexes may have minimally two stoichiometries

TABLE 3.
Effect of Dicarboxylic Amino Acids on the Lipophilicity (R_M value) of Chlorhexidine (C = Concentration of Amino Acid mM).

$$R_M = a + b.\log C$$

Amino acid	n	a	b	s_b	$r_{calc.}$	$r_{99\%}$
L-Glu	5	0.91	-0.52	0.05	0.9795	0.9587
D-Glu	5	0.99	-0.57	0.03	0.9812	0.9587
L-AsP	7	1.02	-0.73	0.02	0.9979	0.9507
D-Asp	7	1.10	-0.70	0.04	0.9813	0.9507

depending on the amino acid - chlorhexidine ratio. At low amino acid concentrations the amino acid - chlorhexidine ratio is lower in the complex than at higher concentrations resulting in the deviation from the linearity.

Calculations show that the logarithmic correlation fits well to the experimental data the significance level being in each case over 99% (Table 3).

The change of amino acid concentration accounts for about 95 - 99% of the lipophilicity change of chlorhexidine (see r values). No significant difference was found between the slope (b) values of L- and D-forms that is the interaction of chlorhexidine with Asp and Glu not stereospecific or the stereospecificity is such low that it is under the detection limit of our method. The L- and D-forms of Asp had a significantly higher effect on the R_M value of chlorhexidine than the corresponding forms of Glu (the calculated t values were 3.32 and 2.60 for L- and D-forms, respectively, the tabulated t value for 95% significance level is 2.23). It means that chlorhexidine preferably

interacts with the more acidic (43) and less lipophilic (44) Asp proving again the hydrophilic character of the interaction.

Our results prove that chlorhexidine can bind to various proteins, the binding sites are probably the acidic amino acid side chains in the proteins. The interaction is of hydrophilic character and its strength depends on the type and concentration of the ions in the environment. We assume that these protein - chlorhexidine interactions may have a role in the biological activity of chlorhexidine.

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LIQUID CHROMATOGRAPHIC ANALYSIS OF CHLORHEXIDINE AT SPECIFIC SITES IN THE SALIVA FILM AFTER APPLICATION OF A TOOTH-BONDED DELIVERY SYSTEM

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ABSTRACT

This paper describes the use of an HPLC assay to investigate whether a chlorhexidine-containing tooth-bonded delivery system produces antibacterial concentrations in the surrounding saliva film.

Saliva (<1.5 µl) was collected from ten sites in the mouth at eight times over a period of four days following application of tooth-bonded delivery systems in two dentally healthy subjects. Analysis of chlorhexidine concentrations in these samples showed a non-uniform distribution of chlorhexidine in the saliva film. Antibacterial concentrations were produced in the area immediately surrounding the delivery system (20 ± 4 and 28 ± 12 µg/ml for Subjects One and Two respectively) whereas concentrations at more distant sites remained low.

INTRODUCTION

Chlorhexidine is an antibacterial agent used in the treatment of plaque-initiated gum diseases such as gingivitis and periodontitis. It has a wide spectrum

of antibacterial activity [1, 2] and has demonstrated effectiveness as an agent for supra-gingival plaque control [3-5]. It is usually administered as a mouthrinse or topical solution and its effectiveness is, in part, due to its ability to reversibly bind to the tissue surfaces in the mouth which can provide antibacterial effects for up to 24 hours in some individuals [6, 7]. However, for sustained anti-plaque effects, twice daily administration is usually recommended [8] and therefore good patient compliance is required. Some problems identified with the use of chlorhexidine mouthrinses include the bitter taste imparted by the high drug concentration (0.2 %w/v) and tooth discolouration that occurs with prolonged use [9-11]. Also, mouthrinses have been shown to have little or no effect on the subgingival microflora as the drug cannot penetrate to this site [12]. To improve the delivery of chlorhexidine for the treatment of gum diseases, systems that employ smaller quantities of chlorhexidine and deliver the drug directly to diseased sites have been investigated. To date these systems have been evaluated *in vivo* by analysis of the rate at which drug is released from the delivery system along with assessment of clinical and microbiological effects [13-22]. Analysis of drug concentrations has been limited to measurement of concentrations in total saliva [23] because of the lack of chlorhexidine assays with suitable sensitivity which would allow determination in the small samples, typically <1.5 μl, that can be collected from some sites in the mouth.

This paper describes the use of a recently developed assay [24] to determine chlorhexidine concentrations in the saliva film at different distances from a tooth-bonded delivery system.

MATERIALS AND METHODS

Chemicals and reagents

Chlorhexidine diacetate B.P. was purchased from ICI Chemicals (Wellington, New Zealand). Poly(ε-caprolactone) was purchased from Polysciences (Warrington, U.S.A.). The internal standard, benzethonium chloride was AnalaR grade purchased from Sigma Chemical Co. (St Louis, MO, USA). Acetonitrile,

and glacial acetic acid were HPLC grade purchased from Ajax Chemicals Pty. Ltd. (Auburn, N.S.W., Australia) and sodium laurylsulphate was HPLC grade purchased from BDH chemicals Ltd (Poole, England). Sodium hydroxide was AnalaR grade purchased from BDH chemicals. Deionised water was produced with a Millipore Milli-Q system (Bedford, MA, U.S.A.).

Equipment

A Mettler five decimal place analytical balance (Mettler AT201) was used to determine the mass of samples collected from the saliva film.

A Spectra Physics HPLC system was used for the analysis of chlorhexidine in saliva samples. It comprised a SP8800/8810 ternary pump, a Spectra System UV 2000 dual wavelength detector, a SP4400 Chromjet integrator and a Rheodyne injector with a 50 μl sample loop. The stainless steel column, 10 cm x 2.1 mm i.d. was packed with 5 μm C18 ODS-B Exsil purchased from HiChrome Ltd. (Berkshire, England).

Preparation of the chlorhexidine delivery system

Chlorhexidine diacetate was dissolved in water and sufficient sodium hydroxide was added to increase the pH to 12. The precipitate (chlorhexidine base) was collected, dried then recrystallised twice from methanol. The final product was dried to constant weight in a vacuum oven, then sieved using Endecott sieves (Endecott Ltd., London, England) and the particle size fraction 63-125 μm was collected.

Duplicate films of poly(ε-caprolactone) containing 20%w/w chlorhexidine were prepared by solvent evaporation. Poly(ε-caprolactone) (0.96 g) was dissolved in dichloromethane (8 ml) and chlorhexidine (0.24 g) was added as a powder, stirred, then poured into an aluminium ring (7.6 cm diameter) placed on a silanised glass plate. Dichloromethane was evaporated at 25°C for 24 hours, then a vacuum was applied for a further 12 hours. The resulting films were stored in a desiccator with silica gel, at room temperature, until required.

Characterisation of the chlorhexidine delivery system

Drug loading of films was determined by cutting discs (0.5 cm diameter) and dissolving the poly(ε-caprolactone) in chloroform (0.5 ml), then extracting the chlorhexidine into 1%v/v glacial acetic acid (5 ml) by shaking for 20 minutes. The aqueous and organic phases were separated by centrifugation at 2500 rpm using a Megafuge 1.0R centrifuge (Heraeus Sepatech), then chlorhexidine concentrations in the aqueous portions were determined by UV spectroscopy (λ =254nm).

In vitro release of films was determined by cutting five discs (0.5 cm diameter) from each film and attaching them to individual teflon discs with a silicone adhesive (Bostick RTV sealant). These were immersed in sodium citrate/sodium hydroxide buffer (5 ml, pH 6.6, 0.1 M sodium citrate) and placed in a shaking water-bath (Grant Instruments Ltd.) at 37°C and 100 oscillations per minute. At sampling times, which were determined so that the chlorhexidine concentration in the release medium did not exceed 15% of its maximum solubility, the buffer was removed and replaced with fresh pre-warmed buffer. Chlorhexidine released was measured by UV absorbance spectroscopy (λ =254nm). Calibration curves for chlorhexidine in sodium citrate/sodium hydroxide buffer were linear over the range of chlorhexidine concentrations 1 to 16 μg/ml chlorhexidine (R^2>0.99). Control experiments showed that poly(ε-caprolactone) or the silicone adhesive did not interfere with the assay.

Attachment of the chlorhexidine delivery system and determination of chlorhexidine concentrations in the saliva film

Ethical approval for this part of the study was obtained from the Southern Regional Health Authority (Otago, New Zealand).

One section was cut from each film to approximately 2.5 x 4 mm and trimmed to allow attachment to the buccal surface of the left lower first molar in two dentally healthy subjects. Sections were weighed then attached to the tooth surface using a dental adhesive system (Scotchbond, 3M Pharmaceuticals). The subjects did not brush their teeth or eat breakfast prior to collection of morning samples and refrained from using toothpastes, mouthrinses or brushing the tooth to which the delivery system was attached during the study.

Saliva (<1.5 μl) was collected on Periopaper strips (Harco Electronics, Winnipeg, Canada*) from the saliva film at ten sites in the mouth prior to and one (t1) and three (t2) hours after attachment of the chlorhexidine-containing film, then again on the morning of the following four days (t3, t5 t6 and t8) and in the afternoon on the second (t4) and fourth (t7) days. Chlorhexidine concentrations were determined using the HPLC method previously described [24]. At each sampling time teeth were observed for the presence of plaque. Film sections were removed from the teeth after saliva samples had been collected on the morning of the fifth day and the remaining chlorhexidine was determined by dissolving the poly(ε-caprolactone) in chloroform (0.5 ml) then extracting the chlorhexidine into 1%v/v glacial acetic acid (2 ml). Chlorhexidine in the aqueous portion was determined by absorbance spectroscopy (λ=254nm).

RESULTS AND DISCUSSION

Characterisation of the chlorhexidine delivery system

The drug load of films, as determined by extraction of chlorhexidine, did not differ from the theoretical loading calculated from the weight of starting materials by greater than ±1.5%. Duplicate films contained 19.9 ± 0.4 (mean ± s.e.m., n=4) and 20.3 ± 0.8 (n=5) %w/w chlorhexidine. *In vitro* release profiles are shown in Figure 1. Films released 33.0 ± 0.9 (mean ± s.e.m., n=5) and 31.1 ± 0.7 (n=5) of the drug load over four days and the difference between films was not significant ($p>0.05$).

Chlorhexidine concentrations in the saliva film after bonding of the chlorhexidine delivery system to a tooth

The sections attached to teeth weighed 1.4 mg and contained approximately 280 μg chlorhexidine. They released 24 and 48% of their drug loads over the study in Subjects One and Two respectively. The consistancy of *in vitro* release (Figure 1) would suggest that this variation in the percentage released *in vivo* was

*current supplier is IDE Interstate (Amityville, U.S.A.)

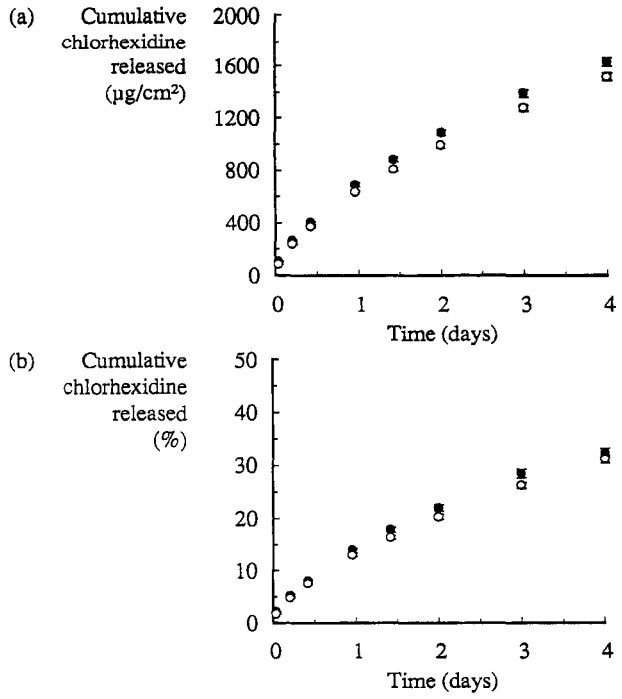
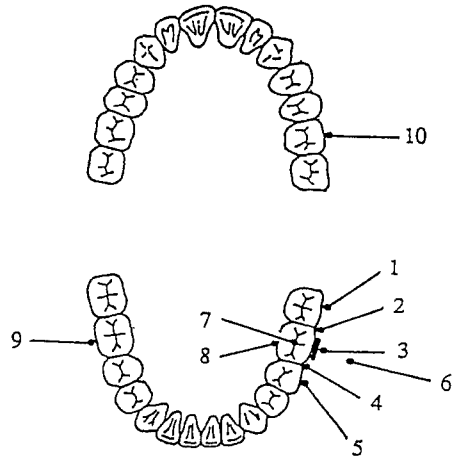


FIGURE 1: *In vitro* release profiles for chlorhexidine release from films containing 20%w/w chlorhexidine in poly(ε-caprolactone). Film 1 (•) and Film 2 (o). Error bars represent the standard error of the mean (n=5).

due to differences in conditions within the mouths of the subjects rather than differences in the film sections.

Sites in the oral cavity from which saliva was collected are shown in Figure 2 and the chlorhexidine concentrations measured at these sites, for times t1 to t8, are shown in Figure 3. Typically, the highest chlorhexidine concentrations were measured directly below the tooth-bonded delivery system (Site 3). Concentrations at this site were 20 ± 4 and 28 ± 12 (mean ± s.e.m., n=8) μg/ml for Subjects One and Two respectively while lower concentrations were measured at the more distant sites. Considerable variability was, however, observed in the



Site	Description of site
1	Buccal gingival margin of the lower left second molar
2	Buccal interdental space between the lower left first and second molars
3	Buccal gingival margin of the lower left first molar *(i.e. immediately below the tooth-bonded delivery system)*
4	Buccal interdental space between the lower left second pre-molar and the first molar
5	Buccal gingival margin of the lower left second pre-molar
6	Lower left buccal sulcus
7	Occlusal surface of the lower left first molar
8	Lingual gingival margin of the lower left first molar
9	Buccal gingival margin of the lower right first molar
10	Buccal gingival margin of the upper left first molar

FIGURE 2: Sites in the oral cavity for saliva collection.

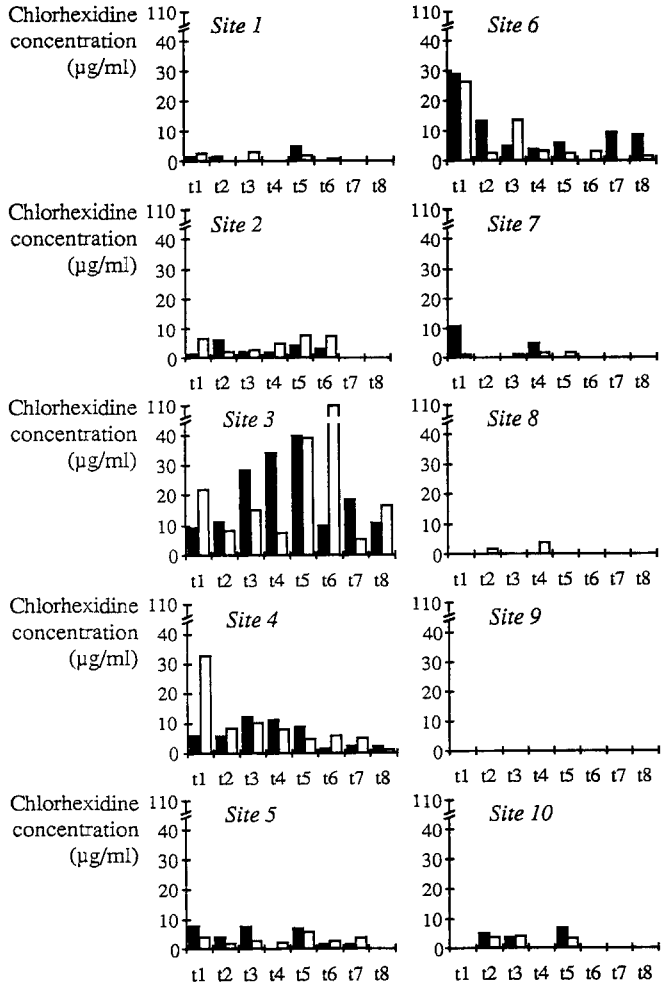


FIGURE 3: Chlorhexidine concentrations in the saliva film at Sites 1 to 10 (times t1-t8) in Subject One (■) and Subject Two (□).

concentrations measured at Site 3. This variability was not unexpected as the subjects continued normal oral function throughout the study, with the exception that they did not brush their teeth or eat breakfast prior to collection of the morning samples and refrained from using toothpaste or brushing the tooth to which the delivery system was attached. Normal oral functions such as movement of the cheek on the film surface and mastication, is likely to affect the drug release from the delivery system as well as clearance of the released drug. These processes will vary between and within subjects and could contribute to the variability in both the percentage drug released from the film sections *in vivo* and the saliva chlorhexidine concentrations at individual sites. It is interesting to note that despite a lack of control over subjects oral function the chlorhexidine concentrations measured immediately below the delivery system (Site 3) were in the order of those reported to inhibit the growth of plaque bacteria *in vitro* [2, 25, 26]. Examination of tooth surfaces throughout the study confirmed the localised anti-plaque effect of this tooth-bonded delivery system as no plaque was observed on the buccal side of the left lower first molar in either subject. Subject Two did show some plaque accumulation a tooth away, on the buccal side of the left lower second molar. In the absence of oral hygiene plaque would be expected to accumulate over the period of this study, however it appears that chlorhexidine released from the tooth-bonded delivery system was sufficient to prevent this occurring in areas immediately adjacent to the delivery system.

CONCLUSION

Measurement of chlorhexidine concentrations in the saliva film surrounding a tooth-bonded delivery system showed that effective concentrations were maintained immediately adjacent to the delivery system for a period of four days. At other, more distant, sites chlorhexidine concentrations were lower, thus indicating the tooth-bonded delivery system may be useful for inhibiting plaque-growth at specific sites in the mouth where gum disease exists. This may avoid the side effects that relate to high saliva chlorhexidine concentrations evident with conventional delivery systems.

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DETERMINATION OF DICLOFENAC SODIUM IN HUMAN PLASMA BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

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ABSTRACT

Following a detailed study, a simple, rapid and accurate reversed-phase liquid chromatographic method has been developed for the determination of diclofenac sodium in human plasma. The sample is separated isocratically within 7 min using an octadecyl-bonded silica column and a mobile phase of methanol and sodium acetate buffer (68:32, v/v; pH 4.2). The compounds were quantitated using a ultraviolet detector operated at 274 nm which allowed determination of 0.10 - 2.50 μg/ml of diclofenac sodium with high reproducibility. The limit of detection is 0.03 μg/ml. Intra-day and inter-day coefficients of variation for assaying the plasma sample containing 0.20 μg/ml concentration of diclofenac sodium were 7.4% (n=9) and 7.6% (n=7), respectively. The extraction efficiency of diclofenac sodium were 91.3 - 93.2% for plasma. The method has been used to determine diclofenac sodium in the plasma samples from ten volunteers and provide data on the pharmacokinetics of the drug.

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INTRODUCTION

Diclofenac sodium (sodium [o-(2,6-dichloroanilino)-phenyl] acetate) is a relatively safe and effective non-steroidal drug with pronounced antirheumatic, antiinflammatory, analgesic, and antipyretic properties (1). It has been widely used for several years in the treatment of degenerative joint diseases and other arthritic conditions (2,3).

A few procedures have been reported for quantitation of diclofenac sodium in human plasma, including thin-layer chromatography (TLC) (4), gas chromatography (GC) (5-7) and high-performance liquid chromatography (HPLC) (8-15). TLC lacks the sensitivity and accuracy required for the analysis of diclofenac sodium in pharmacokinetic investigation. The GC methods are highly sensitive and specific. However, all require extensive sample preparation by extraction and derivatization prior to GC separation. In recent years, HPLC methods have been used for the determination of diclofenac sodium alone or together with its metabolites in body fluids. Nevertheless, some procedures have to employ the complex instrumentation and installation (8,9) or use expensive electrochemical detection (10,11) or fluorimetric detection (12,13). These hardly meet the needs of simplicity and rapidity for clinical drugs monitoring.

This paper describes a simple and accurate method based on one organic extraction step with hexane-isopropyl alcohol and quantitation by reversed-phase liquid chromatography with ultraviolet detection. It has been used to determine diclofenac sodium in the plasma of ten volunteers who had taken diclofenac sodium tablets, and provides data on the pharmacokinetics of the drug.

EXPERIMENTAL

Apparatus

The analysis was performed with a high-performance liquid chromatograph consisting of a Waters Model 510 pump, a U6K injector, a 490E programmable multiwavelength detector operated at 274 nm

and 0.015 AUFS and a Baseline 810 Chromatography Workstation (Waters Assoc., Milford, MA, U.S.A.). Chromatographic separations were carried out on a Spherisorb ODS column (200 x 4.6 mm I.D.; particle size 10 μm; Dalian, China) at ambient temperature. A Model ϕ71 pH meter (Beckman Instruments, Fullerton, CA, U.S.A.) with a pencil combination Beckman eletrode was employed for the pH measurement of the different solutions.

Standards and reagents

Diclofenac sodium (99.9%) and Diphenylamine (the internal standard) were supplied by Mingxing Pharmaceutical Factory (Guanzhou, P.R.China) and Nanxian Reagent Factory (Shanhai, P.R.China), respectively. Diclofenac sodium tablets were provided by BEIJING CIBA-GEIGY Pharmaceutical (Beijing, P.R.China). HPLC-grade methanol (Linhai Chemicals Factory, Zhejiang, P.R.China) and sodium acetate (Shanhai Chemical Reagent Factory, Shanhai, P.R.China) were used to prepare the mobile phase. All chemicals, except where otherwise stated, were of analytical grade, and water used in this assay was doubly distilled.

Mobile phase

The mobile phase was a methanol-buffer mixture (68:32, v/v) which had been passed through a 0.45-μm membrane filter (Millipore, Bedford, MA, U.S.A.) and then degassed before use. The buffer was 0.05 M sodium acetate, prepared by dissolving 6.8 g of sodium acetate in 1000 ml of water and the pH adjusted to 4.2 with hydrochloric acid. Mobile phase flow-rate was 1.40 ml/min with a typical back-pressure of 11.5 MPa.

Preparation of solutions

A 1.00 mg/ml diclofenac sodium stock standard solution was prepared by dissolving 100 mg of diclofenac sodium in 100 ml of methanol and storing in a refrigerator. A 1.00 mg/ml diphenylamine stock solution was prepared by dissolving 100 mg of diphenylamine in 100 ml of methanol and storing in a black box in a refrigerator.

Analytical procedure

A 0.5-ml volume of a plasma sample was placed in a test tube, and 0.6 ml of 1 M phosphoric acid added. After vortex mixing for 10s, 5ml hexane-isopropyl alcohol (95:5, v/v, containing 30 ng/ml diphenylamine as an internal standard) were added and vortex mixed for 1 min, then centrifuged for 10 min at 1000 g. 4 ml of the organic layer was collected, evaporated to dryness with air at 40 °C and 150 µl of the HPLC mobile phase added to dissolve the residue. After 30 s of vortex mixing, 25 µl of this sample solution were injected into the HPLC system.

RESULTS

Chromatographic separation

Figure 1 shows typical chromatograms of a standard solution and plasma samples. Under the chromatographic conditions described, diclofenac sodium and diphenylamine (internal standard) had retention times of approximately 4.8 min and 6.4 min, respectively. It can be seen, from Figure 1, good separation and detectability of diclofenac sodium in serum was obtained with minimal interference from serum components. Hence it is relatively easy to estimate the peak area with accuracy.

Precision

Intra-day reproducibility studies, evaluated by assaying 9 plasma samples containing 0.20 µg/ml concentration of diclofenac sodium, yielded a coefficient of variation of 7.4%. Inter-day reproducibility studies, evaluated by assaying the same concentration 7 times over a 7-day period, was 7.6%. At a concentration of 0.80 µg/ml, the intra-day and inter-day coefficient of variation were 3.7% and 2.5 %, respectively.

Linearity and detection limit of method

A series of the solutions containing 0.10, 0.50, 1.00, 1.50, 2.00 and 2.50 µg/ml of diclofenac sodium were prepared to study

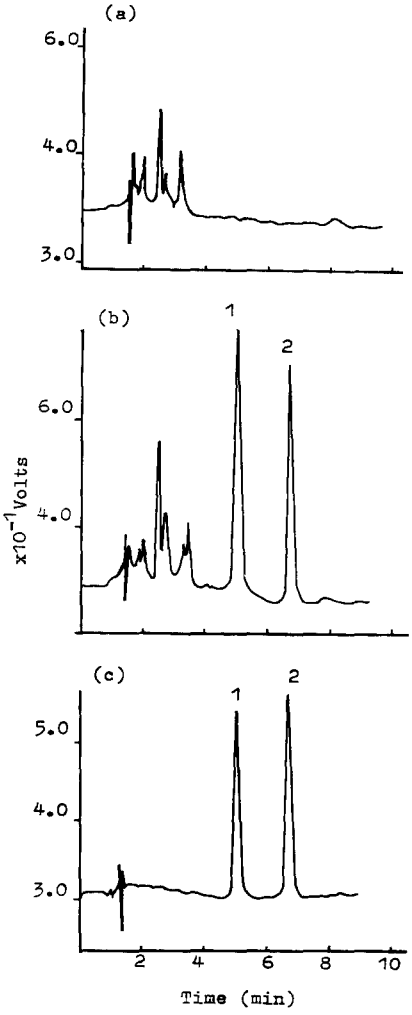

Fig. 1. Chromatograms of diclofenac sodium in human plasma and the standard solution containing 0.80 μg/ml diclofenac sodium.
(a) a plasma extract of a blank plasma.
(b) a plasma extract containing diclofenac sodium (2 h after oral administration of 50 mg of diclofenac sodium).
(c) standard solution.
1, diclofenac sodium; 2, diphenylamine.

the relationship between the peak area ratio of diclofenac sodium to diphenylamine and the concentrations of diclofenac sodium under selected conditions. The results showed that the peak area ratio was linearly related to the diclofenac sodium concentration for the range 0.10 - 2.50 μg/ml. The linear equation for the concentration versus the peak area ratio was Y=0.90X+0.02 with a correlation coefficient of 0.9994. The detection limit was 0.03 μg/ml.

Extraction efficiency

Extraction efficiencies of diclofenac sodium and the internal standard were determined by comparing peak areas of the analytes from extracted plasma standards to those from a chromatographic standard solution prepared in mobile phase at the equivalent concentration and chromatographed directly. Mean (n=7) percent recoveries (S.D.) of diclofenac sodium were 91.3 (10.1) and 93.2 (3.9) for the low (0.20 μg/ml) and high (1.20 μg/ml) concentrations, respectively. The recovery of internal standard (0.20 μg/ml) from plasma was 97.3 (9.1).

Interferences

The interference of other commonly encountered medications on the HPLC chromatogram was studied using aspirinum, chlorprophenpyridamine, ibuprofen, acidum pipemidicum, norfloxacin, ofloxacin, lomefloxacin and ciprofloxacin. No interference was observed on the detection of diclofenac peak.

Application

Ten healthy male Chinese volunteers aged 22.9±s0.7 and weighing 67.7±s4.7 kg entered the study. All volunteers gave their written consent and underwent a physical examination. There were no abnormal findings in liver and kidney functions in particular. After 12 h of overnight fasting, the volunteers received an oral dose of single 75-mg diclofenac sodium, in a randomized crossover study design. Blood samples (2.0 ml) were taken before medication and after 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8 and 10h and the plasma separated by centrifugation and was then frozen. Fig. 2 illus-

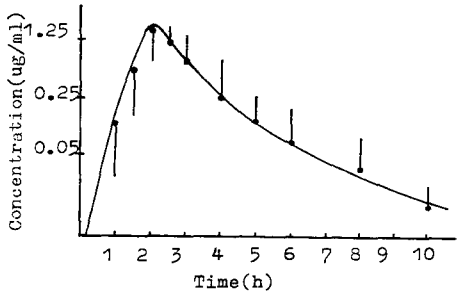

Fig. 2. Mean plasma levels of diclofenac sodium after oral administration of 75-mg of diclofenac sodium to ten human volunteers. n=10, x±s.

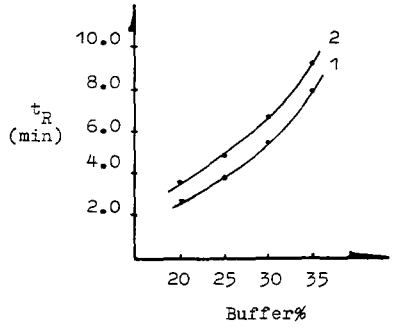

Fig. 3. Dependencies of the retention times of diclofenac sodium and diphenylamine on the composition of the mobile phase. 1, diclofenac sodium; 2, diphenylamine.

trates the plasma concentration versus time profile for diclofenac sodium in the ten volunteers.

DISCUSSION

To achieve optimum resolution, the composition of mobile phase, buffer concentration and pH were all studied systematically. A 200 x 4.6 mm I.D. octadecylsilane column was chosen, with various proportions of methanol in aqueous sodium acetate buffer and different pH values as the mobile phase. At a given buffer pH and composition of mobile phase, neither retention time changed noticeably with varying buffer salt concentration. Fig. 3 shows the dependency of the retention times of diclofenac sodium and diphenylamine on the composition of the mobile phase. Both retention times are reduced with increase in methanol content. On the other hand, the retention time of diclofenac sodium is also reduced with a increase in buffer pH (Fig. 4). However, the retention time of diphenylamine was unaltered. In addition, it has been found in the experiment that no changes in the detector response (peak area) were observed with varying composition of the mobile phase and pH. Thus, the retentions of diclofenac sodium and diphenylamine can be easily controlled within a large appropriate range by adjusting the pH or methanol content of the mobile phase. In this method the concentration of sodium acetate buffer solution was selected as 0.05 M and the pH was adjusted to 4.2 with hydrochloric acid. The ratio of sodium acetate buffer solution and methanol was selected as 32:68 (v/v), delivered at a flow-rate of 1.40 ml/min at ambient temperature.

There are several methods described that dichloromethane (10) and hexane (14) can be used as the organic solvents for the extraction of diclofenac sodium from plasma. However, it has been observed in the experiment that the emulsive phenomenon was too frequent to operate when dichloromethane was used as the extractant. When hexane was selected as the extractant, it was also not completely out of the emulsive phenomenon. However, when the hexane used was mixed with a small amount of isopropyl alcohol the emulsive phenomenon can be completely removed. In the present

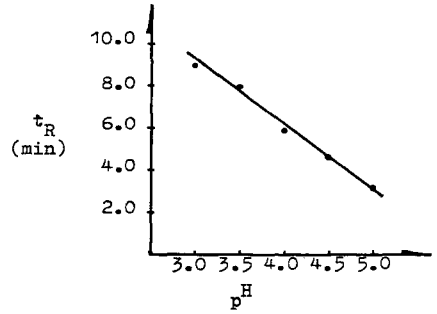

Fig. 4. Dependence of the retention time of diclofenac sodium on pH of the buffer solution.

method, the ratio of hexane and isopropyl alcohol was selected as 95:5 (v/v).

To obtain higher extraction efficiency for diclofenac sodium from plasma, a study was made of the effect of the amount of phosphoric acid added in the plasma on the extraction efficiency. The results indicated that the extraction efficiency is exceedingly low when the organic extractant is used for the extraction alone. However, it is favorable for improving the extraction efficiency to acidify the plasma before the extraction. The results showed that the extraction efficiency increased with increasing the volume of phosphoric acid (1 mol/L) added, but the responses were unchanged when the acid volume added above 0.5 ml. In this work the volume of phosphoric acid was selected as 0.60 ml.

The pharmacokinetics of diclofenac sodium were studied in 10 healthy Chinese volunteers. After single oral administration of 75 mg diclofenac sodium, the data obtained were fitted with PKBP-N1 program (16) on computer. In Table 1 are reported the pharmacokinetic parameters of diclofenac sodium administered orally to ten volunteers. The results showed that the dispostion of diclofenac sodium was conformed to a two-compartment model. Peak plasma drug concentration occur 1.8 h after ingestion and the

TABLE 1. Pharmacokinetic parameters of diclofenac sodium after administering an oral dose of single 75-mg to ten healthy Chinese volunteers

	α (1/h)	β (1/h)	K_a (1/h)	K_{12} (1/h)	K_{21} (1/h)	K_{10} (1/h)	$T_{a1/2}$ (h)	$T_{\alpha\ 1/2}$ (h)	$T_{\beta\ 1/2}$ (h)	$AUC_{0\rightarrow\infty}$ (ug/ml.h)	T_{max} (h)	C_{max} (ug/ml)
1	2.37	0.60	3.07	0.57	1.34	1.06	0.23	0.29	1.16	2.92	2.08	1.39
2	1.91	0.45	2.00	0.34	0.61	1.40	0.35	0.36	1.55	2.66	2.00	1.48
3	2.03	0.34	2.24	0.37	0.45	1.55	0.31	0.34	2.01	3.22	2.06	1.82
4	1.86	0.41	2.13	0.26	0.50	1.51	0.32	0.37	1.70	4.09	1.58	2.26
5	1.31	0.47	1.91	0.21	0.71	0.85	0.36	0.53	1.49	4.39	1.50	1.72
6	1.72	0.25	2.89	0.53	0.43	1.01	0.24	0.40	2.73	8.31	1.86	4.12
7	1.17	0.36	1.40	0.12	0.42	0.99	0.49	0.59	1.94	4.06	2.28	1.42
8	1.90	0.44	3.10	0.35	0.59	1.39	0.22	0.37	1.59	2.95	1.32	1.82
9	2.53	0.51	2.60	0.58	0.77	1.69	0.27	0.27	1.36	2.69	1.50	1.60
10	1.35	0.32	1.70	0.24	0.43	0.99	0.41	0.52	2.17	2.84	1.63	1.04
$\bar{x}$	1.82	0.42	2.30	0.36	0.63	1.24	0.32	0.40	1.77	3.81	1.78	1.87
$\pm$SD	0.44	0.10	0.59	0.16	0.28	0.29	0.09	0.11	0.46	1.70	0.32	0.86

mean peak plasma concentration achieved is 1.87 ug/ml. Moreover, The results implied that diclofenac sodium is absorbed repidly, distributed widely in the body and also eliminated at a fairly rapid rate.

CONCLUSION

The method provided excellent recovery and good precision, and is simple and reliable in both its chromatographic conditions and sample preparation procedure. Furthermore, the analytical procedure is easy to handle and because of the short time between two injections it is very suitable for the routine determination of a large number of sample. It has been successfully applied to the analysis of plasma sample obtained during the pharmacokinetic study of diclofenac sodium in ten healthy volunteers participating in a diclofenac sodium single oral dose clinical trial.

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DETERMINATION OF HYDROXYL RADICAL FORMATION IN THE TESTES OF CADMIUM-TREATED MICE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An application of high performance liquid chromatography (HPLC) with electrochemical detection to investigate the hydroxyl radical production in the testes of cadmium-treated mice is described. Salicylate was used as a free radical trapping agent to trap hydroxyl radicals in vivo in the testes of cadmium-treated mice. Using this HPLC method, the products formed by hydroxyl radical addition to salicylate, 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihyroxybenzoic acid (2,5-DHBA), were separated and quantitated. It was found that the concentrations of both 2,3-DHBA and 2,5-DHBA in the testes of cadmium-treated mice were significantly higher than that without the treatment of cadmium. This study demonstrated that the tissue damage induced by cadmium was the result of the increase in the production of hydroxyl radicals.

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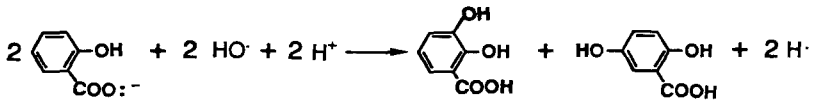
INTRODUCTION

Hydroxyl radicals ($HO^{\cdot}$) and superoxide are two oxygen radicals which have been implicated as possible etiological agents in the development of several disease or pathological conditions such as in aging, [1] arthritis, [2] carcinogenesis, [3] tumor promotion, [4] hyperbaric oxygen toxicity, [5] radiation injury, [6] ischemic injury to heart, brain and other tissues and the toxic action of certain chemicals. [7], [8] Superoxide radicals are not as reactive as hydroxyl radicals in aqueous solution. In comparison, hydroxyl radicals are highly reactive and short lived in both chemical [9], [10] and biological systems, [11] and present at very low concentrations in tissues in normal conditions. Hydroxyl radicals can cause tissue injury through a variety of pathways including damaging DNA, inactivating specific proteins or via lipid peroxidation of cell membrane components, and disrupting the interstitial matrix by degradation of hyaluronic acid and collagen. [12]

Cadmium is a toxic trace metal. Occupational and environmental pollutants are the main sources of cadmium exposure. Cadmium is toxic to virtually every system in the animal body, whether ingested, injected, or inhaled. The toxic effects of exposure to cadmium include anemia, dermatitis, testicular degeneration or atrophy, reduced growth rate, liver and kidney damage, cardiovascular disorders, pulmonary edema and emphysema, teratogenic malformations and increased mortality. [13], [14] Cadmium induced lipid peroxidation has been observed in numerous tissues either in vivo or in vitro. [15], [16] The relationship between cadmium toxicity and $HO^{\cdot}$ is not clear because $HO^{\cdot}$ is difficult to detect and quantitate in vivo. Salicylate is non-toxic at a low concentration and has been used to trap $HO^{\cdot}$ in animals. [12], [17] Two main products

are 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihyroxybenzoic acid (2,5-DHBA) formed by hydroxyl addition to salicylate. [18]



2,3-DHBA 2,5-DHBA (1)

This study was to use salicylate as a radical trapping agent to detect HO· in vivo and provided a direct evidence for pathogenetic role of the hydroxyl radical in the testis toxicity caused by cadmium.

MATERIALS AND METHODS

The HPLC instrumentation was from Waters (Waters, Division of Millipore, Milford, MA, USA), which included a 501 HPLC pump, an U6K injector with a 500 µl sample loop, a 460 electrochemical detector with a glassy carbon working electrode, an auxiliary electrode and a Ag/AgCl reference electrode. Data acquisition and processing was accomplished by a Waters Baseline 810 chromatographic work station which included a NEC PowerMate SX/16 computer and a system interface. The column used was a Hibar RT Lichrosorb-RP-18 (10 µm, 250 x 4 mm) (E. Merck, Gibbstown, NJ, USA). The voltage in the electrochemical detector was set at 0.8 V. The injection volume was 50 µl and the flow rate of the mobile phase was 0.8 ml/min. The mobile phase was 0.03 M citric acid and 0.03 M acetic acid, and prepared by titration with sodium hydroxide to pH 3.0 followed by titration with sodium acetate to a final pH of 3.6.

The statistic analysis of the experimental date of 2,3-DHBA and 2,5-DHBA in testes were carried out using Student "t" test. The results were considered significant at $P < 0.05$ and very significant at $P < 0.01$.

All the reagents were of analytical grade. Cadmium chloride ($CdCl_2$), trichloroacetic acid (TCA), sodium salicylate and its monohydroxylated products, 2,3-DHBA and 2,5-DHBA were obtained from Sigma Chemical Company (St. Louis, MO). Water was from a Millipore Milli-Q system (Millipore, Milford, MA, USA).

The calibration curves of the DHBAs were built with 2,5-DHBA and 2,3-DHBA standards in 10% TCA solution. The concentrations of the two DHBAs in the tissue extracts were determined by comparison of the peak areas of the extracts with that of the calibration curves of 2,5-DHBA and 2,3-DHBA.

Animal Preparation

Male CD-1 mice with body weights of 30-35 g were used in all experiments. They were housed in groups of 5 mice/cage, kept at a constant room temperature and maintained on a controlled environment with a 12 h light : 12 h dark cycle. Food and water were provided *ad libitum*. After one week adaption, the mice were divided into two groups of 5 mice each. Cadmium chloride solution (2 mg/kg) was injected to cadmium treatment group in s.c. once a day for 7 days. Control group was given equal volume of saline.

Sample Preparation

After 7 days cadmium treatment, the mice were given i. p. sodium salicylate solution (100 mg/kg), 30 min prior to killing mice by cervical dislocation. The testes

were removed immediately, weighted and placed in ice-cooled 10% TCA solution (1.5 ml/200 mg tissue). The testes were homogenized in a polytron homogenizer (Polytron model # PT 10/35, Brinkman Instruments, Switzerland) 20 seconds. The homogenate was centrifuged at 2,000 RPM at room temperature for 10 minutes. The supernatants were filtered with a 0.22 μm Millipore filter, and then 50 μl of this solution was injected into the HPLC system.

Recovery Assay

2,5-DHBA and 2,3-DHBA solutions at various concentrations were spiked into the blank tissues and then extracted with the same procedure described above. The concentrations of the DHBAs in the tissue extracts were determined by comparison of the HPLC peak areas of the extracts with that of the calibration curves of 2,5-DHBA and 2,3-DHBA. The recoveries of 2,5-DHBA and 2,3-DHBA were 92.1% and 91.4%.

RESULTS AND DISCUSSION

As shown in Figure 1a, 2,5-DHBA and 2,3-DHBA were not found in the blank extracts of mice testes which were not treated with salicylate and cadmium. Figure 1b shows the chromatogram of the control extract, which was from the mouse given only salicylate (100 mg/kg). Figure 1c shows the chromatogram of the extract of cadmium-treated mouse. The peaks of 2,5-DHBA and 2,3-DHBA in the control testes (Figure 1b) are much smaller than that in the cadmium-treated testes (Figure 1c). Figure 1d shows the chromatogram of the 1 μM standard extract of 2,5-DHBA and 2,3-DHBA, which was obtained by spiking standard of 2,5-DHBA and 2,3-DHBA into a testis tissue and then extracted.

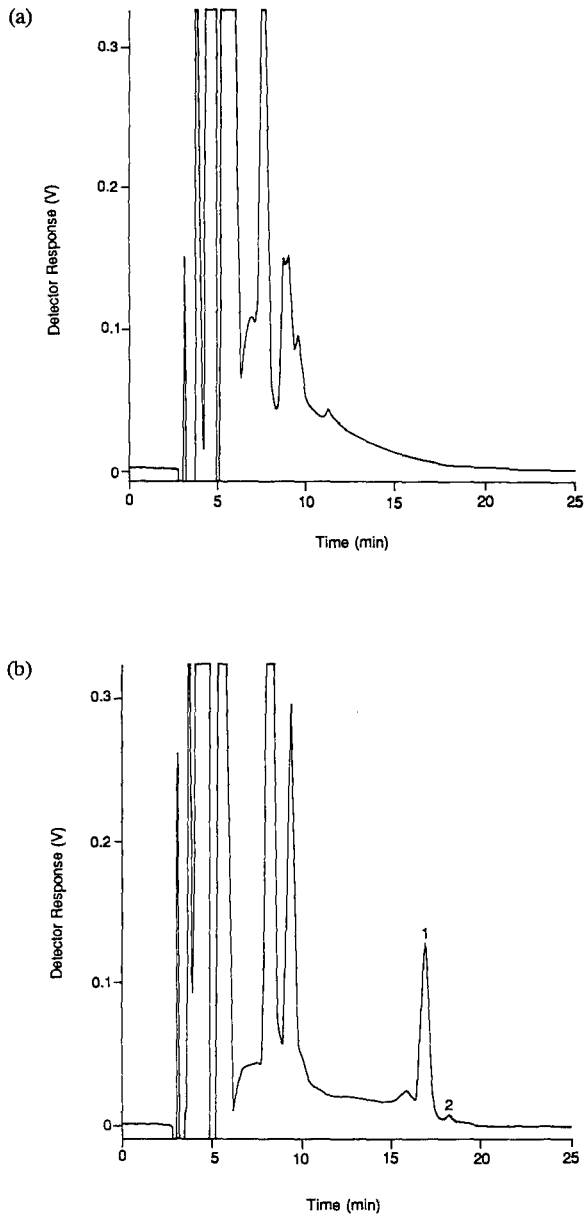
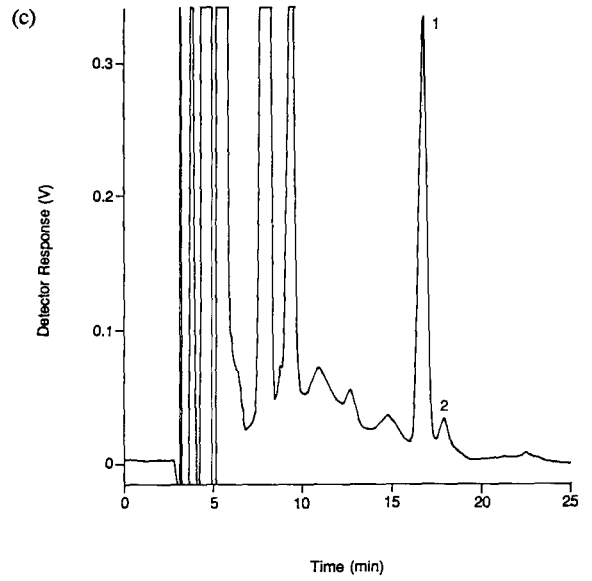


Figure 1 Chromatograms of the testes extracts in mice (a) a blank mouse; (b) a control mouse (treated with salicylate); (c) a salicylate- and cadmium-treated mouse; (d) 1 μM standard of 2,5-DHBA and 2,3-DHBA in testis tissue. Principal peaks, 1: 2,5-DHBA; 2: 2,3-DHBA



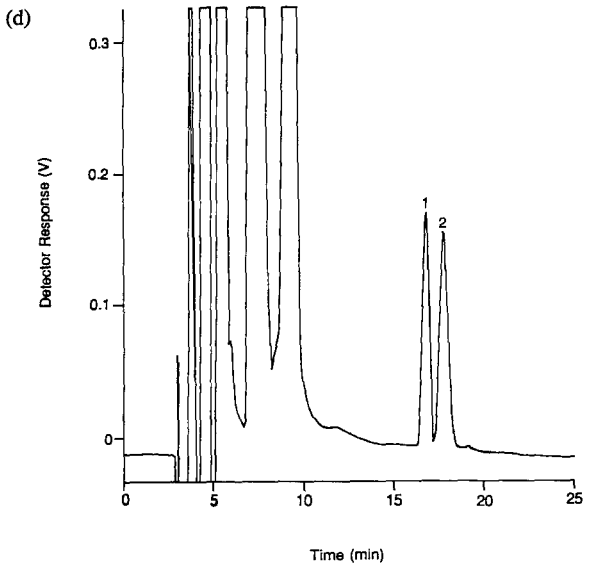


Figure 1 (continued)

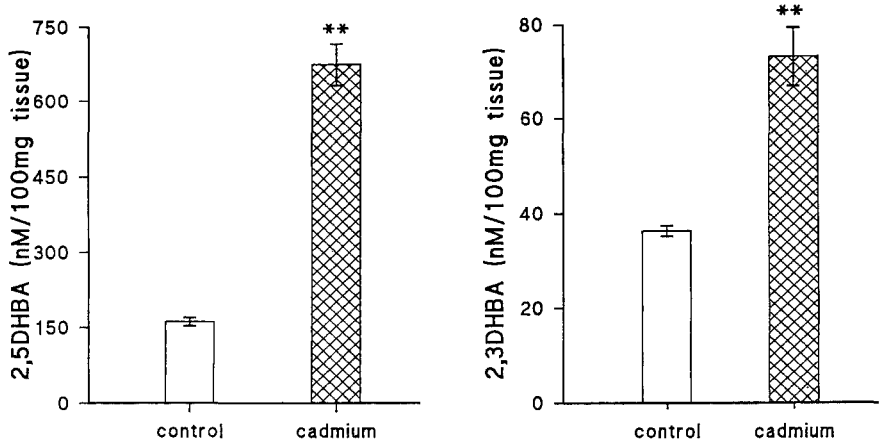


Figure 2 Mean (± SE) level of 2,5-DHBA and 2,3-DHBA in the testes of cadmium-treated mice (n=5) and control mice (n=5).

Figure 2 shows the mean ± SE (SE, standard error) concentrations of 2,3-DHBA and 2,5-DHBA in the testes of the control mice and and cadmium-treated mice. The mean ± SE of 2,3-DHBA and 2,5-DHBA in the control group was 36.34 ± 1.11 and 161.4 ± 68.33 nM/100 mg tissue. The mean ± SE of 2,3-DHBA and 2,5-DHBA in the cadmium treated group were 73.02 ± 6.25 and 671.32 ± 42.12 nM/100 mg tissue. The changes in the concentrations of 2,3-DHBA and 2,5-DHBA in the cadmium treated group in comparison with that in the control were 2.01 and 4.16 times, indicating significant increases.

The experiments were carried out to determine the possibility of further metabolism of the adducts, 2,5-DHBA and 2,3-DHBA, in testes. Testis tissues were spiked with 2,5-DHBA and 2,3-DHBA standard and extracted, and then stored at 4 °C for one week. The concentrations of 2,5-DHBA and 2,3-DHBA in this sample was

TABLE 1 The Level of 2,5-DHBA and 2,3-DHBA in Mouse Testes Homogenates During One Week at 4°C

day	2,5-DHBA Level (nM/100mg tissue)	2,5-DHBA change (%)	2,3-DHBA Level (nM/100mg tissue)	2,3-DHBA change (%)
1	161.46	0.00	36.34	0.00
2	159.97	0.92	35.31	2.83
3	155.67	3.58	32.97	9.27
4	149.50	7.41	32.52	10.51
5	146.51	9.26	31.05	14.56
6	134.56	16.66	30.20	16.90
7	132.32	18.25	29.76	18.11

determined every day by HPLC. As shown in Table 1, there is no significant change in the concentrations of DHBAs in the testes homogenate in 24 hours.

2,5-DHBA was the only product formed In an enzymic salicylate metabolism in vivo. [19] A previous study of the reaction of hydroxyl radicals with salicylate in vitro indicated that both 2,5-DHBA and 2,3-DHBA were formed in almost equal amounts. [19] Our results indicated that the ratio of 2,5-DHBA and 2,3-DHBA was 9.19 and the increases of 2,5-DHBA and 2,3-DHBA were 4 and 2 folds in the testes of cadmium treated mice. This conclusively demonstrates that the testis toxicity caused by cadmium is related to the formation of the hydroxyl radicals. While the detail mechanism of cadmium-mediated hydroxyl radical generation remains to be investigated, one possibility may involve the interaction of cadmium with ferritin and

other iron-containing proteins, resulting in the release of iron. The released iron reacts to form hydroxyl radicals via the Fenton reaction as suggested previously. [20]

Cadmium exerts its toxic effects on testicular vascular endothelium which could lead to ischemia, hypoxia, lipid peroxidation followed by generation of highly reactive hydroxyl radicals in the testicular tissues. [21], [22] The high content of polyunsaturated fatty acids in a testis rendered this organ particularly susceptible to peroxidative damage. It was found that the lipid peroxidation increased in homogenates of the testes after cadmium treatment of male rats. [23] Since thiol agents protect testis toxicity caused by cadmium, the testis damage is apparently oxidative in nature. It is quite possible that cadmium toxicity in testes is mediated by oxygen free radicals.

Salicylate as a specific hydroxyl radical trap is used and it produces stable hydroxylation products. Our results show that there was a 4-fold and 2-fold increase in 2,5-DHBA and 2,3-DHBA, demonstrating direct evidence for the involvement of hydroxyl radicals in cadmium toxicity in vivo. Hydroxyl radicals are extremely reactive with a number of compounds, including lipids and proteins. The presence of $HO^{\cdot}$ in the testes provides an important information for further study of the mechanism of testis toxicity caused by cadmium.

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PRECONCENTRATION, SAMPLE CLEAN-UP, AND HPLC DETERMINATION OF PHENOL AND ITS CHLORO, METHYL, AND NITRO DERIVATIVES IN BIOLOGICAL SAMPLES. A REVIEW

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ABSTRACT

This critical review shows the different high performance liquid chromatography methods proposed for phenol and its chloro, methyl and nitroderivatives determination in biological samples, with special attention to the sample treatment.

INTRODUCTION

Phenol denatures and precipitates cellular proteins, and thus may rapidly cause poisoning [1]. It is metabolized by conjugation to yield phenyl glucuronide and phenyl sulphate [2]. In addition, phenol (and *p*-cresol and 4-ethylphenol) is produced in the gut by microbial degradation of tyrosine [3]. So, for urine samples obtained from healthy, unexposed men, concentration values from 4.5 to 10 ppm were obtained [4]. Acid-labile phenol conjugates are present endogenously in serum at concentrations of about 0.1 μg/mL. Niwa et *al* [5] quantified serum phenol and *p*-cresol in uremic patients on hemodialysis. Concentrations of phenol and *p*-cresol in uremic serum were significantly higher than those in normal serum. Reduction rates of phenol and *p*-cresol by hemodialysis were lower than those of urea and creatinine, suggesting a protein-binding property of phenol and *p*-cresol. Phenol and its derivatives are among the most toxic and widely spread pollutants in industrial

effluent and natural waters. Due to their toxicity, measurement in biological materials (expired air, blood, serum and urine) would be of interest. In addition, they are also metabolites of other toxic compounds (*e.g.* benzene), and their measurement would indicate exposure. An excess of respiratory cancer has been associated with phenol in Finnish woodworkers [6]. Moreover, phenol is a metabolite of benzene. Benzene is considered as a group I carcinogen [7]. It is also a constituent of engine emissions and tobacco smoke. The most commonly used method for biological monitoring of benzene exposure is measurement of phenol in urine. In the body, benzene is metabolized by the microsomal cytochrome P-450 monooxygenase system into benzene epoxide [8]. This benzene epoxide is metabolized in three different pathways which end in excretion of *trans,trans*-muconic acid, phenylmercapturic acid and different phenols. By using enzymes it is possible to differentiate between conjugated (glucuronides and sulphate esters) and unconjugated metabolites. About 30% of retained benzene gives rise to phenol *in vivo*. Therefore, the measurement of excreted urinary phenol has applications in the evaluation of exposure [9], but urinary phenolic compounds are not useful biological markers for exposure to benzene below 1 ppm. No significant increase in phenolic compounds was observed after exposure to benzene (0.01-0.63 ppm) for both smoking and non smoking workers [10].

For cresols, only *p*-cresol is endogenously produced in normal subjects, and may be present in urine at concentrations of 20 to 200 μg/mL (mainly in conjugated form) [1]. *o*- and *m*-cresol are reported to occur in the urine as a result of exposure of an individual to toluene [11] and not as a result of the microbial degradation of tyrosine. Consequently, the occurrence of *o*- and *m*-cresol would be expected to be rare, except in those individuals subjected to toluene exposure. However, only a small fraction of the inhaled vapour is oxidized at the aromatic ring with the production of cresols.

The urinary concentration of *p*-nitrophenol has been proposed for assessing the exposure to nitrobenzene [12], which is used in many industrial processes. Skin is the principal route of exposure to nitrobenzene solution [13].

The respiratory and dermal absorption of chlorophenols results in measurable levels of these compounds in blood and urine of occupationally exposed individuals [14]. Food and drinking water are considered to be the major sources of chlorophenols in serum, urine and adipose tissues of general population. Pentachlorophenol concentration of 10 μg/Kg in food

and of 60 μg/Kg in drinking water have been reported [15]. This can be due to an average daily intake of 1-6 μg pentachlorophenol. A large proportion of pentachlorophenol is excreted in urine, which leads to average levels of 1-6 ppb [16]. The presence of pentachlorophenol in urine has been shown to be a sensitive indicator of human exposure not only to those but also to other organochlorine compounds being metabolized to chlorophenols, *e.g.* hexachlorocyclohexane and chlorobenzenes. Once adsorbed in the body, a significant amount of the chlorophenols may be retained due to the formation of lipophilic conjugates of fatty acids [17]. An accumulation of such conjugates in membranes of tissues and their further metabolism may have deleterious consequences. Selective toxicity of palmitoylpentachlorophenol was found in exocrine pancreas in rats [18]. In urine samples from people working in the wood industry, concentrations of 0.1-10 ppm of pentachlorophenol have been found [19]. About 25% of the pentachlorophenol present in urine is excreted as its glucuronide [20]. Therefore, in order to measure the total amount of pentachlorophenol in urine, hydrolysis is necessary before analysis. Acid hydrolysis has been used, but 70-95% of pentachlorophenol is present in the precipitate formed upon hydrolysis. 2,4-dichlorophenol has been also determined as a common metabolite of the β-blocker B 24/76 [21].

The United States Environmental Protection Agency list eleven substituted phenols, with a variety of substituent such as chloro, methyl and nitro groups, as priority pollutants phenols (PPP) [22], as shown in table I. Analytical procedures are needed to detect and quantify phenolic compounds at very low concentrations. The present study reviews the determination of phenol and its chloro, methyl and nitro derivatives by high performance liquid chromatography (HPLC) in biological samples. This technique is more suitable than gas chromatography for polar analytes with low vapour pressure (as in the case of phenols) in aqueous samples. Our objective is to consider the different HPLC methods proposed in the recent literature (especially referred to the eleven PPP) with attention to all the different kind of biological samples studied, and the preconcentration techniques used. A previous review of 1984 was centered around the determination of priority toxic pollutants in urine, where chlorinated phenols were considered [23]. As it can be seen in figure 1, about 20% of the published works in the last 10 years, as reviewed by Chemical Abstracts, about phenol and its derivatives are directly related with biological samples.

Table 1
The Eleven Priority Pollutants Phenols (PPP) Listed by the United States Environmental Protection Agency.

Phenol
2-chlorophenol
4-chloro-3-methylphenol
2,4-dichlorophenol
2,4-dimethylphenol
2,4-dinitrophenol
2-methyl-4,6-dinitrophenol
2-nitrophenol
4-nitrophenol
Pentachlorophenol
2,4,6-trichlorophenol

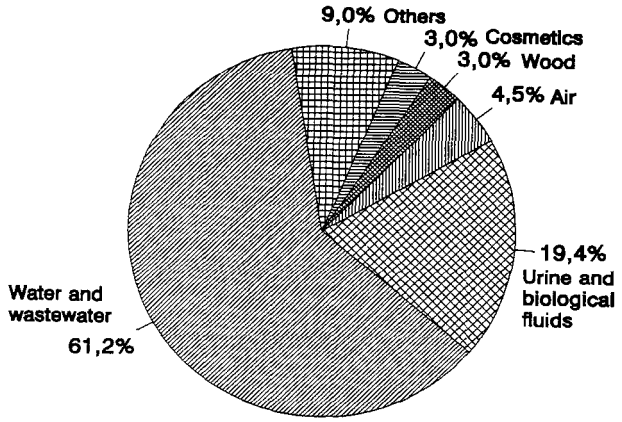

Figure 1 Distribution of the HPLC procedures for the analysis of phenols according to the samples assayed during the last decade.

SAMPLE TREATMENT

Preliminary treatment is often necessary to both separate and preconcentrate the analytes, although direct aqueous sample injection has been also used. Generally, hydrolysis and/or sample clean-up procedures are carried out prior to the chromatographic separation. Direct injection of enzymatic hydrolysed urine samples have been used [24]. After acetonitrile deproteinization, plasma phenol was determined [25]. Diluted urine samples after filtration and hydrolysis were injected [26], and Ogata et *al.* [27] diluted the urine sample and after centrifugation injected the supernatant. Although for phenol determination, enzymatic hydrolysis (Helix Pomatia) was necessary. Automatic HPLC methods, not involving solvent extraction, described [28]. Table 2 summarizes the different methods.

Steam distillation.

The quantitative performance of steam distillation-extraction for different types of organic substances at concentrations ranging from tens of ppm to ppb has been studied in [29]. A theoretical model is introduced, describing the recovery of different classes of organic compounds as a function of the process time. A 100% recovery is obtained within 20 min. for most substances. The effect of concentration, process time and solvent used were also studied. Norwitz et *al.* [30] studied the distillation of 42 phenolics, and the recoveries are related with the hydrogen-bondings (inter and intra molecular) present. As applications, a small steam-distillation scheme for concentrating phenol, *p*-cresol and 4-ethylphenol from faeces and urine samples was described [31]. Recoveries from 99.4 to 101.9% were obtained for the three analytes for added concentrations ranged 0.1-1000 nmol/g wet weight sample. For urine samples it was found that acid hydrolysis did not result in the loss of free phenols, and the recoveries were 100.1-101.7%. Steam distillation has been also used for the determination of phenol and cresols in urine [32]. After enzymic hydrolysis of urine (β-glucuronidase), a 1 mL aliquot was submitted to steam-distillation in a micro-Kjendahl apparatus for phenol and *p*-cresol extraction [33]. Recoveries were 95 to 107%.

Liquid-liquid extraction

The extraction of 25 monohydric alkylphenols with different solvents (*n*-pentane, tetrachlorometane, trichlorometane, dichlorometane, benzene and diethyl ether) from water

Table 2
Analytical Properties of the Different Methods Proposed for the Analysis of Phenols by HPLC

Analyte	Sample	Hydrolysis	Sample clean-up	Stationary phase	Mobile phase	Detector	Detection limit	Notes	Reference
26DCP PCP 235TCP 2345TeCP	Urine	HCl 1 h. 100°C	on-line trace enrichment on a styrene-divinylbenzene polymer	LiChrosorb Si-60 (5μm)	*n*-hexane-toluene-glacial acetic acid (74:25:1)	ECD	1 ppb of PCP	0.4 mL sample Normal phase LC	[41]
PCP 2346TeCP	Urine	HCl 1 h. 100°C	on-line post-column extraction (LiChrosorb RP18)	LiChrosorb RP18 (5 μm) Spherisorb C1 (5μm)	MeOH-1 mM H_3PO_4 (80:20)	UV ECD	40 ppb of PCP (UV) 1 ppb of PCP (ECD)	2.3 mL of sample 2,3,4,6-TeCP not found	[15]
Phenol 4-nitrophenol	Urine	Enzymic (Helix Pomatia Juice) 37°C Overnight(6 h.)	Filtered (0.22 μm)	ODS-2 (5 μm)	MeCN-1% H_3PO_4 10:90 (Screening) 30:70 (simultaneous specific determination of phenol and 4-nitrophenol)	UV (265, 280 nm)		Other compounds assayed	[26]
Phenol	Mice urine	Enzymic (β-glucuronidase and/or arylsulphatase) 37°C 48 h.	Anion exchanger and diethyl ether extraction	Nucleosil ODS (5 μm)	Phosphate (pH = 3.4)-MeOH (70:30)	UV (270 nm)	36 mg/L	Other compounds assayed	[8]
Phenol *p*-cresol 4-ethylphenol	Faeces and urine	H_2SO_4 100°C 30 min.	Steam distillation	RP-8 (5 μm)	H_2O-MeCN-HAc (89:9:1)	Fluorescence λ_{exc} = 275 λ_{ems} = 300	<0.1 pg/μL		[31]

Phenol	Urine	HCl 90°C 90 min.	Diethylether extraction after sodium sulphate addition	Partisphere 5 C18 (5 μm)	10 mM sodium acetate buffer (pH = 3.4 or 3.8)-MeCN Gradient	Fluorescence λ_{exc} = 274 λ_{ems} = 298 UV (280 nm.)	0.3 mg/L	Other compounds assayed	[10]
Phenol *o*-,*m*-,*p*-cresol *p*-nitrophenol	Urine	Enzymic (β-glucuronidase-arylsulphatase) 37°C 12 h.	CH_2Cl_2 extraction and sodium hydroxide back extraction	Pecosphere 3x3 C18 (3 μm)	MeOH-H_2O-H_3PO_4 (30:70:0.1)	UV (215 nm)	0.5 mg/L (phenol and cresols) 1 mg/L (*p*-nitrophenol)	*p*-aminophenol also assayed	[9]
Phenol *p*-cresol	Urine	Enzymic (β-glucuronidase) 37°C 20-24 h.	Steam-distillation	RSil C18LL	MeOH-20mM NaH_2PO_4 (pH=4) (12:13)	UV (285 and 295 nm)		Phenol concentration in some urine samples decreased during storage	[33]
Fatty acid (C_{16} and C_{18}) conjugates of 24DCP, 245 and 246TCP, 2346TeCP and PCP				Reversed phase C18 (5 μm)	MeOH-H_2O (39:1)	UV (210 nm)			[17]
Phenol 4-nitrophenol				LC-SAX (5 μm)	0.05 M ammonium formate buffer (pH=4.5)-MeCN (60:40)	UV (254)	0.9 ng/μL	Glucuronide and sulfate conjugates Anion exchange LC	[2]

at mg/L concentration was compared [34]. The recoveries were obtained relative to *n*-octadecane, being the lowest for phenol and its lower alkyl derivatives. A simple extraction by ethyl acetate was used to measure phenol and *p*-cresol in uremic serum [5]. *p*-nitrophenol was extracted from urine samples with dichloromethane, after hydrolysis with hydrochloric acid [35]. Enzymatic hydrolysis of phenol, cresols and *p*-nitrophenol with β-glucuronidase-arylsulphatase for 12 h. at 37°C was made [9]. After hydrolysis, the analytes were extracted with dichloromethane, and then the organic phase was extracted with 0.2 M sodium hydroxide before injection. The recovery was about 95% for phenol and cresols and 90% for *p*-nitrophenol. Urine samples, after acid hydrolysis, were saturated with sodium sulphate and extracted by diethyl ether. The recovery for phenol was over 90% [10]. n-hexane extraction was used in [14]. The recoveries ranged from 79 (2,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol and 2,3,4,5-tetrachlorophenol) to 87% (2,4-dichlorophenol) when 1 mL of sample was used, and from 72 (pentachlorophenol) to 90% (2,4-dichlorophenol, 2,4,6-trichlorophenol and 2,4,5-trichlorophenol) when 5 mL of sample was used. Ethyl ether was used for determination of pentachlorophenol in human urine [36]. Mice urine hydrolysed with β-D-glucuronidase was extracted with ethyl ether for unconjugated metabolites of benzene, including phenol [38].

Solid-liquid extraction

A global study of some chemically modified resins for phenolic extraction has been made in [37, 39]. C_{18} reversed phase extraction used Sep-Pak C_{18} cartridges [14]. The recoveries ranged from 59 (2,3,4,5-tetrachlorophenol) to 89% (pentachlorophenol) when 1 mL of sample was used, and from 69 (2,4,6-trichlorophenol) to 96% (pentachlorophenol) when 5 mL of sample was used. Pentachlorophenol in urine was extracted in a Baker-1 C18 extraction column and eluted with the mobile phase used in the HPLC separation. The recovery was 89 to 96% [40]. 4 mL of the hydrolysed mice urine (with β-glucuronidase and/or arylsulphatase) was purified and separated by solid-phase extraction with an anion exchanger, followed by extraction with diethyl ether [8].

Column switching

On-line post column liquid extraction was used, before electron-capture detection [15]. After hydrolysis of urine samples, the preconcentration was made. Recovery for

pentachlorophenol at 10 ppb level was 70% and at 100 ppb level was 75%. For 2,3,4,6-tetrachlorophenol, the recovery was 85%. Same authors, after hydrolysis of the urine, made on-line trace enrichment of 100 ppb of 2,6-dichlorophenol, and 10 ppb of 2,3,5-trichlorophenol, 2,3,4,5-tetrachlorophenol and pentachlorophenol using a pre-column packed with a styrene-divinylbenzene copolymer. For the preconcentration of 0.4 mL of sample, recoveries of the chlorophenols at the 10 ppb levels varied from 60 to 80% [41].

DETECTION

Figure 2 summarized the detectors usually employed in the HPLC phenol determination. When biological samples are studied, electrochemistry detectors are used less frequently.

UV detector is the most employed because of its universality. Phenol and chlorinated phenols show absorbance maxima at 220 and 250/320 nm. Direct determination of phenol and chlorophenols is possible if the preconcentration step leads to a higher concentration level. Nitrophenols can be detected in the visible spectra (405 nm.). PCP was determined by UV detection (254 and 313 nm.), with detection limit of 0.4 mg/L [36]. Absorbance at 270nm. was used for determination of benzene metabolites in urine of mice. The detection limit for phenol was 36 mg/L [8]. A wavelength of 210 nm. was used for monitoring fatty acid conjugates of chlorinated phenols [17], and of 215 nm. was used for the determination of phenol, *o*-, *m*- and *p*-cresol, *p*-aminophenol and *p*-nitrophenol in urine [9]. The detections limit were 0.5 mg/l for phenol and cresols and 1 mg/L for the others. 265 and 280 nm. were used for determination of urinary metabolites of benzene, nitrobenzene, toluene, xylene and styrene [26]. 254 nm. has been used with a detection limit of 0.25 mg/L for pentachlorophenol [40], and for phenol determination [38]. 225 nm. for phenol determination [27], with a detection limit of 0.4 ng/μL. 285 nm. for phenol and 295 nm. for *p*-cresol in [33].

The fluorescence detector provides better detection limits than UV detector (table 2). Only phenol and cresols show native fluorescence. Dansyl chloride is used to convert phenols into fluorescent dansyl derivatives. A complete study of a two-phase dansylation procedure for phenolic compounds is described in [42], but we have not found any work which

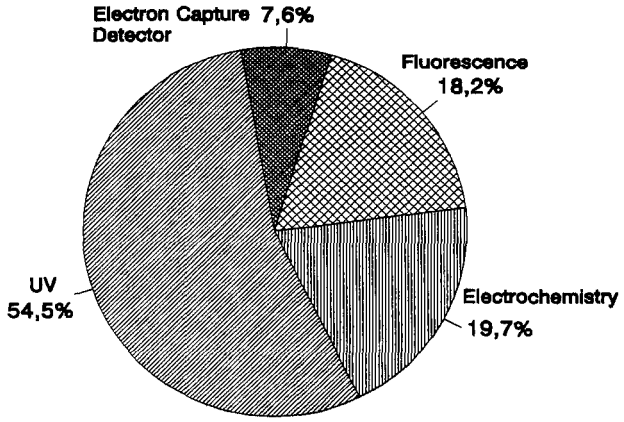

Figure 2 Distribution of the HPLC procedures for the analysis of phenols according to the detection system used during the last decade.

describes an application to biological samples. Fluorescence detection for identification of phenol and *p*-cresol was used [5]. A λ_{exc} = 274 nm. and λ_{ems} = 298 nm. were used, with a detection limit for phenol of 0.3 mg/L [10], and λ_{exc} = 275 nm. and λ_{ems} = 300 nm. for monitoring phenol, *p*-cresol and 4-ethylphenol. The sensibility was of 10 pg. (in 100 μL) [31].

The only described work with electrochemistry detector in biological samples showed electrochemistry detector 30 times more sensitive than UV detection for phenol determination in plasma [25].

Experimental modifications have to be made in order to use electron capture detector (ECD) in liquid chromatography. On-line post-column extraction in column liquid chromatography with a coupled ECD is proposed [15]. Detection limit of 1 ppb for PCP in urine was achieved, when 40 ppb was the detection limit with UV detection. On-line trace enrichment on a reversed phase pre-column for normal phase LC with ECD is described in [41], for low ppb determination of chlorophenols in urine. On-line electron-capture detection was used for determination of ppb levels of PCP in liver [43].

The mass spectrometry has been only used for confirmating phenol and *p*-cresol [5]. The radiochemistry detector has been used for quantification of radiolabelled benzene

metabolites in liver tissue extract or urine of rats exposed to ^{3}H-benzene in air (47.6 ppm) for 6 hours. UV absorbance at 265 nm. was also registered [44].

CONCLUSIONS

A review has been made of the procedures proposed for determination of phenols in biological fluids. Hydrolysis and sample clean-up procedures, and also preconcentration, are generally required for successful determination (table 2). Three types of analytical columns are proposed: normal phase, reversed phase and anion exchange, the second type being most popular. The UV detector is the most used. However, it does not always provide good sensibility, and then other kind of detectors have been tested, as fluorescence and electron capture detectors. The major number of papers referred to urine samples, as can be seen in table 2, where ppb detection limits has been found.

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MEASUREMENT OF NIMODIPINE METABOLISM IN RAT LIVER MICROSOMES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An isocratic, rapid, sensitive and selective reversed phase high-performance liquid chromatographic (HPLC) assay with ultraviolet detection has been used to quantify the in vitro nimodipine metabolism in rat liver microsomes. (±)Nimodipine and its metabolites were separated on a C18 HPLC column maintained at 40°C using a mobile phase consisting of ammonium acetate 0.05M pH 6.6 and methanol (40:60 v/v). The within- and between-run coefficients of variation (CVs) were < 4.2 % in the concentration range of 0.5-50 μM. The limit of detection for nimodipine and ist metabolites was in the range of 30 - 80 nM. The formation of nimodipine metabolites may be described by a sigmoid Vmax model according to Hill equation corresponding to enzyme kinetics associated with positive allosteric effect. The assay was accurate, selective and may be used in studies investigating the interaction of drugs, and substances found in daily food e.g. flavonoids, with nimodipine metabolism.

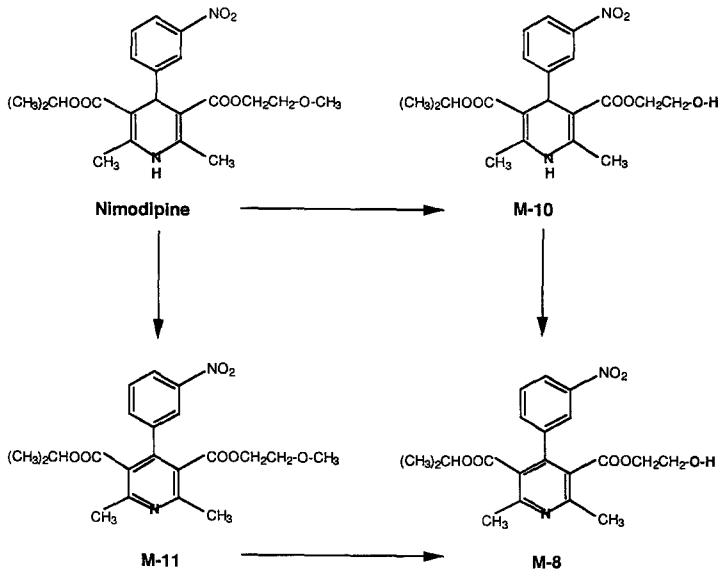

FIGURE 1. Metabolism of nimodipine.

INTRODUCTION

Nimodipine [Bay e 9736, (±)3-isopropyl 5-(2-methoxyethyl) 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-pyridine-3,5-dicarboxylate] is a potent calcium antagonist of the class of dihydropyridines (1). It has been shown in man that nimodipine may dilate the cerebral arterioles and increase cerebral blood flow. The drug is effective in the treatment of a range of cerebrosvascular disorders. However, the actual interest in the drug focuses on its use in the prevention and treatment of the delayed ischaemic neurological deficits that frequently occur in patients with subarachnoidal haemorrhages resulting from sustained cerebral vasospasm (2). In mammals, the biotransformation of nimodipine is complex (1,3). Similar to other dihydropyridine derivatives, nimodipine is primarily biotransformed by the microsomal oxidase system in the liver by dehydrogenation of the 1,4-dihydropyridine nucleus, which leads to the formation of metabolite M11, and/or by O-demethylation, ester-cleavage producing metabolites M10, M9, respectively (1- 6). The metabolites M10 and M11 may be further biotransformed to the common metabolite M8 (Fig.1) by ring- oxidation and side chain-demethylation, respectively.

When nimodipine is administered to rats or humans the metabolites M8, M10 and M11 are detectable in the plasma. The areas under the plasma concentration curves (AUC) of M8

and M10 are higher than that of M11 (4-6). Assuming that the clearance and subsequent reaction rates of these metabolites are similar, these data suggest that the O-demethylation of nimodipine is an important pathway for the elimination of the drug. In man, it has been well documented that the nucleus oxidation of dihydropyridine drugs is mainly catalyzed by a specific enzyme cytochrome P-450 3A (CYP3A) (7). In the rat, this oxidation is similar to that in man but the participation of cytochromes UT-A (cyp 2C11) and PCN-E (cyp 3A) was also reported (8). In contrast to the nucleus dehydrogenation, little attention was given to the oxidative cleavage of side-chain carboxylic esters and of ethers bound to the dihydropyridine ring. The identity and number of CYP(s) involved in these reactions are still unknown (7, 9). In order to investigate the metabolism of nimodipine in detail a rapid and selective analytical assay is needed. A number of chromatographic assays have already been developed to measure nimodipine and its metabolites in human and animal body fluids (1-6, 9-11). In these assays, a clean-up step, e.g. liquid-liquid extraction with diethyl ether-hexane (1:1) or ethyl acetate, was necessary. This manipulation is time consuming and requires the use of ambre glass ware to prevent the light catalyzed dehydrogenation of the the dihydropyridine nucleus. A one step analytical assay with direct injection would be an advantage. Therefore, we have developed a HPLC method with direct injection to investigate nimodipine biotransformation in *in vitro*.

MATERIALS AND METHODS

Reference Compounds

Reference substances (±)nimodipine (Bay e 9376) and its metabolites M8 (Bay o 1762), M10 (Bay m 5397) and M11(Bay m 8922) were supplied by Bayer AG (Wuppertal, F.R.Germany).

Chemicals

Methanol (E. Merck, Darmstadt, F.R.G.) was HPLC gradient grade and ammonium acetate solution 5 M (Fluka, Buchs, Switzerland) was analytical grade. Other chemicals such as acetone, D,L-isocitric acid trisodium salt dihydrate and magnesium chloride were of the highest commercially available purity (E. Merck, Darmstadt, F.R.G.). Isocitrate dehydrogenase from pig heart and NADP disodium salt were purchased from Boehringer (Mannheim, F.R.G.). Naringenin was obtained from Roth GmbH (Karlsruhe, F.R.G.), α-naphthoflavone and quinidine base 97% from Aldrich Chemie (Buchs, Switzerland), (±) verapamil hydrochloride,

troleandomycine, quinine and erythromycine from Sigma Chemie (Buchs, Switzerland), midazolam and its metabolites α-hydroxy- and 4-hydroxy-midazolam from Hoffman-La Roche (Basel, Switzerland) and cyclosporine A from Sandoz (Basel, Switzerland). Rat liver microsomes were prepared from male Sprague Dawley rats according to a method described previously (12). The microsomal protein concentration was assayed by a modification of Lowry's method (13).

Standard Samples

For daily calibration of chromatography 10 µmol of each reference substance were dissolved in 1 ml of acetone. The stock solution was diluted with bidistilled water to the desired concentrations. The content of the organic solvent was 50% (v/v) in the final solutions. Standard samples were then treated in the same manner as the unknown samples.

Instrument Parameters

A HPLC system (Waters 625, Milford, MA, USA) consisting of a solvent delivery pump model 625, a photo diode array detector model Waters 991 set at 218 and 238 nm, an autosampler model WISP 700 and a column oven operated at 40°C were used. Signals were processed and recorded by a data management system (Waters software version 6.22, Milford, MA, USA). Nimodipine and its related compounds were separated on a Spherisorb S3 ODS II (100 x 4 i.d.mm) column (Knauer AG, Berlin, F.R.G.).

Mobile Phase

The mobile phase was a mixture of 400 ml of diluted ammonium acetate solution (0.05 M, pH 6.6) and 600 ml of methanol and saturated with helium for 10 min at a flow rate of 100 ml/min before use. For the routine analysis a flow rate of 0.5 ml through the column was maintained at a pressure of 85-95 bar.

Stability Test

The stability of nimodipine (1.0 - 50 µM) and its metabolites (0.5 - 20 µM) in phosphate buffer 0.1M pH 7.4 was assayed in 1.5 ml polypropylene test tubes (Eppendorf, Vaudaux Co., Schönbuch, Switzerland) by exposing the aqueous solutions to the laboratory light for 0, 2, 4, 8, 16 and 32 hours at room temperature. A further test was performed by exposing the

spiked samples containing nimodipine without microsomal protein in ambre polypropylene test tubes (Eppendorf, Vaudaux Co., Schönbuch, Switzerland) at 37°C to the laboratory light for 0-120 minutes.

Recovery Test

For recovery experiments, 10 µmol of each standard were dissolved in 1 ml of methanol. The stock solution was diluted with water to the desired concentrations. The content of the organic solvent was 50% (v/v) in the final solutions. The analytical recoveries of three nimodipine concentrations (0.5, 5.0, 50 µM) and three metabolites concentrations (0.5, 2.5, 5.0 µM) were determined in three differents occasions by comparing the peak heights of spiked and protein precipitated samples with those of corresponding amounts dissolved in methanol:bidistilled water (1:1).

Selectivity and Limit of Detection Test

In order to investigate the possible interference by other drugs, each test compound was dissolved in phosphate buffer and 2 nmol were injected separately onto the column. The signals were recorded for 1 hour. The limit of detection was determined by diluting a sample of 1 µM nimodipine and its metabolites with phosphate buffer. The diluted samples were then treated and analyzed in the same manner as the unknown samples. The detection limit of the assay was defined as a signal-base line ratio 3:1.

In vitro Nimodipine Metabolism Assay

In a 1.5 ml amber polypropylene test tube (Eppendorf, 2000 Hamburg 65, F.R.G.), nimodipine (concentration range 1.0 - 50 µM) was added as a 25-fold concentrated solution in 50% acetone:bidistilled water and incubated in 0.1 M sodium phosphate buffer, pH 7.4, in the presence of a NADPH generating system (0.5 IU isocitrate dehydrogenase, 1 mM NADP, 5 mM sodium isocitrate and 5 mM magnesium chloride). After a preincubation of 2 minutes at 37°C, reactions were started by the addition of microsomal protein previously kept in ice. The final volume was 0.5 ml. After incubating at 37°C for 5 minutes under an air atmosphere, reactions were stopped by the addition of 0.5 ml of ice-cold methanol, followed by vortexing for 15 seconds and placing in ice. After a brief centrifugation at 10'000 x g for 5 minutes (0 °C), 100 µl of the upper phase were placed into a conical vial, and a volume of 40 µl was injected onto the HPLC column. The reproducibility of the assay was investigated 7 times on the same day by using the same rat liver microsomes and 15 µM substrate.

In order to investigate the formation kinetics of the metabolite M8 nimodipine was replaced in the metabolism reaction by M10 and M11 in two separated experiments. The other conditions were unchanged.

Data Analysis

The calibration graphs of nimodipine (0.2 - 50 μM) and its metabolites (0.2 - 20 μM) were constructed daily using triplicates of 6 different concentrations. The concentration of substances in the biotransformation reaction was quantified by comparing the peak-heights of the corresponding signals with those of the calibration graph. The metabolite concentrations were then transformed to reaction rates by taking into account the incubation time and the amount of microsomal protein. Reaction rates against corresponding substrate concentrations were fitted by nonlinear regression and using the allosteric Hill equation $V = V_{max} * C^N / (C^N_{50} + C^N)$ (14), where V is the reaction rate at the substrate concentration C, V_{max} is the maximal velocity, C_{50} is the substrate concentration at half V_{max} and N is the parameter describing the sigmoidicity of the curve. The apparent constants C_{50}, N and V_{max} were directly estimated by using the Profit software package (QuantumSoft, 8023 Zurich, Switzerland). Results were reported as mean ± standard deviation (SD) of at least three experiments performed in duplicate.

RESULTS

The typical HPLC chromatograms for reference substances dissolved in mobile phase (A) and for *in vitro* nimodipine metabolism reactions before (B) and after (C) incubation are shown in Fig.2. The retention times of M8, M10, M11 and nimodipine were 6.8, 5.9, 14.2 and 11.8 minutes, repectively. Their corresponding retention capacity factors (k') were 3.9 , 3.3, 9.1 and 7.4, respectively. In comparison with Fig. 2A, the metabolites M10 and M8 produced by *in vitro* reactions (Fig.2C) were not separated with base line resolution. The spectrum analysis revealed that the signal corresponding to M10 was pure and that of M8 was contaminated by unknown substance(s). The purity of the M8 signal was 89 ± 6% (n=5). In the metabolism assay investigating the formation of M8 from M10 the respective signals were separated with base line resolution.

In the routine analysis, samples were injected every 16 minutes and in a run of 20 hours no interfering signals, that would hamper nimodipine metabolites quantification were observed. Calibration curves were linear in the concentration range 0.2-50 μM of nimodipine and 0.2-20 μM of its metabolites. The correlation coefficients were > 0.999. Least squares linear regression analysis of the standard calibration plots resulted in the following equations:

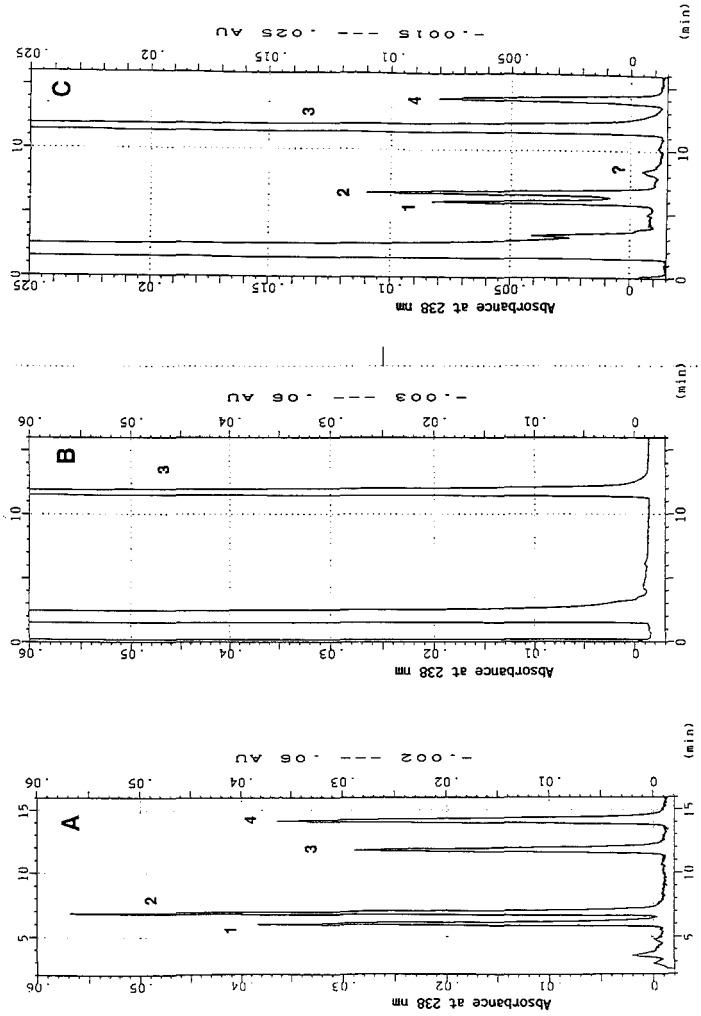

FIGURE 2. Separation of nimodipine and its metabolites by HPLC technique. (A) reference substances; (B) before incubation; (C) after incubation. Peak identification: (1) M10, (2) M8, (3) nimodipine; (4) M11; (?) unknown product.

TABLE 1

Precision and Accuracy of the Analysis of Nimodipine and its Metabolites.

		Within-day assay (n=3)			Between-day assay (n=5)		
compound	added (μM)	found (μM)	CV (%)	bias (%)	found (μM)	CV (%)	bias (%)
M8	0.65	0.64	3.77	-1.54	0.68	2.07	4.85
	1.62	1.62	2.50	0.10	1.72	1.92	6.13
	3.23	3.08	2.22	-4.64	3.25	2.64	0.73
M10	0.61	0.64	3.89	4.92	0.63	2.80	3.21
	1.53	1.53	2.77	0.00	1.56	2.45	2.22
	3.05	3.06	2.18	0.29	3.14	2.23	2.91
M11	0.59	0.58	3.78	-1.69	0.59	3.45	0.23
	1.48	1.55	2.99	4.66	1.46	2.54	-1.49
	2.97	2.92	2.31	-1.74	3.01	1.86	1.50
Nimod.	0.59	0.63	4.12	6.66	0.61	3.80	3.60
	1.48	1.58	3.66	6.76	1.47	3.21	-0.97
	2.95	2.83	2.71	-1.55	3.02	2.71	2.23
	14.8	14.5	2.21	-1.55	15.5	2.11	4.89
	44.4	44.6	1.97	0.49	45.3	2.35	2.09

Note: a) CV(%) = SD*100/added. b) bias(%) = (found – added)*100/added

M8: $y = 5.361\ x - 0.004$; M10: $y = 9.736\ x + 0.002$; M11: $y = 3.958\ x + 0.009$; Nimodipine: $y = 8.322\ x - 0.077$. The intercept with the y-axis was very close to zero. The within-day precision of the assay was determined for each metabolite by analyzing several spiked samples with 3-5 different concentrations 3 times a day, and reproducibility was evaluated by measuring the same samples on 5 different days. Data in Table 1 show that the between-day precision was somewhat better than that of within-day, this may be due to the too small number of repetition (n) used in the within-day investigation. Generally, for drug concentrations ranging from 0.6 to 40 μM the between-day and within-day coefficients of variation (CVs) of this assay were less than 4.2 %.

The absolute recovery of nimodipine and its metabolites was 97 ± 2 % (n=12). It was independent of the nature of compounds and their concentrations ranged between 0.5 and 50 μM. By injection of 40 μl of the incubation solution onto the HPLC column the limit of detection was 60, 30, 80 and 40 nM for M8, M10, M11 and nimodipine, respectively.

Unlike to other dihydropyridine derivatives, nimodipine and its metabolites are relatively stable for usual laboratory manipulations. Incubated in ambre polypropylene test tubes and in phosphate buffer 0.1 M, pH 7.4, nimodipine and its metabolites were stable for at least 120 min at 37° C. Therefore the metabolism reactions could be carried out in this device and under the laboratory light.

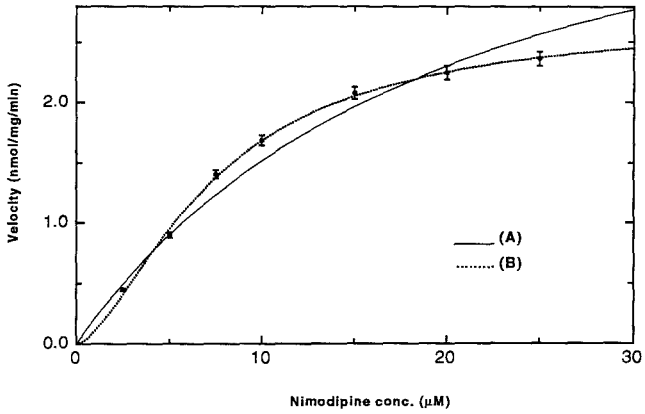

FIGURE 3. Substrate dependent enzyme kinetics of M11 was simulated by Michaelis-Menten (A) and Hill equation (B)

Under the described conditions rat liver microsomes metabolized nimodipine mainly by O-demethylation and by nucleus dehydrogenation leading to the formation of metabolites M8, M10, M11. During the first 15 minutes the concentration of metabolites in the incubation was in the range of nmole/ml and less than 1% of the total nimodipine was metabolized. The concentration of metabolites in the *in vitro* assay is ranked M11 > M8 > M10. The initial rate of the metabolites formation was linear with microsomal protein in the range of 100 - 700 μg per assay and for up to 6 minutes (M11) and 15 minutes (M8 and M10). Therefore 300 μg of rat microsomal protein and 5 min. incubation time were used in all experiments. No nimodipine metabolites were detected when one of the components, such as microsomal protein, substrate or NADP were omitted from the incubation.

The substrate dependent kinetics of nimodipine metabolites formation mediated by rat liver microsomes were studied in the substrate concentration range between 1.0 and 50 μM. The formation kinetics of the primary metabolites M10, M11 may be better described by a sigmoid V_{max} model equivalent to the Hill equation, $V = V_{max} * C^N / (C^{N_{50}} + C^N)$ (14), rather than by the classic Michaelis-Menten model (Fig. 3). Data analysis of different rat liver microsomes preparations revealed that the apparent C_{50} and V_{max} were in the range 9.1-37.1 μM and 0.5-2.6 nmol/min/mg protein, respectively. The value of N varied from 1.0 to 1.5. The formation of the secondary metabolite M8 from M10 and M11 obeyed Michaelis-Menten enzyme kinetics. The apparent kinetic parameters Km (Vmax) were 9.34 μM (1.03 nmol/mg/min) and 21.4 μM (1.83 nmol/mg/min) for M10 and M11, respectively. The enzyme kinetic parameters of primary

TABLE 2

Enzyme Kinetic Parameters of Nimodipine Metabolism in Rat Liver Microsomes .

Rat	M10		M11	
	C_{50}(N)	V_{max}	C_{50}(N)	V_{max}
A	9.13(1.48)	0.51	18.90(1.19)	1.72
B	9.75(1.24)	0.59	16.63(1.24)	1.23
C	19.80(0.96)	0.94	37.08(1.04)	2.61

Note: C_{50} (μM) V_{max} (nmol/mg/min) N (sigmoidicity)

TABLE 3

Specificity of Nimodipine Assay.

Compound	Retention time (min)
M8	6.8
M10	5.9
M11	14.2
Nimodipine	11.8
Cyclosporine A	----(*)
Erythromycine	----(*)
Midazolam	12.5
α-OH-Midazolam	6.8
4-OH-Midazolam	5.5
α-Naphthoflavone	----(*)
Naringenin	----(*)
Quinidine	12.0
Quinine	14.5
Troleandomycine	----(*)
Verapamil	13.0

Note: (*) no signal was detected after 60 minutes.

metabolites M10 and M11 investigated with three different rat liver microsomes samples are summarized in Table 2. Reproducibility of the reaction was investigated 7 times by using the same rat liver microsomes sample and 15 μM substrate. Under our standard conditions the precision of Michaelis-Menten parameters for M8, M10 and M11 formation reaction was between 3.3% and 4.4 %.

The assay is specific for the measurement of nimodipine metabolites in drug interaction studies. In fact, compounds such as cyclosporine A, erythromycine, midazolam, α-naphthoflavone, naringenin, quinidin, quinine, troleandomycine and (±)verapamil added to the nimodipine metabolism reactions did not interfere with the detection of metabolites M8, M10 and M11. However, the midazolam metabolites α-OH-midazolam and 4-OH-midazolam have shown chromatogaphical interferences with M8 and M10, respectively. Data are summarized in Table 3 .

DISCUSSION

The present HPLC assay is suitable for *in vitro* metabolism studies of nimodipine. Any inexpensive HPLC system can be used. However, the use of a photo diode array (PDA) detector set to detect simultaneously at two wavelengths has some advantages over the variable single wavelength detector, because the absorption maxima of dihydropyridine derivatives are at 238 nm, whereas their pyridine oxydative forms absorb maximally at 218nm. Thus, the assay allows to quantify nimodipine and its metabolites more accurately and in parallel. Another advantage is that the purity of the signals (e.g. M8) can be assessed. In our method the nature of the substance(s) which interfered with the signal of M8 was not clear. However in the metabolism assay investigating the formation of M8 from M10 these signals were resolved with base line separation (data not shown). It is therefore possible that other nimodipine metabolite(s) e.g. M9, are formed and coeluated with M8. The use of peak height for the quantitation may already minimize the error of the M8 concentration. Moreover, by using a short column filled with 3 μm ODS material and a low flow rate, we have further improved the detection limit of nimodipine and its metabolites. The major advantage of the present method is that the assay does not require a clean-up procedure. After protein precipitation by methanol, followed by a brief centrifugation, the sample is injected directly onto the HPLC column by an autosampler. The assay shows a stable baseline, allowing the overnight analysis of a large number of samples. However, in order to maintain good stability in routine analysis, the analytical column must be washed after about 80 injections (by back flush).

The present assay has been successfully used in the investigation of nimodipine metabolism in rat liver microsomes. Under the described conditions, nimodipine is biotransformed rapidly to metabolites M8, M10 and M11. The substrate dependent kinetics of

nimodipine metabolites formation may be described by enzyme kinetics with positive allosteric effect (14). In this model, the K_m of Michaelis-Menten kinetics is replaced by C_{50} and N may indicate the number of substrate molecules binding to isoenzyme(s) involved in the reaction. When N=1, the equation returns to that of Michaelis-Menten enzyme kinetics. In our data, N varies from 1.0 to 1.5 indicating that nimodipine may bind to an allosteric site and then potentiate the activity of CYP isoform(s) involved in the nimodipine metabolism. The apparent constant C_{50} was in the range of 9.1-37.1 μM and the V_{max} was between 0.5 and 2.6 nmol/min/mg protein. The formation of the secondary metabolite M8 from the primary metabolites M10 and M11 was also rapid. In a detailed kinetic analysis it was found that M10 competitively inhibits the formation of M8 from M11 and vice versa.

In conclusion, we developed a reliable, sensitive and technically simple method for the *in vitro* determination of nimodipine and its metabolites. The present assay does not only allow to investigate nimodipine metabolism, but also to study the interference of drugs or substances found in daily food, e.g. flavonoids, known as enzyme inhibitors in the biotransformation of dihydropyridine drugs (15), as well.

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AN ISOCRATIC HPLC-ECD ASSAY OF URINARY NORMETANEPHRINE AND METANEPHRINE

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ABSTRACT

This report describes a procedure to quantify simultaneously urinary normetanephrine (NMTN) and metanephrine (MTN) using an isocratic HPLC-ECD methodology. An aliquot of urine (5ml) was adjusted to pH of 1.0, spiked with a known concentration of internal standard, (3-methoxy-4-hydroxy benzylamine, MHBA) and the two metabolites were hydrolyzed in a boiling water bath. The metabolites were adsorbed on a Biorex-70 column and eluted with ammonium hydroxide. Final extraction was carried out in a mixture of ethyl acetate and acetone(2:1, v/v). After drying the extract under nitrogen, it was dissolved in mobile phase, filtered through 0.2μ filters, and injected into a 4 μ Nova-Pak, C_{18} column of the HPLC system. Mobil phase for elution contained citric acid, sodium acetate, EDTA-Na_2, sod. octyl sulfate, dibutylamine, methanol 2% and isopropanol 2%. Peaks were detected by the electrochemical detector at a potential of + 0.55V and characterized using the retention times obtained from HPLC profiles of the standards. Calibration of HPLC was performed by spiking 5 ml of metabolite free urine (MFU) with known amount of standards of NMTN, MTN and MHBA and injecting the extract to obtain chromatographic profile. Concentrations of the metabolites were calculated on a pre-programmed data module using ratio of the areas of the analytes to that of the internal standard. This assay showed a linear relationship between 5-80 ng/ml for NMTN and 5-90 ng/ml MTN. Sensitivity of the method was below 5.0 ng/ml. The total elution time was 15 min. Six to eight urine samples could be extracted and assayed in one day.

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INTRODUCTION

It is now well recognized that in order to investigate the total body catecholamine excretion, urinary catecholamines and their metabolites should be assayed. Metanephrine, normetanephrine and 3-methoxytyramine are the basic metabolites of catecholamines: epinephrine, norepinephrine and dopamine, respectively. These metabolites along with other metabolites of catecholamines, viz., vanillylmandelic acid (VMA) and 3- methoxy- 4-hydroxyphenylglycol (MHPG) account for over 90% of the total catecholamine metabolites (1,2) and are excreted in urine. Determination of urinary metanephrines gives the best measure of both the central and peripheral metabolism of catecholamines and has been helpful in the diagnosis of pheochromocytoma, neuroblastoma and other diseases of neural crest origin (3,4,5). More recently, determination of urinary metanephrine and normetanephrine has also been used in the diagnosis of hypertension (6,7), myocardial infarction (8), and muscular dystrophy (9) since these disease states bring about various changes in urinary excretion of catecholamines and their metabolites. For example, studies show that the mean excretion rate of normetanephrine by hypertensive individuals is greater (upto 600 μg/24 hrs) than that of the normal persons (upto 300μg/24 hrs) (10). In addition, urinary metanephrines and normetaneprine determinations may be valuable in differentiating patients with affective disorders (11,12). To detect relatively small changes in the concentration of these metabolites it is essential to quantify reliably the normal range of urinary metanephrine and normetanephrine concentrations. The spectrophotometric procedures used earlier to determine urinary metanephrines were not sufficiently specific or sensitive to quantify samples having low levels of metanephrine concentrations. Methods involving gas chromatography-mass spectrometry and mass fragmentography (13,14,10) are highly specific and sensitive but involve very expensive equipment and are time consuming. Assay methods involving high performance liquid chromatography equipped with the electrochemical detector (HPLC-ECD) have become available and provide speed and accuracy in the determination of very small

quantities of these compounds present in biological fluids. In our earlier publications, we described HPLC-ECD techniques to assay urinary norepinephrine, epinephrine, dopamine, MHPG and VMA (15,16). We describe here a highly sensitive and specific isocratic HPLC-ECD assay procedure for the determination of nanogram quantities of metanephrines in urine.

MATERIAL AND METHODS

Chemicals and reagents

Normetanephrine hydrochloride and metanephrine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). 3-methoxy-4-hydroxybenzylamine and sodium octylsulfate were obtained from Aldrich Chemical Co. (Milwaukee, WI). Biorex-70 ion exchange columns were purchased from the Biorad Laboratories (Richmond, CA). Sodium acetate, citric acid, dibutylamine, ethylenediamine tetracetate (NA_2EDTA) were obtained from Eastman Kodak Co, (Rochester, NY). All other chemicals were of HPLC grade and were obtained locally.

HPLC Equipment

The liquid chromatography (HPLC) system (Water's Associate, Milford MA) consisted of an injector (Model U6K), solvent delivery system (Model 590) and an electrochemical detector (Model 460), equipped with a glassy carbon working and auxiliary electrodes and silver-silver chloride reference electrode. Water's data module 740 was used to integrate the peaks and calculate concentrations of metabolites in samples on the basis of prior calibration with known amount of standards. The chromatography column 4μ Novapak, C_{18} reversed phase, 3.5 cm x 150 mm (Water's Associate) was used.

Standard Solutions

Stock solutions (1 mg/ml) of normetanephrine (NMTN), metanephrine (MTN) and 3-methoxy 4-hydroxybenzylamine (MHBA) were prepared in HPLC grade water. Stock solution of each standard was divided into 1-2 ml aliquots and kept frozen at -80°C. Frozen standards

were thawed, and diluted with water just before use to prepare aqueous working standard mixture containing in 20 µl 0.4 ng NMTN, 0.8 ng MTN, and 1.2 ng internal standard, MHBA. Internal standard MHBA was added to urine samples before hydrolysis.

Urine Collection

Urine samples either 24 hours or spot collections were obtained and acidified to maintain pH< 2.0 with 1N HCL.

Metanephrine-free urine

In order to obtain a metanephrine-free urine (MFU), a pooled sample of urine was adjusted to pH 10.6 (metanephrines are unstable at alkaline pH) and exposed to air and light for at least 5 days for degradation of metabolites. The pH was then adjusted to 6.5 and the sample was centrifuged at 15,000 rpm for 10 minutes. From the supernate an aliquot (5 ml) was taken and used for extraction and carried through all the steps given below for hydrolysis and extraction of metanephrines from urine samples. The final extract when injected showed a HPLC profile without any detectable peak of either NMTN or MTN. The pool supernate obtained after centrifugation of MFU was then divided into 5ml aliquots and stored at -80°C and used whenever required for calibration. Another 5 ml aliquot of MFU was then spiked with known concentrations of standards for calibration of the instrument and treated similar to the samples.

Mobile Phase

Elution of metanephrines from the HPLC column was achieved using a mobil phase of the following composition: Sodium acetate 0.05M, citric acid 0.05M, sodium octyl sulfate 0.5mM, Na_2EDTA 0.075M, dibutylamine 0.5mM, methanol 2% and isopropanol 2%. The solution was adjusted to pH 3.5, filtered through 0.45 µm filter and degassed by sonication for 30 seconds at room temperature before use.

Extraction Procedure

Metanephrines are excreted in urine in conjugated form either as glucuronides or sulfates. They must be acid hydrolysed before they can be adsorbed on the resin column used to remove interfering substances present in urine. To hydrolyse the conjugated compounds in urine a 5ml aliquot of urine was placed in a screw cap tube, internal standard solution 50µl containing 600 ng of MHBA was added and pH adjusted to 1.0 with concentrated hydrochloric acid. The tubes were placed in a boiling water bath for 30 minutes and then were cooled to room temperature. After centrifugation at 4000 rpm for 5 minutes the pH was adjusted to 6.5 and hydrolysed urine samples were diluted with 15 ml phosphate buffer, pH 6.5, run through cation exchange, Biorex-70, columns and allowed to drain completely. Columns were washed twice with 5ml distilled water. Catecholamines were eluted with 5 ml of 4% boric acid. After the columns ceased to drip the eluates they were washed again twice with 5ml distilled water. The columns were then placed over a screw-cap tube containing 2.5g NaCl. Metanephrines were eluted with 5ml of 4N ammoniuim hydroxide into the screw cap tube. The eluate was extracted twice with 4 ml of a mixture of ethylacetate: acetone (2:1 v/v). Extraction was performed by mixing on a shaker for 10 minutes. After centrifugation, the organic layer from both of the extractions was pooled and 4ml aliquot was evaporated to dryness at 40^0C under a stream of nitrogen gas as described by Shoup and Kissinger (17). The residue was reconstituted with mobile phase (200µl), vortex mixed and allowed to stand for 30 minutes. The extract was filtered through 0.2µ filter (Whatman filters) attached to a syringe and diluted 1:10 with mobile phase. Injections of 20µl of the diluted extract were applied to the HPLC system.

Calibration of HPLC with MFU spiked with standards

Calibration of HPLC was performed by spiking a 5 ml aliquot of MFU. Frozen MFU was thawed and 50 µl of a mixture containing 200ng NMTN, 400ng MTN, and 600ng MHBA

was added. Hydrolysis and extraction procedures were followed as described above for the urine samples. The extracted residue was dissolved in 200 µl mobil phase, filtered through the syringe filter, diluted 1:10 and 20µl aliquot was injected to obtain discrete peaks for calibration. The data module was programmed for calibration using the known concentrations of NMTN, MTN and MHBA and the area of their peaks. Another 20µl injection of the same extract was used to ascertain stability and reproducibility of retention times. Based on calibration, calculation of metabolite concentrations (ng/ml) in urine samples were performed by ratio method, where area of peak of internal standard (MHBA) to that of sample is used to calculate the concentration of analytes in the sample.

Extracts from urine samples were then injected and based on the previous calibration, calculations were made by the data module using the ratio of the integrated areas of peaks of individual analytes to that of internal standard, MHBA of known concentration.

Chromatographic analysis were validated and linearity curves were prepared with a series of aqueous standards of concentrations ranging from 5ng-100ng/ml of both NMTN and MTN.

RESULTS AND DISCUSSION

Typical elution profiles of NMTN, MTN and MHBA are shown in the chromatograms presented in Fig. 1 (A,B, C and D). The retention times of NMTN, MTN and MHBA were found to be 8.319, 10.655 and 16.557 minutes respectively (Fig. 1A). An aliquot of MFU was taken through the whole assay procedure. The extract so obtained was diluted, and 20µl of this extract was injected into the HPLC.

The method described in this paper includes important modifications for improving the sensiyivity as well as specificity over the procedures described earlier (17,18,19,20). In our procedure we used 4µ Novapak C_{18} column and a diffetent mobile phase for the separation of NMTN, MTN and internal standard MHBA than that used by Shoup and Kissinger (17) and

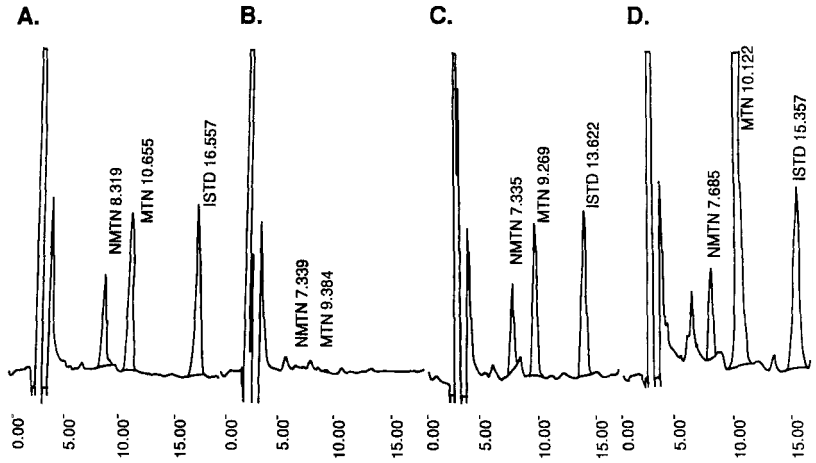

Fig 1: HPLC chromatograms showing peaks with their respective retention times for NMTN, MTN and internal standard. MHBA aqueous standards of known concentrations (A) NMTN and MTN free urine (MFU) extract (B), MFU spiked with known concentrations of standards (C), profile of extract from a urine sample (D).

by Orsulak et al (18). The quality of chromatogrphic profiles in Fig. 1A-1D show well separated peaks and good resolution of analytes of interest. The peak of MTN is separated from NMTN by two minutes and MHBA is separated from MTN by approximately four minutes. Although the total time of resolution and order of appearance of peaks of NMTN, MTN and MHBA are similar to the one obtained by Radial-pak C_8 cartridge used by Orsulak (18) our chromatograms show a clear separation of analytes due to increased differences in retention times. Furthermore, there were no other discrete peaks between NMTN and MTN and no other peaks such as tyramine appeared after the internal standard.

The chromatographic profile of MFU extract shows (Fig. 1B) essentially the baseline with some noise and no identifiable peaks of either NMTN or MTN or any other interfering substances. Fig 1C shows the profile of MFU spiked with known concentration of NMTN, MTN and MHBA. A chromatographic profile of the extract obtained from an aliquot of a

spot collection of a urine sample and taken through the entire extraction procedure is shown in Fig. 1D. The characterstic peaks for NMTN, MTN and MHBA obtained from this extract had retention times in the same range as obtained with those for aqueous standards given in Fig. 1A.

Standard curves for NMTN and MTN were generated and linearity was established by spiking a wide range of concentrations of NMTN and MTN (5-100 ng/ml) and a constant concentration (60 ng/ml) of MHBA to a 5 ml aliquot of MFU. After the assay procedure involving isolation of metanephrines by ion exchange columns and extraction with a mixture of ethylacetate-acetone the diluted extract was injected and calibration curves were obtained by plotting the ratios of the areas of NMTN and MTN to that of MHBA against the corresponding concentrations of each analyte. As shown in plot (fig. 2) the detector sensitivity for NMTN and MTN was identical at lower concentrations (5-20 ng/ml) whereas with increasing concentration (20-80 ng/ml) the detector sensitivity for MTN was higher than that for NMTN. However, the curves for NMTN and MTN plateaued at a concentration of 80 and 90 ng/ml respectively.

The data from the linearity of standards suggest that in cases where levels of these metabolites are expected to be high in urine, such as in some disease states, the starting volume of urine should be reduced, or, alternatively, the final extract should be diluted more before injection into HPLC to maintain the detector sensitivity within the linear range of detectable concentration (5-80 ng/ml).

HPLC analysis of all diluted extracts was carried out at a potential set at +.55V and sensitivity at 0.2 nAFs with a flow rate of mobile phase maintained at 0.5 ml/minute. This potential was found to be optimum after investigating the responses of both NMTN and MTN at different potentials (+ 0.45 to ± .65, data not given). The pH of mobile phase ranging between 3.35 to 5.0 was tested to evaluate the optimal pH for eluting the analytes. The most

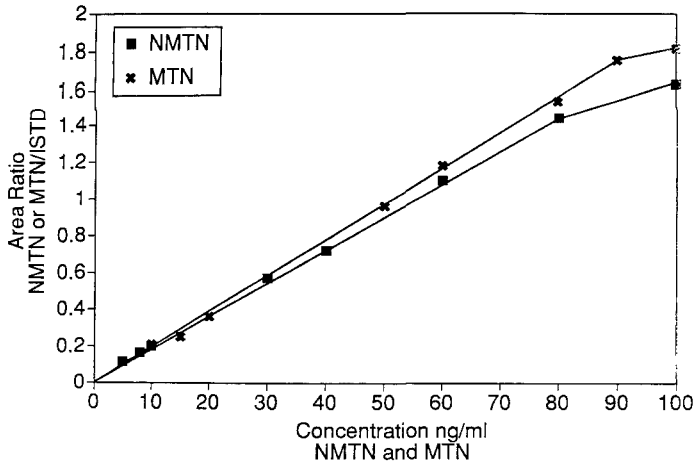

Fig 2: Standard curve of NMTN and MTN showing a linear relationship between various concentrations of NMTN (5- 80 ng/ml) and MTN (5- 90 ng/ml) and the ratio of the areas of peaks of NMTN and MTN to that of internal standard (MHBA).

effective pH was 3.5, which was maintained throughout the analysis. Isopropanol 2% and methanol 2% in mobile phase were found to be a good combination to obtain sharp and well separated peaks of analytes with stable retention times. The use of a detergent, sodium octylsulfate 0.5 mM, provided further stability and high efficiency in separating the peaks, and maintained the order of retention time of the compounds being eluted such as the peak of MHBA always appearing after those of NMTN and MTN. Before injections of aqueous standards or extracts of samples the mobile phase was allowed to flow for at least 4-6 hours to equilibrate the column and to stabilize the detector response. Occasionally a new lot of mobile phase was pumped overnight and recirculated but was discarded when samples were analysed.

Addition of 2.5g NaCl to the aqueous solution before extraction is an important step for increasing the recovery since it aids in the "salting out" of the aqueous phase, and in the

solute transfer to organic phase. Using ethylacetate in combination with acetone (2:1, v/v) enhances extraction due to polarization of ethylacetate by acetone. Recoveries were also optimized by the solvent volume used for extraction: thust two aliquots of 4 ml of solvent mixture, compared to one 8ml aliquot was found to be better for good recovery of various concentration of compounds added for standard curve, apparently due to a better distribution of analytes in the solvent mixture. Addition of internal standard to samples before extraction normalized the recoveries and improved precision and provided a convenient and consistent method for calculating the results in the data module. Any variability due to transient changes in column performance, in the detector sensitivity because of changes in electrical impulses, or temperature variation is also reduced, since both the internal standard and the analytes will undergo parallel changes at the same time.

Within run (intraassay) chromatographic determinations (N=7) for normetanephrine 20 ng/ml and metanephrine (40 ng/ml) gave a coefficient of variation of 7.23 and 4.72% respectively. When aliquots (N=8) of the same sample were extracted separately (interassay), a coefficient of variation for NMTN (20 ng/ml) and MTN (40 ng/ml) was 8.8 and 6.7%, respectively. Analysis of spot collected urine from normal subjects with no dietary restrictions gave values of NMTN 11.9±.64 and MTN 253.9±2.12 ng/ml urine.

One of the salient features of this method is the improvement in sensitivity for detecting the analytes of interest. The sensitivity of the present method is higher than described in earlier methods by Shoup and Kissinger (17) and by Orsulak et al. (18), about several hundred fold higher than that reported by Dutrieu et al (19) and Flood et al (20). This increase in sensitivity was achieved by using a different HPLC column (4μ Novapak C_{18}), and a new mobile phase for eluting the analytes. Another salient feature of this method is that the concentration of metanephrines were measured by the internal calibration technique using MFU devised in our laboratory. This method of calibration and standardization, where MFU

is spiked with known concentration of standards offers a simplified way of calculating the results as compared to the standard addition technique used in earlier studies (18) where urine containing metanephrines is further spiked with standard and the values are calculted by difference. An aliquot of MFU was always tested before spiking with standards. A sample of MFU was discarded if chromatogram showed any peaks, since these peaks can interfere in the calculation of the final results. The procedure described provides simultaneous analysis of normetanephrine and metanephrine in a single chromatogram run in 15 minutes. As many as six to eight samples can be extracted concurrently and be analysed chromatographically in one day. Samples containing as low as 10 ng/ml and as high as 80 ng/ml of both NMTN and MTN can be measured by this method. This increased sensitivity may be helpful in distinguishing normal subjects from those with more subtle derangements in catecholamine metabolism.

ACKNOWLEDGEMENT

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IN VITRO STUDIES OF THE OXIDATIVE METABOLISM OF L-737,415, A C5-CYCLOALKYLAMINE-1,4-BENZODIAZEPIN-2-ONE CCK_B RECEPTOR ANTAGONIST

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ABSTRACT

In an effort to investigate and characterise the metabolism of the C5-cycloalkylamine-1,4-benzodiazepin-2-one CCK_B receptor antagonist L-737,415 a study was undertaken using rat liver microsomes. A procedure is described in which the metabolism of the unlabelled compound is firstly investigated analytically under a controlled set of conditions, then scaled up to allow further metabolite elucidation. We also report the use of HPLC with diode array detection, and thermospray LC-MS and LC-MS-MS, to detect and characterise the observed metabolites. From the UV spectra obtained with diode array detection and fragmentation analysis from LC-MS-MS it is demonstrated that, contrary to other known benzodiazepinones such as diazepam or the CCK_B antagonist L-365,260, metabolism seems to occur predominantly at the C5 substituent. Further work in which urine from rats dosed with L-737,415 was analysed indicated that the *in vitro* microsomal assay provides a good model for *in vivo* metabolism for this class of compounds.

INTRODUCTION

Cholecystokinin (CCK) is a 33 amino acid polypetide hormone which occurs in numerous molecular forms throughout both the central and peripheral nervous systems. CCK exerts a variety of actions on peripheral organs, such as regulating pancreatic secretion and gut motility, and may also function as a neurotransmitter or neuromodulator in the CNS [1,2]. The actions of CCK are mediated by two receptor subtypes designated CCK_A and CCK_B [3], with the majority of the central receptors being of the CCK_B subtype.

A number of non-peptidic CCK_B receptor antagonists have been reported. Structures based upon the natural product asperlicin [4] have given rise to a series of 1,4-benzodiazepin-2-ones, including MK-329 and L-365,260 (Figure 1)[5-7]. Further modifications of this latter structure to increase affinity, selectivity and solubility have led to a series of modifications at the C5 position of the benzodiazepine in which the phenyl ring of L-365,260 has been replaced by a cycloalkylamine to generate an amidine. The homopiperidine L-737,415 (Figure 2) is one example from this series showing an improved profile [8].

Typical benzodiazepines, such as diazepam, are known to undergo a number of biotransformations of which N1 demethylation, C3 oxidations and C5-phenyl oxidations are characteristic [9]. Additionally, investigation of the metabolic fate of L-365,260 has demonstrated that in the dog two hydroxylated metabolites were present, namely the 5-(3-hydroxyphenyl) and the N'-(3-methyl-4-hydroxy-phenyl) analogues identified by NMR [10].

EXPERIMENTAL

Materials

L-737,415 was synthesised in-house as previously described [8] with identity and purity confirmed by NMR, MS, HPLC and elemental analysis. Acetonitrile, ammonium acetate and methyl t-butyl ether were of HPLC grade, obtained from Fisons (Loughborough, UK). NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (Poole, UK). TRIS, EDTA, potassium chloride and potassium phosphate were all of Analar

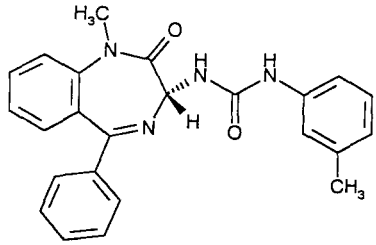

Figure 1. Structure of L-365,260.

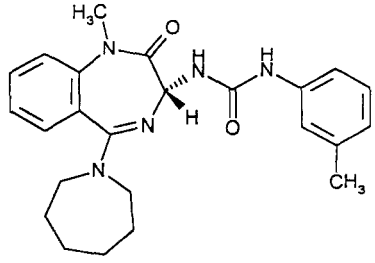

Figure 2. Structure of L-737,415.

grade from BDH (Poole, UK). (+)-Sucrose was from Janssen Chimica sourced through Cambio (Cambridge, UK). Water was of Millipore MilliQ grade and all solvents were filtered using a glass Millipore system with a 0.45μm filter. Male Sprague-Dawley rats (200-250g) were purchased from Bantin and Kingman (Hull, UK).

Instrumentation

An HP1090M series high performance liquid chromatograph (Hewlett Packard, Avondale, USA) was used for HPLC. The system comprised an autoinjector, consisting of a Rheodyne 7010 injection valve fitted with a 250 μl loop, an

autosampler and a DR-5 solvent delivery system. Detection was by UV at 230nm with a bandwidth of 10nm using a built-in linear photodiode array detector saving peak-controlled spectra and data was processed using a 79994A PASCAL workstation. The column was a Spherisorb S5P (250 x 4.6mm i.d.) from Phase Separations Ltd (Deeside, UK) with a mobile phase of 32% MeCN in 100mM NH_4OAc at pH 6.8 and a flow rate of 1.0ml min^{-1}. Analyses were performed at ambient temperature.

Mass spectrometry was performed by interfacing an HP1090L high performance liquid chromatograph (Hewlett Packard, Avondale, USA) to a VG Quattro triple stage mass spectrometer (VG Biotech, Manchester, UK) through a thermospray interface. HPLC mobile phase conditions were modified to 50% MeCN in 100mM NH_4OAc at pH 6.8 and a flow rate of 0.8ml min^{-1}. The source temperature was 250°C and the ion repeller 120V. The capillary was maintained at 240°C with a collision energy of 200V and air as the collision gas in the collision hexapole.

L-737,415 administration

Three male Sprague-Dawley rats (200-250g) were dosed i.v. at 1mg/kg with L-737,415 dissolved in 10mM HCl at 3mg/ml. Animals were allowed water *ad libitum* in a metabowl and urine was collected from t = 0 to 4 hours.

Preparation of microsomes

Whole livers, each weighing approximately 10g, were obtained from freshly euthanised male Sprague-Dawley rats. The livers were minced with scissors and homogenized in 25ml of cold 50mM TRIS containing 1.15% potassium chloride, pH 7.4, using a Potter-Elvehjem teflon pestle homogeniser. The homogenate was centrifuged at 40,000g at 4°C for 20 min and the supernatant portion filtered through cheesecloth and then re-centrifuged at 150,000g at 4°C for 60 min. The supernatant was discarded and the microsomal pellet suspended in 25ml of cold 10mM EDTA containing 1.15% potassium chloride, pH 7.4, and then re-centrifuged at 150,000g at 4°C for 60 min. The supernatant was discarded and the final microsomal pellet resuspended in 10ml of cold 10mM potassium phosphate buffer containing 250mM sucrose, pH 7.4.

Microsomal Incubations

Microsomal incubations were conducted at pH 7.4 under aerobic conditions at 37°C. Initially 100µl of a 250µM dimethylsulphoxide solution of L-737,415 (i.e. 10µg) was incubated with 100µl of microsomes (ca. 2mg protein) in the presence of a NADPH generating system consisting of 100µl of 50mM glucose-6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, 100µl of 10mM NADP and 100µl of 50mM magnesium chloride. The mixture was made up to a final volume of 1ml with 100mM potassium phosphate buffer, pH 7.4, giving a final concentration for L-737,415 of 25µM.

To produce sufficient quantities of metabolites for structural identification the incubation was scaled up with 13mg of L-737,415 being incubated with 8ml of microsomes in a total volume of 100ml. The a final concentration for L-737,415 was 310µM and the concentrations of the other reagents the same as in the initial incubation.

Sample preparation

Samples from the initial incubation were prepared for HPLC analysis by taking a 100µl aliquot at the desired time and quenching metabolism by the addition of an equal volume of acetonitrile. Samples taken at t=0, 30, 60 and 240 mins and samples were stored at -20°C prior to analysis by HPLC. Vortex mixing and centrifugation of this sample separated the denatured protein from the supernatant. The supernatant could then be directly injected onto the HPLC system. Parent compound and metabolites were extracted from both the scaled-up microsome incubation and the urine using liquid-liquid extraction. Typically, 5ml of sample was taken and basified by addition of 100µl 0.1M NaOH, then extracted by vortex mixing with 10ml MTBE. After centrifugation the organic layer was removed and evaporated under a stream of dry nitrogen. Samples were reconstituted in mobile phase and injected directly.

RESULTS

HPLC analysis of parent compound in the initial incubation showed L-737,415 to be rapidly metabolised under oxidative microsomal conditions with 25µM

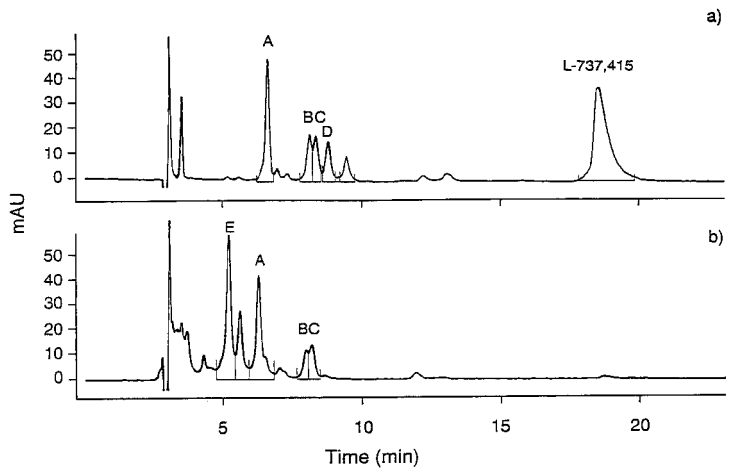

Figure 3. HPLC analysis of L-737,415 metabolites: (a) microsomal metabolites; (b) urinary metabolites. For conditions see text.

substrate. After 60 min, only 10% of the parent compound remained unmetabolised and at 240 min no parent compound was detected.

In order to obtain sufficient material for LC-MS and LC-MS-MS the experiment was scaled-up as described above. At this higher concentration the proportion of parent compound metabolised was lower, but significant concentrations of metabolites were present after 4 hours. Samples were initially analysed using HPLC with diode array detection. Four main groups of peaks were identified from the chromatograms as being potential metabolites and were designated A,B,C and D (Figure 3a). By saving peak controlled spectra using the diode-array detector, we were able to obtain some spectral information on these peaks. The UV spectrum of L-737,415 is characterised by having a λ_{max} at 238nm and an extended UV absorption to above 310nm (Figure 4a). The UV spectra of metabolites A, B, C and D (Figure 4b) are essentially identical to that of L-737,415. This indicates that metabolism is not affecting the chromophore of the molecule, hence aromatic oxidation is unlikely to be a significant metabolic pathway.

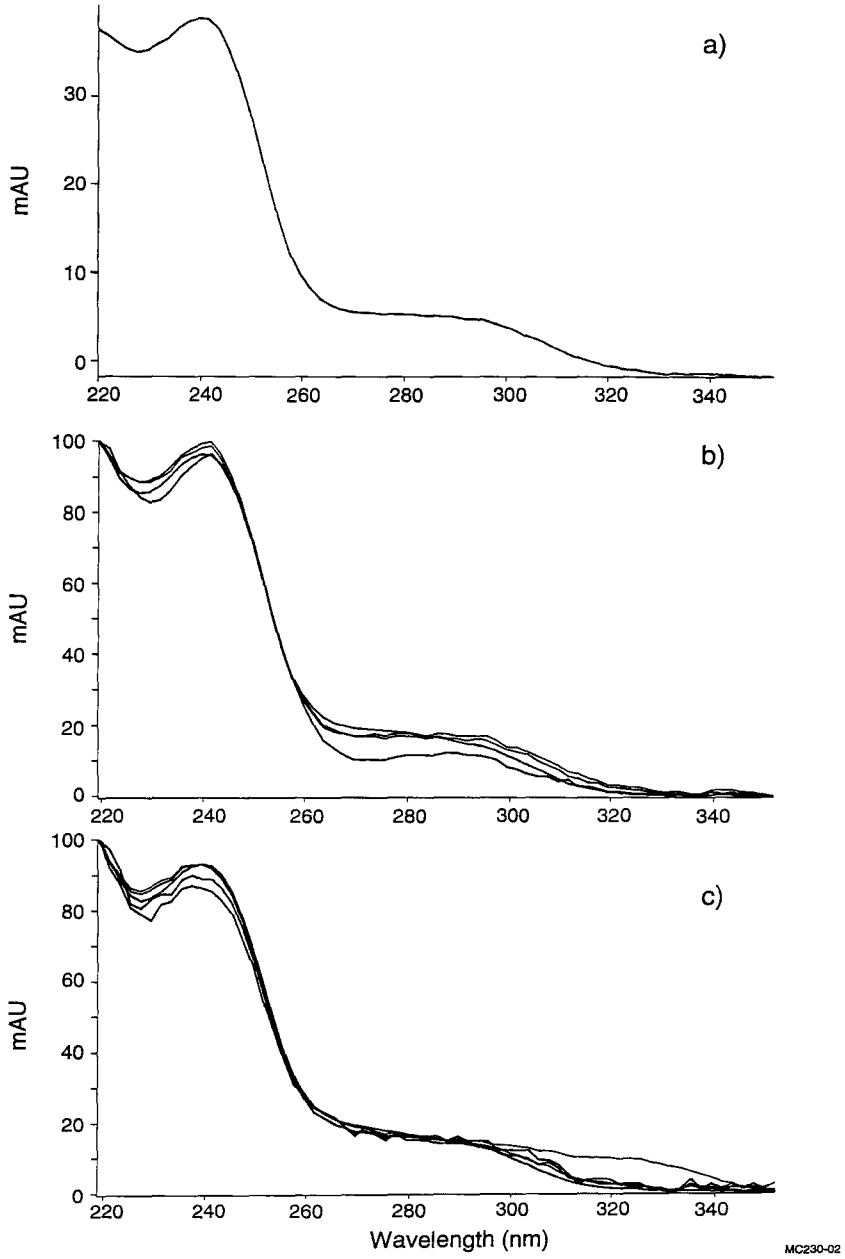

Figure 4. UV spectra of L-737,415 and its metabolites: (a) L-737,415; (b) microsomal metabolites; (c) urinary metabolites.

No parent compound was detected in the extract of combined urine. A number of metabolites were identifiable by HPLC-diode-array of which A, B and C correspond to those produced *in vitro* (Figure 3b). UV spectra of these metabolites again indicate their close relation to parent compound (Figure 4c). A further additional metabolite was seen only in the urine and was designated E. The presence of the metabolites A, B and C in both samples is good evidence that the major metabolic pathways are mediated through hepatic oxidation.

The fragmentation pathway for L-737,415 was elucidated using LC-MS-MS daughter ion scans of the molecular ion m/z 420. It was found that with 50V collision energy only a small amount of fragmentation was observed (Figure 5a) but by increasing the collision energy to 200V further fragments were obtained (Figure 5b) allowing the complete assignment of the pathway (Figure 6). Essentially the fragmentation of L-737,415 involved loss of the tolyl-urea moiety [M - 150] followed by loss of CO with concomitant ring contraction [M - 178]. Finally, the homopiperidine ring was lost [M-275] to produce a fragment of m/z 145.

Full scan LC-MS was performed in order to determine molecular ions for the metabolite peaks. Metabolite A was found to be [M + 14], metabolites B and C [M + 16], metabolite D [M + 18] and metabolite E [M + 30]. The fragmentation pathways of these metabolites were elucidated using LC-MS-MS daughter ion scans of the molecular ion and major fragments. The results from these studies are summarised (Table 1). Crucially the mass differences between the parent and metabolite fragments were maintained until the final fragmentation, leading to a fragment of m/z 145 for A, B, D and E and m/z 160 for metabolite C. This clearly demonstrated the position of metabolism to be on the cycloalkylamine ring.

DISCUSSION

Structures for each of the metabolites are postulated based on the observation that metabolism occurs at the C5 cycloalkylamine ring (Figure 7). Metabolite A, with [M + H] of m/z 434, is 14 amu heavier than parent compound and is likely to correspond to a lactam. Metabolites B and C, with [M + H] of m/z 436, are 16 amu heavier than parent compound indicating ring hydroxylations. Metabolite D, with [M + H] of m/z 438, is 18 amu heavier than parent compound indicating formation of a ring opened amino alcohol. This conclusion is supported by the benzylic cleavage of the secondary amine to form the m/z 160 rather than the usual

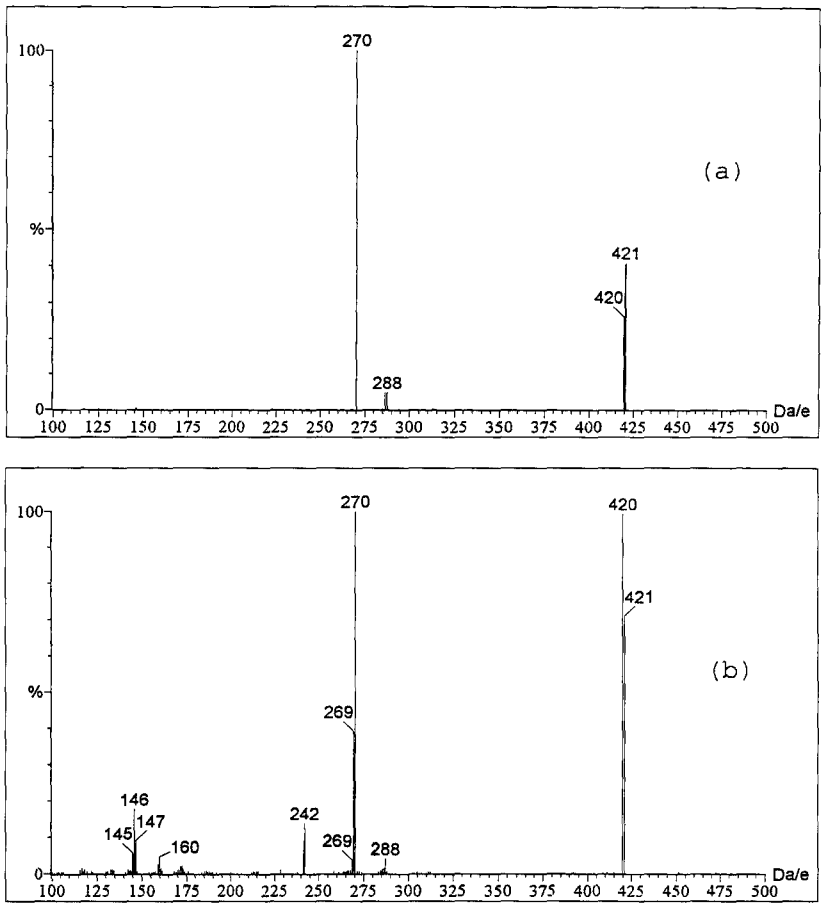

Figure 5. Daughter ion mass spectra of L-737,415 of m/z 420 at (a) 50V and (b) 200V collision energy

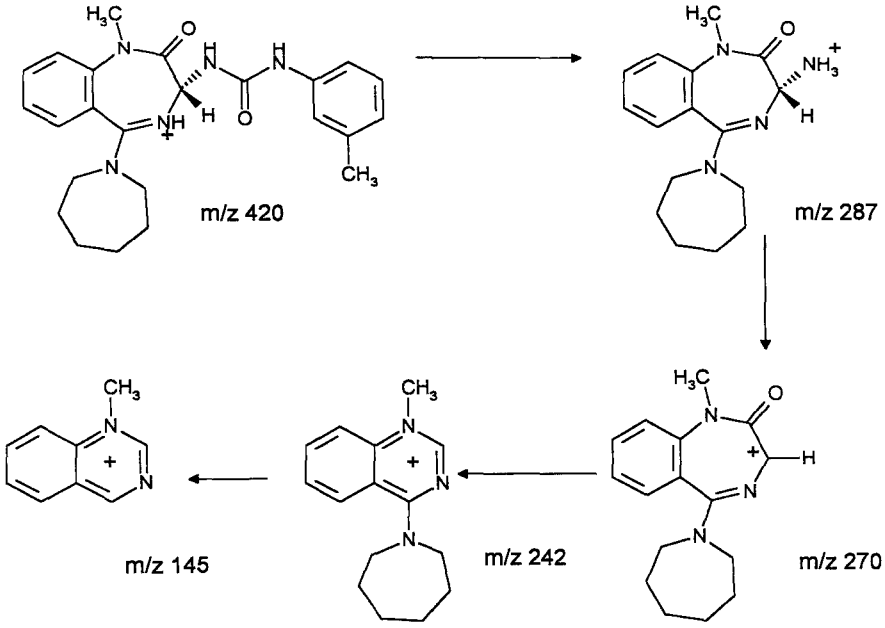

Figure 6. Fragmentation pathway for L-737,415

TABLE 1

Summary of daughter ions observed for L-737,415 and its metabolites

Identity	Molecular ion $[M+H]^+$	Pre-urea fragment	Urea cleavage	Benzo-diazepine	Loss of carbonyl	Loss of amidine
Parent	420	313	287	270	242	145
A	434	327	301	284	256	145
B	436	329	303	286	258	145
C	436	329	303	286	258	145
D	438	331	305	288	260	160
E	450	343	317	300	N.O.	145

N.O. = not observed

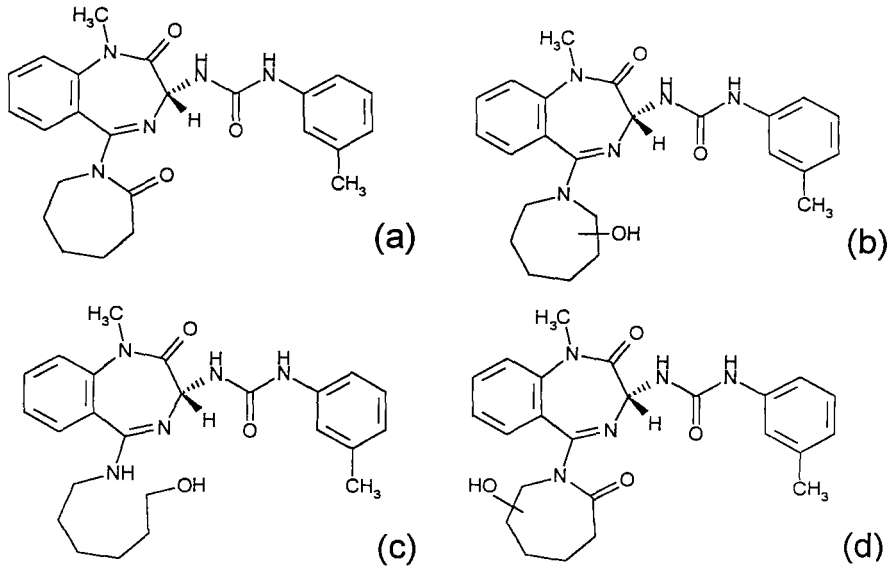

Figure 7. Proposed metabolites of L-737,415; (a) metabolite A, (b) metabolites B and C, (c) metabolite D and (d) metabolite E

m/z 145 ion. Metabolite E, found only in the urine, has [M + H] of m/z 450 and is 30 amu heavier than parent compound. By analogy with the other metabolites, and by consideration of the greater polarity of this compound by HPLC, this is consistent with a hydroxylated lactam.

The evidence consistently suggests that the metabolism of L-737,415 proceeds through oxidative modification of the C5 cycloalkylamine ring, to yield the metabolites A, B, C, D and E (Figure 7). The lack of an exact match of *in vitro* and *in vivo* profiles is perhaps not surprising considering the restricted number of metabolic reactions available *in vitro* compared to the *in vivo* situation. In particular the absence of metabolite D, and the presence of E, in the urine is indicative of metabolism occurring to a greater extent *in vivo* than *in vitro*.

In conclusion, this study has demonstrated the use of scaled-up liver microsomal metabolism assays to produce sufficient quantities of metabolites for structural identification, and the application of LC-MS-MS to obtain rapid information on

the metabolic fate of non-radiolabelled compounds in a pre-development research environment. This provides us with a useful tool for guiding the production of alternative analogues.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(11), 2285-2290 (1995)

LIQUID CHROMATOGRAPHY CALENDAR

1995

JUNE 5 - 8: 5th Symposium on Our Environment / 1st Asia-Pacific Workshop on Pesticides, Singapore. Contact: The Secretariat, 5th Symp on our Environment, Chem Dept, National University of Singapore, Kent Ridge, Republic of Singapore 0511.

JUNE 6 - 8: 28th Great Lakes Regional ACS Meeting, LaCrosse-Winona Section. Contact: M. Collins, Chem. Dept., Viterbo College, La Crosse, WI 54601, USA.

JUNE 11 - 14: 1995 International Symposium and Exhibit on Preparative Chromatography, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JUNE 13 - 16: Capillary Electrophoresis, Routine Method for the Quality Control of Drugs: Practical Approach (in English); L'Electrophorese Capillaire, Methode de Routine pour le Controle de Qualite des Medicaments: Approche Pratique (in French), Monpellier, France. Contact: Prof. H. Fabre, Lab. de Chimie Analytique, Inst. Europeen des Sciences Pharmaceutiques Industrielles de Montpellier, Ave. Charles Flahault, 34060 Montpellier Cedex 1, France.

JUNE 14 - 16: 50th Northwest/12th Rocky Mountain Regional Meeting, ACS, Park City, Utah. Contact: J. Boerio-Goates, Chem Dept, 139C-ESC, Brigham Young Univ, Provo, UT 84602, USA.

JULY 9 - 15: SAC'95, The University of Hull, UK, sponsored by the Analytical Division, The Royal Society of Chemistry. Contact: The Royal Society of Chemistry, Burlington House, Picadilly, London W1V 0BN, UK.

JULY 7 - 8: FFF Workshop, University of Utah, Salt Lake City, UT. Contact: Ms. Julie Westwood, FFF Research Center, Dept. of Chem., University of Utah, Salt Lake City, UT 84112, USA.

JULY 10 - 12: FFF'95, Fifth International Symposium on Field-Flow Fractionation, Park City, Utah. Contact: Ms. Julie Westwood, FFF Researcvh Center, Dept. of Chem. Univ. of Utah, Salt Lake City, UT 84112, USA.

JULY 23 - 27: American Society of Pharmacognosy, 36th Annual Meeting, University of Mississippi, Oxford, Miss. Contact: Russell Cooper, Center for Public Service & Continuing Studies, 14 E.F. Yerby Center, Box 1667, University of Mississippi, University, MS 38677, USA.

JULY 23 - 28: 35th Rocky Mountain Conference on Analytical Chemistry, Hyatt Regency, Denver, Colorado. Contact: Patricia Sulik, Rocky Mt. Instrum. Labs, 456 S. Link Lane, Ft. Collins, CO 80524, USA.

JUNE 25 - 28: Method Development in HPLC, Virginia Tech, Blacksburg, Virginia. Contact: Dr. H. McNair, Chem Dept, Virginia Tech, Blacksburg, VA 24061-0212, USA

AUGUST 13 - 17: ICFIA'95: International Conference on Flow Injection Analysis, Seattle, Washington. Contact: Prof. G. D. Christian, Dept of Chemistry BG-10, University of Washington, Seattle, WA 98195, USA.

AUGUST 14 - 19: 35th IUPAC Congress, Istanbul, Turkey. Contact: Prof. A. R. Berkem, 35th IUPAC Congress, Halaskargazi Cad. No. 53, D.8, 80230 Istanbul, Turkey.

AUGUST 20 - 25: 210th ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 20 - 25: ACS sponsored Symposium on Saponins: Chemistry and Biological Activity, Chicago, Illinois. Contact: G. R. waller, Oklahoma State University, Dept of Chem & Molecular Biology, Stillwater, OK 74078, USA.

SEPTEMBER 4 - 7: 13th International Symposium on Biomedical Applications of Chromatography and Electrophoresis and International Symposium on the Applications of HPLC in Enzyme Chemistry, Prague, Czech Republic. Contact: Prof. Z. Deyl, Institute of Physiology, Videnska 1083, CZ-14220 Prague 4, Czech Republic.

SEPTEMBER 7 - 8: Engineering & Construction Contracting Conference, Phoenician Resort, Scottsdale, Arizona. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA

SEPTEMBER 12 - 15: 5th International Symposium on Drug Analysis, Leuven, Belgium. Contact: Prof. J. Hoogmartens, Inst. of Pharmaceutical Sciences, Van Evenstraat 4, B-3000 Leuven, Belgium.

SEPTEMBER 18 - 21: Safety in Ammonia Plants & Related Facilities, Loews Ventana Canyon, Tucson, Arizona. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

SEPTEMBER 26 - 29: CCPS International Conference on Modelling & Mitigating the Consequences of Accidental Releases of Hazardous Materials, Fairmont Hotel, New Orleans, Louisiana. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

OCTOBER 18 - 21: 31st Western Regional Meeting, ACS, San Diego, Calif. Contact: S Blackburn, General Dynamics, P. O. Box 179094, San Diego, CA 92177-2094, USA.

OCTOBER 23 - 25: 6th Annual Frederick Conference on Capillary Electrophoresis, Hood College, Frederick, Maryland. Contact: Margaret L. Fanning, Conference Coordinator, PRI, NCI-FCRDC, P. O. Box B, Frederick, MD 21702-1201

OCTOBER 22 - 25: 25th Northeastern Regional Meeting, ACS, Rochester, New York. Contact: T. Smith, Xerox Corp, Webster Res Center, M/S 0128-28E, 800 Phillips Rd, Webster, NY 14580, USA.

NOVEMBER 1 - 3: 30th Midwestern Regional ACS Meeting, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr., Miami, OK 74354-3854, USA.

NOVEMBER 1 - 4: 31st Western Regional ACS Meeting, San Diego, California. Contact: T. Lobl, Tanabe Research Labs, 4450 Town Center Ct., San Diego, CA 92121, USA.

NOVEMBER 5 - 7: 30th Midwestern Regional Meeting, ACS, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr, Miami, OK 74354, USA.

NOVEMBER 12 - 17: AIChE Annual Meeting, Fontainbleu Hotel, Miami Beach, Florida. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

NOVEMBER 29 - DECEMBER 1: Joint 51st Southwestern/47th Southeastern Regional Meeting, ACS, Peabody Hotel, Memphis, Tenn. Contact: P.K. Bridson, Chem Dept, Memphis State Univ, Memphis, TN 38152, USA.

DECEMBER 17 - 22: 1995 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1996

FEBRUARY 25 - 29: AIChE Spring National Meeting, Sheraton Hotel, New Orleans, Louisiana. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

FEBRUARY 26 - MARCH 1: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

MARCH 24 - 29: 211th ACS National Meeting, New Orleans, LA. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

MARCH 31 - APRIL 4: 7th International Symposium on Supercritical Fluid Chromatography and Extraction, Indianapolis, Indiana. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

MARCH 31 - APRIL 4: 7th International Symposium on Supercritical Fluid Chromatography & Extraction, Indianpolis, Indiana. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

MAY 7 - 9: VIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Monte Carlo, Monaco. Contact: Prof. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JULY 14 - 18: 5th World Congress of Chemical Engineering, Marriott Hotel, San Diego, California. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 9 - 14: 31st Intersociety Energy Conversion Engineering Conference (co-sponsored with IEEE), Omni Shoreham Hotel, Washington, DC. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 17 - 20: 31st National Heat Transfer Conference, Westin Galleria, Houston, Texas. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 18 - 23: 212th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 1 - 6: 11th Symposium on Quantitative Structure-Activity Relationships: Computer-Assisted Lead Finding and Optimization," Lausanne, Switzerland. Contact: Dr. Han van de Waterbeemd, F. Hoffmann-La Roche Ltd., Dept PRPC 65/314, CH-4002 Basle, Switzerland.

SEPTEMBER 9 - 12: Saftey in Ammonia Plants & Related Facilities, Westin at Copley Place, Boston, Massachusetts. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

OCTOBER 16 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

OCTOBER 24 - 26: 52nd Southwestern Regional Meeting, ACS, Houston, Texas. Contact: J. W. Hightower, Chem Eng Dept, Rice Univ, Houston, TX 77251, USA.

NOVEMBER 6 - 8: 31st Midwestern Regional Meeting, ACS, Sioux Falls, South Dakota. Contact: J. Rice, Chem Dept, S. Dakota State Univ, Shepard Hall Box 2202, Brookings, SD 57007-2202, USA.

NOVEMBER 9 - 12: 48th Southeast Regional ACS Meeting, Greenville, South Carolina. Contact: H. C. Ramsey, BASF Corp., P. O. Drawer 3025, Anderson, SC 29624-3025, USA.

NOVEMBER 10 - 15: AIChE Annual Meeting, Palmer House, Chicago, Illinois. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

1997

APRIL 6 - 11: 213th ACS National Meeting, San Antonio, Texas. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 7 - 12: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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AUGUST 23 - 28: 216th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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MARCH 21 - 26: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 27: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2000

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 20 - 25: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 24: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2002

APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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1. L. R. Snyder, J. J. Kirkland, Introduction to Modern Liquid Chromatography, John Wiley & Sons, Inc., New York, 1979.

2. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in HPLC of Biological Macromolecules, K. M.

Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

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9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used.

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4. **Tables** should be typed on separate pages, one table to a page. A table may not be longer than one page. If a table is larger than one page, it should be divided into more than one table. The word **TABLE** (capitalized and followed by an Arabic number) should precede the table and should be centered above the table. The title of the table should have the first letters of all main words in capitals. Table titles should be typed single line spaced, across the full width of the table.

5. **Figures (drawings, graphs, etc.)** should be professionally drawn in **black** India ink on separate sheets of **white** paper, and should be placed at the end of the text. They should not be inserted in the body of the text. They should not be reduced to a small size. Preferred size for figures is from 5 inches x 7 inches (12.7 cm x 17.8 cm) to 8½ inches by 11 inches (21.6 cm x 27.9 cm). **Photographs** should be professionally prepared *glossy* prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn.

Captions for figures should be typed single-spaced on a separate sheet of white paper, along the full width of the type page, and should be preceded with the word FIGURE and an Arabic numeral. All figures and lettering must be of a size that will remain legible after a 20% reduction from the original size. Figure numbers, name of senior author and an arrow indicating "top" should be written in light blue pencil on the back of the figure. Indicate the approximate placement for each figure in the text with a note written with a light blue pencil in the margin of the manuscript page.

6. The **reference list** should be typed single-spaced. A single line space should be inserted after each reference. The format for references should be as given above.

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