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AMPHETAMINE AND METHAMPHETAMINE DETERMINATIONS IN BIOLOGICAL SAMPLES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY. A REVIEW

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Abstract: This critical review shows the different high performance liquid chromatography methods proposed for amphetamine and methamphetamine determinations. It is directed mainly towards sample clean up and derivatizations steps, because of their significance in such determinations.

INTRODUCTION

Amphetamines are psychoactive substances that have been abused both in society in general and in sports. However, a number of isomeric forms of these sympathomimetic amines have found therapeutic applications as analeptics, stimulants, anorexigens, in combination with antipyretics, etc.

The psychostimulant amines are all synthetic in origin, and possess phenylisopropylamine as common basic structure. Amphetamine is a phenolic derivative with a ramified aliphatic

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amine lateral chain. Amphetamine and methamphetamine both possess an asymmetric carbon which confers a number of isomeric forms (d, l and dl).

The half-life of amphetamine is about 20 hours *in vivo*. The compound is partially hydrolyzed by microsomal liver enzymes, and 30-40% of it is excreted in active form in urine within 48 hours of administration. Urine also contains p-hydroxiamphtamine, 3% as conjugated benzyl methyl ketone, benzoic acid and traces of conjugated 1-phenyl propan-2-ol. Methylamphetamine is fundamentally excreted without modification, with only a small proportion being demethylated to amphetamine (1). The excretion of amphetamines is markedly affected by urine pH (2).

A number of methods have been proposed to assay these analytes. Thus, in 1985 Rasmussen et al. (3) reviewed the analytical techniques developed to detect and identify amphetamines and amphetamine-like substances in non-biological samples. They concluded that infrared and ultraviolet spectroscopy are the most frequently used spectroscopic methods, while thin layer chromatography (TLC) and gas chromatography (GC) are the workhorse separation methods. Budd (4) contrasted the advantages and disadvantages of GC, enzyme multiplied immunoassay technique (EMIT) and radioimmunoassay (RIA). GC was found to be the best method, although the number of analyses obtained was lower than that afforded by the other techniques.

Figure 1 shows the number of publications on amphetamine determination in the literature in the past 10 years (information obtained from Chemical Abstracts). The analytical techniques most used are GC, EMIT, RIA and high-performance liquid chromatography (HPLC). The situation is similar in the case of methamphetamine. These analytical procedures have been applied to pharmaceutical samples and, to a lesser degree, to biological specimens. Most publications to date have employed GC.

In the last few years new methods have been proposed to assay methamphetamine and related

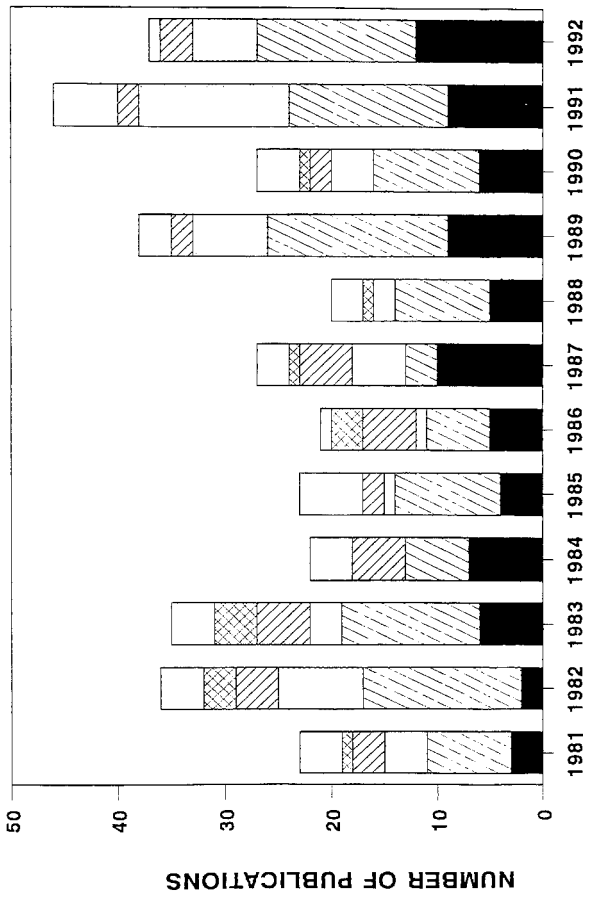

FIGURE 1. Bibliographic evolution of the published papers about the analysis of amphetamine by different techniques during last decade.

compounds by HPLC, this in turn being reflected by an increase in the number of publications. However, only 25% of HPLC papers involve biological samples.

The aim of the present review was to evaluate the different HPLC methods proposed in the literature for assaying amphetamine and methaphetamine samples, in an attempt to establish the most suitable technique.

SAMPLE CLEAN-UP

Table 1 shows that liquid-liquid extraction is used for sample clean-up before HPLC determination. Solvents such as diethyl ether (Et_2O) in strong alkali pH (5-8), and n-hexane (9) have been used in liquid-liquid extraction. Amphetamine and related compounds have pKa values of about 9.9, and an alkaline medium is required.

Ion-pair extraction has been employed by Hoogewijs et al. (10) to analyze basic drugs through the direct injection of extracts into the liquid chromatography column. The extraction efficacy of Na-n-octylsulfate as ion pairing reagent was compared with that of bis (2-ethylhexyl) phosphate. Direct injection of the ion-pairing extracts into the column was possible because the retention behaviour was independent of whether the basic drugs were injected as an ion-pair or as a base.

Reference (11) shows a clean-up procedure based on the precipitation and extraction of methamphetamine with acetone (Table 1).

In recent years, a trend to use phase-solid extraction has been observed. Sekine et al. (12) quantitatively extracted amphetamine, methamphetamine, methylephedrine and p-hydroxymethamphetamine in urine with a Sep-Pak C_{18} cartridge. Each drug could be almost quantitatively extracted at pH 8 and with sample flow rates of between 2.5 and 5.0 ml/min. These authors (13) proposed automatic extraction using an ODS-minicolumn (25 mm x 9 mm i.d.) for analyzing drugs of abuse in biological fluids. Patel et al. (14) isolated amphetamine and methamphetamine from urine using polymer-based C_{18} extraction cartridges. The eluent

was injected directly into the HPLC column for analysis. The extraction principle involved hydrophobic interaction using ion pairing with hexanesulfonic acid before sample application. The extraction was linear between 5.0 and 25 μg/ml.

A simple and reproducible column extraction procedure, using a silica based mixed phase bonded chromatographic column was described for the screening and confirmation of drugs in horse urine by TLC and gas chromatography-mass spectrometry (GC-MS), respectively. Amphetamine and methamphetamine exhibited $< 25\%$ recovery by liquid-liquid extraction when dichloromethane (DCM)-isopropanol (1:3 v/v) were used as extraction solvents; in turn, a recovery of over 85% was achieved by column extraction (15).

Farrell and Jefferies (16) used solid-phase extraction for sample clean-up in an investigation of different HPLC methods for analyzing amphetamines (Table 1).

Recently, Helmlin and Brenneisen (17) extracted phenylalkylamine derivatives, such as methylenedioxymethylamphetamine (MDMA) and methylenedioxyamphetamine (MDA), from urine samples on an Adsorbex SCX (100 mg) cation exchange solid-phase extraction column. Recoveries higher than 98% were obtained (Table 1).

In other procedures the sample was subjected to analyte derivatization, followed by liquid-liquid extraction of the reaction products to remove excess reagent and undesired compounds present in the sample (18,19). Suitable detection limits are achieved by these procedures (Table 1).

Some authors (20) have used urine samples containing methamphetamine directly without either derivatization or extraction.

Other procedures have been described (21-23) to determine amphetamine in physiological fluids using on-line solid phase derivatization and reversed-phase liquid chromatography. The samples were filtered (21-22) or diluted (23) prior to injection in the system. Koning et al. (34) also used dilution of the sample and injection into an on-line derivatization system with naphthalenedialdehyde as fluorigenic label.

TABLE 1. Analytical properties of the different methods proposed for the analysis of amphetamine and methamphetamine by HPLC.

Drug separated	Source	Sample Clean-up	Derivatization	Stationary phase	Mobile phase	Detector	Detection limit	Note	Ref
Methamphetamine	Urine and Plasma	----------	NQS	μBondasphere C_{18} 5 μm	acetonitrile-methanol-0.01M sulfuric acid (20:20:70)	EC	1 ng/mL	The amine-derivatives was extracted with (n-hexane-ethyl acetate) 1:1. Spiked urine samples.	19
Methamphetamine	Urine	----------	----------	Asahipak GS-320M	0.02M $NaHCO_3$ 0.02M Na_2CO_3(8:2) 40°C 1.0 mL/min	UV 207 nm	--------	Linear interval 1-10 μg/mL.	20
Amphetamine	Urine	Filtration at pH=10	Polymeric reagents contained an activated ester linkage to the 9-fluororenyl group (FMOC-Cl).	LichroSpher C_{18}, 5μm	50/50, 80/20, acetonitrile/water	UV 254 nm Fluorescence (265/320nm)	0.1 μg/mL.	% of derivatizations are 90%-70% for 1st and 2nd amines. Spiked urine samples.	21
Dl-amphetamine	Urine	Filtration at pH=10	FMOC-L-proline	C_{18}	(40-48%) acetonitrile-water	UV, Fluorescence	50 ng/mL	The linearity of the overall measurement was 3-4 orders of magnitude of concentration. Spiked urine samples.	22
Amphetamine	Plasma	Dilution	9-fluoreneacetyl-tagged (FA)	Supelcosil LC-ABZ, 5 μm	Step gradient from 1 mM SDS in ACN-water(10:90 v/v) to 1 mM SDS in ACN-water (55:45 v/v) for analytical separation	Fluorescence (254/313 nm)	0.2 μg/mL in plasma	On line derivatization in ACN:water (10:90 v/v). Linear range 2-40 μg/mL with 1 mM SDS at 75°C for 8 min. Spiked plasma samples	23
Amphetamine	Human and horses urine	Concentration in precolumns switching technique	----------	Spherisorb ODS-2, 5μm	0.001M perchloric acid-0.1M sodium perchlorate in water/MeOH	UV 210 nm	20 ng/mL	Microbore HPLC gradient. Determination limit 0.1 μg/mL. Spiked urine samples.	24
Amphetamine, Methamphetamine	Urine	Switching technique with two precolumns	----------	C_8 column and silica column	Buffer pH=6.5 and acetonitrile	UV 210 and 235 nm	0.2 μg/mL	Linear range 0.3-10 mg/L. Suspected urine samples MA: 8.2 mg/L; A: 1.8 mg/L.	25
Amphetamine	Standard	--------	Naphthalene-2,3-dialdehyde, Anthracene-2,3-dialdehyde.	Lichrosorb RP-18 5μm	Acetonitrile-water(70:30) containing 2.5mM imidazole	Fluorescence, Chemiluminescence with peroxyoxalate	$> 10^{-8}$M	The derivates were extracted with toluene:hexane (1:1) and diluted with dichloromethane	33
Amphetamine	Plasma	Diluted as much as 200- fold	Naphthalenedialdehyde	ODS-2-Chromospher C_{18}, 5μm	Gradient elution A- THF-potassium phosphate buffer (pH=6.8 0.05M) (5:95) B-acetonitrile-MeOH-potassium phosphate buffer (pH=6.8 0.05M) (55:10:35)	Fluorescence	50 fmol of A.(50 μL sample injected)	Automated precolumn derivatization. This reaction is carried out in aqueous solution at pH 9.3 in 15 min. Linear range 10 fmol-100 pmol injected.	34
Amphetamine, methamphetamine	Standards	---------	9-fluorenyl methyl chloroformate (FMOC-Cl)	Nucleosil C_{18} (NM) 5μm	acetonitrile-water (58:42, v/v)	UV, 265 nm	---------	On line preconcentration and derivatization. Linear in the range $2x10^{-8}$ - $1x10^{-7}$ M	36

TABLE 1 (continued).

Drug separated	Source	Sample Clean-up	Derivatization	Stationary phase	Mobile phase	Detector	Detection limit	Note	Ref
Amphetamine (A)	Urine	Liquid-liquid (L/L) 1) Et_2O 2) HCl	o-phthaldialdehyde (OPA)	RP-18 (10μm)	MeOH:H_2O (75:25)	Fluorescence 450/350nm	Detection limit 0.2μg/mL	Linear interval 0.25-3μg/mL Spiked urine samples	5
Amphetamine, methamphetamine (MA)	Urine	L/L Et_2O, alk. pH	different reagents: dansyl chloride (Dns-Cl), 4-fluoro-7-nitrobenzoxadiazole, naphthalene-2,3-dicarbaldehyde.	Inertsil ODS-2	$1.0x10^{-3}$ M imidazole in acetonitrile-water.	Fluorescence Chemiluminescence after reaction with Bis(2,4,6-trichlorophenyl) oxalate hydrogen peroxide in acetonitrile.	For Dns-derivatives $3x10^{-15}$-$4x10^{-15}$ mol	Dns-Cl was the most suitable. Recovery with derivatization Dns 99.3$\pm$0.7% for (MA). Spiked urine samples	6
Amphetamine, methamphetamine and piperidine	Urine	L/L Et_2O, alk pH	Dansyl chloride	Inertsil ODS-2	Acetonitrile-water(7:3), 1mM imidazole pH 7	Chemiluminescence after reaction with bis(2,4,6-trichlrophenyl) oxalate and hydrogen peroxide	$2x10^{-10}$M (MA) in urine	Sensitivity was higher than that of Simon's reagent test and CG. Suspected urine samples MA: $1.3x10^{-3}$ to $3.6x10^{-8}$M; A: $3.5x10^{-5}$ to $3.3x10^{-6}$M.	7
Amphetamine, methamphetamine	Urine	L/L Et_2O	ABEI N-(4-amino-butyl)-N-ethyl-isoluminol plus N,N'-disuccinimidyl carbonate in presence of methanol and trimethylamine	RP-C_{18}	MeOH:water (54:64) containing 30mM Na-octanesulfonate	Chemiluminescence after reaction with K-ferricianide, NaOH, H_2O_2 and β-ciclodextrin	Detection limit 20 fmol(MA) and 100 fmol(A). 10^{-7}M in urine for (A)	Linear interval until 5 pmol (MA) and 10 pmol(A). Spiked urine samples.	8
Amphetamine, Methamphetamine	Urine Plasma	L/L n-hexane	Sodium β-naphthoquinone-4-sulphonate (NQS)	Wakogel LC 5H 5μm LiChrosorb SI 100 10μm	$CHCl_3$:ethyl acetate:ethanol:n-hexane(25:10:1:50)	UV 450 and 280 nm	MA: 464 nm 5ng 280 nm 2 ng	Linear range in the visible 0.25-2 μg for both A and MA. Suspected urine samples.	9
Methamphetamine	Serum	Precipitation with acetone	ABEI N-(4-aminobutyl)-N-ethylsoluminol plus N,N'-disuccinimidyl carbonate in presence of methanol and trimethylamine. Heat 80° 30'	Shimpack CLC-C_{18}	MeOH:water (54:64) containing 30mM Na-octanesulfonate	Chemiluminescence after reaction with K-ferricianide, NaOH, H_2O_2 and β-ciclodextrin	Detection limit 20 fmol (100 μL of serum were taken)	Linear range 0.05-5 pmol. Comparable sensitivity CG-MS and Fluorescence-HPLC. Recovery for MA was 99%. Addict serum sample, MA: 3.8 μg/mL.	11
Amphetamine	Urine and plasma	Solid phase: XAD-2	OPA, NBD-Cl, NQS, NS, SDDS.	Partisil 5, LiChrospher Si 100, Partisil ODS-2.	Several	UV/ Fluorescence	Urine 4 ng/mL Plasma 20 ng/mL	Addict urine sample, A: 8.5 μg/mL with SDDS derivatization. The best results were obtained with NQS.	16
Phenylalkylamines (amphetamine, methamphetamine etc...)	Biological matrix (urine and plants material)	Solid phase: Adsorbex SCX(100 mg) cation exchange	-------	3μm Spherisorb ODS-1	Acetonitrile:water (72:928 v/v), 5mL ortophosphoric acid (85%), 0.28 mL hexyl amine per 1000 mL	Photodiode array UV 198 and 205 nm		Separation isocratic. Linear graphs	17
Basic Drugs (Amphetamine, Methamphetamine etc...)	Biological fluids	---------	NQS	μBondasphere C_{18}	MeCN-methanol-0.01M sulfuric acid	Electrochemical detection (EC)	< 10 ng/mL	Only 50 μL of biological fluid containing these drugs at > 1μg/mL was required for determination with good accuracy. Spiked urine samples.	18

Slais et al. (24) have screened amphetamines in human and horse urine. Urine samples can be screened for amphetamines without off-line pre-treatment or derivatization by combining the use of a two pre-column series for sample clean-up and enrichment with a period of selective solute displacement. The reproducibility of the peak areas is acceptable for quantifying the amphetamine below the 1 μg/ml level (the detection limit in urine was 20 ng/ml)(Table 1). Another switching system was proposed by Binder et al. (25) for the automated analysis of basic drugs in urine. The authors used two polymeric pre-columns to isolate the drugs, and a reversed-phase column coupled to a silica column produced the analytical separation. A urine specimen which was positive for amphetamines by TLC was analyzed by this procedure. Concentrations were determined by comparison with urine samples supplemented with known concentrations of drugs: concentrations were amphetamine 1.8 mg/l and meth-amphetamine 8.2 mg/l.

A new on-line method for HPLC using zone electrophoretic sample treatment has recently been introduced (26-27).

DERIVATIZATION

Derivatization has long been accepted as an effective modification technique in HPLC, improving overall specificity, chromatographic performance, and sensitivity of the original trace analysis.

Kinberger (5) incubated amphetamine with o-phthalaldehyde (OPA) at room temperature prior to chromatographic analysis. The precolumn fluorescence derivatization with OPA allowed a sensitive HPLC determination of amphetamine in urine samples, as seen in Table 1.

Derivatization of sympathomimetic drugs with OPA in the presence of different thiols (2-mercaptoethanol, ethanethiol and tert-butylmercaptan) has also been used (28). Some structural assignments of the OPA-amino drug adducts were proposed and the electroactive

properties of these substituted isoindolic products were investigated by voltamperometry. HPLC with chemiluminescence detection (CL) is highly sensitive and selective for fluorescent compounds (29). The CL detection thresholds of several fluorescent compounds were 10-100 times lower than those obtained by fluorescence detection.

For the determination of trace levels of amphetamine-related compounds Hayakawa et al. (6) studied fluorigenic derivatization. Bis (2,4,6-trichlorophenyl) oxalate and hydrogen peroxide in acetonitrile were used as post-column chemilumigenic reagent. As precolumn derivatization reagents, dansyl chloride (Dns-Cl), 4-fluoro-7-nitrobenzoxadiazole (NBD-F) and naphthalene-2,3-dicarbaldehyde (NDA) fluorigenic reagent, which reacts only with primary amines in the presence of cyanide, were compared by these authors. NBD-F reacts with both primary and secondary amines; the reaction is very rapid, which constitutes an advantage over NBD-Cl (30,31). The main disadvantage of NBD derivatives was that the sensitivity by chemiluminescence detection was less than that by fluorescence detection. NDA derivatives could be detected with 10 times greater sensitivity than dansyl derivatives, and over 50 times greater than NBD-F derivatives. However, Dns-Cl was the best derivatization agent for simultaneously determining primary and secondary amines. Only diethyl-ether extraction was necessary as clean treatment before Dns-derivatization. The detection limits of Dns-derivatives were 3×10^{-15} - 4×10^{-15} M (Table 1) with chemiluminescence detection (6). The method was more sensitive than GC-MS by a factor of 70 for NDA derivatives and by 3.5 for Dns derivatives. Methamphetamine was detected as low as 1.0×10^{-7} M in urine without any interfering peak at the corresponding retention time. In another paper (7), these authors identified the dansyl derivatives of these compounds by mass spectrometry in the corresponding peaks from suspected human urine. The concentrations of amphetamine, methamphetamine and piperidine in 6 suspected human urine samples were detected in the range 3.5×10^{-5} - 3.3×10^{-6} M, 1.3×10^{-3} - 3.6×10^{-8} M and 7.3×10^{-5} - 2.4×10^{-6} M, respectively. The detection limit for methamphetamine was 2×10^{-10} M in urine (Table 1).

Nakashima et al. (11) determined methamphetamine concentrations in human serum using N-(4-aminobutyl)-N-ethylisoluminol (ABEI) as precolumn labelling chemiluminogenic reagent. The greatest reaction was obtained at 80°C for 30-60 min. This method was very sensitive and could detect as little as 20 fmol of methamphetamine when 100 μl of serum sample were employed (Table 1). A serum sample from a methamphetamine addict, which contained 3.6 μg/ml methamphetamine as determined by GC, was analyzed. The amount of methamphetamine determined was 5.05 $\pm$ 0.07 μg/ml. The sensitivity was comparable that of the GC-MS (32) and HPLC-fluorescence method (6), but slightly lower than that afforded by the HPLC-peroxyoxalate chemi-luminescence method (6). Recently, these authors (8), have used ABEI reagent for the determination of methamphetamine and its metabolite amphetamine in human urine. The reported detection limit is higher for amphetamine, as seen in Table 1.

Naphthalene-2,3-dialdehyde (NDA) and anthracene-2,3-dialdehyde (ADA) as pre-column reagents for the peroxyoxalate chemiluminescence detection of primary amines using reversed and normal-phase liquid chromatography were used by Kwakman et al. (33). The derivatization reaction time was 20 min. at room temperature for NDA and ADA; however, NDA derivatives were stable, while ADA derivatives were unstable - probably owing to oxidation of one of the aromatic rings. A serious disadvantage was the formation of cyanide-induced side-products which were major interferences in reversed-phase chromatography. Unfortunately, for both NDA and ADA amphetamine derivatives in reversed-phase HPLC, the interfering peaks begin to dominate the chromatogram at the 10^{-8} M even with standard solutions, so that real trace - level analysis cannot be carried out.

Koning et al. (34) proposed an automated precolumn derivatization of amino acids, small peptides, brain amines and drugs with primary amino groups (amphetamine in urine or plasma) by reversed phase-HPLC using naphthalenedialdehyde (NDA) as fluorigenic label. The NDA-cyanide combination was transparent to the fluorescence detector in the absence

of analytes, so removal of the excess of reagent was not required after derivatization.

Another fluorescent labelling reagent (4-(N,N-dimethylamino-sulfonyl)-7-fluoro-2,1,3-benzoxadialzole (DBD-F) and a mixture of hydrogen peroxide and bis (4-nitro-2-(3,6,9-trioxadecyloxy-carbonyl) phenyl) oxalate in acetonitrile as postcolumn chemiluminogen reagent were used to detect methamphetamine and related compounds in urine samples by chemiluminescence. The detection limits were 27 and 100 fmol for methamphetamine and amphetamine, respectively (35).

Sulphonate group displacement in an aromatic reagent can be the basis of a derivatization procedure for the determination of low concentrations of amines. The amino groups with Na-naphthoquinone-4-sulphonate (NQS) in alkaline solution form highly colored compounds that could be determined colorimetrically. Endo et al. (9) applied the method to the determination of amphetamine and methamphetamine in urine by normal-phase HPLC. The absorption maxima for the amphetamine and methamphetamine derivatives were 451 nm and 464 nm, respectively. Derivatization resulted in an approximately 25-fold increase in sensitivity in the visible range. Farrell et al. (16) investigated three pre-column derivatization reagents: o-phthalaldehyde (OPA), 4-chloro-7-nitrobenz-2,1,3-oxadiazole (NBD-Cl), sodium naphthoquinone-4-sulphonate (NQS), and two ion-pair reagents, i.e., naphthalene-2-sulphonate (NS) and sodium dodecylsulfate (SDDS). A 24-hour urine sample from a 22-year-old male was analyzed using SDDS derivatization. The urine was obtained 8 hours after an unknown amount of illegally obtained amphetamine had been taken. Urine pH was normal. The concentration of amphetamine was found to be 8.5 μg/ml, corresponding to an original dose of 55 mg of amphetamine sulfate. However, since after an oral dose of 10-15 mg amphetamine sulfate, peak plasma concentrations of 40-50 ng/ml are attained in 1-2 hours, followed by a decrease to about 2 ng/ml after 8-10 hours, only the method employing derivatization with NQS was able to afford the required sensitivity for the quantitative

analysis of urine or plasma samples containing amphetamines (Table 1). It was not suitable for hydroxylated metabolites.

Nakahara et al. (18,19) used NQS to analyze urine and plasma without tedious extraction procedures by HPLC-electrochemical detection. The use of NQS as an electrochemical label for amines satisfies the requirements of a good labelling reagent as regards sensitivity, selectivity, short reaction time, reproducibility, simple sample pretreatment and low background. The detection limit was 1 ng/ml, as indicated in Table 1.

Polystyrene-divinylbenzene-based, o-nitrobenzophenone-attached labelling reagents containing o-acetylsalicyl or fluorenyl tags were designed for derivatization of primary and secondary amines, on line and off line in HPLC with UV/fluorescence detection (21). These particular polymeric reagents had exhibited good thermal and aqueous stability, high percentage derivatization, low detection limits for amines (low-parts-per-billion range), fewer interferences in the final HPLC-UV/FL chromatograms compared with the analogous solution reactions, and faster estimation of nucleophilic analytes via the on-line approach. The method was applied to urine samples, with a minimum of sample preparation prior to direct injection into the on-line derivatization-HPLC system (Table 1).

Maeder et al. (36) proposed an on-line precolumn derivatization method for the determination of low concentrations of amphetamine using an UV detector, and 9-fluorenylmethyl chloroformate (FMOC-CL) as the derivatizing reagent. Quantitative determination of amphetamines as low as $2x10^{-8}$ mol/l (Table 1) could be obtained using this on-line method with preconcentration. The sensitivity of the technique is about 50 times greater than the equivalent off-line method. The technique has been only applied to standards.

The use of 9-fluoreneacetyl (FA) tag on a controlled-pore substrate, for direct injection analysis of amphetamine in plasma has been described by Zhou et al. (23). The derivatized 9-fluoreneacetyl amphetamine was separated by reversed-phase HPLC with a step gradient

and determined by fluorescence detection. This solid-phase reagent combined with a surfactant containing mobile phase provided a sensitive and simple procedure for on-line derivatization in the direct injection analysis of biological fluids.

In recent years, a significant number of advances have been made in chromatographic separation techniques for the resolution of enantiomers, particularly in gas chromatography (37).

D-methamphetamine is a drug of common abuse, while l-methamphetamine is found in nasal spray. Traditional methods for quantification of enantiomers, e.g., chemical resolution or rotation of polarized light, are not adequate for the determination of trace amounts of enantiomers in biological fluids. The methods for the chromatographic resolution of enantiomers fall into three categories. The first involves the conversion of the enantiomers to diastereomers by reaction with a chiral derivatizing agent. The second makes use of the differences in rates of interaction of enantiomers with chiral stationary materials. A less commonly employed third method utilizes an achiral stationary phase and a mobile phase which contains a chiral eluent.

An analytical approach has been developed (22) for on-line solid-phase derivatizations in HPLC with UV-fluorescence detection. The method involves derivatization with a polymeric 9-fluorenylmethyl chloroformate-L-proline (FMOC-L-proline) chiral reagent. The detection limit was 50 ng/ml (Table 1).

Miller et al.(38) separated enantiomers of amphetamines with four chiral reagents: (R)-(+)-1-phenylethyl isocyanate (PEIC), (-)α-methoxy-α-(trifluoromethyl) phenylacetyl chloride (MTPA.Cl), 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC). Reactions were accomplished under mild conditions (25-70°C) and were complete for all substrates within 60 min. The diastereomeric derivatives were separated by reversed-phase HPLC (C_{18}) with methanol-

water mobile phase. In general, HPLC resolution of the diastereomeric reaction products of GITC or AITC and MTPA.Cl with amine substrate was more complete than products of PEIC.

Noggle et al. (39) applied precolumn derivatization of methamphetamine and amphetamine with phenylisothiocyanate. The thiourea products formed have good reversed-phase chromatographic properties and high UV molar absorption. Using similar derivatization procedures, the enantiomeric compounds can be determined for those amines containing a chiral centre. The separation of the diastereoisomeric products of chiral derivatization can be accomplished using achiral C_{18} stationary phases. The enantiomers of ephedrine, pseudoephedrine, amphetamine and methamphetamine were separated using sugar isotyiocyanate 2,3,4,6-tetra-o-acetyl-β-glucopyranosyl isothiocyanate as a chiral derivatizing agent. When (GITC) was used as derivatizing reagent for amphetamine and related compounds, the separation of amphetamine enantiomers was difficult (39-40). This difficulty for separating amphetamine enantiomers was also seen when 4-nitrophenylsulfonyl-l-prolyl chloride (NPSP) chiral derivatizating reagent was used (41).

The enantiomers of primary, secondary and some tertiary amines were resolved as carbamate derivatives formed by reaction with β-naphthyl chloroformate on an available Pirkel-type HPLC chiral stationary phase consisting of (R)-N-(3,5-dinitrobenzoyl) phenylglicine covalently bonded to silica, by using a mobile phase consisting of mixtures of iso-PrOH in hexane (42).

N-(trifluoroacetyl)-l-prolyl-(N-TFA-l-prolyl-) d- and l-amphetamine diastereoisomers were separated by HPLC and confirmed by an interfaced mass spectrometer system, using the commercially available N-3,5-(dinitrobenzolyl)phenylglycine chiral column (43). The use of chiral derivatizing reagent and chiral LC column achieved a better resolution of d- and l-amphetamine in comparison with those previously reported in the literature.

Nagai and Kamiyama (44) assayed the optical isomers of methamphetamine and amphetamine in rat urine using HPLC with chiral cellulose-based columns. Methamphetamine isomers excreted in rat urine were analyzed by the combined use of Chiralcel OB and OJ columns, which offered good peak resolution, l/d chira ratio and retention time. Mixtures of amphetamine and methamphetamine could be separated simultaneously. The analytical time was less than 25 minutes, and the minimum detection limit was 25 ng per 20 μl of urine (1.7 μl/ml).

Conclusions: A review has been made of the procedures proposed for samples clean-up and derivatization steps for the HPLC determination of amphetamine and methamphetamine. Both off-line and on-line procedures are described and generally those steps required for successful determination. Three types of analytical columns are proposed: normal-phase, reversed-phase and chiral columns for enantiomers resolution. Ultraviolet, fluorescence, chemiluminescence and electrochemical detectors have been used. Most of the procedures described in the literature have employed spiked samples with concentrations in the μg/ml range; others (7,11,16,25) had applied procedures to suspected urine samples, the concentrations encountered being very different - probably due to factors such as pH, urine collection time after administration of the drug, etc., which all affect the concentration present in the sample. The lower detection limits encountered are a few ng/ml in biological fluids (Table 1).

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PREPARATION AND CHARACTERIZATION OF CELLULOSE BASED ADSORBENTS FOR LARGE SCALE HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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ABSTRACT

The hydrophobic packings for preparative low pressure hydrophobic interaction chromatography (HIC) on the new granulated cellulose matrix Granocel were prepared by alkylation with phenyl glycidyl ether in the presence of sodium hydroxide. The ligand concentration up to 600 μmol/g was achieved with high reproducibility. The relative amount of eluted model protein at the fixed set of conditions was used as a criterion for the evaluation of the relative hydrophobicity of adsorbents. The relative hydrophobicity was found to depend on accessible, rather than total, amount of coupled ligand as well as on the nature of matrix.

The chromatographic behaviour of adsorbents both in laboratory and in large scale was evaluated by the enrichment of recombinant α_2-interferon from Pseudomonas putida VG-84.

INTRODUCTION

Hydrophobic interaction chromatography is known as a suitable method for protein isolation and purification with regard to the preservation of biological activity. Since salt fractionation is a very common early step in protein fractionation schemes, it is convenient to take the active fraction at high ionic-strength, to adsorb it on a hydrophobic column and after washing develop the column with decreasing gradient of salt.

The development of biotechnology has increased the importance of preparative large scale low pressure HIC. Large scale chromatography requires improved, tailored and cheap support materials, flexible with respect to process optimization. In order to purify the product as effectively as possible, a wide assortment of hydrophobic packings is desirable. If packings with the optimal pore size are used, fractionation of proteins according to their hydrophobicity as well as to their molecular size may be achieved. The ligand concentration at the surface of packings and their accessibility to sorbates plays a great role in optimization of adsorption-desorption process.

However, commercially available assortment of packings for preparative HIC is quite limited. The most of widely used adsorbents are based on Sepharose matrix. Their regeneration with organic solvents is complicated in large scale process.

In this paper the preparation of packings for HIC on the matrix Granocel is reported. Granocel is trade-mark of semi-rigid cellulose gels recently developed in our laboratory. Gels exhibit high mechanical properties in a wide range of pore size and are cheap to produce, therefore are suitable for large scale chromatography. The hydrophobic packings Phenyl-Granocel were prepared by alkylation of cellulose matrix with phenyl glycidyl ether. The effect of ligand concentration and pore size on binding strength and capacity of model proteins was investigated. The suitability of Phenyl-Granocel for the enrichment of α_2-interferon in large scale process was also studied.

EXPERIMENTAL

Equipment

A liquid chromatograph (KOVO, Czechoslovakia) consisting of HPP 5001 precision electromechanical pump, RIDK 102 differential refractometer, LCD

2563 UV-VIS detector and TZ 4620 linear recorder was used for inverse gel-permeation chromatography and for the chromatography of model proteins.

The chromatography of α_2-interferon in laboratory and in pilot scale was performed on a low pressure liquid chromatograph (Pharmacia-LKB, Sweden) constisting of Microperpex S and Minipuls 2 (Gilson, France) peristaltic pumps, gradient mixer GM-1, UV-monitor UV-2 with analitical or industrial flow cell, columns C10/70, XK 50/60, K 100/45, BP 113, fraction collector FRAC-300 and two-channel recorder REC-482, for large scale chromatography was used Bioprocess I (Pharmacia, Sweden).

A Milton Roy spectrophotometer Spectronic 1201 with a 1-cm cell and multichannel spectrophotometer Multiskan MCC (Flow Laboratories) were used for spectrophotometric measurements.

Materials

Cellulose matrix Granocel was synthesized in our laboratory. Phenyl-Sepharose CL-4B was purchased from Pharmacia (Uppsala, Sweden), Phenyl-Silochrom C-80 was from ''Fermentas'' (Lithuania). Dextran kits were purchased from Pharmacia and Serva (Germany). Phenyl glycidyl ether (PGE) was obtained from Reachim (U.S.S.R.) and distilled at 109°C/5mm Hg. Bovine serum albumin (BSA), human serum albumin (HSA) and horse hemoglobin (Hb) were obtained from Reanal (Hungary).

All other chemicals were of analytical or reagent grade.

Methods

Preparation of cellulose matrix Granocel

Cellulose matrix Granocel was prepared by the saponification of acetylcellulose in solution (patent pending). The gel obtained was mechanically cut and the fraction of 100-200 μm was used for the further modification. The matrixes with exclusion limit of $2x10^6$ (Granocel-2000), $5x10^5$ (Granocel-500) and $3x10^5$ (Granocel-300) for dextrans were used at the present work.

The cross-linking of cellulose matrixes was performed by reaction with epichlorohydrin in 1M NaOH at 50°C for 2h.

Coupling of phenyl glycidyl ether

30 g of suction dried cellulose matrix was suspended in 37 ml of 1 M NaOH and 0.76-4.0 ml of phenyl glycidyl ether was added. The slurry was stirred

at 60°C for 5 h. The product was washed thoroughly with acetone and distilled water.

Determination of the degree of substitution

The dried gel (50 mg) was hydrolyzed at room temperature with 2 ml of 9.2 M H_2SO_4 for 15 h. The hydrolyzed gel was diluted to 50 ml with water and the absorbance at 270.5 nm was registered. The absorbance of acid hydrolysed unsubstituted gel is approx. 0.

The solutions of phenol in the concentration range 0.1-1 mM in 0.37 M H_2SO_4 were used for the determination of the molar absorbtivity (ε). The molar absorbtivity 1.18×10^3 M^{-1} cm^{-1} was used in the calculation of the degree of substitution.

Evaluation of relative hydrophobicity and adsorption capacity of adsorbents

The chromatography of model proteins for evaluation of relative hydrophobicity of adsorbents was performed. A 15 x 1 cm I.D. glass column was packed with the gel to obtain a bed volume of 7 ml. The column was equilibrated with 0.05 M sodium phosphate buffer (pH 7.0) containing 1.5 M $(NH_4)_2SO_4$. The column was charged with 50 mg a protein dissolved in 5 ml of the salt-buffer solution at a flow rate of 30 cm/h and washed with 2 bed volumes of the same buffer. The experiments were performed at a room temperature. The adsorbed protein was eluted from the column in two desorption steps: the first with 2-3 bed volumes of 0.05 M sodium phosphate buffer (pH 7.0), and the second with 2-3 bed volumes of 30% (v/v) isopropanol in the same buffer. The concentration of eluted protein in each fraction was determined according to Bradford[1]. The relative hydrophobicity of adsorbents was expressed by the amount of protein desorbed from the column on the first step of elution, calculated relatively to the total amount of adsorbed protein. Elution with 30% (v/v) isopropanol was found to be sufficient to regenerate the adsorbents.

The same chromatographic process for evaluation of adsorption capacity of packings was performed except that the bed volume of column was 2 ml and only one step elution with 30% isopropanol was used. The adsorption capacity was calculated from the amount of protein adsorbed on the column.

Evaluation of porous structure of adsorbents by inverse gel permeation chromatography

Gel filtration of dextran standards in 10 % (v/v) ethanol solution was performed to evaluate the porous structure of adsorbents. The solutions of

dextrans (2.0 mg/ml) were applied to the column (30 x 0.6 cm I.D.) at a flow rate of 25 cm/h. The results were interpreted according to the method described by A.A. Gorbunov et al[2].

Chromatography of α_2-interferon

HIC of α_2-interferon was performed using solutions after cell disruption, centrifugation and precipitation by ammonium sulfate. Precipitated proteins were dissolved in 0.025 M phosphate buffer, pH 7.2. The conductivity of the solutions was adjusted to 90-100 $mScm^{-1}$ with sodium chloride. The solutions of α_2-interferon were prepared in such way that protein concentration was 2 mg/ml (determined according to Lowry[3]) and interferon activity by ELISA - $3x10^7$ IU/ml. The quantity of α_2 -interferon loaded on adsorbent was $2x10^8$ IU for 1 ml of adsorbent. Equilibration of column, load of the sample and desorption were performed at 50 cm/h. The column was equilibrated and washed after adsorption with 6-8 bed volumes of 0.025 M phosphate buffer, pH 7.2, with 3 M sodium chloride (conductivity - 110 $mScm^{-1}$). α_2-interferon was desorbed step wise with 9-13 bed volumes of 0.025 M phosphate buffer, pH 7.2, without sodium chloride. After desorption adsorbent was regenerated without repacking it in the column with 0.1 M NaOH solution. The solution was put into the column from the bottom to the top at a flow rate of 80 cm/h.

The adsorbent of 10-12 ml, packed in 1 cm I.D. column was used for analytical purposes. The column K45/100 or BP/113 of 2-5 l of adsorbent was used for large scale process.

The recovery of α_2-interferon was calculated from the quantity of α_2-interferon remained in the column after washing.

RESULTS AND DISCUSSION

Synthesis of adsorbents

At a present work synthesis of hydrophobic adsorbents was performed by coupling of phenyl glycidyl ether (PGE) on the cellulose matrix in the 1M NaOH solution. The routine procedure was described above, the results obtained are shown in Fig.1.

A linear relationship and a high reproducibility is observed for the ligand concentration up to 600 μmol/g dry gel. So, in this range of the degree of substitution an uniform distribution of the ligands on the gel may be expected[4,5].

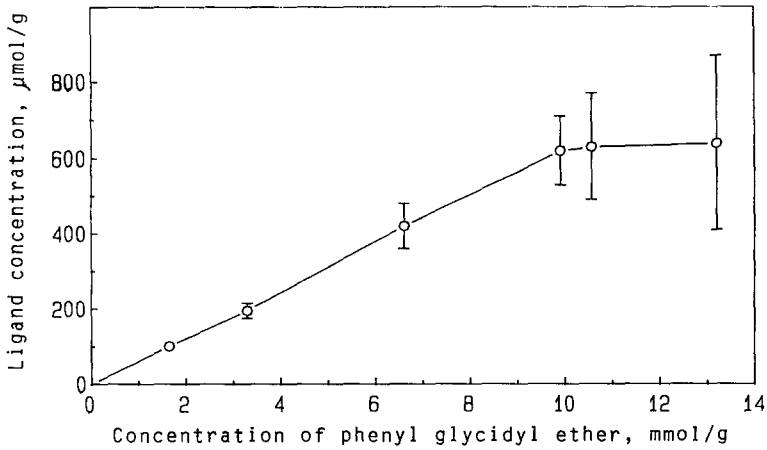

FIGURE 1

Relationship between the amount of phenyl glycidyl ether used during coupling and the degree of substitution. Each synthesis was performed at least five times and standard deviation was calculated (vertical bars).

When the amount of added PGE was more than 10 µmol/g dry matrix, the ligand concentration up to 800 µmol/g was achieved, but the reproducibility of process was unsatisfactory. The same degree of substitution was obtained when matrixes Granocel-2000 and Granocel-300 were used (data not shown).

The coupling yield was found to be low (approx. 6 %). This may be a direct consequence of the low solubility of PGE in the aqueous alkali medium and hydrolysis of oxirane groups in the presence of water. It was found that this serious disadvantage of the method cannot be diminished by the optimization of the synthetic route. It should be emphasized, however, that synthesis of commercially available Phenyl-Sepharose CL-4B, performed in a essentially water-free medium gives a coupling yield at best 50 % and includes tedious procedure of exchanging of the water with an organic solvent[6]. The methods of synthesis of hydrophobic adsorbents suggested later[5,7] are complicated enough too, because the step of activation of the matrix is required.

The flow-resistance of Phenyl-Granocel, synthesized on various matrixes, and Phenyl-Sepharose CL-4B for comparison is shown in Fig. 2. The results

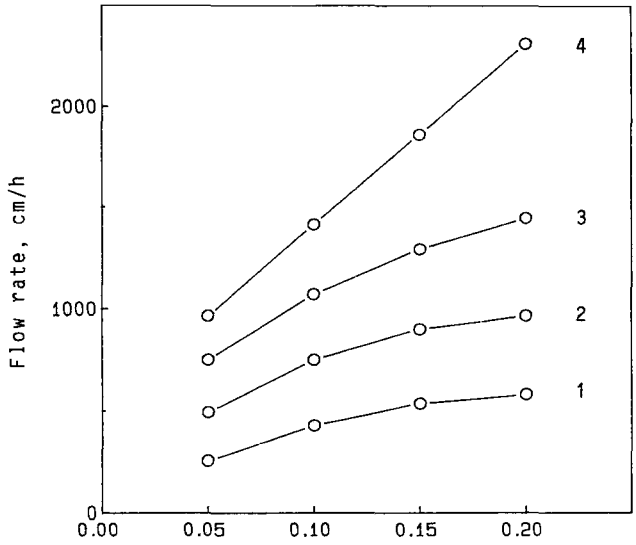

FIGURE 2

Flow characteristics of hydrophobic adsorbents. 1 - Phenyl-Sepharose CL-4B, 2 - Phenyl-Granocel-2000, 3 - Phenyl-Granocel-500, 4 - Phenyl-Granocel-300.

suggest that new-synthesized adsorbents are suitable for low pressure chromatography.

Relative hydrophobicity of Phenyl-Granocel

It is well known, that extent and strength of binding of proteins by the hydrophobic adsorbents depend on the concentration of hydrophobic ligand and therefore the degree of substitution is widely used as a criterion of the hydrophobicity of adsorbents. But there are more factors, such as chemical and physical structure of matrix, an accessibility of ligands to the sorbate molecules, that also affect the strength of hydrophobic interaction. Thus, if the different matrix and (or) different synthetic routes are used, the nearly equally substituted with the same ligand adsorbents may significantly differ in ability to bind proteins.

More criteria are suggested for evaluation of the hydrophobicity of adsorbents: the sorption capacity of hydrophobic probes[8], the partition coefficient

of the amphiphilic probe in the system amphiphilic sorbent-water[9], the energy of interaction between the methylene group and the surface of an adsorbent[10] and some others. However, the evaluation of these criteria is rather tedious.

One of the features of the hydrophobic interaction is that the proteins adsorbed to highly substituted gels are more difficult to elute than they are from gels with lower degrees of substitution[5,11]. This means, that the "difficulty" of elution of model proteins reflects the strength of their binding and so, the amount of eluted protein may be used for comparison of hydrophobicity of various adsorbents.

At the present work the BSA, HSA and Hb were used as a model sorbates. The proteins were adsorbed and eluted at a fixed set of conditions (see Experimental) and the relative amount of bound protein displaced by eluent was determined. Although the values obtained for different proteins differ and depend on the hydrophobicity of protein used (see Table 1), but the same general trend is observed, i.e. the higher ligand concentration leads to higher strength of binding of proteins (the amount of eluted protein decreases). Secondly, it is seen from data in the Table 1, that the protein binding strength depends on the peculiarities of the matrix of adsorbent. Note, that the values of the amount of eluted protein obtained for the adsorbents synthesized on the matrix cross-linked with epichlorohydrin (Phenyl-Granocel CL-500) are appreciably higher than those obtained for the noncross-linked adsorbents with the same ligand concentration and nearly equal pore size. The similar results were obtained on the adsorbents, cross-linked with glutaraldehyde and 2,3-dibrompropanol-1 (data not shown). This decrease of relative hydrophobicity of adsorbents is probably caused by the decrease of accessibility of hydrophobic ligands as a result of cross-linking.

For comparison the results obtained on Phenyl-Sepharose CL-4B and Phenyl-Silochrom C-80 are also given. It is evident, that the relative hydrophobicity of Phenyl-Granocel-500 with substitution degree of 440 μmol/g is very similar to that of Phenyl-Sepharose CL-4B. The relative hydrophobicity of Phenyl-Silochrome C-80 was found to be significantly higher, probably due to the hydrophobic nature of the matrix. So, this rapid and simple test may be used for the qualitative comparison of various commercial and new-synthesized hydrophobic adsorbents.

The dependence of adsorption capacity and the strength of binding of HSA upon the ligand concentration of Phenyl-Granocel-500 is shown in Fig.3.

TABLE 1

Model proteins elution from hydrophobic adsorbents

Adsorbent	Ligand concentration		Relative amount of bound protein displaced by eluent*, %		
	μmol/g	μmol/ml	BSA	HSA	Hb
1. Phenyl-Granocel-500	200	19	98	96	82
2. Phenyl-Granocel-500	380	36	95	82	68
3. Phenyl-Granocel-500	450	44	85	75	53
4. Phenyl-Granocel-500	800	81	79	60	10
5. Phenyl-Granocel CL-500	410	40	97	90	78
6. Phenyl-Granocel CL-500	700	70	95	85	70
7. Phenyl-Sepharose CL-4B	860	40	83	71	56
8. Phenyl-Silochrom C-80	82	27	55	45	36

* 0.05 M sodium phosphate buffer, pH 7.0

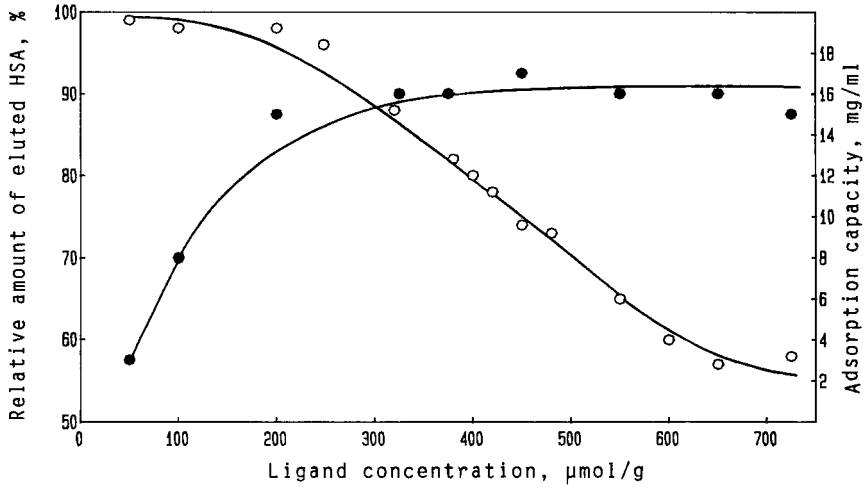

FIGURE 3

Influence of ligand concentration on adsorption capacity (●) of Phenyl-Granocel-500 and relative amount of eluted HSA (o).

It can be seen that the binding capacity is directly proportional to ligand concentration up to 200 μmol/g. At higher ligand concentrations the extent of binding is constant and equal to approx. 16 mg/ml. Otherwise, the relative amount of eluted HSA decreases gradually with increase of ligand concentration within the range of 200-600 μmol/g. So, the dependence of relative hydrophobicity of Phenyl-Granocel on the ligand concentration may be defined exactly in the conditions used.

Chromatography of α_2-interferon on Phenyl-Granocel

In order to study the chromatographic behaviour of synthesized adsorbents and their suitability for use in large scale processes, chromatography of α_2-interferon on the series of Phenyl-Granocel was performed. HIC on Phenyl-Silochrom C-80 or on Phenyl-Sepharose CL-4B is used as one of the first chromatographic step in the scheme of the purification of recombinant α_2-interferon from Pseudomonas putida VG-84[12]. Protein sorption is performed in 0.025 M phosphate buffer solution (pH 7,2) in the presence of 3 M NaCl and the same buffer without salt is used for elution. It should be noted, that the optimum conditions for the use of Phenyl-Granocel were not determined in the present work - both adsorption and elution were performed under condition identical to those in large scale process on Phenyl-Silochrom C-80 or Phenyl-Sepharose CL-4B.

The characteristics of adsorbents used and data of chromatography of α_2-interferon are summarized in Table 2.

As expected, the chromatographic performance is sensitive to relative hydrophobicity of adsorbents. Using columns packed with the samples 1 and 8, which exhibit low relative hydrophobicity, the significant amount of α_2-interferon was detected in unadsorbed fractions. In addition, due to low binding strength of adsorbed protein on these adsorbents, part of adsorbed α_2-interferon was lost by the subsequent washing of the column, therefore the values of recovery are low. The low recovery from the sample 6 with high relative hydrophobicity is obtained too. But in this case the binding strength is seen to be too high to elute the protein with buffer solution. This would suggest, that desorption of α_2-interferon from such adsorbent requires more strong eluent.The influence of pore size of adsorbents on binding capacity of the column was found to be little, except when the adsorbent with average pore diameter of 17 nm,

TABLE 2

Chromatography of α_2-interferon on hydrophobic adsorbents

Adsorbent	Characteristics of adsorbents					Chromatography of α_2-interferon		
	Pore size, nm	Poli-disper-sity	Ligand concen-tration, μmol/g	Relative amount of eluted HSA, %	Adsorption capacity for HSA, mg/ml	Unadsorbed interferon, %	Recovery of inter-feron, %	Enrich-ment, fold
1. Phenyl-Granocel-500	25	1.0	380	82	16	48	44	1.0
2. Phenyl-Granocel-2000	32	1.1	420	78	18	3	79	2.8
3. Phenyl-Granocel-500	23	1.0	450	74	17	7	99	3.2
4. Phenyl-Granocel-500	23	1.0	550	65	16	2	83	3.3
						9	75	3.5*
5. Phenyl-Granocel-2000	28	1.0	600	60	18	0	90	3.8
6. Phenyl-Granocel-500	22	1.0	720	60	15	1	67	3.1
7. Phenyl-Granocel-500	17	1.0	830	55	5	25	79	3.1
8. Phenyl-Granocel CL-2000	28	1.0	680	85	16	28	55	1.8
9. Phenyl-Sepharose CL-4B	-	-	860	83	20	0	96	3.6
10. Phenyl-Silochrom C-80	60	-	82	45	21	2	67	3.2

* The results are obtained in large-scale process after three times of regeneration in column

see sample 7, was used. The adsorption capacities for α_2-interferon and for HSA on sample 7 are significantly lower that these on the other samples despite higher ligand concentration. Results suggest that pore size of 17 nm is not enough to penetrate proteins used.

The enrichment of α_2-interferon in large scale was performed in the column K 45/100 on Phenyl-Granocel-500 (ligand concentration 560 μmol/g). Adsorbent volume - 2.5 l, flow rate 30-60 cm/h. A 3.5-fold enrichment with good recovery was achieved (Table 2). The results obtained are similar to these in laboratory scale.

The gels did not compress noticeably, when packed in the column and used over several runs. The adsorbents have also shown a high resistance to physical attrition which means that production of fines does not occur on repeated handling or mechanical stirring. Consequently the adsorbents can be re-used many times. Another advantage observed for the Phenyl-Granocel adsorbents was their excellent resistance to 1M sodium hydroxide solutions. This feature enables to perform the regeneration process with sodium hydroxide solutions in column without loss in adsorption capacity, recovery and degree of enrichment of α_2-interferon.

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PRE-TREATMENT OF CHIRAL α-AGP COLUMN WITH TRIETHYLAMINE SIGNIFICANTLY IMPROVES THE DETECTION SENSITIVITY OF AN ENANTIOMERIC LEUKOTRIENE ANTAGONIST

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ABSTRACT

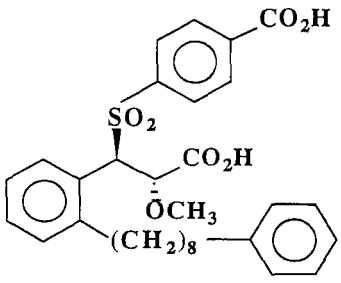
The peptidoleukotriene antagonist, SK&F 107310 and its optical antipode, can be effectively separated on a chiral α-AGP column. Pre-treatment of the chiral α-AGP column with 0.1% v/v triethylamine in the mobile phase significantly improves the peak shape and detection sensitivity of the undesired enantiomer. Chromatography with the modifier in the mobile phase resulted in elution of the drug substance without retention at the solvent front. Without column pre-treatment, only a broad tailing peak was obtained for enantiomer **2**, making low detection of it unattainable.

INTRODUCTION

Leukotriene D_4, (LTD_4), formed from the metabolism of arachidonic acid by 5'-lipoxygenase, is considered an important receptor-mediator of human

bronchial asthma (1). Considerable efforts are ongoing in the pharmaceutical industry in pursuing specific leukotriene LTD_4 receptor antagonists as potential therapeutics for the treatment of asthma (2).

(R-(R*,S*))-ß-(4(carboxyphenyl)sulfonyl)-α-methoxy-2-(8-phenyloctyl)-benzenepropanoic acid (SK&F 107310) (**1**) is a potent and selective LTD_4 antagonist (3). The molecule has two chiral centers and therefore, four possible optical isomers. In order to evaluate the pharmacological profile of the single enantiomer **1**, we required an enantiospecific HPLC assay method to determine its chiral purity. Although **1** is structurally related to SK&F 106203 (4), our attempts to effect similar enantiomeric separation on all cellulosic columns were not successful. In this communication, we describe an enantiospecific HPLC method for the direct separation of SK&F 107310 (**1**) and its enantiomer **2** on a chiral α-AGP column, and report on a simple column pre-treatment technique that significantly improves the peak shape and, consequently, detection sensitivity of the enantiomer **2**. Without the column pre-treatment, only a broad triangular peak was obtained for the enantiomer **2**, making low detection of it unattainable.



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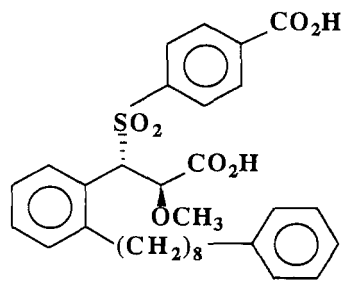

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MATERIALS AND METHODS

The liquid chromatograph system consisted of a Beckman Gold HPLC pump connected to an HP 1050 series autosampler and variable wavelength UV detector. A 4 x 100 mm second generation Chiral α-AGP column (Chrom Tech, obtained through Advanced Separation Technologies, Whippany, NJ) was used. Data was acquired and processed using a WATERS 860 Networking Computer System. All experiments were performed at ambient temperature.

Chemicals

Ethanol (200 Proof dehydrated alcohol, USP Punctilious grade) was obtained from Quantum Chemical Corporation (Newark, N. J.). HPLC water was obtained from J. T. Baker (Phillipsburg, N. J.) and anhydrous HPLC grade potassium dihydrogen phosphate was obtained from Fisher Scientific (Fair Lawn, N.J.). Triethylamine (99+% purity) was obtained from Aldrich (Milwaukee, WI).

SK&F 107310 (**1**) and the free diacid of its optical antipode (**2**) were synthesized in-house in the Department of Medicinal Chemistry. Both compounds were fully characterized by NMR, IR, MS, rotational measurements, elemental analysis and impurity profile by HPLC.

Column Pre-treatment

The mobile phase for the pre-treatment of the chiral α-AGP column consisted of a mixture of 40:60:0.1-ethanol: 20 mM potassium dihydrogen

phosphate buffer (pH 4.6 with no pH adjustment): triethylamine. The flow rate was 0.5 mL/min. The column was washed with this mobile phase for about 40 minutes, and then equilibrated with the assay mobile phase (see below) until a stable base line and constant retention time of **1** was obtained.

Chiral HPLC Assay

The mobile phase for the chiral HPLC assay consisted of a mixture of 40:60 ethanol: 20 mM potassium dihydrogen phosphate buffer (pH 4.6 with no pH adjustment) for Figures 1 and 2. This was changed to 37:63-ethanol: 20 mM potassium dihydrogen phosphate buffer (pH 4.6 with no pH adjustment) for Figure 3. This 37:63 ratio of ethanol:20 mM potassium dihydrogen

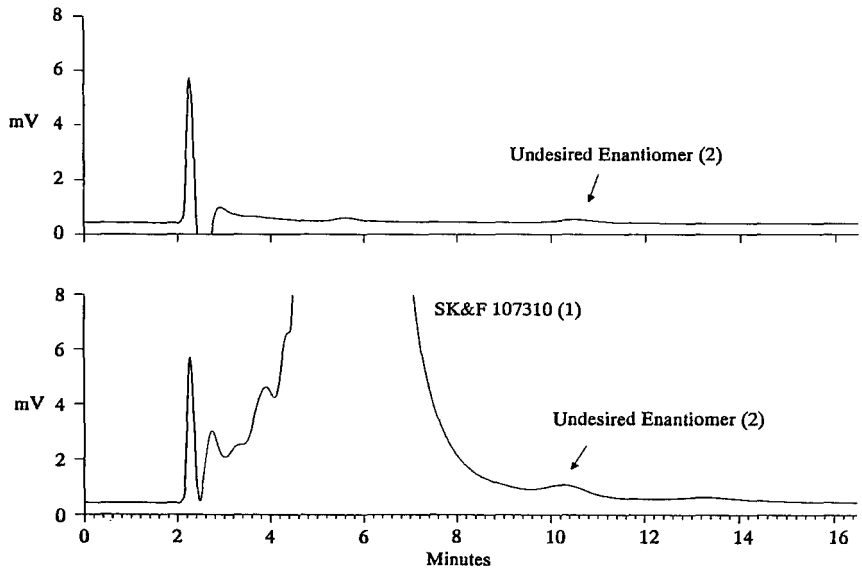

FIGURE 1. Chiral HPLC chromatogram on a brand new untreated Chiral α-AGP column. Top trace: 0.002 mg/ml **2**; Bottom trace: 0.002 mg/ml **2** spiked into 0.53 mg/ml **1** [0.4% w/w spike].

phosphate was also used for the buffer system at different pH (see Table 1), and with the addition of 1.2 mM to 12 mM of N, N-dioctylamine. The flow rate was 0.5 mL/min. UV detection was performed at 215 nm. The injection size was 25 µL unless otherwise indicated in the figure legend. For sample concentration, see figure legends.

Post-assay column care

The dihydrogen phosphate buffer in the chiral α-AGP column was purged with a mobile phase mixture consisting of 37:63-ethanol:water for about 40 minutes. The flow rate was 0.5 mL/min. The column was then stored in a refrigerator.

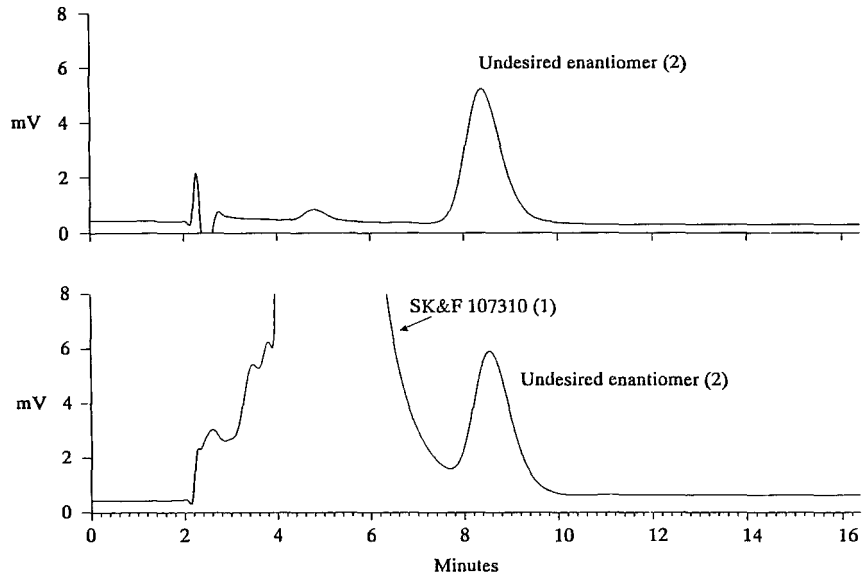

FIGURE 2. Chiral HPLC chromatogram on a pre-treated Chiral α-AGP column of identical sample solution as in Figure 2. Top trace: 0.002 mg/ml **2**; Bottom trace: 0.002 mg/ml **2** spiked into 0.53 mg/ml **1** [0.4% w/w spike].

RESULTS AND DISCUSSION

Protein-based chiral stationary phases have been found to be useful for the chromatographic resolution of a wide range of enantiomeric compounds (5). Good separation of the two optical antipodes **1** and **2** was also obtained with a second generation α-AGP column. However, very broad peak shape for the enantiomer **2** was obtained such that our required low detection of it at 0.5% w/w or lower in SK&F 107310 drug substance was not possible. The very broad peak shape for **2** was not a result of over-loading. At low concentration, the peak shape for **2** was also very broad that only an inflection of base line was observed (see Figure 1). Attempts at various pH adjustments of the mobile phase did not improve the chromatography.

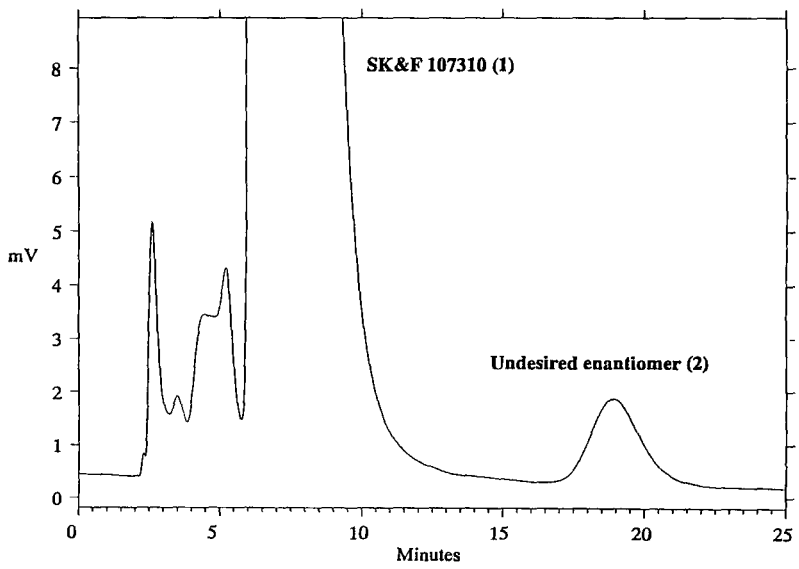

FIGURE 3. Optimized Chiral HPLC chromatogram on a pre-treated Chiral α-AGP column. Spiked at 0.4% w/w level : 0.002 mg/mL **2** spiked into 0.53 mg/mL **1**.

Since α_1-acid glycoprotein (orosomucoid) is known to contain large amounts of neuraminic acid that are located at the very end of the outer chain moiety of the sugar chains (6), we speculated that the broad tailing we observed for **2** may be a result of either residual column silanol effect or perhaps non-specific interaction between the analyte and the readily accessible neuraminic acid of the α_1-acid glycoprotein. We were further encouraged by the report that, although the carbohydrate chain is required for chiral recognition, neuraminic acids of ovomucoid in ovomucoid columns participate in a non-specific retention of acidic residues without affecting chiral resolution of, for example, ketoprofen (7). Accordingly, we attempted modification of the chiral α-AGP column by initially passing through the column for about 40 minutes an eluent that contained an additional 0.1% by volume of triethylamine. After due equilibration with the assay mobile phase (which does not contain triethylamine), the column was then used for analysis. Figure 1 shows the chromatography of a solution of the undesired enantiomer **2** at a concentration that would be equivalent to 0.4% w/w of **2** in **1** (bottom trace), and a 0.4% w/w spike of **2** (same concentration) in **1** (top trace) on an untreated chiral α-AGP column. Figure 2 shows the identical solutions injected after the aforementioned pre-treatment of the same column and analysis after due equilibration. As shown in Figures 1 and 2, the column pre-treatment with triethylamine significantly sharpens the peak shape and, consequently, lowers the detection limit of the undesired enantiomer **2**. The separation can be further optimized by reducing the amount of ethanol in the mobile phase from 40 to 37 %. This is shown in Figure 3. The tailing factor for the enantiomer **2** in this chromatogram is 1.2. Chromatography of the racemic mixture with a mobile phase containing 0.1% v/v triethylamine resulted in elution of the racemic mixture without retention at the solvent front. Similar addition of N,N-dimethyloctylamine in the

range 1.2 mM to 12 mM to the mobile phase (8), also resulted in the elution of **1** and **2** without retention at the solvent front.

We have repeated the same study with both a brand new chiral α-AGP column ourselves, and independently with a separate chiral α-AGP column by another analyst using radiolabelled **1**, and verified the technique to be indeed reproducible. The chiral α-AGP column, once pre-treated, can be stored in a refrigerator for months, and then reused without additional treatment. Indeed, we have validated the method on a pre-treated column that has been used for many prior assays and then stored in a refrigerator for months. The method is precise (% RSD 0.04 for **1** and 0.9% for **2** for twelve replicates), and is linear from 0.1 to 4 % w/w of the undesired enantiomer **2** in **1**. The detection limit is 0.1% w/w of the undesired enantiomer **2** in **1**.

The influence of eluent pH on retention and enantioselectiviety of **1** and **2** is shown in Table 1. As with other acidic solutes on untreated α-AGP column (8), the retentions of both **1** and **2** increase with decreasing pH. Changes in eluent pH also significantly affect enantioseparation and peak shape. Optimum retention, peak shape and separation were obtained at pH 4.6. At pH 3.0, although well separated from each other, **2** had an unacceptable retention time of 94 minute and a peak width of about 28 minutes. At pH 6.0, both **1** and **2** eluted without retention at the solvent front. Less than base-line separation was obatined at pH 5.0. Since the pH of the buffer solution containing 0.1% v/v of ethylamine, and 12 mM of N,N-dimethyloctylamine are 6.7 and 7.2, respectively, the elutions of **1** and **2** without retention at the solvent front are therefore most likely, a result of pH effect.

TABLE 1.

INFLUENCE OF ELUENT[a] pH ON RETENTION AND ENANTIOSELECTIVITY OF ENANTIOMERS 1 AND 2 ON PRE-TREATED α-AGP COLUMN

	pH 3.0[b]				pH 4.0				pH 4.4			
Compound	**k'**	**N**	**R_S[c]**	**Tailing**	**k'**	**N**	**R_S**	**Tailing**	**k'**	**N**	**R_S**	**Tailing**
SK&F 107310 (**1**)	19.8	735		1.5	11.1	867		1.5	3.9	587		1.5
Enantiomer (**2**)	45.9	329	3.9	1.8	22.0	536	3.9	1.4	6.9	398	2.6	1.4

	pH 5.0			
Compound	**k'**	**N**	**R_S**	**Tailing**
SK&F 107310 (**1**)	0.8	579		-
Enantiomer (**2**)	1.1	344	0.7	-

a 20mM potassium dihydrogen phosphate buffer (see Table for pH)-ethanol (63:37 v/v) was used as the eluent. A racemic mixture of **1** and **2** at a concentration of about 0.1 mg/mL was used. The injection size was 5 µL.

b Buffer pH adjusted with either phosphoric acid or sodium hydroxide.

c Resolution factor = $\frac{2 \times [\text{difference of retention times of (+) and (-) isomers}]}{[\text{Bandwidths of the two peaks}]}$

The precise mechanism leading to the sharpening of the peak shape and, consequently, improvements in detection sensitivity that we have observed in the present work, and whether this pre-treatment technique led to a "unique" column, is not clear. Although some applications for acidic compounds, e.g., fenoprofen and naproxen (8), ibuprofen (9, 10) and phenylbutyric acids (10) have been reported, chiral α-AGP column primarily has been used to resolve basic or cationic enantiomers (11-15). The separation factor for enantiomers of ibuprofen on chiral α-AGP column has been reported to increase with increasing concentration of (-) tetrolidine (16), and dimethyloctylamine (8), indicating the distribution of solutes as ion-pairs on the stationary phase. The addition of 1.2 mM dimethyloctylamine to the mobile phase has also been reported to improve the separation and peak shape of ibuprofen enantiomers on chiral α-AGP column (9). Other optimization of detection sensitivity on chiral α-AGP column has included gradient elution with increasing acetonitrile content in the mobile phase, as well as by pH gradient (17). Further work to determine the generality, as well as to probe the mechanistic aspect of our pre-treatment technique, is underway, and the results will be reported in a future communication.

CONCLUSIONS

SK&F 107310 and its enantiomer can be effectively separated on a chiral α-AGP column. Pre-treatment of the chiral α-AGP with triethylamine significantly improves the peak shape and detection sensitivity of the undesired enantiomer. Without column pre-treatment, only a broad tailing peak was obtained for the undesired enantiomer, making low detection of it unattainable.

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ELECTROSPRAY IONIZATION MASS SPECTROMETRY AND HPLC DETERMINATION OF THE PRODUCTS IN THE RADIOLYSIS OF 5-BROMOURACIL, ITS NUCLEOSIDE AND NUCLEOTIDE DERIVATIVES

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ABSTRACT

Radiolysis of 5-bromouracil (BrUr), 5-bromo-2'-deoxyuridine (BrdU) and 5-bromo-2'-deoxyuridine-5'-monophosphate (BrdUMP) has been studied by high performance liquid chromatography and electrospray ionization mass spectrometry. Production of bromide ions and the complementary uracil, 2-deoxyuridine and 2-deoxyuridine-5'-monophosphate has been quantitatively examined. It is concluded that in the presence of hydroxyl radical scavenger, at pH 6 to 10, BrUr, BrdU and BrdUMP quantitatively react with hydrated electron. In the absence of hydroxyl radical (·OH) scavenger in N_2-saturated solutions the yield of bromide ions is about 5.0, indicating that hydroxyl radicals also significantly react with BrUr, BrdU and BrdUMP to eliminate hydrogen bromide. Results from the radiolysis of 1 mM aqueous solutions of BrdU and BrdUMP suggested that abstraction of hydrogen by uracilyl radicals from the sugar moieties is not important. Our mass spectrometry experiments indicated that uracilyl radicals react with the starting substrate to form dimers. The radiolysis of N_2-saturated solutions of nucleosides and nucleotides suggested that the base radicals formed by ·OH addition can react with undamaged reagents to produce dimers.

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INTRODUCTION

Applications of 5-halopyrimidines as radiation sensitizers have been the subject of a large number of investigations because these halopyrimidines are useful tools for studying radiation effects from the model system to whole cells. [1], [2] 5-Bromouracil (BrUr) has been used to localize the site of radiation damage in radiobiological studies by selective attack of hydrated electron (e_{aq}^-) on DNA where it had replaced thymine. [1] In DNA, BrUr can be easily substituted for thymine. Cells (bacterial and mammalian) containing BrUr instead of thymine were deactivated by X-rays considerably faster than those of a control. [3] A similar effect was also observed if BrUr-containing cells were subjected to UV light. [3] In recent years BrUr has been used in clinic. [4], [5] A linear relationship between the cellular radiosensitization factor and the percentage thymidine replacement by 5-bromo-2'-deoxyuridine (BrdU) in human bone marrow cell line was reported. [6]

It is known that the attack of e_{aq}^- on BrUr causes elimination of bromide ion and produces uracilyl radical (Ur·). [1], [7], [8] In the presence of a source of abstractable hydrogen, the uracilyl radical is expected to react to produce uracil (Ur). [1], [9] Improvements in the separation and sensitivity of high performance liquid chromatography (HPLC) methods since the earlier studies now make it possible to investigate the radiolysis of BrUr, and its nucleoside and nucleotide derivatives in more detail. The effort is to provide fundamental data for further exploring the mechanism involved in radiosensitization of DNA by BrdU. We report here the quantitative determination of bromide ion and complementary uracil, 2-deoxyuridine (dU), 2-deoxyuridine-5'-monophosphate (dUMP), produced in the x-ray irradiation of BrUr, BrdU and BrdUMP by HPLC and ion chromatography. Using electrospray ionization mass spectrometry, the products formed in the ^{60}Co γ-radiolysis were identified, which provided valuable information about the reaction mechanisms involved.

MATERIALS AND METHODS

Irradiation was carried out with a Norelco MG 300 X-ray unit (Philips Electronic Instruments) at absorbed dose rates of 1 Gy min^{-1}. Gamma radiolysis was carried out with ^{60}Co γ-ray source at absorbed dose rates of 4 $x10^2$ Gy min^{-1}.

HPLC analysis of bromide, Ur, dU and dUMP The principal components of the HPLC apparatus are a Waters 6000A pump, Waters U6K injector, an optical and a conductivity detector. For detection of bromide ions, a VYDAC anion chromatography column and Waters 431 conductivity detector were used. Complementary Ur, dU and dUMP were determined using Beckman Ultrasphere ODS Column, VYDAC oligonucleotide column, and Linear 206 PHD optical detector. All quantitative results of HPLC were from comparison of peak area with reference samples run under identical chromatographic conditions.

Mass spectrometry analysis of adducts Products formed in the γ-ray radiolysis of the aqueous solutions of BrUr, BrdU and BrdUMP were analyzed by electrospray ionization mass spectrometry (ESI-MS). The instrumentation used in this study and typical operating conditions have been previously described. [10], [11] Electrospray ion production requires two steps: dispersal of highly charged droplets at near atmospheric pressure, followed by conditions resulting in droplet evaporation. A potential difference of 3-6 kV is applied between the capillary and counter electrode located 0.3-2 cm away, producing highly charged liquid droplets of ~ 1 μm diameter. [12] Positively or negatively charged droplets can be produced depending upon the capillary bias. The negative ion mode requires the presence of an electron scavenger such as oxygen to inhibit electrical discharge. [13] The ESE source is mounted 1.5 cm from the entrance of the quadrupole MS. Highly charged ions are sampled through a 1-mm nozzle orifice and 2-mm skimmer and are efficiently transported through a cryopumped region by the radio frequency (rf)

quadrupole lens to a quadrupole mass spectrometer for detection. The mass spectrometer (Extrel Co., Pittsburgh, PA) used for these studies has an effective m/z range of 1700. Scan rates varied between 30 and 2 min over the entire m/z range. With the current data system (Teknivent, St. Louis, MO), peak abundances are collected at integer m/z values. Calibration of the m/z scale for ESE-MS was performed with 5-bromo-2'-deoxyuridine.

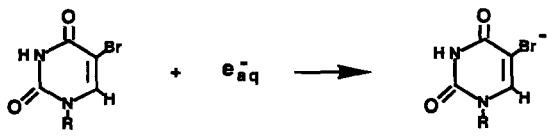
Solutions were prepared in triply distilled water purified by a Millipore Mill-Q Reagent system. The pH was adjusted by addition of Backer Analysis KOH and determined with a Corning 610A pH meter calibrated with VWR buffers. BrUr, BrdU and BrdUMP were from Sigma, 2-propanol, tert-butyl alcohol (t-BuOH) and NaOH from Fisher. The chemicals used in the mobile phases of HPLC were methanol from Burdick & Jackson, acetic acid from Fisher, Na_2HPO_4 from Aldrich and borate/gluconate eluent provided by Waters. Solutions were saturated with nitrogen to purge oxygen. Irradiated samples were covered with aluminum foil to avoid UV exposure and introduced into the HPLC system in a few minutes after completion of irradiation. All experiments were carried out at room temperature (22°C).

RESULTS AND DISCUSSIONS

HPLC Analysis

1. Production of Bromide Ions

It is known from studies of the radiation chemistry of BrUr that e_{aq}^- reacts rapidly with BrUr by dissociative electron capture to eliminate bromide ion and produce the uracilyl radical. [1], [7], [8]



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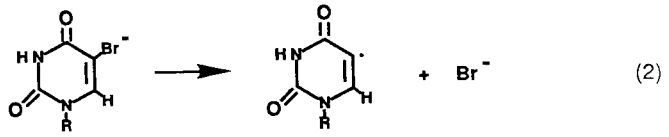


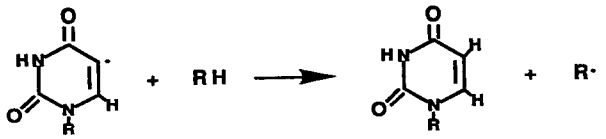
(2)

The production of bromide ion is linear with dose over the range 10 to 100 Gy. The bromide yields of 2.67, 2.71 and 2.45 were determined from the radiolysis of 1 mM BrUr, BrdU and BrdUMP solutions with 0.4 M 2-propanol saturated with N_2 (Table 1).

From the treatment of Balkas, Fendler and Schuler [14] one expects, taking the rate constants typically as 5 x 10^9 M^{-1} s^{-1}, that the radiation yield of products at millimolar solute concentrations should be ~ 2.7. The yields of bromide in BrUr and BrdU equal the yield of hydrated electrons. The yield of bromide ion in the case of BrdUMP is about 10% lower than that in BrUr and BrdU. Since phosphate group is not expected to reduce the electron affinity of the base moieties, [15] this yield-decreasing effect must be due to electrostatic repulsion between the negatively charged reactants, e_{aq}^- and BrdUMP. The chromatograms of bromide ions produced in the irradiation of 1 mM N_2-saturated solutions of BrdUMP with 0.4 M 2-propanol and t-BuOH are shown in Figures. 1a and 1b. Experiments were carried out in both neutral (pH ~6.4) and basic solutions (pH ~10).

2. Conversion of Complementary Uracil and its Derivative Radical

As shown in reactions 1 and 2, Ur· radical and its nucleoside derivative (dU·) and nucleotide derivative (dUMP·) are formed in the reaction of BrUr, BrdU and BrdUMP with e_{aq}^-. In the presence of a hydrogen donor, such as 2-propanol or t-BuOH, these radicals can be converted to Ur, dU and dUMP, i.e.,



(3)

TABLE 1

YIELD [1] OF BROMIDE IONS IN THE RADIOLYSIS OF BrUr, BrdU AND BrdUMP

Solute	pH	$G(Br^-)$	pH	$G(Br^-)$
1 mM BrUr, 2-propanol	6.6	2.67	10.1	5.07
1 mM BrUr, t-BuOH	6.3	2.65	10.2	2.69
0.1 mM BrUr, 2-propanol	6.4	2.20	10.4	3.50
0.1 mM BrUr, t-BuOH	6.3	2.17	10.1	2.20
1 mM BrdU, 2-propanol	6.2	2.71	10.2	5.02
1 mM BrdU, t-BuOH	6.3	2.62	10.3	2.58
1 mM BrdUMP, 2-propanol	6.7	2.45	10.2	4.98
1 mM BrdUMP, t-BuOH	6.5	2.67	10.4	2.40
.16 mM BrdUMP, 2-propanol	6.2	2.09	10.0	3.62
.16 mM BrdUMP, t-BuOH	6.6	2.00	10.1	2.05

1. Yields are given in units of G (molecules/100 eV). Averages of three experiments.

2. In 1 mM solution the concentration of 2-propanol or t-BuOH is 0.4 M. In 0.1 mM solution the concentration of 2-propanol or BuOH is 0.04 M. All solutions were saturated with N_2.

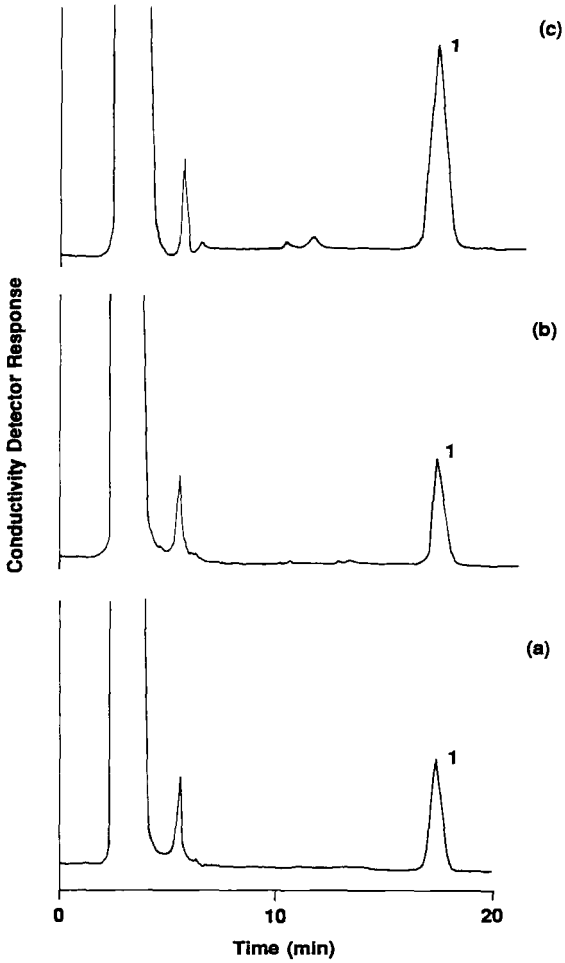


Figure 1 Chromatograms of Bromide Ions Observed in X-Ray Radiolysis of 1 mM BrdUMP Solutions in Neutral pH

(a) with 0.4 M 2-propanol; (b) with 0.4 M t-BuOH;
(c) without alcohol.
Irradiation dose: 20 Gray
Detector: Waters 431 Conductivity
Column: VYDAC oligonucleotide column
Mobil Phase: 0.03 M NaH_2PO_4 and 0.3% acetic acid.
Principle peak: (1) Br^-

The rate constant of hydrogen abstraction of Ur· from t-BuOH is 2.3 x 10^7 M^{-1} s^{-1}. [16] The yields of Ur, dU and dUMP are given in Table 2. The chromatograms of dUMP produced in the radiolysis are shown in Figure 2.

Mass Spectrometry Analysis

1. BrUr

In a pulse radiolysis study of BrUr, Edwin and Schuler [16] suggested that Ur· produced in reaction 1 subsequently reacts with the substrate to give a mixture of radical adducts.

$$Ur\cdot + BrUr \longrightarrow \text{adducts} \qquad (4)$$

Reaction 4 has a rate constant of $\geq 2 \times 10^8$ M^{-1} s^{-1}. [16] Apparently, Ur· can react with the starting BrUr to form a dimmer (BrHUr-Ur). Analysis of γ-radiolysis of 20 mM BrUr solution saturated with N_2 was performed using ESI-MS in positive ion mode. One adduct (BrHUr-Ur) was found in Figure 3 (peak B, m/z: 303, 305). Presumably, Ur· reacts with BrUr to first form a radical adduct that subsequently obtains an electron and then protontes to produce BrHUr-Ur. Radiation significantly increases the amount of non-covalent bond dimers of BrUr (peak C, m/z: 381, 383, 385) though these dimers exist in the control sample their concentration is much lower than that in the irradiated sample. The triplets in peak C are due to bromine isotope.

2. BrdU

Products formed in γ-radiolysis of 20 mM BrdU N_2-saturated solution were examined by ESE-MS in positive ion mode. Peaks B (m/z: 455), C (m/z: 535, 537), D (m/z: 551, 553) and E (m/z: 613, 615, 617) were found in the mass spectrum (Figure 4). Peaks B, C, D and E can be contributed to radiation products: dU-dU, BrHdU-dU,

Table 2

YIELD OF Ur, dU AND dUMP IN THE RADIOLYSIS OF BrUr, BrdU AND BrdUMP

Solute [1]	pH	G [2]	$G(Br^-)/G$	pH	G [2]
1 mM BrUr, 2-propanol	6.6	2.14	0.80	10.1	3.95
1 mM BrUr, t-BuOH	6.3	1.78	0.67	10.2	1.80
0.1 mM BrUr, 2-propanol	6.4	1.61	0.73	10.4	2.82
0.1 mM BrUr, t-BuOH	6.3	1.35	0.62	10.1	1.47
1 mM BrdU, 2-propanol	6.2	2.20	0.81	10.2	4.11
1 mM BrdU, t-BuOH	6.3	1.70	0.65	10.3	1.55
1 mM BrdUMP, 2-propanol	6.7	2.01	0.82	10.2	3.95
1 mM BrdUMP, t-BuOH	6.5	1.60	0.60	10.4	1.85
.16 mM BrdUMP, 2-propanol	6.2	1.67	0.80	10.0	2.82
.16 mM BrdUMP, t-BuOH	6.6	1.38	0.69	10.1	1.43

1. In 1 mM solution the concentration of 2-propanol or t-BuOH is 0.4 M. In 0.1 mM solution the concentration of 2-propanol or BuOH is 0.04 M. All solutions were saturated with N_2.

2. Yield of Ur, dU and dUMP.

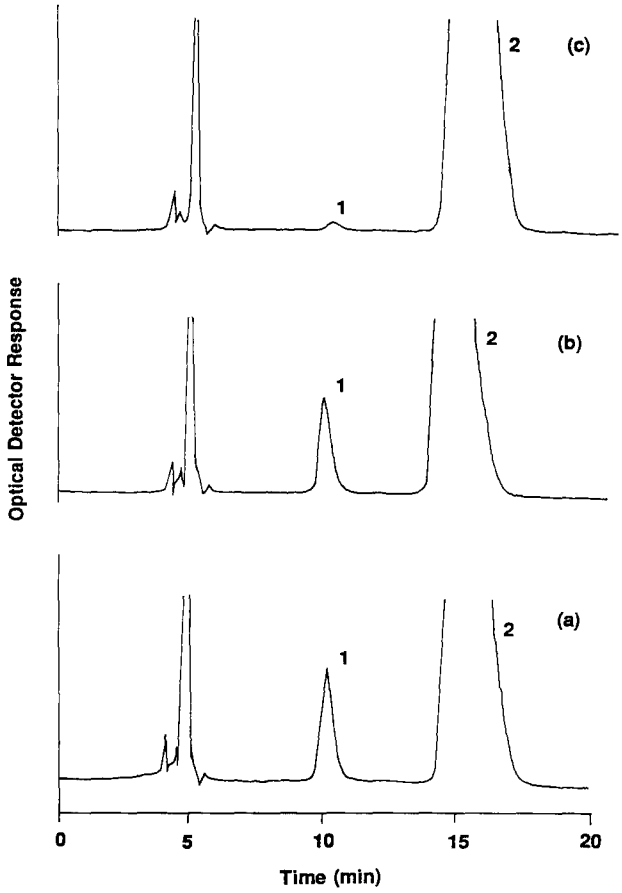


Figure 2 Chromatograms of dUMP Observed in X-Ray Radiolysis of 1 mM BrdUMP Solutions in Neutral pH

(a) with 0.4 M 2-propanol; (b) with 0.4 M t-BuOH;
(c) without alcohol.
Irradiation dose: 20 Gray
Detector: Linear 206 PHD optical at 210 nm
Column: VYDAC 304OL54
Mobil Phase: 0.4 M NaH_2PO_4 and 0.3% acetic acid
Principle peak: (1) dUMP; (2) BrdUMP

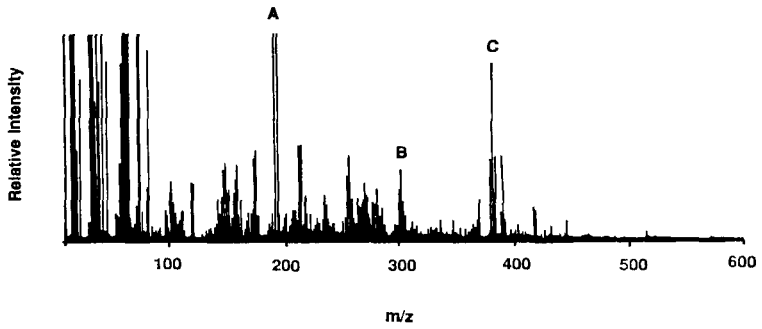
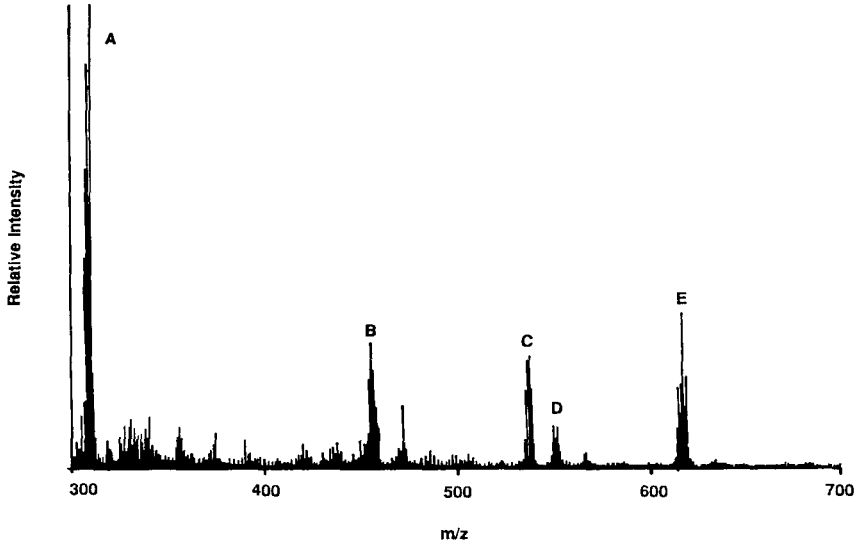


Figure 3 Mass Spectra of Radiolysis of BrUr

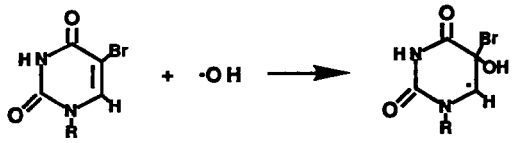
20 mM BrUr irradiated with ^{60}Co at dose 2 x 10^3 Gray.
Principle peaks: A: BrUr, B: BrHUr-Ur, C: $(BrUr)_2$



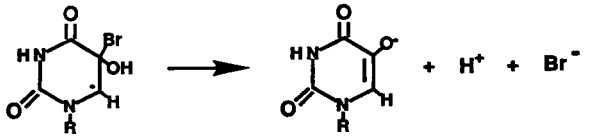
Figures 4 Mass Spectra of Radiolysis of BrdU

20 mM BrdU irradiated with ^{60}Co at dose 2 x 10^3 Gray.
Principle peaks: A: BrdU, B: dU-dU, C: BrHdU-dU
D: BrHdU-OdU, E: $(BrdU)_2$

BrHdU-OdU and non-covalent dimer ($(BrdU)_2$). In N_2 saturated solution, hydroxyl radicals were produced as well as hydrated electrons, and ·OH can react with BrUr, BrdU and BrdUMP rapidly. [17]

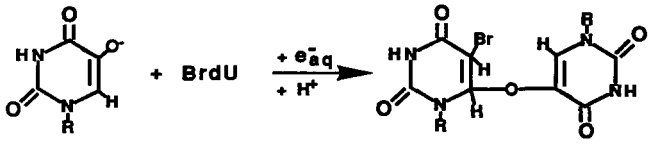


(5)



(6)

The radical produced by oxidative dehydrohalogenation of bromouracil (reaction 6) has been characterized by ESR experiments. [17] BrHdU-OdU must be produced by addition of ·OdU to the starting BrdU to form a radical adduct, dUO-BrdU· which is presumably reduced and protonated to form BrHdU-OdU.



(7)

Peak B can be contributed by the product of the combination reaction of dU·. In addition to radical combination product (dU-dU), radical adduct (BrHdU-OdU) and non-covalent dimer ($(BrdU)_2$), adduct (dU-BrHdU) was also produced in the radiation (peak C). The adduct (BrHdU-dU) must be formed in a similar reaction as in the case of BrUr as discussed above. The list of m/z of MH^+ is given in Table 3.

TABLE 3

m/z OF MH^+ AND M^- OF NUCLEOBASES, NUCLEOSIDES, NUCLEOTIDES AND THE PRODUCTS IN RADIOLYSIS

	m/z of MH^+	m/z of M^-
Br	80, 82	
Ur	113	
BrUr	191, 193	
dU	229	
BrdU	307, 309	
dUMP		307
BrdUMP		385, 387
BrHUr-Ur	303, 305	
$(BrUr)_2$	381, 383, 385	
BrHdU-dU	535, 537	
BrHdU-OdU	551, 553	
dU-dU	455	
$(BrdU)_2$	613, 615, 617	
dUMPO-BrHdUMP		355, 356
dUMPOH		323

3. BrdUMP

Products formed in γ-radiolysis of 20 mM BrdUMP aqueous solution saturated with N_2 were analyzed by ESE-MS in negative ion mode. Three major products, dUMP (peak A, m/z: 307), dUMPOH (peak B, m/z: 323) and dUMPO-BrHdUMP (peak C, m/z: 355, 356) were found in Figure 5. Production of dUMP in this experiment condition is expected. The concentration of BrdUMP is 20 times higher and the radiation dose is 10 times higher (200 x 10^3 rads) than that in HPLC experiments. dUMPOH is possibly formed by the reduction and protonation of a radical (·OdUMP), similar to the one formed in reaction 6, which is a very similar reaction mechanism involved in the oxidation of bromophenols by hydroxyl radical. [18] This radical (·OdUMP) apparently can also react with the starting BrdUMP to form dUMPO-BrHdUMP. The relative importance of these two competitive reactions is not known. Non-covalent dimer, $(BrdUMP)_2$, was not found in the radiolysis, which indicated that phosphate group prevent the formation of the dimer. This must be due to steric hinderance of the phosphate group and electrostatic repulsion between the negatively charged phosphate groups.

Comparisons and Discussions

In Table 1, one can see that the yields of bromide, at neutral pH, obtained from the radiolysis of 1 mM BrUr, BrdU and BrdUMP solutions N_2-saturated in the presence of hydroxyl radical scavenger, 2-propanol or t-BuOH, are about 2.7, showing that the reaction of e_{aq}^- with BrUr, BrdU and BrdUMP is quantitative and the elimination of bromide ion does not depend on the substituted groups. [19] This is not surprising because sugar moiety has very low reactivity with e_{aq}^-. [20] In the presence of alcohol, Ur·, dU· and dUMP· mainly undergo hydrogen abstraction. With 0.4 M 2-propanol in 1 mM solutions of BrUr and BrdU, the yields of Ur, dU and dUMP are about 2.15 and the ratio of the yields of bromide and Ur, dU or dUMP is about 0.80 (Table 2). With t-BuOH

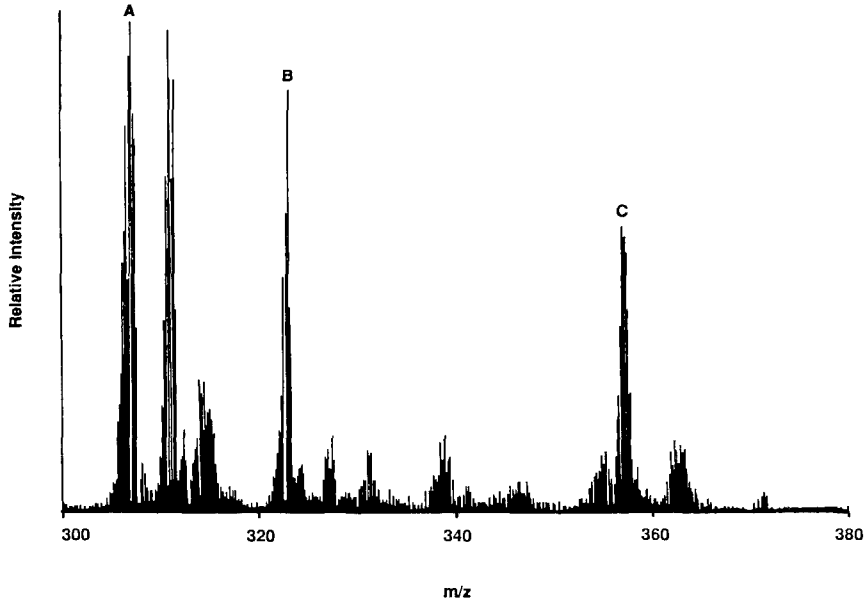


Figure 5 Mass Spectra of Radiolysis of BrdUMP

20 mM BrdUMP irradiated with ^{60}Co at dose 2 x 10^3 Gray.
Principle peaks: A: dUMP, B: dUMPOH, C: dUMPO-BrHdUMP

the yields of Ur, dU and dUMP are lower than those with 2-propanol and the ratio of the yields of bromide and Ur, dU or dUMP is only about 0.65 (Table 2). The chromatograms of dUMP obtained from 1 mM BrdUMP solutions with 2-propanol and t-BuOH were shown in Figures 2a and 2b. Studies of the reaction of halogen substituted benzoic acid indicated that 2-propanol is about an order of magnitude more reactive as a hydrogen donor than t-BuOH in neutral solution. [18] [21] The rate constants for hydrogen abstraction of carboxyphenyl from t-BuOH and 2-propanol are 0.32 and 5.5 x 10^6 M^{-1} s^{-1}. [21] The rate constant for hydrogen abstraction of Ur· from 2-propanol is, unfortunately, not available. From the pseudo-first-order rate constants of reaction 4 and the hydrogen

abstraction from t-BuOH, we estimated that less than 5% of Ur· will undergo addition reaction (reaction 4). The yield of uracil formed in the radiolysis is only about 65% of that of bromide ion. The indication is that the rate constant of reaction 4 should be about 3 x 10^9 M^{-1} s^{-1} or that the rate constant of the hydrogen abstraction from t-BuOH should be only about 2.0 x 10^6 M^{-1} s^{-1} which is not unreasonable value when compared with that of the hydrogen abstraction of carboxyphenyl from t-BuOH. Other unknown competition reactions may also be involved with Ur·, dU· and dUMP .

The experiments were also carried out in basic solutions of BrUr, BrdU and BrdUMP (see Table 1). The yields of bromide ions with t-BuOH as a hydroxyl radical scavenger were found to be about 2.67, showing that the reaction of e_{aq}^- with BrUr and BrdU, and the elimination of bromide basically do not depend on the solution pH. This result is similar to that found in the study of the reaction of e_{aq}^- with halogen substituted benzene, phenol and benzoic acid. [18] In basic solutions (pH ~ 10.5) with t-BuOH as a hydrogen donor, the yields of bromide ions and complementary Ur, dU and dUMP are similar to those found in neutral solutions (see Tables 1 and 2). With 2-propanol the yields are significantly higher than that with t-BuOH. The high yield of bromide must be due to that the isopropyl radical anion produced in the radiolysis reduces BrUr, BrdU and BrdUMP to form extra bromide as in the case of the reaction of halobenzonate with isopropyl radical. [18] Chain reactions are involved.

$$(CH_3)_2CHO:^- + \cdot OH \longrightarrow (CH_3)_2\dot{C}O:^- + H_2O \qquad (8)$$

$$(CH_3)_2\dot{C}O:^- + BrUr \longrightarrow (CH_3)_2C{=}O + Br^- + Ur\cdot \qquad (9)$$

$$(CH_3)_2CHO:^- + Ur\cdot \longrightarrow (CH_3)_2\dot{C}O:^- + Ur \qquad (10)$$

TABLE 4

PRODUCT YIELDS IN THE X-RAY RADIOLYSIS OF BrUr, BrdU AND BrdUMP IN THE ABSENCE OF ALCOHOL

Solute [1]	pH	$G(Br^-)$	G [2]	pH	$G(Br^-)$	G [2]
1 mM BrUr	6.4	4.92	0.48	10.3	4.89	0.51
0.1 mM BrUr	6.2	3.81	0.35			
1 mM BrdU	6.8	5.05	0.47	10.1	5.02	0.45
0.1 mM BrdU	6.4	3.90	0.39			
1 mM BrdUMP	6.5	5.12	0.50	10.4	5.08	0.49
0.1 mM BrdUMP	6.7	3.92	0.42			

1. All solutions were saturated with N_2.
2. Yields of Ur or dU or dUMP.

In the absence of alcohol the yields of Ur, dU and dUMP are very low ($G \leq 0.5$, Table 4). Figure 2c shows the signal of dUMP produced in 1 mM N_2-saturated solutions of BrdUMP. The results indicate that hydrogen abstraction from the sugar moiety is not an important process for dU· and dUMP·. This is expected because the unpaired electron of Ur· is expected to be localized at C_5 in a σ orbital. As result the reactivity of this radical is about as reactive as the phenyl radical. [16], [21] Our mass spectrometry experiments suggest that Ur· and dU· can react with the BrUr and BrdU to form dimers.

In the absence of alcohol, hydroxyl radicals react with BrUr, BrdU and BrdUMP to produce radicals as shown in reactions 5 and 6. Mass spectrometry experiments suggest

that radicals produced in the case of BrdU and BrdUMP can react with starting substrates to form stable adducts.

Figure 1c shows the chromatogram of bromide ion obtained from the same irradiated sample as in Figure 2c. The yield of bromide ions was about 5.0 in 1 mM N_2-saturated solutions of BrUr, BrdU and BrdUMP (Table 2). As shown in Table 1, the yield of bromide in the reaction of e_{aq}^- with BrUr, BrdU and BrdUMP is about 2.7, which amounts to 54% of G(Br^-) 5.0. This result indicates that in the absence of hydroxyl scavenger 46% of G(Br^-) 5.0 is contributed from the reactions of hydroxyl radicals and hydrogen atoms with BrUr, BrdU and BrdUMP.

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CHROMATOGRAPHIC ANALYSES OF THE EFFECTS OF GLUTATHIONE, CYSTEINE AND ASCORBIC ACID ON THE MONOPHENOL AND DIPHENOL OXIDASE ACTIVITIES OF TYROSINASE

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ABSTRACT

The effects of glutathione, cysteine, and ascorbic acid on the monophenol and diphenol oxidase functions of tyrosinase were assessed by high performance liquid chromatography with electrochemical detection (HPLC-ED) at both oxidative and reductive potentials. The enzyme-catalyzed hydroxylations of tyrosine to dopa and tyramine to dopamine were inhibited completely by glutathione and cysteine, but not by ascorbic acid. However, the rates of oxidation of dopa and dopamine were enhanced approximately 5% by cysteine and 75% by glutathione. There was no chromatographic evidence to indicate that either thiol reduced o-quinones back to their respective o-diphenols, a reaction that was documented for ascorbic acid. Glutathione and cysteine each formed sulfhydryl conjugates with the oxidation products of both dopa and dopamine. The thiol-mediated alterations in tyrosinase activity are likely due to the direct interactions of these sulfhydryl compounds with the enzyme, suggesting that the

availability and relative quantities of glutathione and cysteine at the sites of o-quinone formation may have a profound effect on quinone cytotoxicity. Under certain conditions the nucleophilic addition of glutathione and cysteine to o-quinones may represent a mechanism for regulating quinone cytotoxicity. However, glutathione-enhanced diphenol oxidase activity can potentiate cytotoxic damage by generating oxyradicals, depleting cells of o-diphenols, and lowering the level of glutathione available for antioxidant activity.

INTRODUCTION

Tyrosinase (EC 1.14.18.1) plays a critical role in the biosynthesis of melanins by catalyzing two rate-limiting reactions, the initial hydroxylation of tyrosine to dopa (monophenol oxidase), and the ensuing oxidation of dopa to o-dopaquinone (diphenol oxidase). O-quinones derived from o-diphenols such as dopa and dopamine are extremely reactive substances that either undergo nucleophilic addition with sulphydryls forming thiol conjugates and precursors of pheomelanin, or become oxidized to indolequinones that form eumelanin (1). Alternation between eumelanogenesis and pheomelanogenesis is controlled by several factors including the availability of sulfhydryl compounds, the level of activity of tyrosinase and ancillary enzymes (dopachrome tautomerase and peroxidase), and the availability of certain metal ions, such as copper and iron, which accumulate in pigmented cells (1-3).

There is considerable interest in knowing how sulfhydryl compounds, such as glutathione and cysteine, modify melanogenic processes in both malignant melanocytes (4-11), and melanotic catecholaminergic neurons that are believed to be selectively destroyed in patients with Parkinson's disease (12-19). Conflicting reports are found in the literature concerning the actual mechanism(s) of interaction of these thiols with tyrosinase. While some studies show glutathione and/or cysteine to have no inhibitory effect on tyrosinase activity (20), other reports indicate that these compounds inhibit the enzyme (8, 21-26), suppress catechol oxidation (27), and interrupt melanogenesis (7, 26, 28). Jara *et*

al. (2) reported that both tyrosine hydroxylase and dopa oxidase functions of melanoma tyrosinase were inhibited by cysteine and increased by glutathione. With mushroom tyrosinase they reported increased monophenol oxidase activity with both thiol compounds, but their effects on the diphenol oxidase function of the enzyme were not investigated.

In this investigation high pressure liquid chromatography with electrochemical detection (HPLC-ED) was used to elucidate the differential effects of glutathione, cysteine, and ascorbic acid on both the monophenol and diphenol oxidase activities of mushroom tyrosinase. By employing extremely sensitive electrochemical methods enzyme incubation times are reduced. This essentially eliminates both the effects of reaction products derived from autoxidation, and the need for diphenol cofactors that otherwise compete with the monophenol substrate for the active site of the enzyme. In addition, the HPLC-ED methods provided a mechanism for continuous monitoring of the exact content of substrate, thiols, and products. The data obtained by these methods provide convincing evidence that at physiological concentrations, glutathione and cysteine are incapable of suppressing tyrosinase-mediated oxidations of dopa and dopamine, and in the case of glutathione, this function of the enzyme is enhanced significantly.

MATERIALS AND METHODS

5, 6-Dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) were obtained from Regis Chemical Company (Morton Grove, Illinois). Mushroom tyrosinase (EC 1.14.18.1, 3300 units/mg) and all other chemicals were obtained from Sigma Chemical Company (St. Louis, Missouri). The oxidations of dopa and dopamine were monitored spectrophotometrically (Δ OD 480 nm) by a Virion spectrophotometer interfaced with a recorder, and electrochemically by reversed-phase, ion-pairing high pressure liquid chromatography. The HPLC-ED system consisted of a Bioanalytical Systems (West Lafayette, IN) LC-4B electrochemical detector equipped with a glassy

carbon working electrode and a Ag/AgCl reference electrode. Oxidative potentials (+700 mV or +850 mV) were employed to monitor the oxidation of o-diphenols (dopa and dopamine) and to detect their immediate electrochemically oxidizable reaction products (leucodopachrome, leucodopaminechrome, and/or 5,6-dihydroxyindoles). A reductive potential (-100 mV) was employed to detect electrochemically reducible compounds, such as o-quinones, dopachrome and dopaminechrome, produced during the reactions. Separations were achieved at 40°C by a BAS Phase-II, 3-μm ODS reverse phase column (3.2 mm I.D. x 10 cm). A Gilson (Madison, WI) 712 HPLC System Controller was used to integrate peak areas. The standard mobile solvent system used throughout the study was comprised of 25 mM citrate buffer (pH 3.0) containing 2.5% acetonitrile, 0.5 mM sodium octylsulfate, and 0.7 mM Na_2EDTA. Two additional chromatographic conditions were used to identity electroactive reaction products. The second mobile phase was similar to the first except the concentration of citric acid was reduced to 15 mM. In the third solvent system 35 mM NaCl substituted for the acetonitrile. Electroactive components were identified by their co-elution with authentic standards under all three different chromatographic conditions, and amplification of their peak dimensions in proportion to the amount of standard co-injected with the reaction samples. The flow rate was maintained at 0.8 ml/min. Where appropriate, standard curves were prepared for each component in the reaction mixtures, and the data analyzed by linear regression. Calibration curves were calculated prior to and after each test using varying amounts (4 calibration points) of each component in the reaction mixtures. Known amounts of different catechols were occasionally incorporated in reaction mixtures to serve as internal standards. The correlation coefficient of the calibration curves established for the standards typically was greater than 0.98.

Enzyme activity was measured in incubation mixtures containing 20 μg enzyme protein and various concentrations (0-6.6 mM) of ascorbic acid, glutathione, or cysteine in a total volume of 300 μL in 0.16 M potassium

phosphate buffer (pH 7.2). Non-enzymatic oxidations of dopa and dopamine were initiated by incorporation of 0.3 mM $NaIO_4$ into the standard reaction mixture. For tyrosinase-mediated oxidations of the monophenols tyrosine and tyramine the concentration of substrate used was 1 mM, and for the diphenols dopa and dopamine the concentration was 0.3 mM. To clearly evaluate the different effects of ascorbic acid and thiol compounds on the monophenol and diphenol oxidase functions of tyrosinase, no diphenol cofactor was used in the monophenol assays. 5 mM stock solutions of each substrate as well as all other components used in the reaction mixtures were prepared daily, just prior to each assay, in ultrapure reagent-grade water obtained with a Milli-Q system (Millipore, Bedford, MA) and kept at 4°C protected from light. The incubations were performed at 22°C. All reactions were stopped by removing 40 μL aliquots from the incubation mixtures at specified times and placing each into 60 μL cold (4°C) 0.2M perchloric acid. Stopped reactions were stored at 4°C and analyzed immediately after each assay was performed. 5 μL of each stopped reaction mixture was injected for HPLC-ED analyses. All experiments were replicated at least 3 times. Quantitative electrochemical measurements of monophenol oxidase activity were made by monitoring the rates of conversion of tyrosine to dopa and tyramine to dopamine, and for diphenol oxidase activity the conversions of dopa and dopamine to their respective o-quinones were determined by measuring substrate depletion. Activity was expressed as pmols of either product or substrate change per min under the standard conditions established for the study.

The methods of Li and Christensen (29) were employed with slight modifications to verify the identity of those oxidized products derived from tyrosinase-catalyzed oxidations of dopa and dopamine that were detected by HPLC-ED in the reductive mode (-100 mV). Briefly, 1.3 mM ascorbic acid or $NaBH_4$ was incorporated into reaction mixtures at different times post-incubation with tyrosinase. 5μL of the treated samples were analyzed at an oxidative potential of +850 mV. Ascorbic acid reduces o-quinones back to their respective

o-diphenols without affecting aminochromes, whereas $NaBH_4$ reduces both o-quinones and the aminochromes, leucodopachrome and leucodopaminechrome.

Results are presented as the means $\pm$ SEM of the determinations specified. Differences between mean values were evaluated using the Student's *t*-test, and Duncan's new-range multiple comparisons test. Difference between 2 means was considered significant when $P < 0.05$.

RESULTS

Suitability of HPLC-ED Methods

The addition of tyrosinase into reaction mixtures containing either monophenols or o-diphenols but lacking antioxidants resulted in the solutions first turning pink, and then dark brown to black within 30 min. These color changes were not evident when antioxidants were incorporated into the reaction mixtures. The conventional spectrophotometric methods (ΔA 475 nm) used to monitor dopachrome formation provided some measure of the rate of formation of certain pigment precursors, but they were inadequate for assessing the roles of antioxidants in these reactions because thiols formed colorless reaction products (i.e. cysteinyl- and glutathionyl- conjugates with o-diphenols), and ascorbic acid reduced o-quinones back to o-diphenols. The HPLC-ED methods employed in this investigation effectively detected changes in the levels of monophenol and diphenol substrates and their principal electrochemically oxidizable and reducible products, and provided quantitative data with which to compare the effects of ascorbic acid, glutathione, and cysteine on both monophenol and diphenol oxidase functions of mushroom tyrosinase.

Monophenol Oxidase Activity

Tyrosinase-catalyzed hydroxylations of tyrosine and tyramine assessed at oxidative potentials documented both the depletion in the levels of these

monophenol substrates (detected at +850 mV), and the concurrent formation of their respective o-diphenols, dopa and dopamine (detected at either +700 mV or +850 mV) (Fig. 1). In control mixtures lacking antioxidants, tyrosine and tyramine were rapidly hydroxylated to dopa and dopamine, respectively. The o-diphenols in turn were oxidized further by tyrosinase to o-quinones and subsequently by non-enzymatic mechanisms to precursors of eumelanin, thereby diminishing the levels of dopa and dopamine in the analities.

There was no HPLC-ED evidence of monophenol oxidase activity in reaction mixtures containing glutathione or cysteine in concentrations ranging from 30 μM to 6.6 mM. In the presence of these thiols, substrate levels remained unaltered and no o-diphenols were produced during 30 min incubations. In reaction mixtures containing ascorbic acid the levels of dopa and dopamine produced in 3 min assays were 5 to 7 times greater than in the control incubations lacking ascorbic acid (Fig. 2). These elevated levels resulted from ascorbic acid reducing the o-quinones back to their respective o-diphenols, and not from any alteration in the activity of the enzyme. This was verified by HPLC-ED which in the reductive mode (-100 mV) showed no o-quinone formation, and in the oxidative mode (+850 mV) showed the rates of substrate depletion to be identical in both control and ascorbic acid incubation mixtures (not presented). Thus, monophenol oxidase activity was inhibited by glutathione and cysteine, but not by ascorbic acid.

Diphenol Oxidase Activity

Oxidative potentials also were used to study diphenol oxidase activity, with rates of reaction determined on the basis of the depletion of each o-diphenol substrate. A reductive potential (-100 mV) was used to monitor the formation of oxidized products derived from the o-diphenols. In control mixtures the rates of oxidation of dopa and dopamine averaged 298 pmol/min and 453 pmol/min, respectively (Table 1). Very little diphenol oxidase activity was detected in

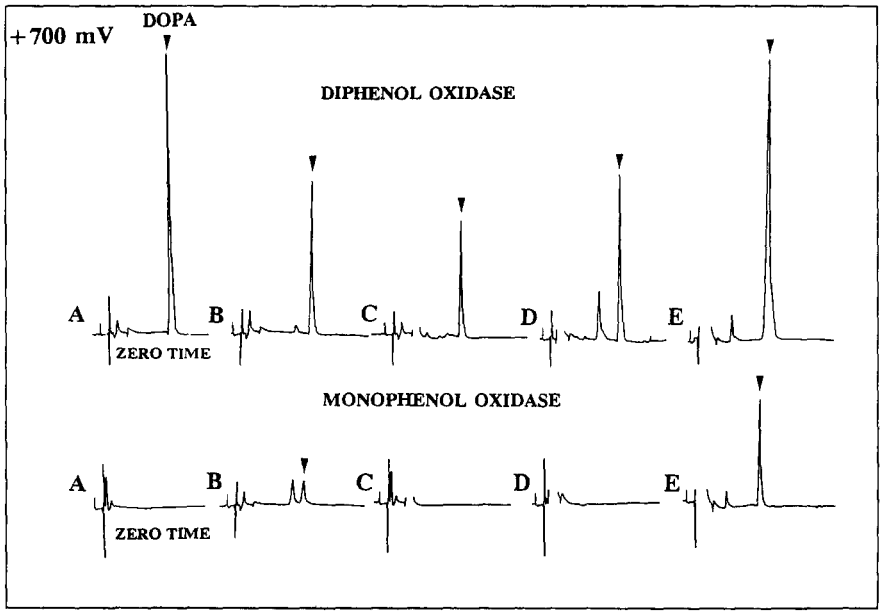

Figure 1. Representative HPLC-ED chromatograms obtained in the oxidative mode (+700 mV; 500 nA) illustrating the tyrosinase-catalyzed hydroxylation of tyrosine (monophenol oxidase function) and the oxidation of dopa (diphenol oxidase function). Control, buffer-mediated incubations are illustrated in (B) of both upper and lower series of chromatograms. Note the absence of dopa production in monophenol oxidase incubations containing glutathione (C) and cysteine (D), and the elevated level of dopa (arrow) with ascorbic acid (E). Diphenol oxidase activity, calculated by the rate of substrate delpletion, was enhanced by glutathione (C). Cysteine-mediated diphenol oxidase reactions (D) are similar to control reactions (B). Reaction mixtures contained 0.3 mM dopa or 1 mM tyrosine, 20 μg enzyme, and 1.3 mM antioxidant, and were incubated at 22°C for 1 min. The peaks produced by the antioxidants were removed to better accommodate the graphic representations.

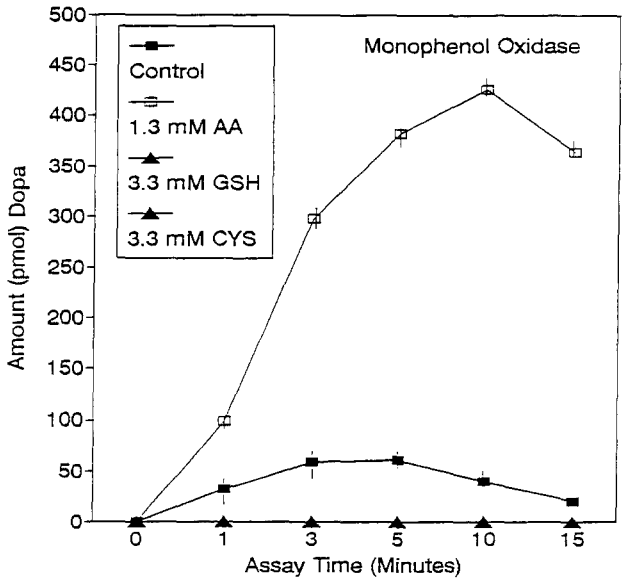

Figure 2. The effects of ascorbic acid (AA), glutathione (GSH) and cysteine (CYS) on the tyrosinase-catalyzed hydroxylation of tyrosine. Elevated levels of dopa with ascorbic acid result from the reduction by the antioxidant of dopaquinone back to the o-diphenol. Diminished levels of dopa in control incubations lacking antioxidant result from the enzyme-mediated oxidation of dopa to dopaquinone. Both thiol compounds used, glutathione and cysteine, inhibited the monophenol oxidase function of the enzyme (see chromatograms Figure 1C, D). Reaction mixtures (total volume 300 μL) were comprised of 1 mM tyrosine and 20 μg enzyme protein in 0.16 M phosphate buffer (pH 7.2). 5 μL of stopped reactions were injected for HPLC-ED analysis at +850 mV and 100 nA. Data points represent the means of 4 tests.

reaction mixtures containing ascorbic acid due to the regeneration of dopa and dopamine by ascorbic acid which reduced their o-quinones (Fig. 3). Unlike their role in inhibiting the monophenol oxidase function of tyrosine, neither glutathione nor cysteine could block the enzyme-catalyzed oxidations of dopa or dopamine. Instead, both thiols increased the diphenol oxidase function of the enzyme. With cysteine the rates of oxidation of dopa and dopamine were increased slightly

TABLE 1. The Effects of Glutathione, Cysteine, and Ascorbic Acid on Tyrosinase-catalyzed Oxidations of Dopamine and Dopa.

	Dopamine	Oxidation		Dopa	Oxidation	
	pmol/min	± (SE)	%	pmol/min	± (SE)	%
Controls	453	(5.6)	100	298	(1.1)	100
GSH	793	(7.2)	175	515	(3.9)	173
CYS	466	(3.2)	103	313	(2.3)	105
AA	12	(2.2)	3	5	(0.5)	2
GSH + AA	738	(3.7)	163	453	(4.0)	152

Reaction mixtures containing 0.33 mM substrate and 3.3 mM of components listed were incubated for 1 min at 22°C. 5μL aliquots were analyzed by HPLC-ED at +850 mV and 100nA.

(approximately 5%), but they were not significantly higher than reaction rates in control incubations lacking antioxidants. However, with glutathione the diphenol oxidase function of tyrosinase was enhanced significantly ($p < 0.05$), causing an increase of approximately 75% in the rates of oxidation of dopa and dopamine (Table 1).

Since diphenol oxidase activity was enhanced by glutathione, we next investigated how ascorbic acid would interact with the thiol in an environment where o-diphenols were oxidized to o-quinones. In reaction mixtures containing 3.3 mM ascorbic acid and glutathione, dopa oxidase and dopamine oxidase activities were virtually similar to those in reaction mixtures containing only glutathione (Table 1). These data clearly establish that the glutathione-mediated enhancement of the diphenol oxidase function of tyrosinase was not diminished

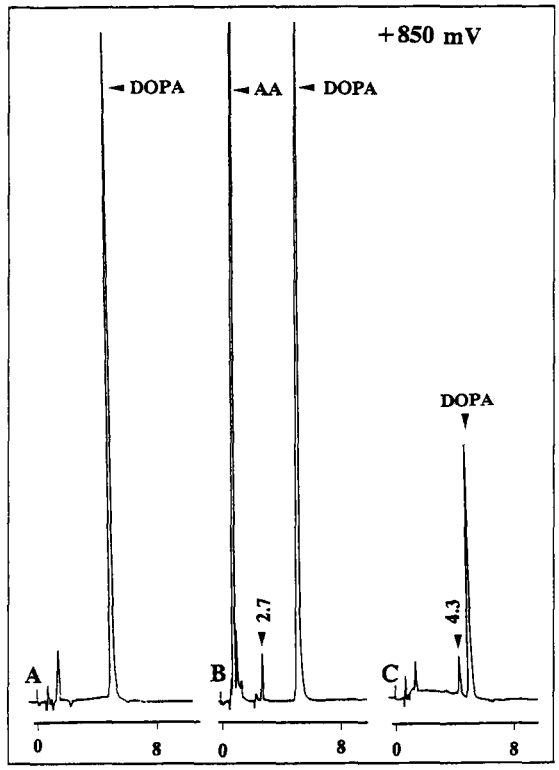

Figure 3. Representative HPLC-ED chromatograms obtained in the oxidative mode (+850 mV; 500 nA) depicting the extent of dopa oxidation in reaction mixtures containing 1.3 mM ascorbic acid (AA) and in those lacking the antioxidant. Dopa level at time zero (A) represents 0.6 nmol substrate. Less than 5% of the o-diphenol is oxidized in 1 min when ascorbic acid is present in the incubation mixture (B), whereas approximately 50% of the substrate is oxidized in control incubations lacking the antioxidant (C; and Fig. 4). A single oxidation product with a retention time of 2.7 min was detected in ascorbic acid reaction mixtures, whereas a product with a retention time of 4.3 min was formed in mixtures lacking antioxidant.

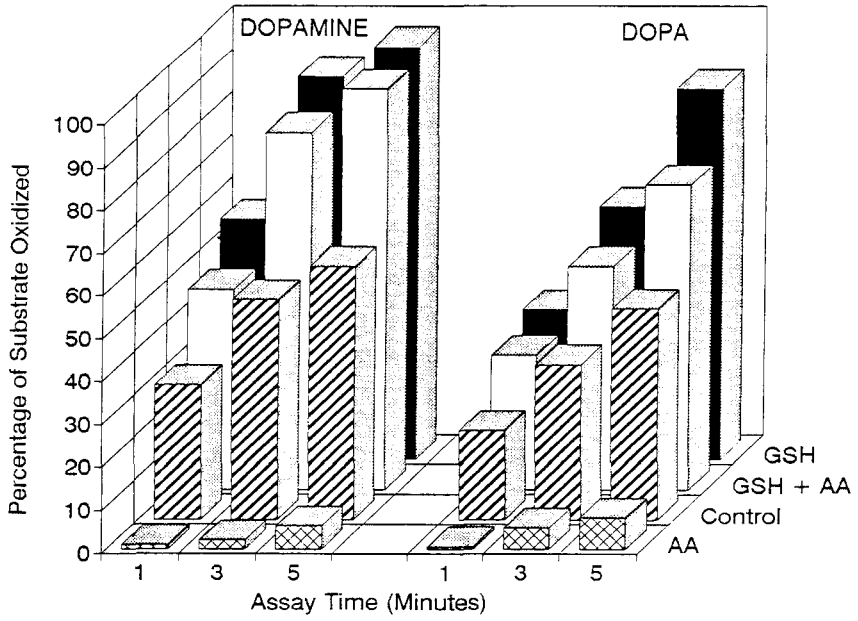

Figure 4. Histograms illustrating the separate and combined effects of ascorbic acid (AA) and glutathione (GSH) on the diphenol oxidase function of tyrosinase. Reaction mixtures contained 0.3 mM substrate (dopa or dopamine), 3.3 mM glutathione, and 6.6 mM ascorbic acid. Each bar represents the mean of 4 tests. Each experimental group is statistically different ($p < 0.05$) compared to controls, but comparative data involving GSH and GSH + AA are not significantly different. Data were obtained by HPLC-ED in the oxidative mode (+850 mV; 100 nA).

significantly by ascorbic acid. Identical results were obtained when the concentration of ascorbic acid was doubled (6.6 mM), and the time of incubation extended to 5 min (Fig. 4).

The effects of different concentrations of glutathione and cysteine on the diphenol oxidase activity of tyrosinase were investigated using dopa as substrate and incubating the reaction mixtures for 1 min (Table 2). With cysteine incorporated into reaction mixtures in concentrations ranging from 30 μM to 6.6

TABLE 2. The Effects of Varying Concentrations of GSH and Cysteine on the Tyrosinase-catalyzed Oxidations of Dopa.

	Dopa Oxidation pmol/min ± (SE)	
Controls	**309 (3.3)**	
Concentration (mM)	**GSH**	**Cysteine**
6.6	**510 (5.4)**	**299 (5.3)**
3.3	**508 (6.2)**	**301 (4.4)**
1.0	**514 (2.2)**	**307 (3.3)**
0.3	**450 (4.2)**	**302 (2.9)**
0.06	**320 (3.6)**	**300 (2.0)**
0.03	**314 (3.7)**	**301 (3.3)**

Reaction mixtures contained 0.3 mM DOPA and were incubated for 1 min at 22°C. 5μL aliquots of the stopped reactions were analyzed by HPLC-ED at +850 mV and 500 nA.

mM there was no statistically significant alteration in dopa oxidase activity. With glutathione, statistically significant ($p < 0.05$) increases in dopa oxidase activity were noted when the concentration of this thiol in reaction mixtures was 0.3 mM and higher.

Detection of O-Diphenol Products by Reductive Electrochemistry

Under reductive HPLC-ED conditions (-100 mV; 500 nA) the tyrosinase-catalyzed oxidation of dopa produced a single compound with a retention time of 1.1 min. With dopamine as substrate a single product also was detected but with

a retention time of 2.5 min (Fig. 5). Both o-diphenol derivatives also were generated non-enzymatically by $NaIO_4$-mediated oxidations of the o-diphenols. No electrochemically reducible compound was produced when reactions were initiated with ascorbic acid, glutathione or cysteine present in incubation mixtures in concentrations ranging from 30 μM to 6.6 mM. Once the compounds were produced in control mixtures, subsequent attempts to reduce them by adding ascorbic acid, glutathione or cysteine to the reaction mixtures as early as 1 min post-incubation were unsuccessful. However, both compounds were completely reduced upon the addition of 1.3 mM $NaBH_4$ to the reaction mixtures. Analyses of the $NaBH_4$-treated reaction mixtures at oxidative HPLC-ED conditions (+850 mV) showed that the o-diphenol substrates were rejuvenated to nearly their initial levels (Fig. 5). The stability of the two compounds after treatment with ascorbic acid eliminates the $NaBH_4$-reducible substances as o-quinones, since the latter are reduced by ascorbic acid. Since $NaBH_4$ has the ability to regenerate o-diphenols by reducing aminochromes (Li and Christensen, 1993), the two reducible compounds produced by the enzyme-mediated oxidations of dopa and dopamine are dopachrome and dopaminechrome, respectively.

Detection of O-Diphenol Products by Oxidative Electrochemistry

HPLC-ED analyses of tyrosinase-catalyzed oxidations of dopa at an oxidative potential (+850 mV; 500 nA) showed a single product with a retention time of 4.3 min (Fig. 3). This electrochemically oxidizable compound was identified as 5,6-dihydroxyindole (DHI) in co-elution experiments using the authentic standard and three different solvent systems (Fig. 6). A single electrochemically oxidizable product also was derived from the oxidation of dopamine, but this substance could not be identified. The incorporation of thiols into reaction mixtures generated o-diphenol-sulfhydryl conjugates that were detected at oxidative potentials (Figs. 7, 8). With dopamine as substrate, a single electrochemically oxidizable compound was formed with each thiol. The retention time for the cysteinyldopamine conjugate was 23.1 min (Fig. 7A), and

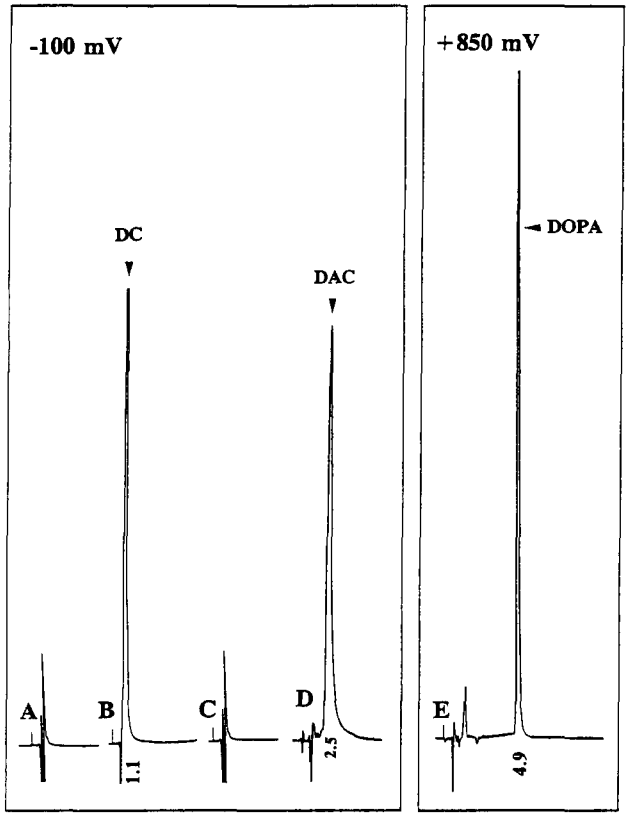

Figure 5. Chromatograms A - D obtained in a reductive mode (-100 mV; 500 nA) showing glutathione suppression of electrochemically reducible compound in tyrosinase-catalyzed reactions of dopa (A) and dopamine (C). Dopachrome (DC) and dopaminechrome (DAC) were produced in 1 min assays when the antioxidant was not incorporated into the reaction mixtures (B and D). The aminochromes were identified by $NaBH_4$ reduction to o-diphenols, a reaction detected at +850 mV. $NaBH_4$ reduction of DC formed a compound (4.9 min) identified as dopa (E). Under identical conditions $NaBH_4$ reduction of dopaminechrome (D) produced dopamine (not presented).

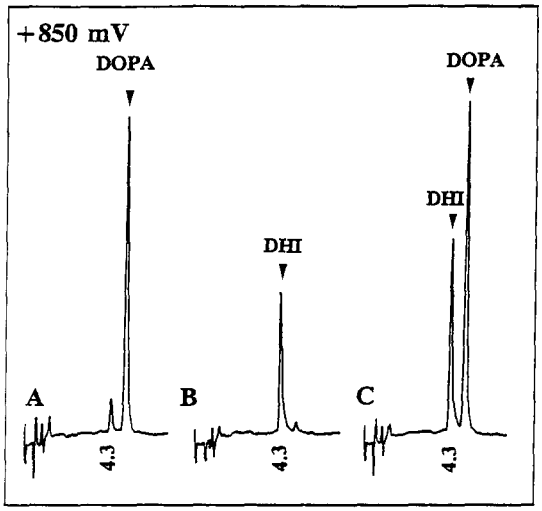

Figure 6. (A) Chromatograph showing the presence of a single oxidation product with a retention time of 4.3 min obtained during the tyrosinase-catalyzed oxidation of dopa (1 min incubation) in phosphate buffer (7.2 pH). (B) 0.2 nmol of authentic standard DHI. (C) Chromatogram showing co-elution at 4.3 min of the oxidation product with 0.2 nmol of DHI standard incorporated into 5 μL sample that was injected. Co-elution was demonstrated with three solvent systems (see Materials and Methods).

40.6 min for the glutathionyldopamine conjugate (Fig. 8A). With dopa as substrate, three electrochemically oxidizable compounds were generated with glutathione, and two with cysteine. One compound with a retention time of 3.7 min was common to glutathione- and cysteine-mediated oxidations of dopa (Figs. 7B and 8B). The oxidation of dopa in the presence of ascorbic acid also produced a single unidentified compound with a retention time of 2.7 min (Fig. 3).

DISCUSSION

Eumelanin and pheomelanin originate from a common metabolic pathway involving two sequential tyrosinase-catalyzed reactions, the conversion of tyrosine

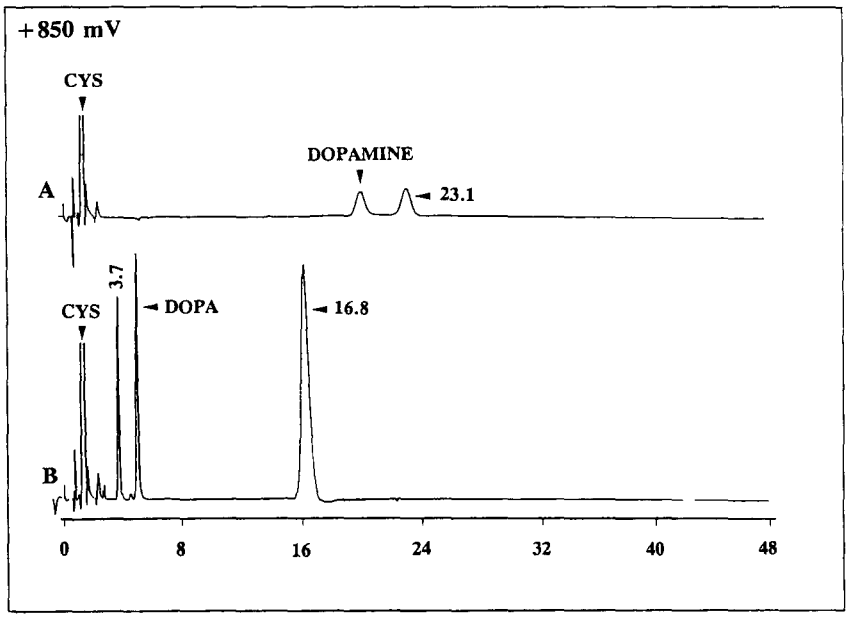

Figure 7. Chromatograms of tyrosinase-catalyzed oxidations showing the formation of a single cysteinyldopamine conjugate at 23.1 min (A), and two cysteinyldopa conjugates at 3.7 and 16.8 min (B). The compound with a renetion time of 3.7 min was also formed in glutathione-mediated oxidations (see Figure 8B).

to dopa, and the oxidation of dopa to dopaquinone. Ensuing spontaneous reactions involving cyclization and oxidative polymerization convert o-quinones into precursors of eumelanin. An alternate pathway is provided by thiols which react with o-quinones to produce colorless addition compounds and precursors of pheomelanin. The capacity of cells to transiently modify their biosynthetic activity to alternate between the synthesis of eumelanin and pheomelan may be important either in their defense against cytotoxic quinone-generated intermediates derived from oxidant stress, or in generating such molecules for protection against pathogens. Several enzymes play pivotal roles in regulating melanin biosynthesis

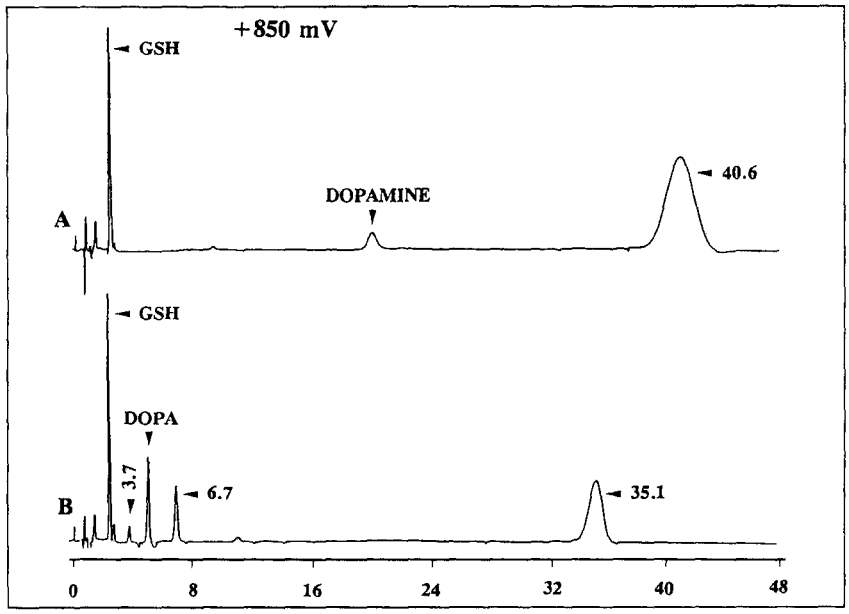

Figure 8. Chromatograms of tyrosinase-catalyzed oxidations showing the formation of a single glutathionyldopamine conjugate at 40.6 min (A), and three glutathionyldopa conjugates at 3.7, 6.7, and 35.1 min (B).

including tyrosinase, peroxidase, dopachrome tautomerase, sulphydryl-metabolizing enzymes (i.e., glutathione-reductase and gamma-glutamyl transpeptidase), and catechol-o-methyltransferase (3, 30, 31). Regulatory roles have been ascribed to antioxidants also, but the mechanism(s) by which these compounds interact with o-quinones has not been completely elucidated.

In this investigation quantitative HPLC-ED data establish that ascorbic acid maintains the level of o-diphenols in tyrosinase-catalyzed reactions by reducing the o-quinones that are produced, and not by modifying the activity of the enzyme. Glutathione and cysteine were found to inhibit the monophenol oxidase function of tyrosinase and increase the diphenol oxidase function. The diphenol oxidase function of tyrosinase was enhanced approximately 5% by cysteine and

75% by glutathione. Both thiols formed catechol-thiol conjugates during tyrosinase-catalyzed oxidations of dopa and dopamine. These reactions proceeded with no electrochemical evidence of thiol regeneration of o-diphenols, a function readily documented with ascorbic acid. Thus, nucleophilic addition of glutathione and cysteine may be the primary if not sole mechanism by which these thiols engage the oxidation products of dopa and dopamine.

The fact that the glutathione-mediated enhancement of diphenol oxidase activity was not altered significantly by ascorbic acid suggests that glutathione may be more effective than ascorbic acid in engaging o-quinones. However, when glutathione was added to reaction mixtures containing o-dopaquinone or o-dopaminequinone, there was no detectable evidence the thiol reduced these compounds to their respective o-diphenols. These findings indicate that the glutathione-mediated enhancement of diphenol oxidase activity results from a specific interaction of glutathione with tyrosinase, and that thioether formation occurs rapidly without the generation of free o-quinones. When monophenols are involved, glutathione or cysteine interact with the enzyme to inhibit tyrosine hydroxylation. It is not known how these varied activities are produced, but since sulfhydryl compounds form stable complexes with copper ions (2, 22, 24, 32), the thiols likely interact differently with the copper active site of tyrosinase during monophenol and diphenol oxidase activities. The enzymatic activities of tyrosinase are dependent on its capacity to coordinate phenolic substrates at the binuclear copper active site, a reaction sequence that requires a concomitant copper-mediated, four-electron reduction of dioxygen. Thiols may inhibit monophenol oxidase function by interfering with the incorporation of an oxygen atom into the aromatic ring, and enhance diphenol oxidase activity by a redox exchange involving the substrate and the binuclear copper ions at the active site of the enzyme.

Our results of the effects of thiols on the diphenol oxidase function of tyrosinase support in part the findings of Agrup *et al.* (20) who showed no inhibition of the dopa oxidation by cysteine. Our chromatographic data clearly

establishing thiol-suppression of monophenol oxidase activity and thiol-enhancement of diphenol oxidase activity of mushroom tyrosinase are in contrast to those of Jara *et al.* (2) who reported that both functions of melanoma tyrosinase were inhibited by cysteine but enhanced by glutathione. This disparity with respect to diphenol oxidase activity is attributed to their assessment of rates of reaction only after cysteine was exhausted from the reaction mixtures, at which time dopachrome appears. The extremely sensitive electrochemical methods employed in this investigation for diphenol oxidase activity provided a quantitative assessment of the actual changes in the amount of o-diphenol substrate consumed. Our assessment of thiol-mediated suppression of monophenol oxidase activity was based on the absence of detectable changes in substrate and the fact that no electrochemically active products were evident under either oxidative or reductive modes.

Regardless of the mechanism(s) involved, glutathione and cysteine can, under certain circumstances, serve as a detoxification mechanism by both inhibiting tyrosine hydroxylation and scavenging potentially cytotoxic o-quinones and other eumelanogenic quinoids to form pheomelanin (31). However, since cysteinyldopas have the ability to bind to proteins through their sulfhydryl groups (33), thiol-catechol conjugates may be as potentially damaging to cells as quinone oxidation products. Also, the availability of glutathione during the oxidation of dopa or dopamine will accelerate the formation of o-quinones, possibly depleting cells of these o-diphenols and potentiating quinone cytotoxicity by generating reactive oxygen species. In this regard it is of interest to note that correlations have been made by numerous investigators between neurodegenerative conditions in the substantia nigra of Parkinson's patients and the presence of neuromelanin in the catecholaminergic neurons targeted for destruction (34). Neuromelanin is a mixed-type melanin comprised of oxidation products of dopamine and cysteinyldopamine (35). Although the mechanism by which dopamine is oxidized in the substantia nigra is still unclear, the immediate oxidation product is o-dopaminequinone. Alterations in brain levels of glutathione or cysteine would

have a pronounced effect on the rate of oxidation of the o-diphenol and the pathophysiological manifestations of such a reaction.

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HIGH PERFORMANCE AFFINITY CHROMATOGRAPHY FOR ANALYSIS OF HUMAN ANTITHROMBIN III

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SUMMARY

Antythrombin III (AT III) is one of the most important regulators of the coagulation system. Improper concentration of AT III in blood is a great risk and often leads to thrombotic complications.

In routine laboratories there is a choice between two types of methods (functional and immunology) of AT III examination which are time consuming.

The paper deals with the AT III analysis by means of high performance affinity chromatography (HPAC). The results presented in the paper indicate high sensitivity of HPAC for AT III analysis which can be performed in a few minutes.

INTRODUCTION

α_2-globulin, known also as Antithrombin III (AT III) is the main inhibitor of blood coagulation [1]. This glycoprotein, synthetized in the liver is characterized by molecular weight from 58.000 to 67.000 and consists of a single polipeptide chain and about 15 % of carbohydrate [2,3]. The correct

concentration of At III in the blood plasma ranges from 85 to 115 % of the standard amount which equals 25-30 mg/100 cm^3 [2,3].

Reduced concentration of AT III in blood is a great and well established risk and can lead to many thrombotic complications resulting in the blood clot formation in a vascular bed [4].

The decrease of AT III biosynthesis is innate or caused by chronic acute liver diseases or by medical treatment with drugs like L-asparginase and oestrogen-progestogen [5]. The recognition of AT III deficit due to its increase consumption has also been demonstrated in sepsis and major trauma [6,7].

The functional methods (amidolytic or coagulative ones) and immunology methods (includig immunoelectrophoresis, laser nephelometry and radioimmunodiffusion on NOR-Plate-Partigen-Antithrombin III) are the main methods employed for AT III level determination in the routine analyses [8-10].

The comparison of the fibrinogen clotting time in the investigated and standard plasmas (which contains the correct AT III amount) is the coagulation method principle [8]. This measurement takes about 2 hours and requires at least 2 ml of plasma.

The amidolytic method is based on the thrombin deactivation process by AT III in the presence of heparin [9]. The AT III amount is estimated indirectly from the residual (not blocked) thrombin activity. The latter is analyzed in the second step, titrating an incubation mixture by a chromogenic substrate which reacts specifically with this enzyme. The experiment lasts at least 24 hrs even with the applicaton of the automated Hitachi 717 analyzer [11].

The competition of labelled and non-labelled antigen molecules with a limited number of bonding centers in the antibody molecule is the principle of the immunology method [10]. It is assumed that the labelled and non-labelled antigen exhibits the same skill at binding antibody. During the analysis the amount of antibody and labelled antigen is always constant. Only the amount of the investigated antigen changes. A typical immunology procedure demands about 7.5 μl of the investigated plasma and 4.4 μl of the labelled antigen (which is rather expensive substance). When electrophoresis is used for separation of the immunology mixture the analysis takes about 45 hrs.

As results from the above the analysis time of AT III by one of the described routine methods ranges from a few to several hours. The accuracy of these

methods is about 3 % which corresponds to about $\pm$ 1 mg of AT III per 100 cm^3 of blood at normal AT III blood level.

It is commonly known that antithrombin III neutralizes the active coagulation factors presented in plasma [1-3]. This process is accelerated by heparin. The affinity of antithrombin III to heparin is also revealed in the artificial system in which heparin is chemically bonded with a support surface [12,13]. In other words AT III can be estimated directly in plasma by means of affinity chromatography [14]. In the well selected chromatography system and conditions the precision of analysis can be considerably high and analysis time relatively short.

The results discussed in the paper not only show the possibility of AT III analysis directly in blood by means high performance affinity chromatography (HPAC) but also allow to conclude about the time consumption and sensitivity of this method.

EXPERIMENTAL

Materials

Controlled porous glass (CPG) preparation. Vycor glass composed of 10 % Na_2O, 35 % B_2O_3 and 55 % SiO_2 was used as the starting material for preparation of controlled porous glass. A portion of this glass (fraction 40-80 μm) was heated at 557 °C for 72 hours. The thermally treated glass was next converted into porous sorbent by proper leaching with 3 N H_2SO_4 and 0,5 N NaOH according to the procedure previously described [15,16]. The final material was characterized by the following data: the specific surface area S_{BET} = 72.6 m^2/g, the pore volume V_p = 1.13 cm^3/g and the pore diameter D = 60.0 nm.

Preparation of silica-based supports. By the deposition of polysaccharide on silica surface the advantages of the traditional soft gel affinity supports with the excellent mechanical properties of silica supports were combined. The introduction of a hydrophilic and non-ionic polymeric layer on the silica surface minimizes the specific adsorptions and gives a high concentration of activable hydroxylic functions. The obtained porous glass was covered for this reason with a double layer of polysaccharide. The starting material was preliminarily impregnated with positively charged DEAE-dextran to neutralize the

cation exchange capacity of CPG. 1.5 g DEAE-dextran (Pharmacia Fine Chemicals) (MW = 500.000) in 25 ml bidistilled water (pH had been adjusted to 11.5) was added to 10 g of CPG at room temperature. After water evaporation (in vacuum conditions) the support was dried at 80 °C for 15 hours. The former layer was cross-linked by diethylenglycol diglycidyl ether (EDG-2) (IChP, Warsaw, Poland) in the aqueous media. The solution consisting of 10 ml 1 N NaOH, 0.56 ml EDG-2 and 0.023 g $NaBH_4$ in 100 ml bidistilled water was used for cross-linking. The mixture was stirred at 40 °C for 30 minutes. After the reaction the material was washed and dried. The second layer of polysaccharide was introduced following the procedure described above, but instead of DEAE-dextran, pure dextran (Polfa, Kutno, Poland) (average molecular weight = 110.000) was employed and only 0.14 ml EDG-2 was applied during the cross-linking procedure. Evaporation of water was performed at 80 °C at the atmospheric pressure.

Before heparin bonding the prepared support was activated by EDG-2 according to the conditions described elsewhere [17].

Heparin immobilization. In order to immobilize high molecular weight heparin (Polfa, Poland), 5 g of the modified CPG was treated with the solution consisting of 5 ml 0.1 M Na_2CO_3 buffer, pH = 8.7 and 2 ml heparin. The material with heparin was stirred at room temperature for 48 hours. After washing and drying the obtained affinity sorbent was put into the chromatographic column.

Preparation of blood plasma. A standard procedure was used for blood preparation [18]. Blood taken from vein was immediately mixed with sodium citrate (concentration 3.8 %) at the volume ratio 9:1. Stabilized in such a way blood was next centrifuged at 1.500 - 2.000 rpm over 10 - 15 min. The separated plasma was directly injected on the column.

Methods

Pore volume and mean pore diameter in the obtained CPG were calculated from Mercury porosimetry data. Measurements were performed with a Mercury porosimeter Type 2000 (Carlo Erba, Milan, Italy).

The specific surface area investigations were carried out by the BET method (low temperature adsorption of nitrogen). A nitrogen Sorptomat (type 1800 from Carlo Erba) was used for this purpose.

The chromatographic investigations were carried out with a Shimadzu liquid chromatograph (Japan). The HPLC apparatus consisted of an LC-9A high pressure pump, a gradient system and UV-VIS photodiode array detector SPD-MGA connected to an IBM AT PC PCD-2M data system from Siemens (Germany).

100 µl volumes of AT III standard solution (Institute of Blood-Derivatives, Production Division of Immuno AG, Vienna, Austria) or blood plasma were injected (by means of Rheodyne 7125 injection valve) onto a column (120 x 4 mm) packed with affinity sorbent and pre-equilibrated with initial buffer (0.02 M disodium phosphate, pH 7.4 - buffer A) at a room temperature and at a flow rate 1 ml/min. The adsorbed AT III was then eluted using a salt gradient (0.02 M disodium phosphate, 2 M sodium chloride, pH 7.4 - buffer B). In these conditions the AT III and accompanying substances were eluted on their respective affinity sorbents.

RESULTS AND DISCUSSION

Controlled porosity glasses represent siliceous materials which are characterized by a very narrow pore size distribution. Due to this feature their porous structure is far better penetrated by the chromatographed molecules compared to the structure of silica gel. Controlled porosity glass applied in the investigations possesses the mean pore diameter equal 60 nm. Thus the structure of such a material is accessible for AT III molecules even after deposition of dextran layers (which changes the CPG surface character and screen the denaturative interaction between the separated molecules and bare CPG surface) and coupled heparin molecule.

The chromatogram of AT III standard obtained with the application of the sorbent described above is presented in Fig. 1. The first part of the chromatogram is connected with substances which accompany the AT III and do not exhibit the affinity for the immobilized heparin. The second concentration zone with the maximum at 14.9 min. corresponds to AT III. It appears about 4 minutes after the beginning of NaCl gradient rise (NaCl gradient starts in the 10-th minute). Table I contains the retention data of AT III standard after successive injections. These values prove the reproducibility of the analysis.

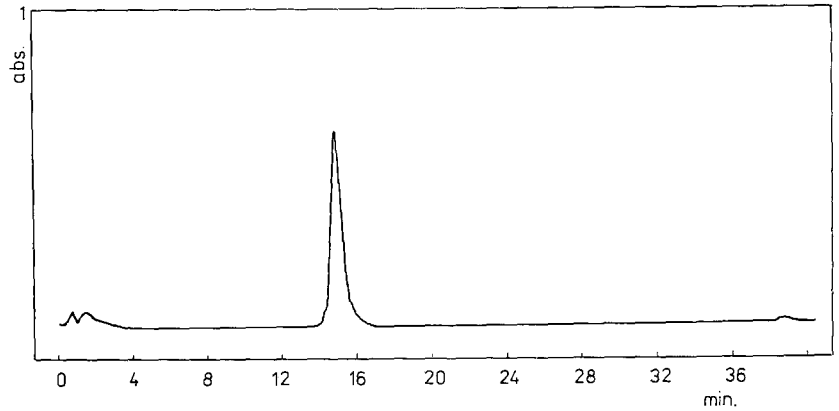

Figure 1. Elution of human antithrombin III from a column packed with the sorbent composed of heparin chemically bonded to a dextran layer immobilized on CPG.

Column: 120 x 4 mm,

Sample: 100 µl of AT III standard (50 I.U./ml),

Buffers to the elution: Buffer A - 0.02 M disodium phosphate, pH 7.4

Buffer B - 2.0 M NaCl in 0.02 Na_2HPO_4, pH 7.4

Elution: 0.0 - 10.0 min - buffer A,

10.0 - 30.0 min - linear gradient from 0 to 100 % of buffer B

Flow rate: 1 ml/min,

Detector wave length: 280 nm.

As mentioned before the possibility of the prepared sorbent employment in the AT III analysis in a human plasma was one of the main tasks of the presented investigations. The blood plasma is a complex mixture containing substances which can affect the separation shown in Fig. 1. Fig. 2 presents the chromatogram of a human plasma obtained on the same column as in Fig. 1. As in Fig. 1, in Fig. 2 two concentration zones are distinctly seen. The second peak with the retention time 14.8 min corresponds to AT III contained in the plasma. The first one represents the other compounds of plasma which do not show the affinity for chemically bonded heparin. In comparison to the previous

Table I. Retention data and features of AT III zones obtained after successive injections.

N°	Retention time [min]	Concentration [%]	Peak purity index	Max. wave length [nm]
1	14.85	93.745	0.9475	231
2	14.83	94.917	0.9037	233
3	14.85	93.743	0.9472	230
4	14.81	94.931	0.9107	230
5	14.86	94.989	0.9487	237
6	14.70	93.435	0.9245	220
7	14.73	92.498	0.8984	233
8	14.77	93.263	0.8883	226
9	14.87	95.327	0.8924	234
10	14.77	93.427	0.8706	225

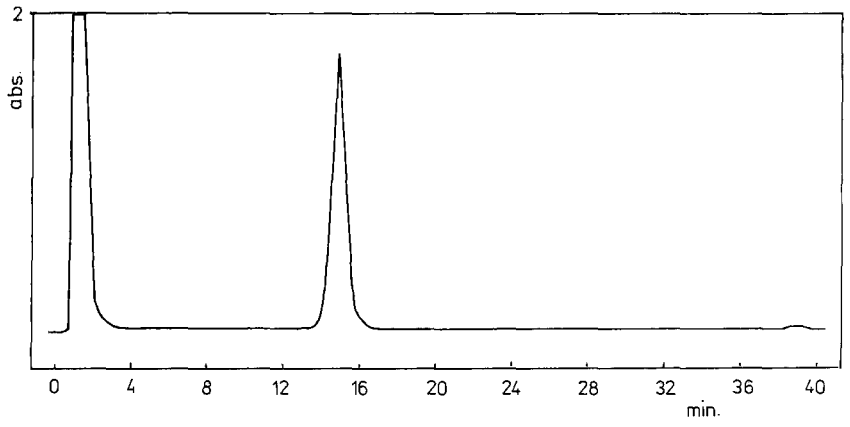

Figure 2. Elution of human plasma (sample 100 µl).

Sorbent, column and conditions - see Figure 1.

chromatogram it is significantly larger which can be seen if one takes into account a number and concentration of plasma compounds (except AT III). The result seen in Fig. 2 proves the utility of high performance affinity chromatography (with immoblized heparin in the column) for AT III analysis even in such a complex mixture as blood (after centrifugation of red cells).

The conditions and system used in the separations shown in Figures 1 and 2 allow for AT III analysis in 30 minutes. In the first 10 minutes the isocratic elution with initial buffer (buffer A) runs. From the 10-th to the 30-th minute the NaCl concentration rises (linear gradient from the initial buffer to the same buffer containing 2 M NaCl - the linear concentration increase of buffer B in buffer A). The column regeneration (the flushing of the column with the initial buffer) begins in the 30-th minute. As appears from the chromatograms (Figures 1 and 2) nothing is eluted from the column between the 3-rd and 13-th minute. The scanning from 195 to 380 nm leads to the same conclusion. This suggests the possibility of the analysis time decrease e.g. by the earlier start of the NaCl gradient in the mobile phase. Figure 3 presents the chromatogram of AT III standard where the gradient of buffer B starts in the 3-rd minute. In the 8-th minute a mobile phase contains 50 % of B, and in the 9-th minute buffer B reaches 100 %.. As it is seen the chromatogram is similar to that in Figure 1. The significantly shorter analysis time (less than 8.5 minutes) is the only difference. The retention time of the AT III peak maximum equals 7.33 minutes and the AT III zone starts to appear about 6.7 minutes after its injection. Considering the chromatogram in Figure 3 it is seen that the AT III analysis can be carried out even in shorter time and using lower NaCl concentration. The latter conclusion is confirmed by the chromatogram shown in Figure 4 where the gradient of B eluent is steeper and in 7-th minute the mobile phase contains 50 % of B. For the next 2 minutes the mobile phase composition comes back to the initial buffer. Comparing Figures 3 and 4 similarity of the both chromatograms is shown. Still shorter AT III retention time is the only difference. Almost the same peak areas (representing AT III) indicate the same amounts of the AT III eluted.

Figure 5 presents the chromatogram of AT III at the very steep NaCl gradient. It starts in the 3-rd minute and finishes in the 6-th minute (100 % of B). The

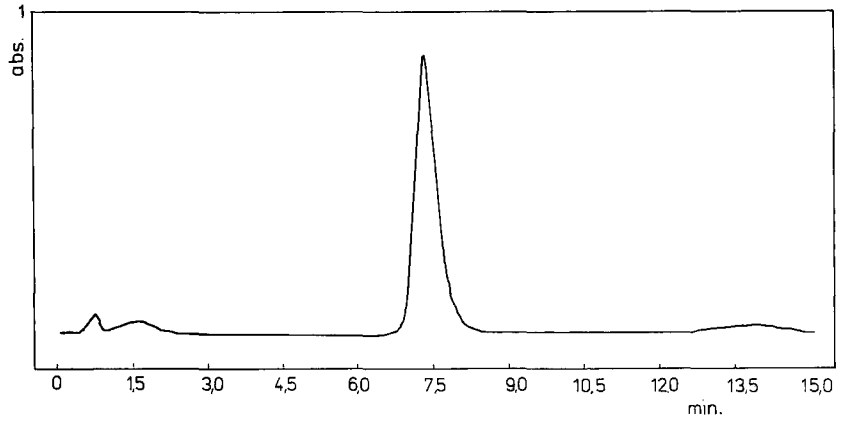

Figure 3. Elution of AT III standard.

Sorbent, column and conditions - see Figure 1.

Elution: 0.0 - 3.0 min - buffer A,

3.0 - 8.0 min - linear gradient from 0 to 50 % of buffer B,

8.0 - 9.0 min - linear gradient from 50 to 100 % of buffer B.

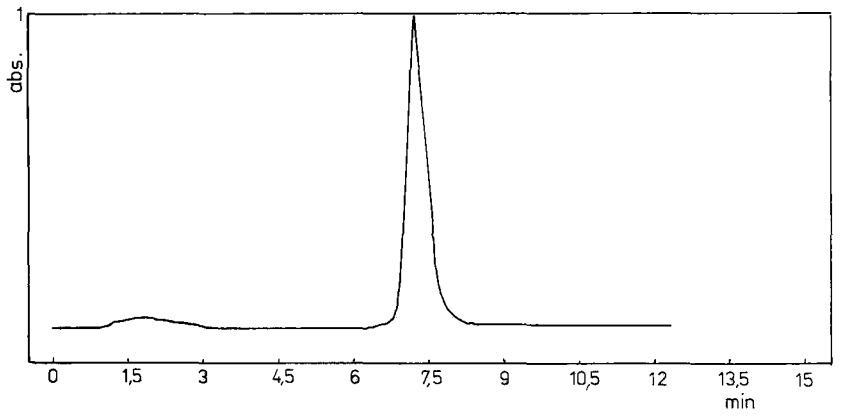

Figure 4. Elution of AT III standard.

Sorbent, column and conditions - see Figure 1.

Elution: 0.0 - 3.0 min - buffer A,

3.0 - 7.0 min - linear gradient from 0 to 50 % of buffer B.

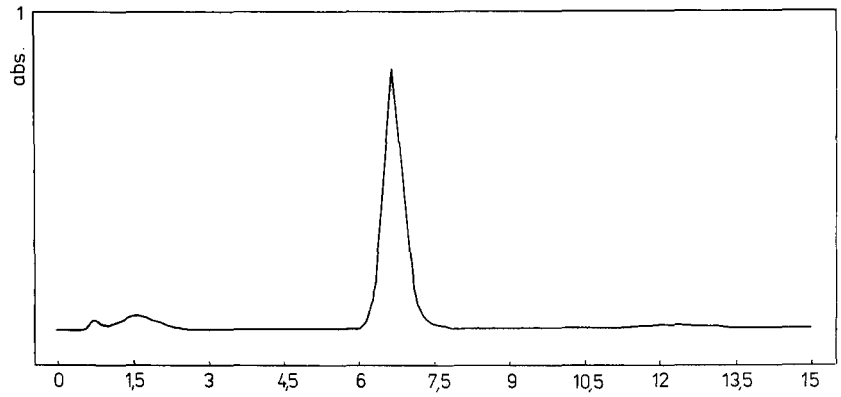

Figure 5. Elution of AT III standard.

Sorbent, column and conditions - see Figure 1.

Elution: 0.0 - 3.0 min - buffer A,

3.0 - 6.0 min - linear gradient from 0 to 100 % of buffer B.

retention time of AT III equals 6.68 minutes. Practically it is the shortest retention time that can be observed with the column and the flow rate used in the presented experiments and at the beginning of the NaCl gradient in the 3-rd minute (just after the elution of the substances which accompany the AT III). The further diminution of the analysis time is possible by the decrease of the column length (e.g. to 3 or 5 cm) and by the increase of the mobile phase flow rate.

Taking into account the last remarks and the results shown in Figures 3 - 5 it is seen that the AT III analysis can be carried out by means of high performance affinity chromatography within less than 5 minutes. It is a considerable advance compared to the quickest, classic methods described above [8-11].

The chromatograms shown in Figures 1 - 5 were performed employing 0.02 M Na_2HPO_4, pH 7.4 buffer as the initial mobile phase. It can be easily found that besides Na ions, which are contained in both eluents, blood possesses, among others, Cl ions, which are the components of the AT III eluting buffer. Their concentration in blood (95 - 107 mmol/dcm^3) should not practically influence the separation, all the more the small plasma volume (100 μl in the case of the

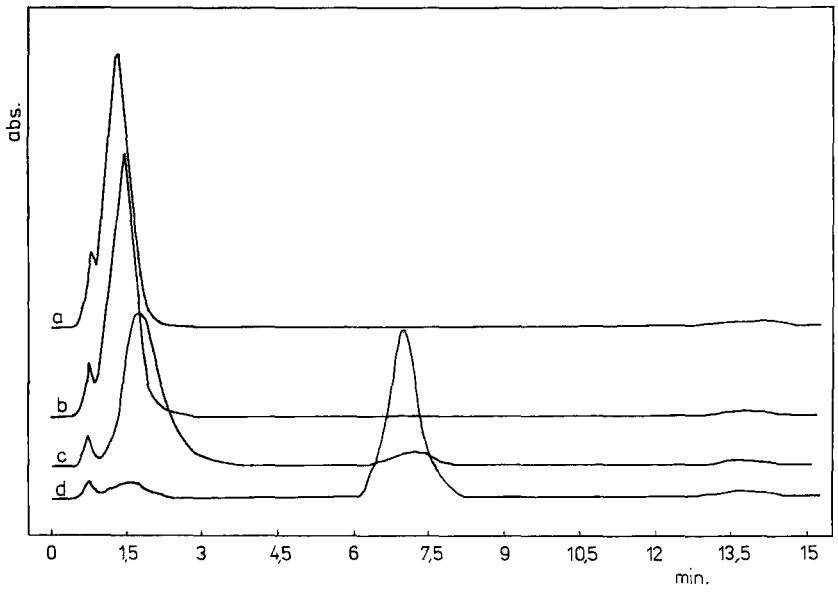

Figure 6. Chromatograms of AT III standard.

Sorbent, column and conditions - see Figure 1.

a) Elution: 0.0 - 3.0 min - 15 % of buffer B in buffer A,
3.0 - 8.0 min - linear gradient from 15 to 100 % of buffer B.

b) Elution: 0.0 - 3.0 min - 10 % of buffer B in buffer A,
3.0 - 8.0 min - linear gradient from 10 to 100 % of buffer B.

c) Elution: 0.0 - 3.0 min - 5 % of buffer B in buffer A,
3.0 - 8.0 min - linear gradient from 5 to 100 % of buffer B.

d) Elution: 0.0 - 3.0 min - 2 % of buffer B in buffer A,
3.0 - 8.0 min - linear gradient from 2 to 100 % of buffer B.

above shown chromatograms) is diluted in the mobile phase after injection. However, the problem appears what the maximal NaCl concentration in the initial buffer is which does not change the separation.

Figure 6 presents the chromatograms of AT III obtained at the same shape of gradient (the increase of B buffer begins in the 3-rd minute of analysis time) but with the various concentrations of NaCl in the initial buffer. (The latter is expressed as the percent of B buffer in buffer A). As results from Figure 6, at the 15 and 10 % of B buffer in buffer A (chromatograms a and b) the separations are very bad and not acceptable from the analytical point of view. A similar situation is in the case when the initial buffer contains 5 % of B. The appearance of a small concentration zone after 7.2 minutes is the only main difference among a, b and c chromatograms. The concentration 2 % of B in the initial eluent leads to the chromatogram d (Figure 6) which is very similar to those in Figures 3 - 5.

The quantitative analysis is based on the relation between the amount the injected substance and the dimension of the signal response which shows the presence of the substance leaving the chromatographic column. The linearity of such a relationship is very useful, especially in the routine analysis. The dependences of AT III peak area vs. the volume of the injected AT III standard or plasma are shown in Figures 7 and 8 respectively. These relationships were performed at 280 nm wave length and at the same detector sensitivity as well as the same gradient of the mobile phase. As results from the last Figures the deviation from the linearity of these plots occurs for the samples exceeding 50 µl. Thus, employing the system used the sample volumes lower than 50 µl should be injected or the dilution of the analysed preparate is desirable.

Table II lists the dilution degree of AT III standard (Table II a) or human plasma (Table II b), the elution volumes of AT III peak, the concentration of AT III in the eluted zone and the properties of AT III zone. These data indicate the independence of the retention of α_2-globulin vs. dilution.

The investigations of the sensitivity of the HPAC method, carried out using the equipment described in Experimental, shows that the AT III can be examined in the human plasma diluted more than 10.000 times. In other words AT III can be analyzed using less than 10 nl of blood, hence in the much smaller amount than the classical method requires.

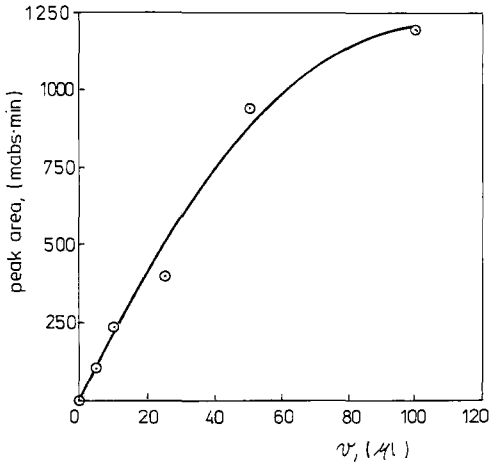

Figure 7. AT III peak area vs. the sample volume of AT III standard (activity - 50 I.U./ml).

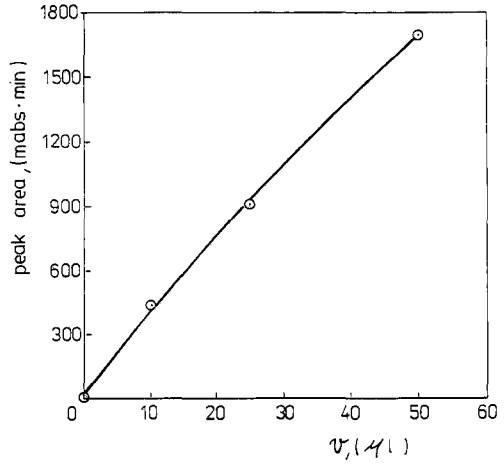

Figure 8. AT III peak area vs. the volume of human plasma.

Table II. Retention data of AT III peak in the diluted AT III standard or human plasma and features of AT III zone

Table II a. Data for AT III standard.

N°	Dilution degree	Retention time [min]	Concentration [%]	Peak purity index	Max. wave length [nm]
1	Pure standard	7.24	99.8	0.9276	225
2	2 times	7.21	100.0	0.9124	223
3	4 times	7.12	100.0	0.9410	227
4	10 times	7.27	100.0	0.9085	222
5	20 times	7.27	99.5	0.9659	219

Table II b. Data for human plasma

1	Pure plasma	7.19	99.5	0.9194	221
2	2 times	7.17	99.7	0.9212	224
3	4 times	7.19	99.8	0.9164	218
4	10 times	7.32	100.0	0.9202	227

CONCLUSIONS

1. Heparin chemically bonded to a dextran layer immobilized on CPG exhibits affinity for the human antithrombin III.
2. The other substances presented in blood plasma do not disturb the elution of AT III.
3. The application of the proper NaCl gradient and short chromatographic column can reduce significantly the AT III analysis time.
4. The sensitivity of high performance affinity chromatography in relation to AT III analysis exceeds many times that of classical methods

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USE OF ADSORPTION CHROMATOGRAPHY ON SEPHACRYL S-500 FOR IMPROVED SEPARATION OF ISOFORMS OF SOLUBLE PHOTOSYNTHETIC CATALYSTS FROM CYANOBACTERIA

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ABSTRACT

Sephacryl S-500, a gel-filtration matrix, adsorbs water-soluble proteins at high concentrations of ammonium sulfate. It has been used here as a matrix for adsorption chromatography, separating isoforms of cytochrome c_{553} and ferredoxin from *Oscillatoria sp.* and *Microcystis aeruginosa*, respectively. The isoforms of these proteins were separated at 35% and 40% saturated ammonium sulfate, respectively. Using this adsorption chromatography, we have purified high levels of individual isoforms with improved resolution over conventional procedures such as polyacrylamide gel electrophoresis and ion-exchange chromatography.

Introduction

Cytochrome c_{553} and ferredoxin are valuable tools for studying structure-function relationships, and both are small, water-soluble proteins acting as photosynthetic electron carriers (1). While ferredoxin is common among photosynthetic organisms, cytochrome c_{553} has only been found in eukaryotic algae and

cyanobacteria. The presence of isoforms of either protein in the same organism has been reported in a number of species (2-5). For examination of individual isoforms, large amounts of purified proteins are required. Most methods of purification rely on the surface charges of individual isoforms. In certain cases, the proteins show very similar charge and have to be purified by hydrophobic chromatography (6) or reversed-phase HPLC (7). These methods entail the use of expensive gel matrices or equipment and have not been widely adopted. Recently, we found that Sephacryl S-type gels adsorb proteins at high ammonium sulfate concentrations and such adsorption can be used with advantages for protein separation (8). Here, the separation of isoforms of cytochrome c_{553} and ferredoxin on Sephacryl S-500 has been studied using a high concentration of ammonium sulfate. The results show that this gel matrix provides a convenient and inexpensive way to purify individual isoforms which are not readily resolved by ion-exchange chromatography or gel electrophoresis.

MATERIALS AND METHODS

Sephacryl S-500 was purchased from Pharmacia. All other chemicals were of analytical reagent grade. *Oscillatoria sp.* and *Microcystis aeruginosa* were grown and harvested according to Kang et al. (9) Protein purifications were conducted at 4°C.

Purification of ferredoxin from *Microcystis aeruginosa*

The packed cells (28 g fresh wt.), resuspended in 50 mM NaCl-50 mM Tris/HCl (pH 7.5), were sonicated for nine 1-min intervals with a Sonifier cell disruptor B-30 (power at two-fifths maximum). After centrifuging at 15,000 rpm for 10 min, the

pellets were resuspended and sonicated again. These steps were repeated several times until the supernatant turned pale blue. All the soluble extracts were pooled, made to 35% $(NH_4)_2SO_4$ saturation and centrifuged for 20 min at 40,000 rpm. The resulting supernatant was adjusted to 60% $(NH_4)_2SO_4$ saturation and centrifuged again for 20 min at 40,000 rpm. The final supernatant was loaded to a Sephacryl S-200 column (1.5 X 17.5 cm) previously equilibrated with 60% $(NH_4)_2SO_4$ saturation in the NaCl/Tris buffer. Ferredoxin and other proteins adsorbed to the column were eluted with a 500 ml gradient of $(NH_4)_2SO_4$ decreasing from 60% to 35% saturation in the NaCl/Tris buffer. Fractions containing ferredoxin were pooled, dialyzed several times against a Tris/HCl buffer (50 mM, pH 7.5), concentrated with an Amicon flow-cell (10 kDa cut-off) and passed through a Sephadex G-50 column (2.5 X 90 cm) washed with the Tris/HCl buffer. Ferredoxin was finally adsorbed to a DEAE-Sephacel column (1.5 X 6 cm), previously equilibrated with the Tris/HCl buffer. After washing the column with 250 mM NaCl followed by 280 mM NaCl in the Tris/HCl buffer, proteins were eluted by a 400 ml 280-330 mM NaCl gradient. The protein, eluted at around 300 mM NaCl, was used for SDS and non-denaturing gel electrophoresis, IEF and adsorption chromatography on Sephacryl S-500.

Purification of cytochrome c553 from *Oscillatoria sp.*

The packed cells (30 g fresh wt.) were broken after several cycles of freezing and thawing in 50 mM Tris/HCl-50 mM NaCl (pH 7.5) and centrifuged for 10 min at 15,000 rpm. The initial purification of the soluble extract was similar to that of *Microsystis aeruginosa*. This included fractionation at 35% and

75% $(NH_4)_2SO_4$ saturation and Sephacryl S-200 chromatography. The latter was developed with a 400 ml $(NH_4)_2SO_4$ gradient decreasing from 60% to 30% saturation in 100 mM Tris/HCl, pH 8.6. Fractions containing the cytochrome were pooled, dialyzed several times against 5 mM Mes-Tris, pH 6.0, and adsorbed to a CM-Sephadex column (1 X 6 cm), previously washed with the dialyzing buffer. The cytochrome, eluted with a Tris-HCl buffer (25 mM, pH 7.5), was concentrated by an Amicon flow-cell (10 kDa cut-off), and passed through a Sephadex G-50 column (1.5 X 30 cm) washed with the Tris-HCl buffer. Fractions containing the cytochrome were pooled and used for SDS and non-denaturing gel electrophoresis, IEF and adsorption chromatography on Sephacryl S-500.

Analytical methods

Protein concentration in column effluents was monitored at 206 nm using an LKB UV detector. Cytochrome concentration was estimated by a reduced minus oxidized spectrum, using 20 $mM^{-1}cm^{-1}$ as the difference absorption coefficient at the 553 nm (10). The absorption difference (ascorbate minus ferricyanide) was made at 553 nm relative to a line drawn through the absorbances at 542 and 561 (11). Ferredoxin was estimated from the oxidized spectrum of the sample at 423 nm using an extinction coefficient of 9.7 mM^{-1} cm^{-1} (12). All estimations were made at room temperature with a Shimadzu UV-160 spectrophotometer.

Polyacrylamide gel electrophoresis

Non-denaturing and SDS gel electrophoresis were performed in either 10 or 15% acrylamide gel rods using the high pH buffer system of Hames (13). After electrophoresis, the gels were stained for proteins using Coomassie brilliant blue G-250 (14). IEF gel electrophoresis was conducted on gel rods as described by Righetti and Drysdale (15).

Adsorption chromatography on Sephacryl S-500

This was conducted at room temperature using a Sephacryl S-500 column (16.5 x 1.5 cm). For cytochrome c_{553}, the column was washed with 100 mM Tris/HCl (pH 8.0), 200 mM NaCl and $(NH_4)_2SO_4$ adjusted to 35% saturation. For ferredoxin, the washing buffer and $(NH_4)_2SO_4$ were adjusted to pH 8 and 40% saturation, respectively. The flow-rate was maintained at 11 ml h^{-1}. Protein samples were prepared in small volumes of washing buffers.

RESULTS

Tables 1 and 2 summarize the purification of ferredoxin and cytochrome c_{553}, respectively. At the early stage, high concentrations of ammonium sulfate were used to precipitate the large amounts of colored pigments interfering with the purification. Prior to dialysis, the ammonium sulfate supernatant was adsorbed on Sephacryl S-200 and the adsorbed proteins were subsequently fractionated by a decreasing salt gradient. Ferredoxin and cytochrome c_{553}, both eluted between 35% to 45% ammonium sulfate saturation, were found to be completely free of the remaining pigments which remained tightly bound at these concentrations. The two proteins were also recovered in much smaller volumes than that of the starting ammonium sulfate supernatant. This cut down the amount of time and buffers needed for dialysis.

Ferredoxin eluted from the final DEAE-Sephacel column as a broad band characterized by a distinct shoulder and a main peak (Fig.1). The proteins corresponding to the peak and shoulder were pooled and analyzed by gel electrophoresis. The results show a single protein band on 15% SDS and 10% non-denaturing gels and two very close bands on a 15% non-denaturing gel. An unsuccessful

Table 1

Purification of ferredoxin from *Microcystis aeruginosa*

Fraction	Total protein(mg)	Ferredoxin (mM)	% recovery	Degree of purification
Crude extract	3585	-	-	-
35% $(NH_4)_2SO_4$	1696	-	-	-
60% $(NH_4)_2SO_4$	164	-	-	-
Sephacryl S-200	92.1	2.5×10^{-3}	100	1
Sephadex G-50	11.0	0.64×10^{-3}	26	2.5
DEAE-Sephacel	3.09	0.28×10^{-3}	11	4.5

Table 2

Purification of cytochrome c_{553} from *Oscillatoria sp.*

Fraction	Total protein(mg)	cytochrome (mmol)	% recovery	Degree of purification
Crude extract	1080	-	-	-
35% $(NH_4)_2SO_4$	500	-	-	-
75% $(NH_4)_2SO_4$	30.6	-	-	-
Sephacryl S-200	20.99	1.4×10^{-4}	100	1
Sephadex G-50	5.1	1.0×10^{-4}	71	4.2
DEAE-Sephacel	1.0	0.8×10^{-4}	57	4.5

- Prior to the Sephacryl step, blue phycobiliproteins in the extract interfered with ferredoxin determination.

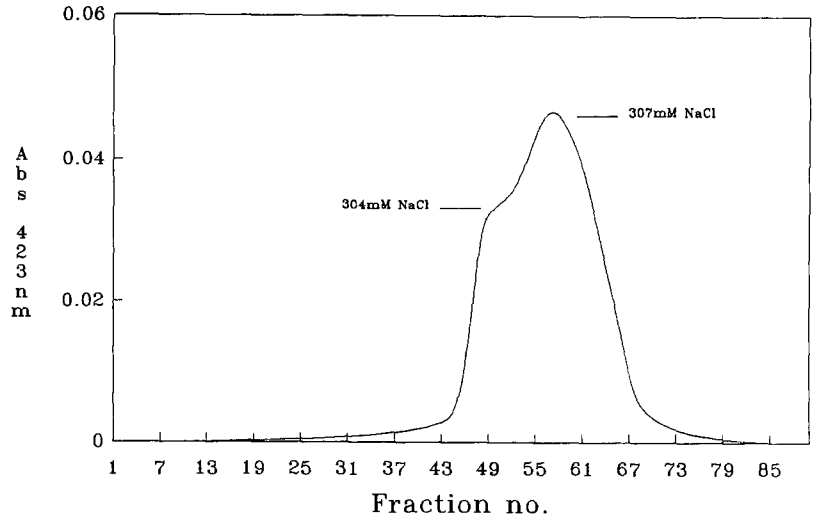

Fig. 1. Elution profile of *Microcystis aeruginosa* ferredoxin from DEAE-Sephacel column (1.5 X 6 cm). Ferredoxin was eluted with a 500 ml linear salt gradient from 280 mM to 330 mM NaCl in 50 mM Tris/HCl, pH 7.5.

attempt was made to resolve the protein on an IEF gel. As the protein approached its pI value on the gel, the low pH enviroment caused it to denature and form a broad yellow smear at the lower portion of the gel.

Cytochrome c_{553} eluted as a single band during CM-Sephadex chromatography. After the final Sephadex G-50 column chromatography, the purified protein was subjected to gel electrophoresis. It migrated as a single band on 15% non-denaturing and SDS gels. The protein focused to a pink band near the alkaline end of an IEF gel and showed a pI value of 9.3.

Figure 2 shows the chromatographic behaviour of the purified ferredoxin and cytochrome c_{553} on a Sephacryl S-500 gel column washed with high concentrations of $(NH4)_2SO4$. The results show that the isoforms of these proteins were clearly resolved.

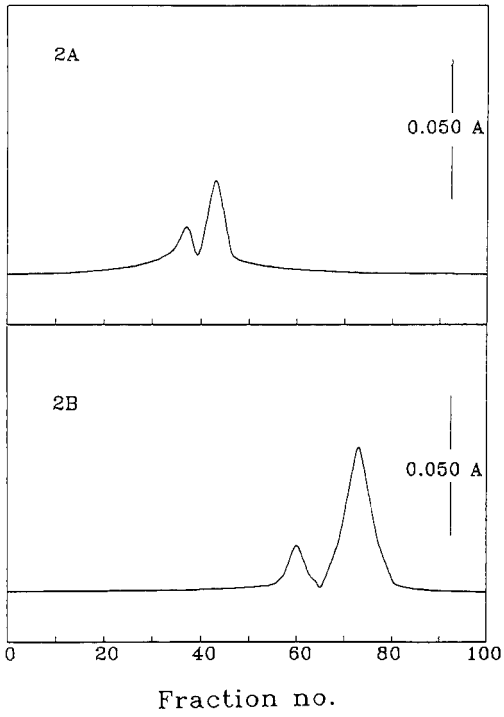

Fig. 2. Elution profile of purified proteins from Sephacryl S-500 (1.5 X 16.5 cm) in the presence of ammonium sulfate at 35% saturation in 200 mM NaCl, 50 mM Tris/HCl. (A) *Microcystis aeruginosa* ferredoxin, 0.8 mg, was separated at pH 7.5. (B) *Oscillatoria sp.* cytochrome c_{553}, 1.4 mg, was separated at pH 8.0. The elution was monitored at 206 nm.

We concentrated the peak fractions of individual ferredoxin isoforms and ran them separately on 15% non-denaturing gels. Figure 3 shows the electrophoretic profiles of individual isoforms and a sample containing a mixture of the two isoforms. The results clearly show that the first and second isoforms eluted off the Sephacryl S-500 column correspond to the fast and the slow-moving species on the non-denaturing gel, respectively.

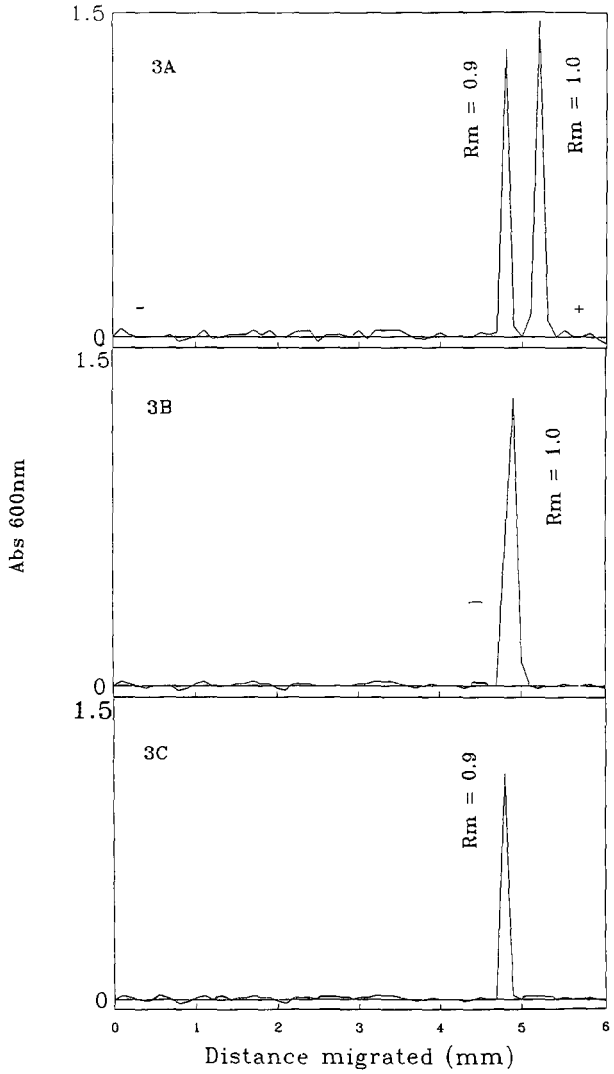

Fig. 3 Densitometric scans at 600 nm of *Microcystis aeruginosa* ferredoxin on nondenaturing 15% PAGE gels. Protein bands were stained with Coomassie Blue. (A) shows a mixture of the two ferredoxin isoforms, about 2 ug each . (B) and (C) represent about 2 ug each of the first and second ferredoxin isoforms, respectively. Rm is expressed as distance migrated by the protein band divided by the distance migrated by the dye front.

We also ran the two separate forms of cytochrome c_{553} through reversed-phase HPLC and found them eluted at different retention times (result not shown).

DISCUSSION

The present work exploits the adsorption phenomenon on Sephacryl S-500 for protein separation. This procedure provides a better separation for the two isoforms of ferredoxin from *Microcystis aeruginosa*, compared with gel electrophoresis. Moreover, it gives a higher yield of the purified proteins. At high concentrations of ammonium sulfate, hydrophobic interactions contribute to protein adsorption on Sephacryl S-type gels (8). These interactions are most likely different between individual isoforms, thus allowing them to be separated on Sephacryl S-500.

Hydrophobic chromatography has been used previously to separate two isoforms of spinach ferredoxin, but this required a hydrophobic matrix, TSK-gel Phenyl-5PW (6). On the contrary, Sephacryl S-500 is less costly and is commonly used for gel-filtration chromatography. Its rigid matrix also allows high flow-rates.

The isoforms of cytochrome c_{553} from a local strain of *Oscillatoria sp.* were not resolved by either ion-exchange chromatography or gel electrophoresis, probably because of their very similar surface charges. This agrees with an early study which found that reversed-phase HPLC had to be used for separating two isoforms of cytochrome c_{553} from an American strain (7). Since reversed-phase HPLC results in protein denaturation, a more gentle procedure such as the one described here should be useful for functional studies.

Adsorption chromatography was used not only in the final separation of the ferredoxin or cytochrome c_{553} isoforms but also at the early stage of purification. Right after the ammonium sulfate step, the soluble proteins were adsorbed and subsequently fractionated on Sephacryl S-200. This step, designed to remove the remaining colored pigments which interfered with the purification, worked better with a continuous gradient of decreasing ammonium sulfate. The salt gradient seemed to work against the separation of the isoforms of ferredoxin and cytochrome c553 on Sephacryl S-type gels. These proteins were eluted off Sephacryl S-200 as broad bands. Individual isoforms of these proteins separated somewhat better when Sephacryl S-500 was used instead, but the resolution was still poor. It is noteworthy that Sephacryl S-200 is less hydrophobic than Sephacryl S-500 (8), and this may explain why individual isoforms were not separated at all on this matrix. However, Sephacryl S-200 was chosen in preference to Sephacryl S-500 at this stage of purification because of its greater flow-rate and smaller elution volumes for the proteins.

Apart from Sephacryl S-type gels, there are other gel-filtration matrices known to adsorb proteins in the presence of high concentrations of ammonium sulfate (16-19). Very few have been exploited for protein separation based on their adsorptive properties. Here, we demonstrated that Sephacryl S-500 could be useful for purifying isoforms in a preparative scale.

CONCLUSION

Cyanobacterial cytochrome c_{553} and ferredoxin isoforms were separated successfully using adsorption chromatography on Sephacryl S-500. This method achieves better resolution and

higher yields of purified proteins than gel electrophoresis. Adsorption chromatography, like reversed-phase HPLC separates proteins according to their relative hydrophobicities and does this without denaturing the protein. Herein lie its advantages in the separation of proteins that differ appreciably in hydrophobicities. Prior to the submission of this manuscript, we have succeeded in extending the present procedure to separation of isoforms of ferredoxin from a variety of high plants.

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DETERMINATION OF ALIPHATIC ALDEHYDES C_1 - C_4 IN WASTE GAS BY HPLC

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ABSTRACT

A simple and rapid method for the determination of aliphatic aldehydes (C_1 - C_4) in waste gas was developed. Sample collection and derivatization are performed directly in a sorption tube containing Chromosorb P impregnated with 2,4 - dinitrophenylhydrazine and catalyst. This methods allows direct injection of a methanol extract of a sorption tube into a chromatographic column. Detection limit of aldehydes is below 1 mg/m^3 for a 10 l sample. The technique was used to measure aldehyde emissions from wood fired furnace.

INTRODUCTION

Aldehydes represent a class of chemical compounds of partially oxidized organic substances which react more or less rapidly to form further products. they are produced by many industrial processes and combustion sources. Aldehydes are known contributors to photochemical smog and irritants

of the skin, eyes and nasophatyngeal membranes (1,2). Formaldehyde has been identified as a suspected carcinogen, and for this reason the maximum tolerable limit in air must be low (1.2 mg/ m^3) (3).

Concern about environmental pollution and occupational hazards due to the presence of aldehydes in air has led to the development of analytical method for the determination at trace level. The best known method for the determination of aldehydes at trace levels by HPLC is the derivatization which uses 2,4-dinitrophenylhydrazine (DNPH) as reactant (4). Numerous studies have been published which use DNPH as the derivatizing reagent for the collection of carbonyl compounds in impingers and cartridges with subsequent analysis by HPLC (5-7). The anthrone reagent has been utilized as a selective reagent for derivatizing the unsaturated aldehydes to produce fluorometric derivatives (8). Several other techniques have been reported for the derivatization of aldehydes (9-11).

The aim of this paper is to describe a method for the determination of aliphatic aldehydes (C_1 - C_4) by HPLC in which the sample collection and the derivatization reaction are performed directly in a tube containing a sorbent which is impregnated with DNPH reagent. This technique was used to measure aldehyde emissions from wood fired furnace.

EXPERIMENTAL

In sampling the highest efficiency of aldehydes trapping from the waste gas using a sorption equipment is required.The sampling system consists of the sampling probe from stainless steel (inside diameter 10 mm). The suction tube is heated to such a degree that the temperature of the aspirated gas stream corresponds to that in the waste gas channel.The waste gas passed trough 2 sorption tubes ordered one after other in order that the determination of aldehyde sampling could be tested. The flowmeter was used

for the measurement of the waste gas volume. For the measurement of the temperature of the gas in front of the sorption tube a thermocouple was used. An oil diffusion pump was used as a suction aggregate. The emission gas passed trough the sorption system at the rate about 200 ml/min. After 10 minutes the pump is shut off and sample volume as well as temperature and barometric pressure at the sampling site is recorded. The sorption system consists of sorption tubes which are filled with Chromosorb P impregnated with DNPH (50 g Chromosorb P, 50 ml 0.5% DNPH in acetonitrile and 0.3 ml conc. phosphoric acid).

The contents of the sorption tubes were extracted with 3 x 1 ml of methanol. Both sorption tubes of a sample are to be measured separately. If the amount of aldehydes in second sorption tube exceed 10% of the amount found in the first tube then the sample volume of the waste gas must be lower.

The HPLC equipment consisted of a Waters 510 HPLC pump, a sample valve VALCO (10 µl loop), a Waters 484 variable wavelength detector. 2,4-dinitrophenylhydrazones of aldehydes were separated on a 100 x 3 mm I.D./ Tessek SGX C18 column, 5 µm with isocratic elution at 0.5 ml/min. The eluent was methanol - water (7 : 3, v/v). The temperature of column was 25°C. The injected volume was 10 µl and spectrophotometric detection took place at 355 nm.

The standard 2,4-dinitrophenylhydrazones were synthesized by the reaction of carbonyl compounds with DNPH in the presence of hydrochloric acid that promotes the protonation of the carbonyl group. The reaction products were purified by recrystallization from methanol. Standard solutions were prepared by dissolving weighed amounts of the pure hydrazones in methanol

All chemicals must be of analytical grade, distilled water is used for the solutions. Water deionized with synthetic resins may contain formaldehyde.

RESULTS AND DISCUSSION

The chromatographic system was designed for a complete separation of all 2,4-dinitrophenylhydrazones of aldehydes expected as products of the wood fired furnace. Care must be taken to optimize HPLC conditions for sensitivity and selectivity of the separation process. Some absorption maxima of 2,4-dinitrophenylhydrazones can be seen in Fig.1, from which the maxima at 215 and 355 nm are most advantageously. Many organic substances absorb at 215 nm and an interference can be occurred. For this reason the detection will be more selective at 355 nm and possibility of the interference with other compounds is considerably lower. The qualitative evaluation was carried out on the basis of a comparison of the elution time of the standard and the sample. In our experimental conditions, retention times were reproducible within 4.5%.

Prior to measurements the blank value of the sorption tubes employed must be determined. For this purpose the sorption tubes are submitted to the complete analytical

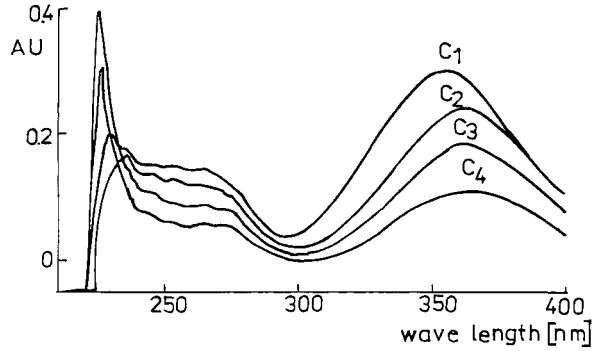

FIGURE 1
Absorption spectra of 2,4-dinitrophenylhydrazones of aldehydes C_1 - C_4.

procedure. The average blank value is to be considered in the calculation of the results.

The calibration mixtures are analyzed by using at least three hydrazone/solvent dilution ratios for each aldehyde. Calibration curves relating the concentration of hydrazone to the ratio of the peak area of the compound to that of the internal standard are constructed by regression analysis. These calibrations cover the range of hydrazone concentrations in samples listed in Table 1. Fig.2 illustrates the separation of hydrazones of C_1 - C_4 aldehydes in

TABLE 1
Determination of aldehydes in the samples

sample	sampling volume [l]	concentrations of aldehydes [mg/m^3]		relative standard deviation (3 measurements)
1	8.0	C_1	1.18	0.06
		C_2	1.30	0.06
		C_3	--	
		C_4	--	
2	6.0	C_1	1.57	0.07
		C_2	0.62	0.08
		C_3	--	
		C_4	--	
3	8.5	C_1	0.71	0.07
		C_2	--	
		C_3	--	
		C_4	--	
4	8.6	C_1	0.83	0.07
		C_2	--	
		C_3	--	
		C_4	--	

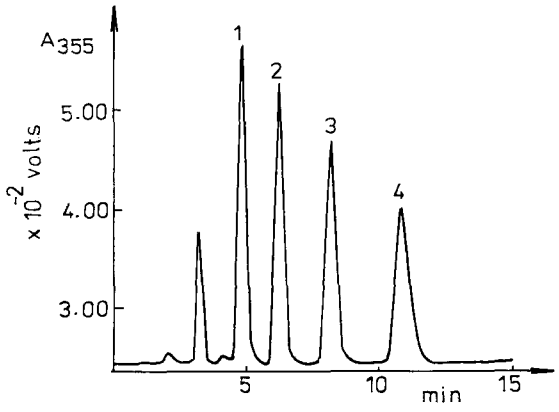

FIGURE 2

Chromatogram of 2,4-dinitrophenylhydrazones of aldehydes separated on a Tessek SGX C18 column: methanol-water (7:3) at 0.5 ml/min.
Formaldehyde (peak 1), acetaldehyde (2), propanal (3) butanal (4), (1 ppm).

less than 15 min. The chromatogram of the sample 1 is shown in Fig.3.

The results of the determination of aldehydes in the samples are given in Tab.1. The concentration of C_3 and C_4 aldehydes was below the limit of the determination. Simultaneously with the extract of the first sorption tube the extract of the second tube was analyzed. It was found that the amount of hydrazones was below the limit of the determination. It can be seen (Tab.1) that the values of the standard deviation depend on the content of aldehydes in the samples. The optimum concentration interval is in the range 0.1 - 100 μg in 1 ml of methanol. The relative standard deviation can be decreased by sampling a greater gas volume but on the other hand the possibility of an interference can increase.

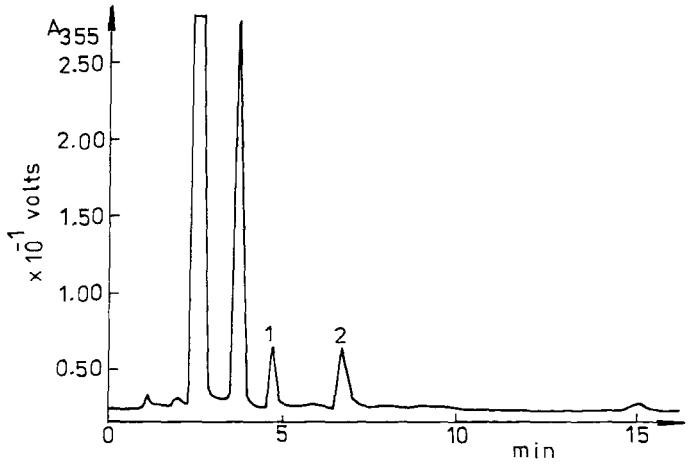

FIGURE 3
Chromatogram of the sample 1.
Formaldehyde (peak 1), acetaldehyde (2).

The limit of the determination depends on the aspir ated waste gas volume. The concentration of aldehydes in the waste gas volume of 1.0 mg/m^3 (sampling volume 10 l) can be determined that is in agreement to the requirements given in literature (3). The absolute limit of the determination - the amount, that can be determined in the injected solution (10 μl) is 1 ng. At this injected amount the height of the peak of hydrazones was five time higher than the noise of the base line.

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QUANTITATIVE HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHIC DETERMINATION OF ORGANIC ACID PRESERVATIVES IN BEVERAGES

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ABSTRACT

A simple quantitative HPTLC method for the determination of sorbic acid, benzoic acid, and dehydroacetic acid in beverages without extraction or cleanup is described. Aliquots of samples and standards are chromatographed on preadsorbent silica gel or C-18 bonded silica gel plates containing fluorescent indicator, and the zones, which quench fluorescence, are compared by scanning densitometry. Recoveries of the acids from wine and juices spiked at 50-300 ppm averaged 98%, and the coefficient of variation of replicate analyses ranged from 2-5%. Commercial sodas containing unknown amounts sodium benzoate and iced teas containing potassium benzoate were analyzed by the method, and the tea analyses were validated by standard addition. The advantages of the TLC method relative to current HPLC and absorption spectrometric procedures are described.

INTRODUCTION

Sorbic acid and benzoic acid are antimicrobial agents that are used in a great variety of foods including juices, iced teas, sodas, and other beverages (1), and sorbic acid (2) and dehydroacetic acid (DHA) (3) have been used as preservatives in wine. The latest methods for quantitative determination of sorbic and benzoic acids in foods and DHA in wine have been based on high performance liquid chromatography (1,3). The AOAC official methods for sorbic acid in wine involve steam distillation followed by UV absorption spectrometry at 260 nm or reaction with thiobarbituric acid and colorimetry at 532 nm (2,4). The only quantitative TLC method for determination of these preservatives was reported for sorbic and benzoic acids in mayonnaise and ketchup by steam distillation, solvent extraction, separation on a CN-bonded silica gel layer with fluorescent indicator, and detection under 254 nm UV light (5). In this paper we report a simple quantitative procedure for sorbic and benzoic acids in fruit juice, iced tea, and soda and sorbic acid and DHA in wines that involves direct spotting of samples on high performance preadsorbent silica gel or C-18 bonded silica gel layers containing fluorescent indicator, and scanning densitometry of quenched zones. The method was validated using juice, iced tea, and wine fortified with 50-300 ppm of the acids, and it was used to analyze commercial iced teas and sodas containing potassium sorbate and sodium benzoate, respectively, added as a preservative.

EXPERIMENTAL

Standards

Standards of sorbic, benzoic, and dehydroacetic acids were obtained from Aldrich (Milwaukee, WI). Stock

standard solutions were prepared containing 25.0 mg/ml of sorbic acid in ethanol, 20.0 mg/ml of benzoic acid in ethanol, and 20.0 mg/ml DHA in acetone. TLC standards were prepared by exact 1:100 dilution of the sorbic acid stock solution with ethanol (250 ng/ul), and 1:10 dilution of benzoic acid and DHA with ethanol and acetone, respectively (2.00 ug/ul).

Thin Layer Chromatography

Normal phase TLC was carried out on 20 x 10 cm Whatman (Clifton, NJ) LHPKDF preadsorbent high performance silica gel plates containing 19 lanes of 9 mm width. Reversed phase TLC was performed on 20 x 20 cm Whatman LKC18F preadsorbent C-18 bonded silica gel layers. Both layers contained a phosphor that fluoresced when irradiated with 254 nm UV radiation.

Sample and standard zones were applied to the preadsorbent as vertical streaks using a 10 ul Drummond (Broomall, PA) digital microdispenser. After spotting, initial zones were thoroughly dried with warm air from a hair dryer; application of sample aliquots greater than 10 ul was facilitated by positioning the hair dryer so that a gentle stream of warm was sweeping over the preadsorbent during the spotting process. Plates were developed for a distance of 7 and 10 cm beyond the sorbent-preadsorbent interface for silica-gel and C-18, respectively, in one compartment of a Camag (Wilmington, NC) twin-trough chamber with a sheet of Whatman No. 1 chromatography paper dipping in a pool of mobile phase in the other compartment in order to saturate the chamber with solvent vapors. The mobile phases were n-pentyl formate-chloroform-formic acid (2:7:1) for silica gel (6) and methanol-0.5 M NaCl (1:1) for C-18 bonded silica gel. Chromatograms were air dried in a fume hood for 20 min,

and acids were detected under 254 nm UV light in a viewing cabinet as dark quenched zones on a bright fluorescent background. Areas of zones were evaluated by scanning each lane using a Shimadzu (Columbia, MD) model 930 densitometer with a UV (deuterium lamp) source and a slit size of 6 mm (height) x 0.4 mm (width), operated in the single beam, reflectance mode. Scanning was performed at the wavelength of maximum absorption, which was determined by measuring the in situ spectrum of a standard zone of each analyte between 200 and 370 nm. Calibration curves were obtained by chromatographing 0.50, 1.00, 2.00, 4.00, 6.00, and 8.00 ul aliquots of TLC standards (125-2000 ng for sorbic acid and 1.00-16.0 ug for benzoic acid and DHA) and calculating the linear regression equation from the scan area and weight data by use of a computer program.

Analysis of Samples

Fortified samples of sorbic acid were prepared at nominal concentrations of 300, 250, and 50.0 ppm by combining 1.20, 1.00, and 0.20 ml of stock standard solution with 98.8, 99.0 and 99.8 ml of beverage, respectively. Fortified samples of benzoic acid and DHA were prepared in volumetric flasks at 250 and 125 ppm by combining 1.25 ml of stock solution with 98.75 and 198.75 ml of beverage, respectively.

Recovery analyses were performed by spotting duplicate aliquots of fortified beverage, duplicate aliquots of standard solution that contained the theoretical weight for 100% recovery and was within the linear calibration range, and duplicate aliquots of blank (unfortified) sample. For analysis of beverages spiked with 250 ppm of sorbic acid, 2.00 ul aliquots of standard, sample, and blank were compared; the standard

and sample would each contain 500 ng of acid if recovery was 100%. For wine containing 300 ppm of sorbic acid, 1.67 ul of spiked sample and blank were spotted along with 2.00 ul of standard. For analysis of a beverage spiked with 50.0 ppm of sorbic acid, 2.00 ul of standard was compared to 10.0 ul of sample. For analysis of benzoic acid or DHA spiked at 250 and 125 ppm, 1.00 ul of standard (2.00 ug) was compared respectively to 8.00 and 16.0 ul of sample. Percent recovery was calculated by comparing the average scan area of acid zones in the beverage chromatograms with the average of the standard scans.

Commercial samples of iced tea containing unspecified amounts of potassium sorbate and sodas containing sodium benzoate as preservatives were analyzed by spotting 0.50 to 8.00 ul aliquots of standard as described above adjacent to 0.50, 1.00, 2.00, 4.00, 8.00, 12.0, and 16.0 ul aliquots of sample. The soda samples were initially decarbonated by stirring and then adjusted to a pH of 2.0-2.5 by addition of concentrated HCl to assure the conversion of benzoate to benzoic acid. The ratio of the average areas of the most closely matching sample and standard zones (spotted in duplicate) was used to calculate the weight (ug) of acid in the sample; as an example, in the analysis of diet cola, the 0.5 ul benzoic acid standard zone was compared to the 8.00 ul sample zone. The weight of acid was divided by the aliquot volume of the sample (ml) to calculate the concentration as ppm. The results for the tea analyses were validated by fortifying the unknown samples with an additional 250 or 125 ppm of sorbic acid and chromatographing the fortified and nonfortified samples in parallel. The percent recovery of the added sorbic acid was calculated as 100 times the difference between the area of the fortified sample zone and the area of the unfortified

sample zone, divided by the area of a standard zone whose weight represented the amount of the standard addition.

RESULTS AND DISCUSSION

Sorbic and benzoic acids and DHA were detected as quenched zones on plates containing a fluorescent phosphor.

The limit of detection was ca. 100 ng for sorbic acid and 1000 ng for benzoic acid and DHA. The lower absolute sensitivity for densitometry of sorbic acid led to a correspondingly lower analysis limit on a concentration basis for the acid in beverages.

On preadsorbent silica gel layers developed with the formic acid solvent, sorbic, benzoic, and dehydroacetic acids formed flat, compact zones with respective R_f values of 0.61, 0.58, and 0.78. On C-18 layers developed with methanol-aqueous NaCl, sorbic and benzoic acids had respective R_f values of 0.44 and 0.59. The ability to separate sorbic and benzoic acids makes the C-18 layer the choice for analysis of products containing both of these preservatives.

The wavelengths used for densitometric quantification, as determined from the maxima of in situ spectra recorded for standard zones, were 230 nm for benzoic acid and 285 nm for sorbic acid and DHA. In addition to the absorption peak at 285 nm, the spectrum of DHA showed a smaller peak at 230 nm. A 4 ug standard zone gave a scan area about 50% larger at 285 nm compared to 235 nm, so the former was used for analysis of samples. To test whether better densitometric results could be obtained by scanning UV absorption rather than fluorescence quenching, a 4 ug standard of DHA was developed on a silica gel plate without fluorescent indicator and scanned at its absorption maximum, 307 nm

(3). The scan area the non-fluorescent plate was only ca. 10% greater than for quenching at 285 nm, and the calibration curve did not have significantly greater linearity. It was concluded that the convenience of being able to view the spot against the fluorescent plate background outweighed the minimal sensitivity advantage of direct scanning of UV absorption.

Calibration curves between 125-2000 ng for sorbic acid and 1-16 ug for benzoic acid and DHA had an average linear correlation coefficients for 45 trials of 0.95, with a range of 0.91 to 0.99. This strong linear correlation allowed quantification to be carried out reliably by comparing the sample area with the area of a closely matching single standard within the linear region. Considering the above calibration ranges and the ability to precisely apply 0.5 to 16 ul of samples, the quantification limits of the method extended from 7.8 to 4000 ppm for sorbic acid and 62.5 to 32,000 ppm for benzoic acid and DHA.

Table 1 shows recoveries obtained for samples of various juices and wines fortified with 50 to 300 ppm of the preservatives. All analyses were done on silica gel except those indicated with an asterisk, which were performed on a C-18 layer. Each analysis was run on a separate plate and was based on the average of duplicate sample and standard scan areas. As one measure of reproducibility, the percent difference between the scan areas of the duplicate samples and standards ranged from 0.30-5.4% for all analyses. Table 1 shows that recovery values ranged from 86 to 111% and averaged 98.0% for all analyses. These recoveries compare well with those reported for HPLC, which ranged from 90-104% for 250 and 50 ppm of sorbic and benzoic acid in juices (1) and 93-109% for 50 and 100 ppm of DHA in wines (2), and with the spectrophotometric results for sorbic acid in wine, which

TABLE 1

AVERAGE PERCENT RECOVERIES FROM FORTIFIED SAMPLES

Beverage	ppm	S	B	D
Apple juice	250	92.1	101	
		98.7	98.0	
		93.0*		
		96.8*		
	125		95.0	
			100	
	50	96.6		
		109		
Pineapple juice	250	106	97.5	
		105	102	
		111*		
	125		101	
			101	
	50	98.2		
		101		
Grapefruit juice	250	102		
		106		
	50	99.5		
		95.8		
		100		
Tomato juice	250	99.8		
		103		
		99.2		
	50	99.7		
		95.7		
Red wine	300	99.7		
		102		
	250			88.7
				86.0
				90.6
	125			90.2
				96.0
White wine	300	99.0		
		98.3		
Gold wine	250			92.8
				89.8
	125			94.7
				92.9
				90.2

S=sorbic acid, B=benzoic acid, D=dehydroacetic acid
* analysis performed on C-18 layer

ranged from 98-104% at concentrations of 200-400 ppm (2,4). They are also consistent with the generally acceptable 85-115% recovery range for ppm level chromatographic analyses performed to establish systematic error. All possible beverages were not tested with each preservative, but rather a reasonable assortment of samples and concentrations was tested in order to illustrate the levels of sensitivity, accuracy, and precision it was possible to achieve with the TLC method.

The method was used to analyze two commercial lemon flavored iced teas, one with added sugar and one containing NutraSweet, both of which listed an unspecified amount of potassium sorbate among their ingredients. The results of triplicate analyses were 223 ppm with a coefficient of variation (CV) of 1.8% for the sugar-containing tea and 102 ppm +/- 2.6% for the diet tea. The accuracy of these results was validated by adding 125 and 250 ppm of sorbic acid, respectively, to the teas and repeating the analyses as described above. The respective recoveries were 95.6 and 96.0%. The potassium sorbate was converted to sorbic acid during development with the acidic mobile phase.

Three canned sodas, a caffeine-free diet cola, a citrus soda, and a cream soda, containing unspecified concentrations of sodium benzoate were analyzed in triplicate, and the following respective concentrations (average +/- CV) were found: 137 ppm +/- 5.2%, 115 ppm +/- 0.87%, and 431 ppm +/- 3.9%. Many sodas contain sodium or potassium benzoate as a preservative, and our results indicate that the additive is present in a wide range of concentrations.

None of beverages used for recovery studies contained the analytes, as shown by the absence of zones with the same R_f value as the standards in the blank

chromatograms. In addition to the analyte zone, chromatograms of red and gold wine spiked at 250 and 125 ppm with DHA had an additional zone with an R_f value of 0.55. This unidentified zone did not interfere with scanning the DHA. For all samples, strongly sorbed, fluorescence-quenching compounds remained in the preadsorbent area and, for larger aliquots of some of the beverages, migrated a short distance beyond the interface into the silica gel area of the plate. Some of the sample chromatograms also had additional quenched zones at the solvent front.

In summary, the HPTLC method allows direct quantification of sorbic, benzoic, and dehydroacetic acid preservatives in a variety of beverages with accuracy and precision adequate for routine analytical use. The HPTLC procedure has several advantages over previously-published methods. The ability to spot multiple samples on a single plate provides higher sample throughput compared to sequential analysis by HPLC, and solvent usage is very low in the twin-trough chamber. The analysis of standards on the same plate under the same conditions as the samples eliminates the need for an internal standard, as used in HPLC (1). Direct injection of samples in HPLC (1,3) can leave irreversibly sorbed residues on top of the column that may affect column performance with later samples, while strongly sorbed residues from directly-spotted samples do not cause problems in TLC, where each plate is used only once. The HPTLC method does not require sample preparation by steam distillation, as do the spectrophotometric methods for sorbic acid in wine (2,4). Beverages could be analyzed for benzoic acid and DHA at lower concentration levels by HPLC than by our TLC method, i.e., 50 ppm (1) and 5 ppm (3), respectively.

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Evan Norfolk performed preliminary comparative experiments on the TLC separation and detection of organic acids.

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IDENTIFICATION OF BARBITURATES USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-PARTICLE BEAM EI/CI MASS SPECTROSCOPY

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ABSTRACT

The procedure described uses a high performance liquid chromatograph (HPLC) coupled to a quadrupole mass spectrometer via a particle beam interface for the determination of barbiturate identity. The object was the development of a general LC-MS method capable of performing routine analysis. The use of the particle beam interface as a sample introduction technique was selected because of its relatively simple nature and the ability to adapt existing HPLC methods with only minor modification. Chemical ionization (CI) using methane as a reagent gas generated base peak molecular ions for all of the barbiturates analyzed. Electron ionization (EI) at 70 eV caused sufficient fragmentation to allow the analyst to discriminate barbiturates when identical molecular weights were encountered. In those case where highly similar EI and CI spectra are produced, chromatographic separation provided clear distinction of analyte identity. Conversely, when very different spectra are encountered, base line resolution of the analytes was not necessary for accurate identification. When nearly co-eluting spectrally similar analytes are encountered, careful evaluation of the EI+ mass spectra revealed the presence of specific mass peaks exclusive to a particular barbiturate. These mass peaks are used to confirm identity.

INTRODUCTION

Mass spectroscopy (MS) represents an ideal detection system for many disciplines. The ability of the technique to provide structural information on an analyte allows the analyst to determine identity without relying on a reference standard for comparison. This is particularly useful when analytes take the form of metabolites, degradation products, or impurities contributed by the manufacturing process. As system hardware and controlling software become more sophisticated and reliable, mass spectrometry is finding its way out of R&D laboratories into more routine environments, such as analytical and quality control labs. In view of an ever stricter regulatory climate for many aspects of pharmaceutical manufacturing, MS may soon be a required technique for such exercises as purity profiles and process validation.

A variety of techniques for the identification of barbiturates are discussed in the literature. UV photodiode array[1-3] and dual wavelength detection[4] can provide very accurate means of identification, but require a known standard and therefore are limited when an unknown compound is encountered. Although some structural deductions can be provided by the UV spectra[5], when an unknown is encountered UV photodiode array is limited to determining what the compound is *not*, and such "negative results" are *not* the goal of an analytical laboratory. A number of GC-MS methods have been documented but many[6-9] require derivatization, although there are exceptions[10,11]. All employ EI or CI (or a combination of both) despite claims that simple EI/CI spectra do not provide readily available identification of barbiturates[12].

There are many HPLC methods that rely on retention matching for peak identification[13-17]. However, the establishment of retention databases[18] for barbiturate identification can be limited by occurrences of coelution and retention wandering that may result from day to day method variations, despite the use of an internal reference standard. Coupled with an appropriate extraction method, a reliable LC-MS method is vastly superior to much more cumbersome clinical techniques, such as immunocytochemistry[19].

Reversed phase liquid chromatography, common in pharmaceutical analysis, was chosen as the inlet system because of the variety of analytes which could be accommodated. LC is also better suited for a number of common sample preparation techniques, such as solid phase extraction. The particle beam interface provides a straightforward way of introducing the sample into a quadrupole mass spectrometer. The interface is a very simple device, essentially it is a mildly heated vacuum assisted desolvation chamber. LC eluent entering the desolvation canister is converted to an aerosol using helium. In the desolvation canister the eluent rapidly evaporates leaving the lower volatility sample as a particle dispersion in the helium and gas phase solvent. The sample particles and gas phase solvent form a jet as they are sprayed out of the other end of the desolvation canister into a partial vacuum. The rapidly expanding gas phase solvent is separated from the sample particles using two skimmer cones. The process results in the sample particles entering the ion source where they are vaporized and ionized. This can result in the production of classical EI spectra which can be matched against entries in a spectral library to aid in sample identification. The system can also be operated in the CI mode. Using a combination EI/CI source the analyst can switch from one ionization technique to the other in a relatively short time. Chromatographic parameters are also retained. This might not be the case if a different "soft" ionization technique, such as FAB or electrospray, were employed.

MATERIALS AND METHODS

The equipment included a TRIO 2000 single quadrupole mass spectrometer equipped with a combination EI/CI source (VG Biotech Division of Fisons Instruments, Altrincham UK). The liquid chromatograph was a Model 1050 pump, autosampler and variable wavelength detector (Hewlett-Packard, Palo Alto CA USA). MS control and spectral processing was via MassLynx™ release 1.5 (VG Biotech). MassLynx™ functions in a Windows™ 3.1 environment (Microsoft Corporation, Redmond WA USA) on a 486/66 MHz PC. Separations were performed using a 2.0 x 300 mm μBondapak C_{18} column (Waters Division of Millipore, Milford MA USA). B&J Brand™ high purity acetonitrile was purchase from Burdick and Jackson (Baxter Healthcare Corporation, Muskegon MI USA). Distilled deionized water

was provided by a NANOpure II water purification system (Barnstead Thermolyne, Dubuque IA USA). Research purity methane was used as the reagent gas for chemical ionization (Matheson, Bridgeport NJ USA). Samples of aprobarbital, butabarbital, and butethal were purchase from Sigma Chemical Co. (St. Louis MO USA). All other barbiturates were provided by Ganes Chemicals (Pennsville NJ USA, see Figure 1 for barbiturate structures).

The mobile phase used was 70/30 water/acetonitrile adjusted to pH 3.0 using formic acid. The mobile phase was filtered and degassed by sonication under vacuum. Samples of barbiturates were prepared at approximately 0.1 mg/ml in mobile phase. UV detection (220 nm) was performed in series with the mass spectrometer. Analyses were conducted at ambient temperature using a 5 µl injection volume. Flow rate was 270 µl/min.

For EI+ spectra, the EI source was tuned using a sample of butalbital (although any of the barbiturates will suffice). Comparison of the "tuned" spectra to an entry in the NIST library was performed to determine accuracy. For CI+ spectra, appropriate reagent gas pressure was determined by monitoring the m/z 19 and m/z 29 peaks. When a 1:1 ratio between the peaks was attained, further tuning of the source was performed using butabital and monitoring for the presence of the molecular ion at M+H. Tuning was always performed using the LC as the inlet system, and the mobile phase as the sample eluent.

RESULTS AND DISCUSSION

Figure 2 is a list of the EI+/CI+ spectra of the barbiturates. Positive ion MS was selected because of its compatibility with the acidic mobile phases commonly encountered in reversed phase HPLC. The CI+ spectra (right column) show the clear base peak generated by the molecular ion adduct M+H. Barbiturates are generally distinguished by the identities of the R-groups at the #5 carbon. Determining the molecular weight of an unknown disubstituted barbituric acid is critical in establishing identity.

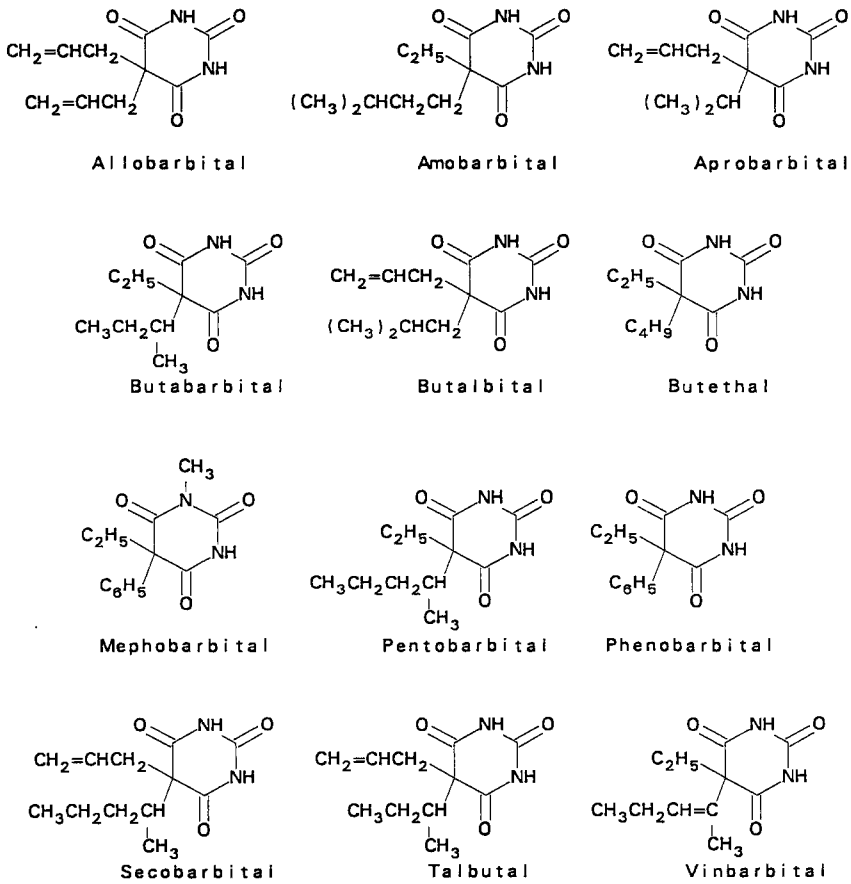


FIGURE 1
Barbiturate Structures

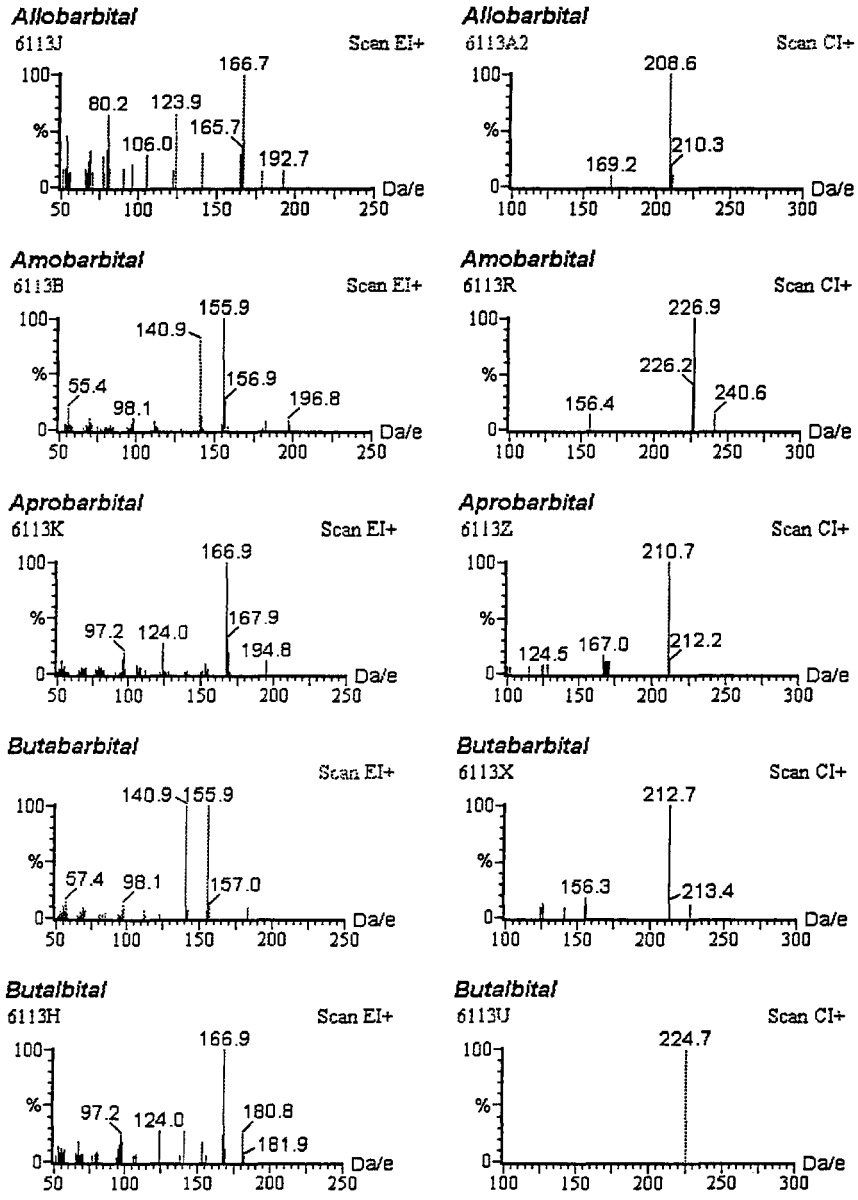
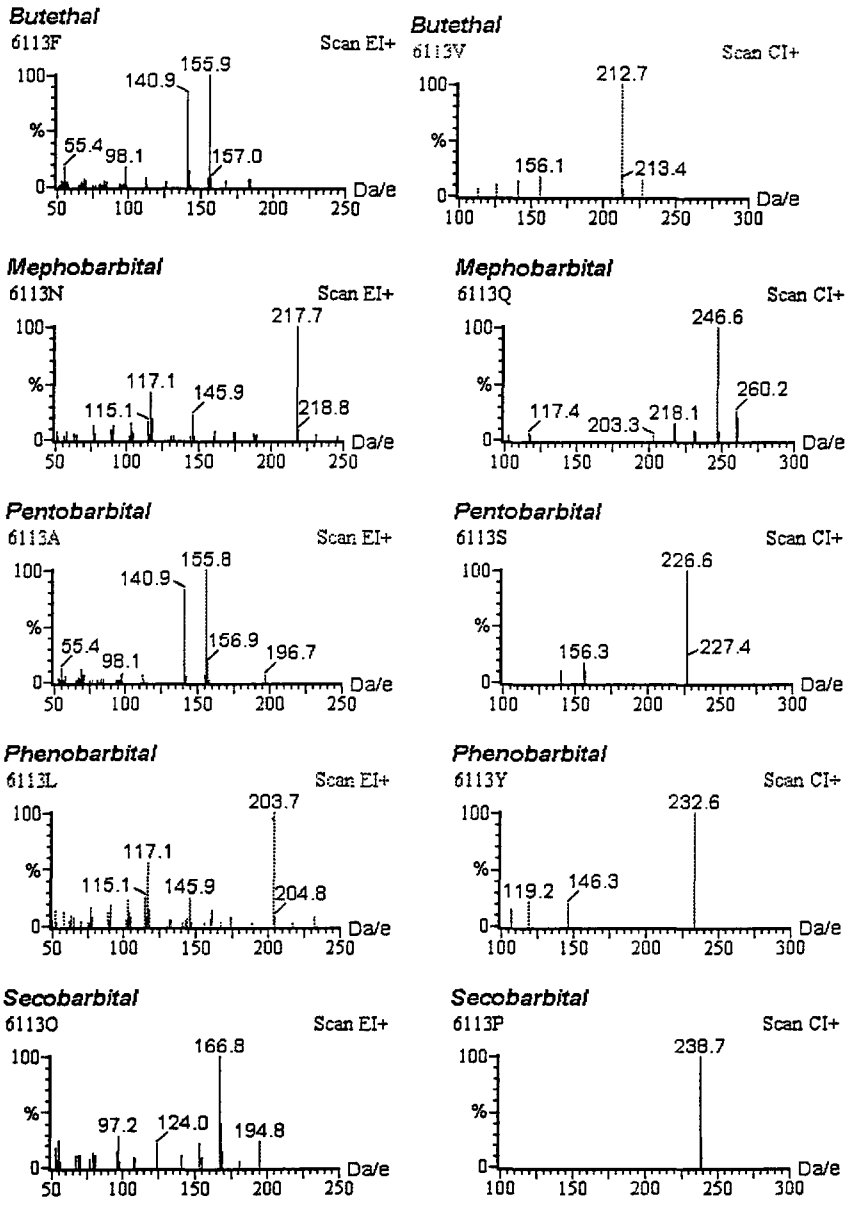


FIGURE 2

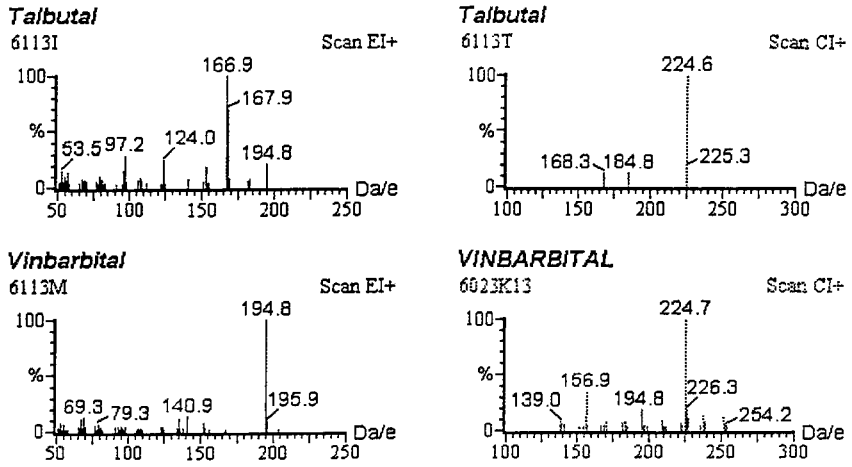
Barbiturate EI/CI Mass Spectra

FIGURE 2 (cont'd)

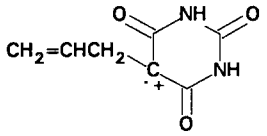


(cont'd)

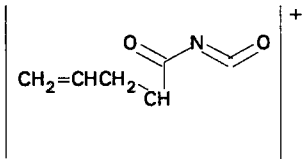
FIGURE 2 (cont'd)



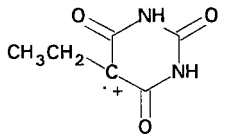
The highly similar EI+ spectra of allobarbital, aprobarbital, butalbital, secobarbital, and talbutal are due to the preferential loss of the alkyl R-groups while the ally R-group (CH_2CHCH_2-, present in all five) is retained along with the intact pyrimidinetrione ring. This generates the base peak at m/z 167, representing the following structure:



Another consistent ion present for these compounds is at m/z 124, caused by ring cleavage and subsequent rearrangement yielding:



Another common base peak observed in the EI+ spectra is at m/z 156, found in amobarbital, butabarbital, butethal, and pentobarbital. In this case the fragment ion is generated due to the preferential loss of the larger alkyl R-group, and the retention of C_2H_5- yielding the following structure:



Along with m/z 156, an equally strong ion is observed at m/z 141, and would be consistent with the loss of CH_3 from the remaining R-group. Other base peaks observed in the EI+ spectra of the remaining barbiturates conform to the same pattern of R-group elimination. In vinbarbital the base peak is at m/z 195 generated by the loss of the alkyl R-group C_2H_5-, again illustrating the preferential loss of an alkyl R-group over an allyl R-group. Phenobarbital exhibits a base peak at m/z 204, consistent with the loss of the alkyl R-group C_2H_5- while the phenyl ring is retained. The same is true for mephobarbital (the higher mass is due to the methylated nitrogen at position #1).

From Table 1, 3 pairs of barbiturates are observed to possess identical base peaks in both the EI+ and CI+ spectra. When a situation like this is encountered, careful evaluation of both spectral and retention information will provide the identity of the eluting compounds. Using the combination of the techniques, the analyst can accurately identify co-eluting multicomponent mixtures as long as the spectra are distinct. This approach has previously been demonstrated using UV photodiode array[20]. Using mass spectroscopy the EI+ spectra is examined for specific mass peaks, i.e., mass peaks that are present exclusively in spectra of one compound, but absent in the spectra of a near or co-eluting compound. Accurate selection of such an identifier peak is in a specific mass spectra is critical. The peak is usually a minor peak (a major peak with an intensity approaching that of the base peak would by definition provide the analyte with a distinct spectra). Spectral processing will compensate for most background elements, but the analyst should observe if a specific low intensity peak is truly part of the spectra, or if it is

TABLE 1
BARBITURATE MASS SPECTRA BASE PEAK SUMMARY

Sample	Molecular Weight* (amu)	Base Peak CI+* (m/z)	Base Peak EI+* (m/z)
Allobarbital	208	209	167
Aprobarbital	226	227	156
Amobarbital	210	211	167
Butabarbital	212	213	156
Butalbital	224	225	167
Butethal	212	213	156
Mephobarbital	246	247	218
Pentobarbital	226	227	156
Phenobarbital	232	233	204
Secobarbital	238	239	167
Talbutal	224	225	167
Vinbarbital	224	225	195

*Rounded to nearest whole number.

noise. Typically this is determined by monitoring the presence of the peak throughout the chromatogram, and noting if the relative abundance jumps sharply when the peak elutes. Figure 3 is the analysis of a sample consisting of equal parts butabarbital and butethal. The UV analog signal shows baseline resolution of the compounds. The TIC shows the broader elution profile associated with peaks as they pass through the desolvation canister of the particle beam. Although not completely resolved, accurate spectra of both compounds can be acquired from this analysis. Also shown is the scan of m/z 167, a mass peak exclusive to butethal. If a less complete resolution had occurred, this would distinguish the identities. Figure 4 is an analysis of butalbital and talbutal. Again the UV analog shows close to baseline resolution of the compounds, but the TIC shows a profile that is poorer than the previous example. In this case, the presence of a mass peak at m/z 195 in the talbutal spectra provides the means of identification. Figure 5 is an example of a true worst case scenario. Amobarbital and pentobarbital are incompletely resolved chromatographically. UV-photodiode array has proven to be extremely accurate at resolving the non-specific spectra of barbiturates[1,2], but this discriminating capability does require that the peaks be chromatographically resolved. In a situation like this UV-photodiode array would be useful in indicating

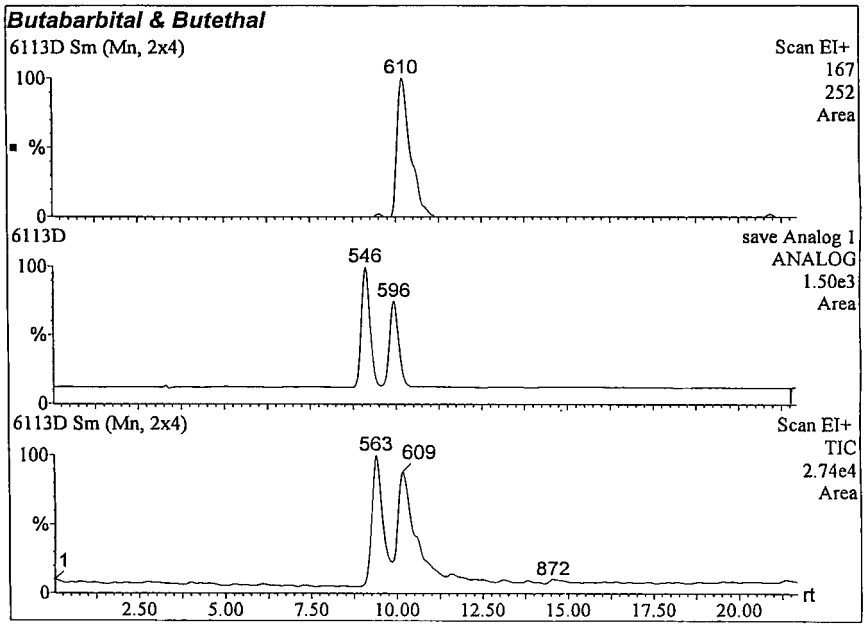


FIGURE 3

Detection of Butabarbital and Butethal Using EI+ Mass Spectroscopy (Specific detection of butethal at m/z 167)

the presence of a co-eluting compound via peak purity determination. Likewise, UV-photodiode array with multicomponent analysis would require a sharper spectral distinction[20] for accurate identification, although it would also indicate problems with eluting peak purity. The TIC shows the peaks merged into one another, the only way to discriminate the two under these circumstances is to monitor for the presence of the fragment ion at m/z 183, found exclusively in amobarbital.

CONCLUSION

The use of HPLC-particle beam EI/CI mass spectroscopy as a qualitative identification technique for barbiturates was successful. For the most part,

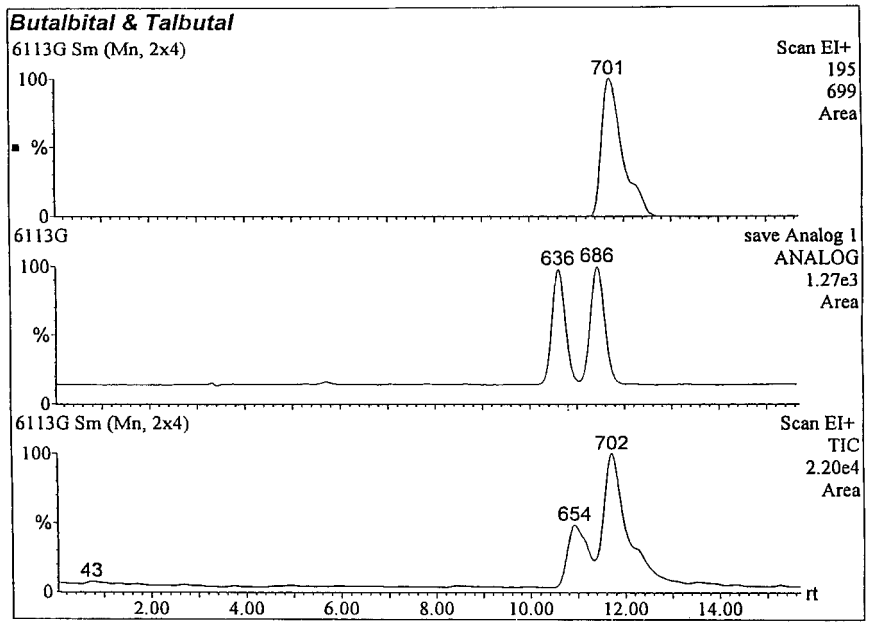


FIGURE 4
Detection of Butalbital and Talbutal Using EI+ Mass Spectroscopy (Specific detection of talbutal at m/z 195)

barbiturates were accurately identified by comparison of the EI and CI spectra. In those cases where spectra are very similar additional characterization via retention differences can be incorporated to enhance peak identification. The use of "identifier" mass peaks in similar spectra proved an accurate tool when chromatographic resolution was incomplete. The use of the particle beam interface should make the technique applicable not only in the pharmaceutical manufacturing or in related clinical assays, but in any industry where reversed phase HPLC is common. Two deficiencies in the technique were noted during the course of experimentation. The first was the sample band broadening associated with the particle beam interface. While representative spectra could be acquired from incompletely resolved peaks (such as Figure 3 and 4), this would

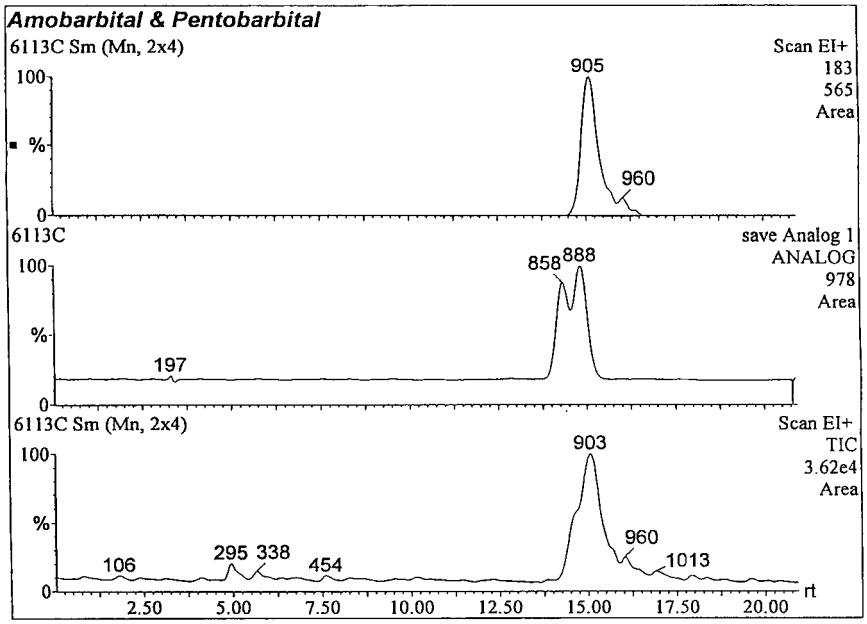


FIGURE 5

Detection of Amobarbital and Pentobarbital Using EI+ Mass Spectroscopy (Specific detection of amobarbital at m/z 183)

limit the technique if applied in a quantitative aspect. Of course, sample resolution could be enhanced by the use of a weaker mobile phase. However, this would extend analysis time and degrade peak shape which would cause a loss in sensitivity and potentially the generation of a non-representative spectra. Also, while not an analytical deficiency, LC-MS tends to increase routine instrument maintenance. LC-MS, particularly LC-CI, is an extremely aggressive technique. Cleaning the EI/CI source was typically carried out on a weekly basis. This in turn increases the wear on the source, and the likelihood that the source might be damaged. While random accidents cannot be avoided, impact on productivity can be limited by appropriate maintenance scheduling.

Further investigations into the technique will focus on quantitative as well as qualitative analyses, and also in establishing minimum concentrations at which the barbiturates can be accurately identified.

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SIMULTANEOUS DETERMINATION OF COCAINE AND BENZOYLECGONINE IN VITREOUS HUMOR BY HPLC

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ABSTRACT

An isocratic HPLC system for quantifying benzoylecgonine (BE) and cocaine (COC) in vitreous humour (VH) was developed. Following solid-phase extraction, the two drugs are chromatographed on a column filled with octadecyl silica packing, eluted with 75% methanol in phosphate buffer of pH 7, and sensed by UV spectrophotometry at 235 and 275 nm, simultaneously. The detection limit thus achieved was 0.5 μg drug per millilitre of VH. By using tetracaine as internal standard, BE and COC can be determined with a coefficient of variation of 6 and 7%, respectively.

INTRODUCTION

Cocaine (COC) is a powerful stimulant and one of the most widely abused drugs by all social classes at present. Cocaine-related deaths are reportedly on the increase [1,2]. Consequently, analyses for COC in biological specimens have major social and legal implications. One of the major metabolites for COC is benzoylecgonine (BE), which must thus also be assayed jointly with the parent drug in comprehensive analyses.

Vitreous humour (VH) is frequently the best or even the sole type of sample available for post-mortem analyses on severely burned, embalmed or

bleeding-shocked bodies. It is a fairly simple matrix compared to blood and urine and is scarcely prone to major post-mortem alteration. The lack of metabolic activity in the eye suggests that drug levels may provide a more accurate indication of body drug concentrations at the time of death.

Only a few of the many analytical procedures available for the determination of COC and BE by using RIA [3], GC–MS [4] and HPLC [5] are concerned with VH [6]. High performance liquid chromatography (HPLC) is particularly effective for the isolation of polar, non-volatile substances such as BE and related products with no derivatization.

This paper reports a straightforward, sensitive reversed-phase HPLC method for the joint determination of COC and BE in VH, based on solid-phase extraction and UV spectrophotometric detection.

MATERIALS

Apparatus

The isocratic HPLC system used was composed of a Model 501 pump, a U6K injector and a 490 programmable multiwavelength UV detector, all from Waters Associates (Mildford), and was interfaced via an appropriate module to a Digital-Professional 350 personal computer for recording.

Reagents

COC and BE hydrochlorides were obtained from the Spanish Ministry of Health. Tetracaine (TET) hydrochloride was complimentarily supplied by Ifesa S.A. Laboratories. HPLC-grade methanol from Merck (Darmstad) and water distilled through a Milli-Q apparatus from Millipore Corporation were also used. A phosphate buffer of pH 7 was made by adding 320 mL of 0.02 M *di*-potassium hydrogen phosphate to 680 mL of 0.02 M potassium dihydrogen phosphate.

Standards

COC and BE stock solutions containing 1 mg/mL of either drug in methanol were used to make working strength solutions of 0.5, 1, 2, 4, 8 and 10 μg/mL by appropriate dilution.

The TET internal standard was made by dilution to 20 μg/mL of a previously prepared stock solution containing 1 mg/mL of the compound.

Vitreous humour samples were prepared as follows: a VH pool was made from drug-free post-mortem specimens that were mixed, centrifuged and refrigerated at 4°C prior to use. Aliquots of 0.5 mL were spiked with 0.5, 1, 2, 4, 8 and 10 μg/mL COC and BE, plus 20 μg/mL TET internal standard. All these solutions were prepared in triplicate.

METHODS

Extraction Procedure

The solid-phase extraction procedure employed used 3-mL Bond-Elut certify™ columns from Analytichem Int. that were held in a Vac-Elut™ system. The sorbent was prepared by adding 2 mL of methanol, 1 mL of water and 0.5 mL of 10 mM phosphoric acid at pH 3.4. The sample was applied after mixing 0.5 mL of COC- and BE-spiked VH with 0.25 mL of 10 mM phosphoric acid. The washing step was effected by adding 1 mL of 10 mM phosphoric acid, 0.5 mL of 0.1 M acetic acid, 0.5 mL of methanol and 0.5 mL of 0.3 M ammonium hydroxide. Elution was done with 2 mL of methanol and the eluate was finally concentrated by evaporation to dryness under a nitrogen atmosphere at 70°C, followed by reconstitution with 500 μL of 20 μg/mL TET in methanol. The pressure used for passing liquids through solid phase cartridge was 2 in of mercury.

Chromatographic Procedure

The analytical column used was a 125 × 4 mm ID reversed-phase column packed with 5-μm Lichrospher 100 RP-18 (Merck, Darmstad). A guard column (4x4 mm) with the same packing was also employed. The mobile phase was prepared from methanol and 0.02 M phosphate buffer in a 75:25 ratio. The flow-rate was 0.6 mL/min and the working pressure 1,000–1,200 psi. Detection was carried out by UV spectrophotometry at 235 and 275 nm simultaneously (0.03 AUFS). The overall analysis time was 11 min and the injected volume 25 μL.

RESULTS

As can be seen in figures 1 and 2, the chromatograms obtained under the above described conditions, at 235 and 275 nm respectively, showed three peaks corresponding to as many compounds with retention times of 2.45 (BE), 6.40 (COC) and 9.40 min (TET).

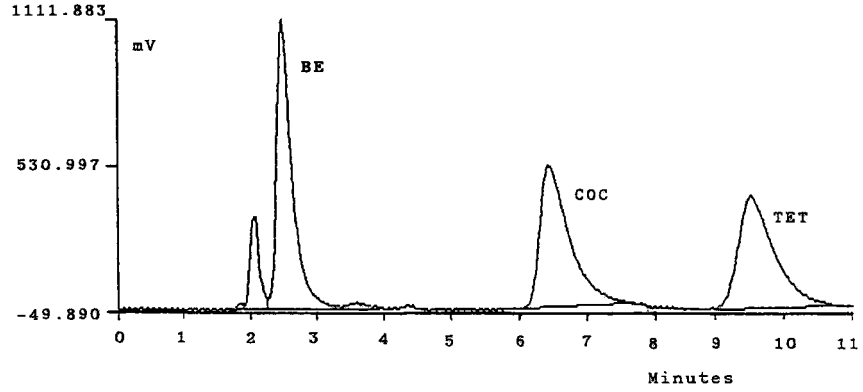

FIGURE 1 — Chromatogram obtained at 235 nm for 10 μg/mL BE (Area: $1.73.10^7$) and COC (Area: $1.80.10^7$) in methanol, and 20 μg/mL TET (Area: $1.54.10^7$) as internal standard.

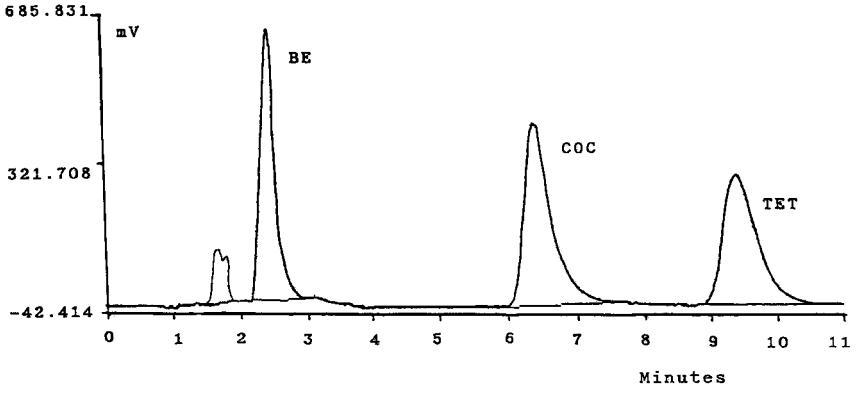

FIGURE 2 — Chromatogram obtained at 275 nm for 10 μg/mL BE (Area: $1.13.10^7$) and COC (Area: $1.32.10^7$) in methanol, and 20 μg/mL TET (Area: $1.42.10^7$) as internal standard.

TABLE 1
Statistical parameters for the determination of COC and BE

	r		Slope		Intercept		SEE	
Compound	MeOH	VH	MeOH	VH	MeOH	VH	MeOH	VH
COC	0.999	0.996	0.131	0.074	−0.035	−0.017	0.009	0.023
BE	0.999	0.997	0.113	0.080	−0.008	−0.008	0.019	0.024

SEE = standard error of estimate

Calibration curves were run by analysing mixtures of methanol and VH spiked with COC and BE at concentrations between 0.5 and 10 μg/mL. The peak area ratios of BE and COC to internal standard (TET) were found to be linearly related to the drug concentrations. Table 1 summarizes the results of the regression analysis performed.

No peaks were obtained at the retention times of BE and COC following solid-phase extraction. Figures 3 and 4 show the chromatograms obtained for a blank of VH containing TET and another of VH spiked with 10 μg/mL BE and COC.

The mean absolute recoveries or extraction efficiencies were 75 and 65% for BE and COC, respectively.

The reproducibility of the proposed method was determined by analysing a sample of VH spiked with 4 μg/mL BE and COC in sixtuplicate. The coefficients of variation for BE and COC were quite acceptable (6 and 7%, respectively).

The detection limit achieved was 0.5 μg of BE or COC per millilitre of VH.

DISCUSSION

The mobile phase used was a modification of that employed by Masoud [7]. After assaying various pH values and methanol contents, we finally chose pH 7 and 75% methanol as optimal for separation of the analytes (Fig. 1).

Of the three compounds tested as internal standards, *viz.* lidocaine, procaine and tetracaine, the last was found to be the most suitable as it was readily

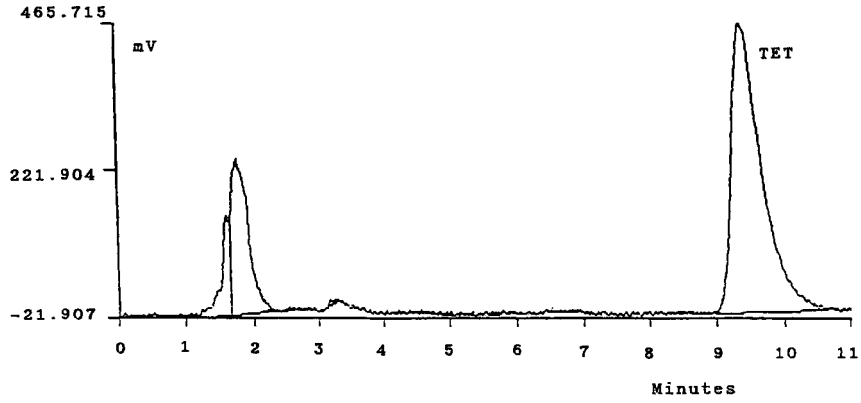

FIGURE 3 — Chromatogram obtained at 235 nm for a blank of VH containing 20 μg/mL TET (Area: $1.56.10^7$).

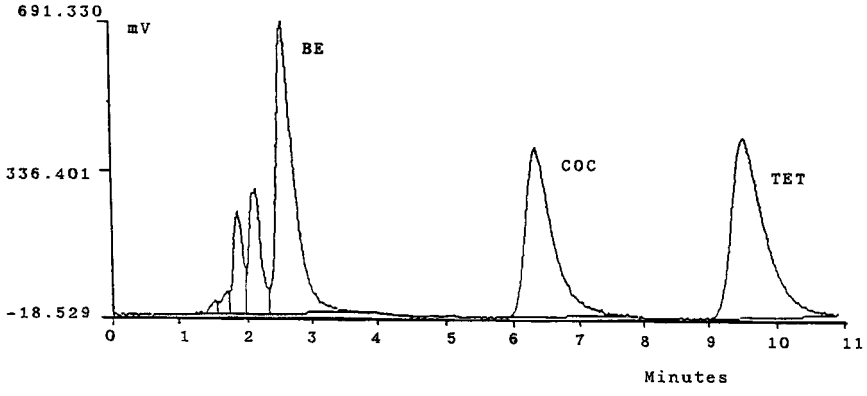

FIGURE 4 — Chromatogram at 235 nm for VH spiked with 10 μg/mL BE (Area: $1.35.10^7$) and COC (Area: $1.24.10^7$) plus 20 μg/mL TET (Area: $1.50.10^7$).

separated from BE and COC and exhibited adequate absorption at 235 nm in the form of a consistent peak.

Of the two optimal wavelengths, 235 and 275 nm, the former resulted in stronger signals (figures 1 and 2), notwithstanding which we chose to use both simultaneously in order to obtain height peak ratios to ascertain the presence of BE and COC in the samples.

Ecgonine methyl ester, which was also assayed, exhibited inadequate absorption for UV spectrophotometric detection.

Solid-phase extraction (figure 4) offers the advantage that it prevents emulsion formation. Also, it has so proved to be more expeditious than traditional liquid-liquid extraction, which usually involves using large solvent volumes, time-consuming centrifugation, back-extraction and evaporation to make the sample ready for analysis.

The detection limit of the method proposed (0.5 μg of drug/mL of VH) happened to be adequate at the sight of the results obtained in our laboratory, where twelve cases of fatal poisoning caused by heroine and cocaine or cocaine alone were studied. This work, not yet published, was carried out using radioimmunoassay and the COC and BE levels ranged between 1.04 and 26.14 μg/mL of VH.

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NOVEL UNIFORMLY SIZED POLYMERIC STATIONARY PHASE WITH HYDROPHILIZED LARGE PORES FOR DIRECT INJECTION HPLC DETERMINATION OF DRUGS IN BIOLOGICAL FLUIDS

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ABSTRACT

Novel separation media for the direct injection of complex samples have been prepared from porous 10 μm uniformly sized poly(glycidyl methacrylate-co-ethylene dimethacrylate) beads. The simple hydrolysis of all epoxide groups produces beads with separation characteristics similar to those of some restricted access media. The use of a novel size-specific modification of porous materials provides separation media with even better chromatographic properties. In this approach, hydrolysis of the epoxide groups to diols can be carried out exclusively within the large pores of the medium through the use of a polymeric catalyst. The epoxide groups remaining in the small pores after hydrophilization of the large pores were then transformed either to hydrophobic C_{18} or phenyl groups, or to more polar diethylamino groups. Due to the size specific characteristics of the modification process used in the preparation of the beads which renders the large pores hydrophilic, no protein interaction with the surface of the pores was observed, the protein recovery was high, and the retention times of proteins and of a non-retained probe was similar. Examples of separations of drugs from plasma document the good efficiency and excellent selectivity of columns packed with the novel separation medium.

INTRODUCTION

Pre-treatment of a sample by deproteinization or solvent extraction typically precedes the determination of drugs, endogenous metabolites or other small molecules in physiological liquids by high-performance liquid chromatography . This pre-treatment prevents accumulation of proteins in the column which would likely lead to clogging and fast deterioration of the separation efficiency. However, such pre-treatment is time consuming and does not always give reproducible and quantitative results. Therefore, stationary phases that allow the direct injection of complex matrices like plasma, serum, saliva, and urine into a column for the direct determination of drugs and metabolites without any pre-treatment have been developed (1,2).

All direct injection stationary phases yet described in the literature (3-11) are based on porous silica that has been modified in order to prevent any contact of the protein molecules with the hydrophobic or charged functionalities that are attached to the surface of the stationary phase. In each case, modification of the pores is uniform and affects all pores regardless of their size. Moreover, some of these silica-based packings have limited working pH range, ionic strength range, or do not allow the use of organic solvent in high concentrations in the mobile phase during the reversed phase chromatography (12). The modifications may also impair diffusion of the low molecular weight compounds that have to be separated, and the slow mass transfer results in lower column efficiencies (about 20 - 30,000 plates/m) (2) when compared to those of typical reversed phase columns.

Polymeric stationary phases have gained considerable popularity in HPLC due to their chemical stability in the entire pH range, broad variety of surface groups chemistries and polarities (13). Styrene-divinylbenzene copolymers are the most often used polymeric stationary phases (14-16). Their highly hydrophobic surface accounts for their extensive use in reversed-phase chromatography and size-exclusion chromatography in non-aqueous media. A search for more hydrophilic stationary phases is still in progress (13-16) in order to develop polymeric media for the separation of water soluble hydrophilic compounds and proteins without adversely affecting their biological activity. Though more rugged in terms of chemical resistance, the polymeric stationary phases are intrinsically less efficient than silica based phases with efficiencies seldom exceeding 30,000 plates/m for 10 μm beads. Despite this limitation, the ability to modify the chemistry within specialized polymer phases remains a very significant advantage that frequently justifies their use.

This paper concerns a new polymeric stationary phase prepared using the concept of pore-size specific functionalization. This novel phase is specifically designed for the direct injection assay of drugs in complex matrices.

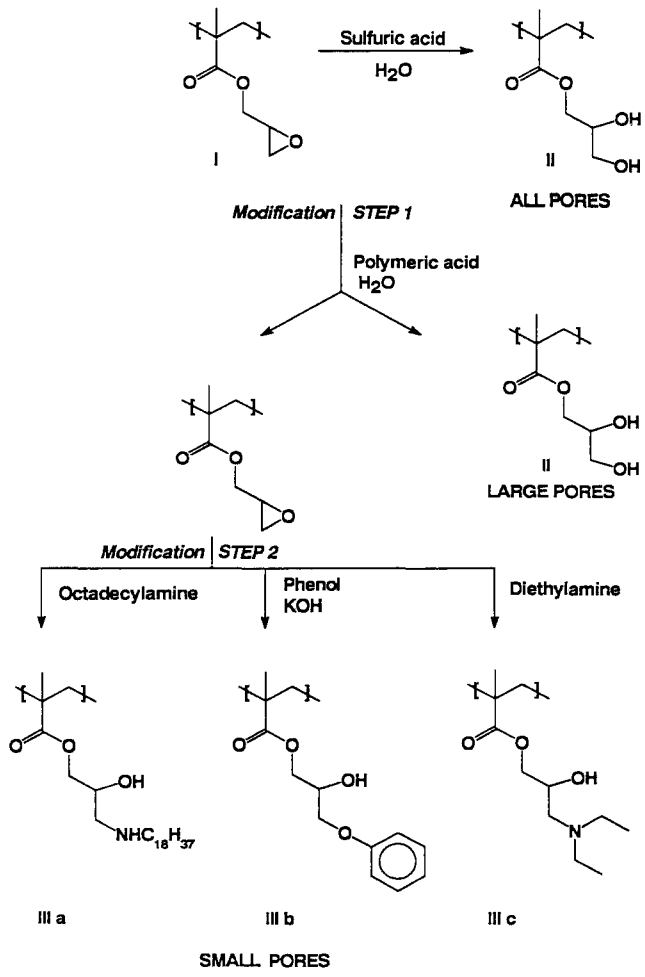
EXPERIMENTAL

Polymer Beads. Uniformly sized 10 μm porous copolymers each with a different but controlled pore size distribution were prepared by a modified shape template swelling and polymerization method described in detail elsewhere (17). Two samples were prepared from glycidyl methacrylate and ethylene dimethacrylate (monomers; 60:40 vol.%) (Resin I). Beads A were prepared in the presence of cyclohexanol as a porogenic solvent, while for beads B, butanethiol was added in order to shift the pore size distribution towards small pores. Table 1 lists the characteristics of both samples of the porous beads. Epoxide groups contents were determined by volumetric titration as follows: the beads were dispersed in 0.1 mol/l tetraethyl ammonium bromide solution in acetic acid and titrated with 0.1 mol/l perchloric acid solution in acetic acid until the crystal violet indicator indicated the blue-green end point. Pore size distributions, pore volumes and median pore diameters were calculated from retention volumes of polystyrene standards in THF (18).

Polymeric Catalyst. Poly(styrenesulfonic acid) PSSA 141 with a broad molecular weight distribution (M_W= 141,000, M_W/M_n = 1.7) was purchased from Polyscience and its molecular weight distribution was determined after an esterification to a poly(styrenesulfonic acid butyl ester).

Hydrolysis. The total hydrolysis of the epoxide groups of **I** into vicinal diol groups proceeds under catalysis with sulfuric acid. Resin **I** (3 g) was suspended in 50 ml 0.1 mol/l aqueous sulfuric acid, stirred occasionally and kept at 60°C for 10 hours to afford diol resin **II** (Scheme 1).

Pore Size Specific Hydrolytic Reactions. The pore size specific hydrolysis of resins **I** was catalyzed with 0.1 mol/l 4-toluenesulfonic acid or with a 1 wt.% aqueous solution of poly(styrenesulfonic acid) containing 0.054 mol/l sulfonic groups. The epoxide resin **I** (3 g) was placed in a 100-ml beaker, 25 ml of aqueous catalyst solution was added and the beaker sealed with Parafilm. The dispersion was then stirred magnetically at ambient temperature for 48 hours. The modified beads were filtered off on a fritted glass filter and washed with water until neutral. After washing with acetone the beads were dried in vacuo at room temperature.


Sulfuric acid
H_2O
I
II
OH
OH
ALL PORES
Modification | STEP 1
Polymeric acid
H_2O
II
OH
OH
LARGE PORES
Modification | STEP 2
Octadecylamine
Phenol
KOH
Diethylamine
OH
$NHC_{18}H_{37}$
OH
OH
N
III a
III b
III c
SMALL PORES

Scheme 1

TABLE 1
Properties of Uniformly Sized Porous Poly(Glycidyl Methacrylate-co-Ethylene Dimethacrylate) Beads

Beads	A	B
Particle size (μm)	10	10
Epoxide groups (mmol/g)	2.7	2.5
Specific pore volume (ml/g)	1.1	1.0
Median pore diameter (nm)	13.8	7.0
Polystyrene exclusion limit MW	3.4×10^5	7.7×10^4

Aminolysis of Epoxide Groups. The resin containing epoxide groups (3 g) was placed in a 100-ml round bottom flask and 20 ml diethylamine was added. The mixture was heated to reflux (55 °C) for 6 hours, the resin was filtered and washed with water. The remaining unreacted epoxide groups were hydrolyzed in 50 ml 0.1 mol/l sulfuric acid at 60 °C for 4 hours. The beads **IIIc** (Scheme 1) were filtered, washed with water, then with methanol and dried.

A slightly modified procedure was used for the aminolysis of beads containing epoxide groups using octadecylamine **IIIa**. The beads (3 g) were admixed to 15 g molten octadecylamine at 70 °C and the mixture was stirred for 16 hours. The reaction mixture was then diluted with 60 ml 1,4-dioxane, stirred for 30 minutes and filtered. The resulting beads IIIa were washed with dioxane and water, and the remaining unreacted epoxide groups were hydrolyzed. The product was washed with water and methanol, and dried.

Reaction with Phenol. The resin containing epoxide groups (3 g) was dispersed in 100 ml 0.01 mol/l KOH containing phenol (3 g). The mixture was heated to reflux for 2 hours then cooled to room temperature. The resulting beads **IIIb** were washed with water and acetone and dried.

Chromatography. The stainless steel columns (150 x 4.6 mm I.D.) were slurry packed at constant pressure (10 MPa) using water as the dispersion liquid.

Testing and separations were carried out using an IBM-Nicolet Ternary Gradient Liquid Chromatograph LC 9560 equipped with a Rheodyne 7125 loop injector. The reproducibility of injection was found to be better than 2.5 %. Elution was monitored by a Hewlett Packard 1050 UV detector at 254 nm or by a Milton Roy refractive index detector. Column efficiency was determined with carbamazepine as a standard using an isocratic elution with an 80:20 mixture of 0.1 mol/l phosphate buffer solution (pH=7.0) and acetonitrile at a flow rate of 1 ml/min and ambient temperature. D_2O was used as an unretained standard for the calculation of capacity factors and column selectivities and its elution was monitored using the refractive index detector.

The plasma protein recovery was calculated from the ratio of the protein peak area measured under standard chromatographic conditions and the peak area of the same amount of protein injected into chromatographic system from which the column was removed and the inlet and outlet capillaries were connected.

The concentration of the bovine plasma (Sigma Chemical Co.) in all samples was kept constant at 70 mg/ml while the concentration of the admixed drugs was 8-40 μg/ml. Actual concentrations of individual drugs are shown in captions to Figures. The ratio between proteins and drugs was similar to that in the actual assays. The injected volume was 20 μl.

RESULTS AND DISCUSSION

Characterization of the Starting Polymer Beads. Two different batches of uniformly sized 10 μm polymer beads were prepared from glycidyl methacrylate and ethylene dimethacrylate. Batch A was prepared with cyclohexanol as a porogenic solvent, while for batch B, butanethiol was added (17) in order to shift the pore size distribution towards small pores (Table 1). The particle size, specific pore volume and polymer composition were kept constant in both polymers. The pore size distributions calculated for beads A and B from chromatographic data (Figure 1) differ considerably in the percentages of macropores (over 50 nm), mesopores (2-50 nm) and micropores (below 2 nm) and allow permeation of proteins to a different extent. For example, the radius of gyration of human serum albumin is 3.1 nm which corresponds to a "solid sphere" radius of approximately 4 nm. Due to the specific pore shape of typical porous separation media, the protein would not be able to penetrate pores with an apparent diameter less then 8 nm (4). The pore size distributions of the beads under study reveals that albumin would penetrate about 60 % of the total

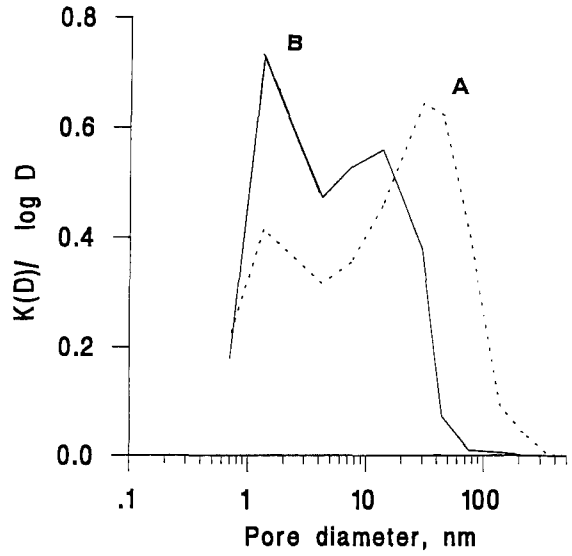


FIGURE 1. Pore size distribution of poly(glycidyl methacrylate-co-ethylene dimethacrylate) beads A and B calculated from inverse size-exclusion chromatography data.

pore volume of beads A but only about 30 % of beads B. It means that about 40 and 70 vol.% respectively of the pores will not come in contact with the protein.

Separation Media for Direct Injection Separations. In the case of complex mixtures of solutes containing molecules with very different properties such as proteins and drugs, it is very difficult to carry out a satisfactory separation. Several approaches have been suggested to avoid the absorption of proteins on silica based reversed phase columns during the separation of complex mixtures but the preparation of these separation media is tedious and their chemical stability is seriously limited (3-11). Typically, these separation media either have hydrophilic outer surfaces and very small pores that totally prevent penetration of large protein molecules into the particles, or all of their pores are covered with hydrophilic moieties. These modification methods frequently decrease the separation efficiency (2).

Evaluation of Totally Hydrolyzed Beads. First, it was determined whether or not the hydrolysis of epoxide groups is sufficient to prevent adsorption of

proteins on the surface of copolymer beads made from glycidyl methacrylate. Such a packing containing only hydrophilic surface chemistry in both large and small pores would not be expected to be suitable for direct injection separation of both proteins and hydrophobic drugs but its properties would be useful for comparison purposes.

To verify this, beads A and B were first fully hydrolyzed using sulfuric acid as a catalyst (Reaction scheme 1) and used for the separation of a mixture of bovine plasma and three drugs in a reversed-phase chromatographic mode with aqueous acetonitrile as the mobile phase. Though no interactions of both plasma proteins and drugs with the surface were expected in fully hydrolyzed beads, the separation of drugs was in fact achieved. However, the separation selectivity in the column packed with hydrolyzed beads A is relatively low, lidocaine and carbazepamine are eluted within the tail of the plasma proteins (peak 1 and 2, Figure 2a).

Since the pore size distribution in hydrolyzed beads B is shifted towards smaller pores, the major portion of these pores cannot be penetrated by the protein molecules. Therefore, the plasma proteins are eluted from beads B within a smaller elution volume than from beads A. This faster plasma elution results in a narrow protein peak that does not tail appreciably. This leads to an almost baseline separation of lidocaine (peak 1) from plasma; the separation selectivity for carbazepamine and phenytoin (peaks 2 and 3, Figure 2b) is also better as compared to that obtained for beads A. This unexpected observation led us to a more extensive examination of the chromatographic properties of the fully hydrolyzed beads B.

Figure 3 shows the dependency of log k' on the volume fraction of acetonitrile in the mobile phase for various compounds including both drugs with relatively low hydrophobicity having a capacity factor extrapolated to pure water as a mobile phase (k_w') lower than 10, and more hydrophobic drugs with k_w' in the range from about 100 to 1000. The linear relationship confirms that the separation in the column packed with hydrolyzed beads B is controlled by interactions characteristic for reverse-phase chromatography.

This unexpected behavior can be explained by the functional heterogeneity of the modified surface. In addition to their hydroxyl groups, the beads contain the hydrophobic hydrocarbon chains of the copolymer and the hydrophobic diester units of the crosslinking agent. The hydroxyl groups generated by hydrolysis of epoxide units cannot cover all of the surface of the pores with a homogeneous hydrophilic layer as their number is insufficient. The

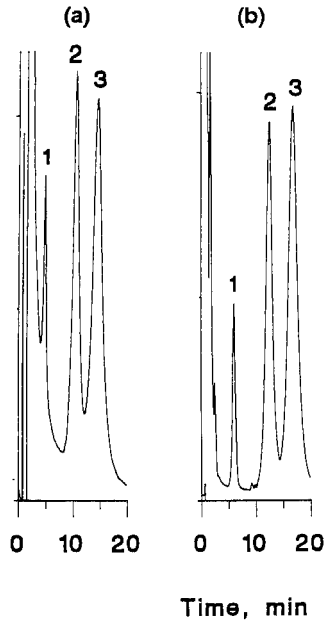


FIGURE 2. Direct injection separation of model mixtures containing drugs and bovine serum plasma.
Column 150 x 4.6 mm I.D. packed with H_2SO_4 hydrolyzed beads A (chromatogram *a*) and beads B (chromatogram *b*), mobile phase, 0.1 mol/l phosphate buffer (pH=7.0) - acetonitrile (80:20), flow rate 1.0 ml/min, UV detection 254 nm, injected volume 20 μl; peaks, 1- lidocaine (20 μg/ml) , 2- carbamazepine (8 μg/ml), 3- phenytoin (40 μg/ml).

surface is not uniformly hydrophilic but also contains hydrophobic domains interspersed within hydrophilic areas. However, the size of hydrophobic domains is smaller than that of the large protein molecules; therefore, the proteins are not adsorbed and their recoveries are high (Table 2).

Figure 1 shows that beads A contain mainly large pores and therefore the actual surface area available for interaction between surface and drug molecules is smaller than in the case of beads B which contain more of the meso- and micropores that are best suited for the separation of small molecules. This conclusion is supported by the higher capacity factors k' exhibited for all drugs by the column packed with beads B (Table 3). The capacity factors also suggest that resolution is better for the column packed with hydrolyzed beads B than

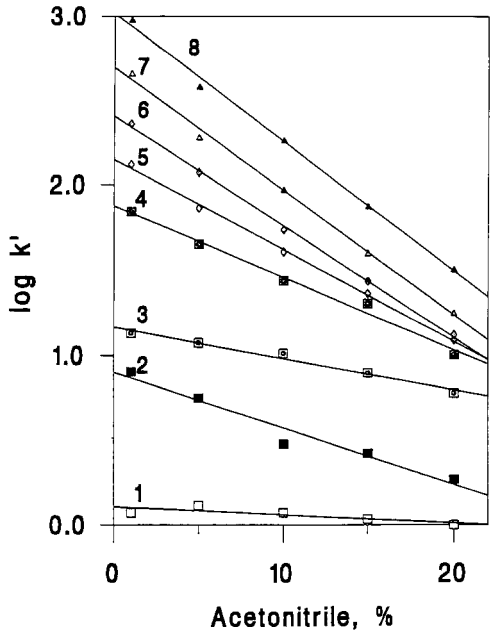


FIGURE 3. Capacity factor k' as a function of acetonitrile concentration in the mobile phase.
Column 150 x 4.6 mm I.D., packing, beads B hydrolyzed with aqueous H_2SO_4 mobile phase 0.1 mol/l phosphate buffer (pH 7) - acetonitrile (80:20), flow rate 1.0 ml/min, UV detection 254 nm; 1- acetylsalicylic acid, 2- sulfamethoxazole, 3- lidocaine, 4- chlorpheniramine, 5- quinidine, 6- carbamazepine, 7- phenytoin, 8- desipramine.

hydrolyzed beads A. This is further confirmed by a measurement of the effect of the linear flow velocity on the plate height (Figure 4). The efficiency of the column packed with beads B is twice as high as that of a column packed with beads A and the slope of the dependency is also smaller for beads B. Overall, hydrolyzed beads A are ill-suited for reversed-phase direct injection chromatography application while hydrolyzed beads B show a good performance. This is further supported by their excellent stability which does not change even after more than 500 repetitive manual injections over a period of three months.

The surprisingly good performance obtained with hydrolyzed beads B in the direct injection separation of plasma samples containing drugs are somewhat

TABLE 2

Protein Recovery from Functionalized Packings Modified in Pore-Size Specific Fashion

Hydrolysis catalyst	Surface derivatization	Protein recovery, %
	Beads A	
none	none [a]	78
Sulfuric acid	none	84
none	Octadecylamine	16
PSSA-141	Octadecylamine	81
	Beads B	
Sulfuric acid	none	100
4-Toluenesulfonic acid	Phenol	96
PSSA-141	Phenol	92
PSSA-141	Octadecylamine	96

[a] Original poly(glycidyl methacrylate-co-ethylene dimethacrylate) beads

TABLE 3
Capacity Factors k' of Drugs for a Column Packed with Beads Hydrolyzed with Aqueous Sulfuric Acid

#	Drug	k'	
		Beads A	Beads B
1	Lidocaine	1.14	2.13
2	Carbamazepine	3.40	5.78
3	Phenytoin	6.13	8.16
4	Theophyline	1.05	1.33
5	Theobromine	1.50	2.36
6	Caffeine	1.81	2.95

Column 150 x 4.6 mm I.D., mobile phase 0.1 mol/l phosphate buffer pH 7 - acetonitrile 80:20 (for #1-3); 99:1 (for #4-6), injection volume 20 μl, detection 254 nm.

similar to those obtained for shielded hydrophobic phases (5-8,11). Since the preparation of our separation medium is much easier, it may compete advantageously with many more sophisticated restricted access media.

Evaluation of Hydrophobic Beads. In contrast to the hydrolyzed beads, modification of the epoxide groups with octadecylamine should result in a material with enhanced hydrophobic interactions. Though the modification increases the overall hydrophobicity, strong adsorption of proteins in the column should also occur. Indeed, the protein recovery decreases drastically and does not exceed 16 %; the major part of the protein remains adsorbed (Table 2). The packing exhibits chromatographic properties similar to those of standard reversed phases with C18 chemistry. Such packings cannot be used for the desired direct injection separation as the proteins accumulate in pores and on the surface of the hydrophobic medium and the column becomes clogged rapidly . The column life-time typically does not exceed 200 injections (19,20).

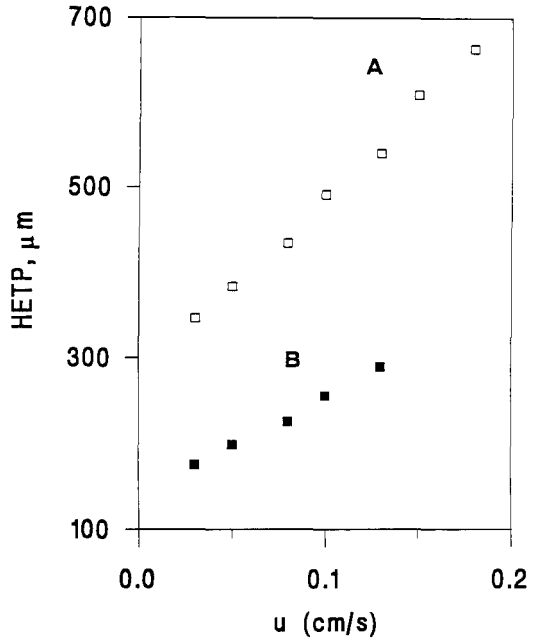


FIGURE 4. Effect of linear flow velocity on plate height.
Column 150 x 4.6 mm I.D. packed beads A or B hydrolyzed with aqueous H_2SO_4, solute carbazepamine, mobile phase 0.1 mol/l phosphate buffer (pH=7.0) - acetonitrile (80:20), UV detection 254 nm, injected volume 20 μl.

Concept of the Pore-Size Specific Modification. An ideal medium for direct injection chromatography should have both hydrophilic pores large enough to accommodate the proteins from a complex sample without risk of adsorption, and small hydrophobic or charged pores to provide for good chromatographic separation of the small molecules present in the sample while excluding proteins. In order to obtain this ideal medium, we have developed a novel concept different from the uniform pore modification processes outlined above. This concept is shown schematically in Figure 5 where the structure of a porous material is depicted as a combination of interconnected large and small pores covered with epoxy groups I, that are susceptible to acid catalyzed hydrolysis to afford diol polymer II (Scheme 1). In instances where the acid catalyst is itself a large molecule such as a polymer, its ability to catalyze reaction of the functional

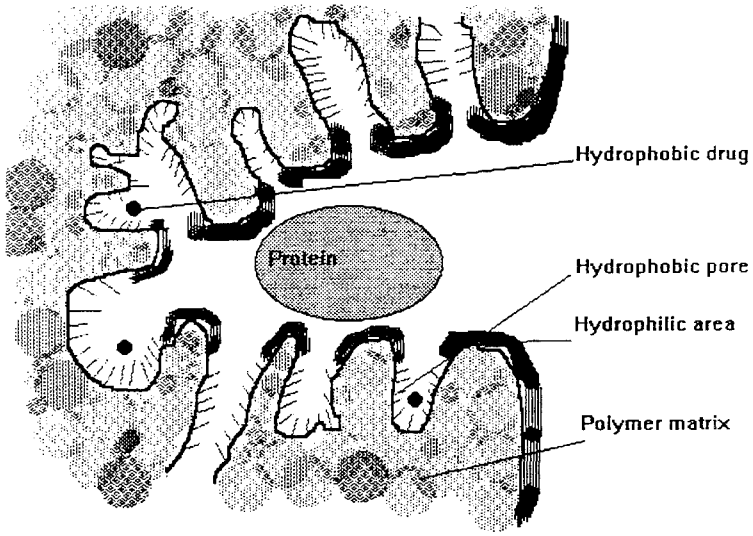


FIGURE 5. Schematic view of a separation medium modified in pore-size selective fashion using a polymeric catalyst

groups contained in the various pores will be controlled by the relative size of the catalyst molecules and the pores.

In the first step, hydrolysis of the reactive epoxide groups is carried out within the large pores only using an aqueous solution of a poly(styrenesulfonic acid) that is too large to penetrate the small pores. In the following step, reaction with a low molecular weight reagent (phenol, amine) transforms the epoxide groups that remained unchanged in the small pores to the desired functionality. In this way, beads with a bimodal distribution of surface chemistries are obtained. The process is simple and can be carried out either using a batch technique or within the column itself.

The actual separation in the bifunctional packing would proceed according to the following mechanism: all components of the injected plasma sample containing blood proteins, drugs, metabolites, etc. can penetrate all pores large enough to accommodate them. Specifically, large protein molecules enter only large pores covered with hydrophilic chemistry. Since they are not retained, they elute from the column at a volume close to V_j. Small molecules enter both large

and small pores. They also interact only weakly with the hydrophilized surface of large pores but they are retained in the small pores where the chemistry is different. Therefore, the small molecules will be eluted later and separated according to their interaction energy with the surface groups of the small pores.

Evaluation of Bimodal Beads for Direct Injection Reversed-Phase Chromatography. We have shown that the uniformly hydrolyzed diol separation media may not be sufficiently hydrophobic for all direct injection separations while the uniformly hydrophobized beads are not suitable for the separation envisioned. Therefore we used the concept outlined above to design beads containing both hydrophilic large pores and hydrophobic small pores with chromatographic properties we believe are close ideal for direct injection chromatography. The reactive epoxide groups located in the large pores were hydrolyzed in a process catalysed with a poly(styrenesulfonic acid) having a molecular weight of 141,000. The unchanged epoxide groups of the small pores were then allowed to react with octadecylamine in one case, or phenol in another, to provide hydrophobic sites.

The data presented in Table 2 confirm that the pore-size selective hydrolysis of large pores prevents the adsorption of proteins. Protein recoveries obtained from beads **IIIa** and **IIIb** are close to those of beads in which uniform hydrolysis of all epoxide groups had been carried out.

The pore-size specific hydrophilization/hydrophobization process further improves the chromatographic properties of the beads (Figures 6 and 7). The more hydrophobic surface of the modified small pores increases the retention and improves the drug separation selectivity compared to similar separations with fully hydrolyzed beads (Figure 3). Once again, the positive effect of the absence of large pores in beads B is confirmed as shown in Figure 6. Thus, the selectivity of drug separation and the peak symmetry of the octadecylamine-modified beads B are better than those for beads A. Therefore, only beads B were used in the rest of this study.

The chromatograms of Figure 7 document the effect of the catalyst size (4-toluenesulfonic acid and PSSA 141) on the chromatographic properties of the packing as a consequence of the different extents of hydrophilization. With 4-toluenesulfonic acid 94 % of all epoxide groups in beads B are hydrolyzed as opposed to only 17 % with poly(styrenesulfonic acid). Therefore, after the second step of pore-size selective modification the number of phenyl groups attached to the surface of the small pores is higher for the beads treated with aqueous poly(styrenesulfonic acid). The beads show higher hydrophobicity, the

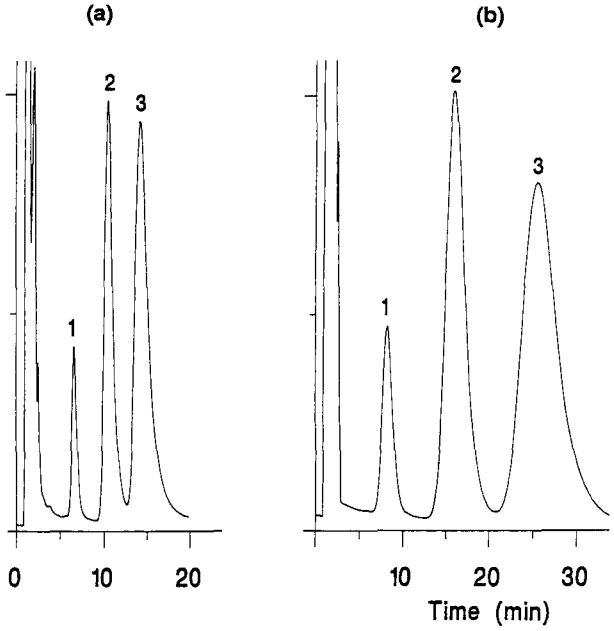


FIGURE 6. Direct injection separation of bovine serum plasma and drugs in a column packed with beads A (*a*) or B (*b*) modified by hydrolysis catalyzed by PSSA 141 then by reaction with octadecylamine.
Column 150 x 4.6 mm I.D., mobile phase, 0.1 mol/l phosphate buffer (pH=7.0) - acetonitrile (80:20), flow rate 1.0 ml/min, UV detection 254 nm, injected volume 20 µl; peaks, 1 - lidocaine (20 µg/ml), 2 - carbamazepine (8 µg/ml), 3 - phenytoin (40 µg/ml).

retention times are longer, and the separation is excellent. Column stability is also very good and equals that of the column packed with hydrolyzed diol beads.

Evaluation of Bimodal Beads for Direct Injection Ion-Exchange Chromatography. Charged compounds can be separated better in an ion-exchange chromatographic mode than in the reversed phase mode. The pore-size specific modification approach may also be used for the preparation of media for ion-exchange chromatography. After the initial hydrolysis catalyzed with polymeric acid, a reaction with diethylamine was employed in the second modification step affecting the small pores. This procedure results in a stationary phase containing diethylamino groups in the small pores (**IIIc**). Figure 8 shows

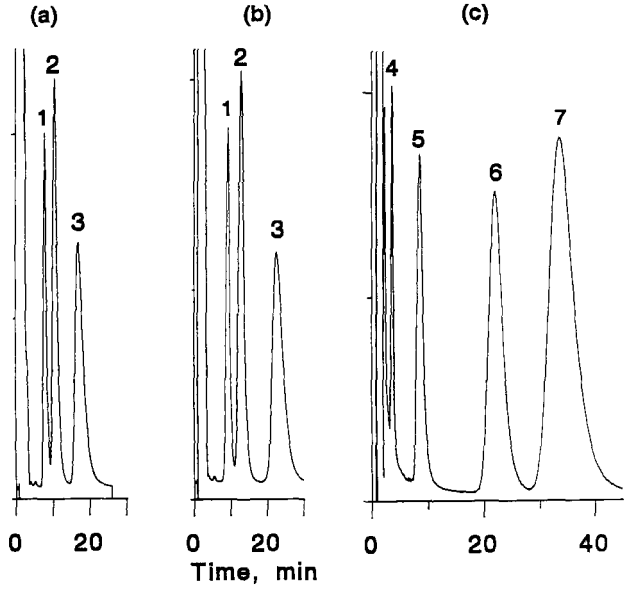


FIGURE 7. Direct injection separation of bovine serum plasma and drugs in a column packed with beads B modified by hydrolysis catalyzed by 4-toluenesulfonic acid (*a*) and PSSA 141 (*b,c*), respectively, then modified by reaction with phenol.
Column 150x4.6 mm I.D., mobile phase 0.1 mol/l phosphate buffer (pH 7) - acetonitrile 99:1 (A,B) or 80:20 (C), injected volume 20 µl, detection 254 nm; peaks, 1- theophyline (10 µg/ml), 2- theobromine (10 µg/ml), 3- caffeine (20 µg/ml), 4- barbital (10 µg/ml), 5- phenobarbital (10 µg/ml), 6- carbamazepine (8 µg/ml), 7- phenytoin (40 µg/ml).

the separation of salicylic and acetylsalicylic acids from blood plasma. The retention times strongly depend on the pH of the mobile phase (Figure 9) and confirm the ion-exchange separation mechanism. The capacity factor for salicylic acid at pH 7 is about 20 times higher than that for acetylsalicylic acid (Table 4). In contrast, a fully hydrolyzed diol column (**II**) or a column containing diol large pores and phenol modified small pores (**IIIb**) do not separate the acids and plasma at all, because there is almost no retention of the two drugs (Table 4). Due to the hydrophilization of the large pores, the protein recovery is again close to 100 % as the proteins cannot come into contact with the charged surface of the small pores.

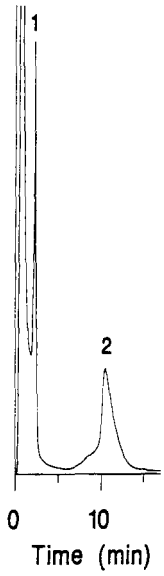


FIGURE 8. Direct injection separation of bovine serum plasma and acidic compounds in a column packed with beads B modified by hydrolysis catalyzed by PSSA 141 then modified by reaction with diethylamine.
Column 100x4.6 mm I.D., mobile phase 0.1 mol/l phosphate buffer (pH 9.3) - acetonitrile (80:20), injected volume 20 µl, detection 280 nm; peaks, 1 - acetylsalicylic acid, 2 - salicylic acid.

TABLE 4

Capacity Factors k' of Acidic Compounds for Three Columns Packed with Beads B Containing Different Functionalities in the Small Pores

	k'		
Compound	II	III b	III c
Acetylsalicylic acid	0.00	0.03	2.63
Salicylic acid	0.30	0.35	50.80

Column 150 x 4.6 mm I.D., mobile phase 0.1 mol/l phosphate buffer pH 7 - acetonitrile, 80:20, injection volume 20 µl, concentration of compounds 2 µg/ml, detection 280 nm.

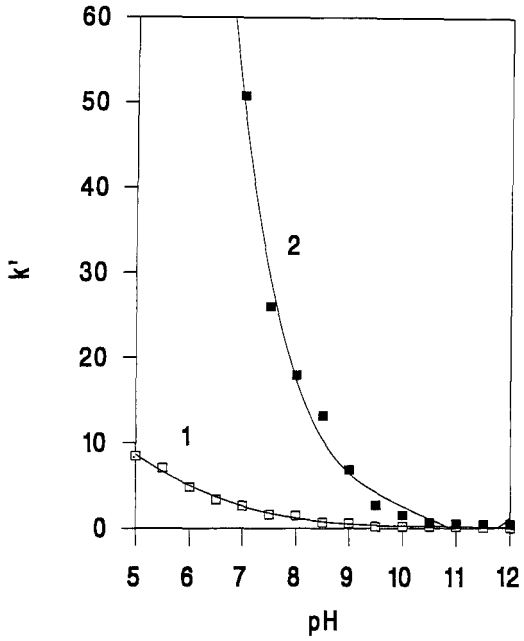


FIGURE 9. Effect of the mobile phase pH on capacity factor k' of acetylsalicylic acid (1) and salicylic acid (2).
Column 150x4.6 mm I.D., packing, beads B modified by hydrolysis catalyzed by PSSA 141 then allowed to react with diethylamine, mobile phase 0.1 mol/l phosphate buffer (pH 5-12) - acetonitrile (80:20), injected volume 20 µl detection 280 nm.

CONCLUSION

A novel polymeric stationary phases for direct injection HPLC has been prepared. The simple hydrolysis of beads having selected pore size distributions provides a medium with separation properties similar to those of commercial restricted access media. Beads with even better selectivities have been prepared by a pore-size specific modification process using catalysts that are able to recognize the sizes of the pores and promote reaction only within those pores, that are large enough to accommodate them.

Though only a few examples of preparation of separation media containing pores with different functionalities have been presented, the pore-size

specific modification method is universal and can be extended to many other combinations of functional groups.

Despite of the use of a polydisperse polymeric acid as a catalyst, the pore-size selective approach proved to be feasible. The pore surface chemistry would be controlled even better if polymeric catalysts with very narrow molecular weight distributions were used. The application of such catalysts is currently under study.

The simplicity of preparation, excellent separation of components of model mixtures similar to plasma samples, high protein recovery and very good column stability makes the novel separation media advantageous over the currently available stationary phases for direct injection.

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DETERMINATION OF GLG-V-13, A NOVEL ANTIARRHYTHMIC AGENT, IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A sensitive reversed-phase HPLC technique with UV detection has been developed to determine the concentration of GLG-V-13 (3-[4-(1H-imidazol-1-yl)benzoyl]-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane dihydroperchlorate) (I), a novel combined class I and class III antiarrhythmic agent, in dog plasma and urine. Alkalinized plasma and urine samples were extracted with chloroform, and the extracts were reconstituted in methanol. An aliquot was injected on to a Waters HPLC system with a 250 x 4.6 mm Ultramex 5 C_6 analytical column (5 μm) and 30 x 4.6 mm Ultramex 5 C_6 guard column (5 μm). The elute was detected by a UV detector at 256 nm. Acetonitrile-methanol-37.5 m*M* phosphate buffer, pH6.8 (27:27:46 v/v)

containing 3.6 mM triethylamine was used as the mobile phase. The average extraction recovery was 89% from plasma and 93% from urine. Good lineary ($r>0.999$) was observed throughout the range of 8 - 8000 ng/ml in plasma and in urine with the quantitation limit of 8 ng/ml. Intra- and inter-assay variabilities were less than 4%. HPLC analysis of plasma and urine samples from a dog treated with I has demonstrated that the method was accurate and reproducible. Preliminary pharmacokinetic results showed that the plasma concentration-time curves fitted a two compartment open model with slow elimination ($t_{1/2\beta}$ 3.0827 h^{-1}); wide distribution (V_c 2.389 L/kg and $V_{d(ss)}$ 3.6808 L/h·kg); and longer mean residual time (MRT 4.7632 h), respectively. It seems that there is a difference in disposition of this compound in pathological dogs compared to normal one.

INTRODUCTION

3,7-Diheterobicyclo[3.3.1]nonane (DHBCN) derivatives have been found to show potential antiarrhythmic properties.[1-5] Several DHBCN derivatives exhibited antiarrhythmic activity in animal models and therefore are viable candidates for the treatment of life-threatening disorders in humans who experence sudden heart attacks or major infarctions of the heart.[6-8] GLG-V-13,3-[4-(1H-imidazol-1-yl)benzoyl]-7-isopropyl-3,7-diazabicyclo[3.3.1] nonane dihydroperchlorate (I) (Figure 1), has been demonstrated to increase the ventricle effective refractory period, to prolong QT duration (increase atria Hispurkinje and Hispurkinje-ventricle interval), to prevent sustained ventricular tachycardia induced by programmed electrical stimulation of infarcted dog heart.[9-10] Compound I was found to have combined class I and class III antiarrhythmic activity without hemodynamic depressant effect.[9-10]

The longer duration of pharmacological effects, low proarrhythmic activity, apparent lack of cardiodepressent effects, and combined class Ib and class III antiarrhythmic actions of I make this compound a very promising candidate as an antiarrhythmic agent. Thus, there is merit in characterizing the pharmacokinetics of I in animals. To date, there are no analytical

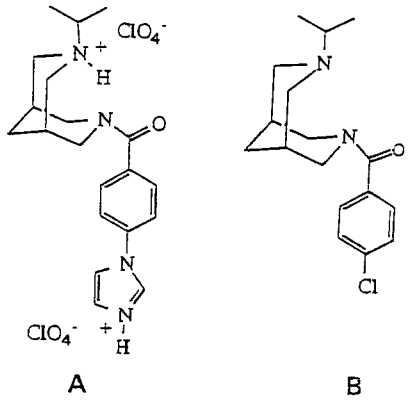

Figure 1. Chemical Structures of GLG-V-13 (A) and the I.S. (B)

methods currently available for analyzing compound I in biological fluids. We now describe a rapid, selective, and sensitive HPLC technique for the determination of this compound in biological fluids, including plasma and urine. Using this method, preliminary pharmacokinetic profiles of I in dogs were characterized.

EXPERIMENTAL MATERIALS AND METHODS

Chemicals

All the reagents used in this study were HPLC grade, and deionized distilled water was used throughout [Milli-Q™ Water system (Millipore Corp., Marlborough, MA)]. Acetonitrile, methanol, chloroform, and potassium phosphate monobasic were obtained from Fisher Chemicals (Fair Lawn, NJ), and Ionate™ triethylamine was purchased from Pierce Chemical Co. (Rockford, IL). Compound I and SAZ-VII-22 [3-(4-chlorobenzoyl)-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane, II], as an internal standard (I.S.) were synthesized by established methods.[11-12]

Standard Solution and Internal Standard

A stock solution of compound I was prepared in methanol at a concentration of 0.4 mg/ml. This stock solution was then diluted further to yield appropriate working solutions for the preparation of the calibration standards.

A stock solution of the I.S. was prepared in methanol (10 μg/ml) for addition to plasma and urine samples.

Extraction Procedures

For the determination of I, II was used as I.S. To 250 μl of dog plasma or urine, 25μl of 10 μg/ml of the I.S. was added. After alkalinization with 100 μl of 5 *M* NaOH, five ml of chloroform was added, and the mixture was mixed for 3 min. Following centrifugation (1000 x g 10 min), the organic phase was transferred into a clean test tube. The supernatant was re-extracted with 1 ml of chloroform. The combined chloroform extracts were evaporated to dryness under a stream of N_2. The residue was reconstituted in 50 μl of methanol, and 35 μl of this solution was injected for HPLC analysis.

Apparatus and Chromatographic Conditions

The HPLC system consisted of a Waters 501 HPLC pump, Waters U6K universal liquid chromatography injector with a 2 ml injection loop, a Model 484 Tunable Absorbance Detector controlled by a Baseline 810 Chromatography Work Station with a NEC Powermate Sx plus a computer, and an NEC Pinwriter P5200 (Millipore, Milford, MA).

The analytical column used was a 250 x 4.6 mm Ultramex 5 C_6 (5 μm) and the guard column was a 30 x 4.6 mm Ultramex 5 C_6 (5 μm). Both columns were purchased from Phenomenex (Torrance, CA).

The mobile phase was acetonitrile-methanol-37.5 m*M* phosphate buffer, pH6.8-triethylamine (27:27:46 v/v) containing 3.6 mM triethylamine. The mobile phase was filtered through a 0.5 μm Millipore filter and degassed before use. The column was eluted under isocratic conditions utilizing a flow

rate of 1.2 ml/min at ambient temperature. The detection wavelength was 256 nm.

Extraction Recovery

The samples (n=5) were prepared to give final concentrations of 100 ng/ml and 4000 ng/ml of compound I and 3 μg/ml of the I.S. in plasma and in urine, respectively. Using the extraction procedure described previously, the samples were extracted in the absence of the I.S. The organic layer was evaporated, and the residues were reconstituted in methanol. The ratio of the peak area of I extracted over that of unextracted equivalent concentrations of agent under identical chromatographic conditions was calculated as extraction recovery.

Calibration Curves

A calibration curve was generated to confirm the linear relationship between the peak-area ratio and the concentration of the agent in the samples. Twenty five μl of a suitable standard solution of compound I and 25 μl of the I.S. working solution were added to drug-free plasma and urine to give compound I concentrations at 8, 40, 80, 200, 400, 800, 2000, 4000, and 8000 ng/ml. Plasma and urine samples with known concentrations of I and the I.S. were extracted as previously described, and standard curves were generated by plotting peak-area ratios (agent/I.S.) against drug concentrations tested. Each standard curve was replicated five times. Linear regression analysis of standard curve was performed using the computer program PHARM/PCS. [13] The concentrations of I in unknown plasma and urine samples were calculated by interpolating the peak-height ratios with the calibration curve.

Intra- and Inter-assay Accuracy and Precision

To evaluate the intra-assay accuracy and precision, I and the I.S. were added to drug-free plasma and urine samples (n=6) at concentrations of 80

and 4000 ng/ml. These standard samples were prepared and stored at -20°C, and analyzed with the unknown samples. The concentrations were calculated using a standard curve. The percentage of the mean concentration determined over the mean concentration added was taken as the accuracy of the method, and the coefficient of variance was used as an index of precision. The inter-assay accuracy and precision were determined similarly over 6 consecutive days. Precision was estimated by determing the inter-assay coefficient of variations (C.V.).

Stability of Compound I

Compound I was added to free-drug dog plasma and urine to a final concentration of 100 and 4000 ng/ml, respectively. An aliquot of plasma or urine was extracted immediately as described above for the determination of compound I, while other aliquots of plasma or urine were stored frozen at -20°C or exposure to light at room temperature (25±2°C). Each month, an aliquot of frozen plasma and urine sample was thawed, extracted and analyzed as described above to evaluate the stability. For the determination of stability of I exposed to light at room temperature, a one day experimental duration was performed (one sample /two hour interval).

Drug Administration and Sampling

One experiment was carried out 24-96 hours after two-stage ligation of the left anterior descending coronary artery in an anesthetized beagle dog. Programmed premature stimuli or rapid intermittent 3-beat-bursts (240-420/min) were delivered to the right ventricle to induce sustained monomorphic ventricular tachycardia. After administration of 3 mg/kg of compound I via an i.v. bolus injection, blood samples (± 5 ml) were collected via vein puncture at 5, 10, 15, 30, 45, 60, 75, 90 and 120 min. Another experiment was carried out on a normal unanesthetized beagle dog with an intravenous bolus injection of 6 mg/kg of I with blood samples (± 5 ml) being

collected up to 12 hr. The samples were heparinized and centrifuged at 20000 x g for 10 min. The plasma fractions were stored at -20°C until analyzed. Urine was collected by catheter and stored at -20°C until analyzed.

RESULTS AND DISCUSSION

Extraction Efficiency

Alkalinization of plasma and urine samples increased extraction efficiency with the pH value for the solution at 12 showing the best extraction recovery. Most of compound I was in a nonionized state at pH value of 12. The use of chloroform to precipitate proteins and to extract compound I directly from plasma offered marked advantage since fewer peaks were found for contaminants. Using trichloroacetic acid (TCA) to precipitate proteins decreased the absolute recovery. This may be due to decomposition of I. This situation was similar to that of some other DHBCN derivatives. [14-16] Anticoagulators, such as EDTA and heparin, did not affect the extraction recovery. Extraction recoveries of compound I were 87.5-91.2% from plasma and 92.2-93.6% from urine, respectively (Table 1). The recoveries of the I.S. were 85% from plasma and 95% from urine.

Chromatographic Separation

Several combinations of acetonitrile, methanol, buffer (with different pH) and triethylamine were evaluated as possible mobile phases. It was determined that the combination described herein was found to be the most suitable for separating I. Under the described chromatographic conditions, a good separation of compound I and its I.S. was achived. The retention times were 11.40 ± 0.44 and 19.07 ± 0.73 min, respectively. At the retention time, the compound I and its I.S. were eluted without an interference peak from the blank plasma and urine (Figures 2 and 3). Varying proportions of

TABLE 1.
Recovery of Compound 1 added to dog plasma and urine (Mean±SD, n = 5)

	Added (ng/ml)	Found (ng/ml)	Recovery (%)
Plasma			
	100	87.3±4.5	87.3±4.5
	4000	36480±1320	91.2±3.6
Urine			
	100	92.2±5.3	92.2±5.3
	4000	37420±1800	93.6±4.5

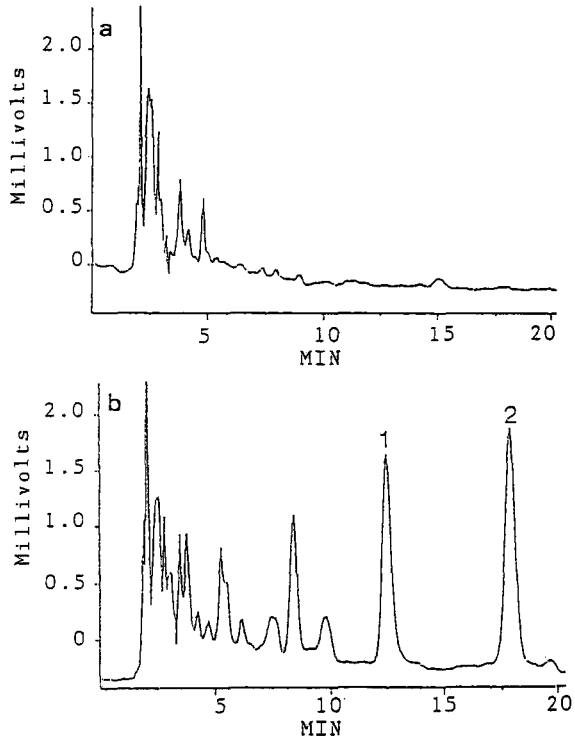

Figure 2. Representative chromatograms from (a) blank plasma and (b) plasma sample 8 hr after i.v. dose of 6 mg/kg of I. See experimental for chromatographic conditions.
Peaks: 1 = I, 2 = I.S.
The estimated concentration of I was 0.27 μg/ml.

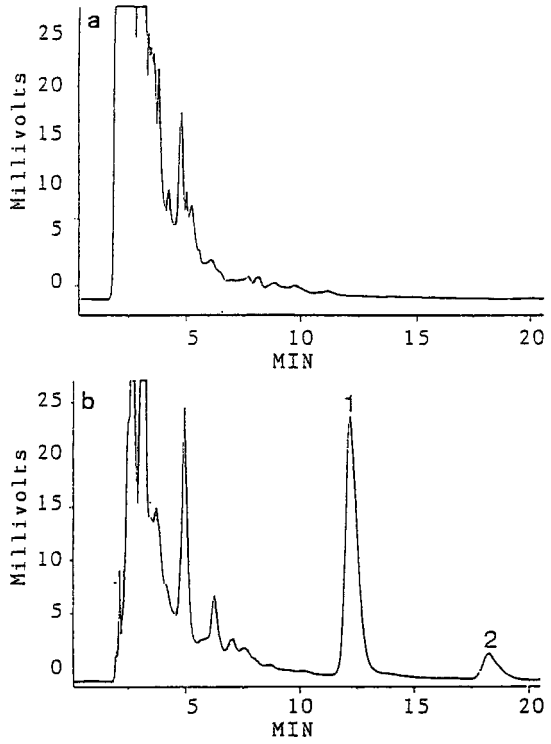

Figure 3. Representative chromatograms from (a) blank urine and (b) urine sample 1 hr after i.v. dose of 6 mg/kg of I (b). See experimental for chromatographic conditions.
Peaks: 1 = I, 2 = I.S.
The estimated concentration of I was 5.1 μg/ml.

triethylamine in the mobile phase changed both retention time and sharpness of the peak for compound I. The pH of the mobile phase is a very important factor influencing the elution of I. If the mobile phase pH was below 6.8, the retention time for I was shorter. There was a concomitant decrease in sensitivity. Neither acetonitrile nor methanol alone was suitable as the strong solvent. These observations were very similar to that with other DHBCN

derivatives. [14-16] Therefore, varying ratio of methanol-acetonitrile-triethylamine-phosphate buffer may be the best combination for separating and analyzing DHBCN derivatives in biological fluids.

Assay Validation

Linearity. Five consecutive standard curves for pure I analyzed on separate days demonstrated a good linear relationship between concentration and peak area. The standard curves obtained from extraction of both dog plasma and urine containing known amounts of I were linear over the concentration ranges tested (8-8000 ng/ml). The range of coefficient of variations was between 1-17%. The calibration curves were found to be linear and could be described by the regression equations: $Y = -0.01492 + 0.5127X$ ($r = 0.9996$) for plasma and $Y = -0.04057 + 0.6557X$ ($r = 0.9995$) for urine, respectively, in which Y was the agent recovered in μg/ml, and X was peak area ratio (agent/internal standard). The limits of quantitation of I were 8 ng/ml in plasma and in urine, respectively. This sensitivity has proved useful in the analysis of pharmacokinetic data of dog plasma and urine after administration of compound I.

Precision and Accuracy. The results obtained indicate that intra- and inter-assay coefficient of variance (C.V.) in plasma and urine were less than 4%. The accuracy of this method was 94-99% (Table 2). These results suggest that the procedures described as above are satisfactory with respect to both accuracy and precision.

Stability of Compound I

It was found that the free amine of compound I turned yellow when exposed to light for 2-3 hr, while compound I (disalt) was not sensitive to light. This experimental observation showed that I was very stable in dog plasma and urine at -20°C for at least 6 months and was also stable when exposed to light at room temperature (25±2°C) for one day. Possibly compound I may be present as the disalt or monosalt in the biological fluid.

TABLE 2.
Intra-assay (Within-day) and Inter-assay (Between-day) Precision and Accuracy of the Determination of Compound I in Dog Plasma and Urine

Concentration (ng/ml)		Accuracy (%)	C.V. (%)
Added	Found		
Plasma			
Intra-assay (n = 6)			
80	76.5	95.6	1.65
4000	3728	93.2	2.26
Inter-assay (n = 6)			
80	79.2	96.8	3.00
4000	3810	95.2	2.29
Urine			
Intra-assay (n = 6)			
80	76.0	95.0	3.14
4000	3780	94.5	1.52
Inter-assay (n = 6)			
80	77.6	97.0	3.53
4000	3960	99.0	1.38

* C.V. = coefficient of variance

Preliminary Pharmacokinetic Studies

The plasma and urine samples were extracted and analyzed as previously described. Representative HPLC profiles of the plasma and urine samples of a dog given I (6 mg/kg) intravenously are shown in Figure 2 and 3.

The plasma concentration-time profiles of I in dogs given intravenous dose of 3 and 6 mg/kg are shown in Figure 4. Data fitting and

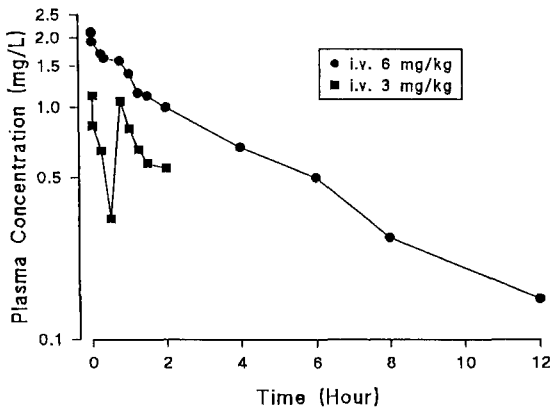

Figure 4. Plasma concentration profiles after i.v. dose of 6 and 3 mg/kg of compound I in a normal dog and in a pathological dog, respectively.

pharmacokinetic parameters calculations were carried out using the Boomer program. [17] The plasma concentration change can be best described as two compartment model with equation: $C = A_1e^{-\alpha t} + A_2e^{-\beta t}$. The calculated pharmacokinetic parameters after iv bolus injection of 6 mg/kg was showed in Table 3. The results show that the compound I has very low elimination rate with $t_{1/2\beta}$ 3.0827 h^{-1} and longer mean residual time (MRT 4.7632 h), which is consistent with the duration of pharmacological effects. It was interesting that the disposition of I was different in the beagle dog under normal and pathological conditions. In a pathological dog, plasma concentration of I durimg 45-60 min was higher than that of 20-30 min. This may be due to the enterohepatic circulation. The exact mechanism is unknown.

The results showed that the HPLC method described above has a lower quantitation limit of 8 ng/ml using a 250 μl sample. As shown in this report, this method is suitable for pharmacokinetic studies of this novel antiarrhythmic agent. Studies of the pharmacokinetic and metabolite(s) profiles of I in animals are in progress.

TABLE 3.
Pharmacokinetic Parameters Following iv Bolus 6 mg/kg of I to One Dog

Parameters	Value
iv bolus (6 mg/kg)	(n = 1)
A1 (mg/L)	0.844
A2 (mg/L)	1.669
α (h^{-1})	6.754
ß (h^{-1})	0.2248
K_{10} (h^{-1})	0.3326
K_{12} (h^{-1})	2.086
K_{21} (h^{-1})	4.575
$t_{1/2\alpha}$ (h)	0.1026
$t_{1/2ß}$ (h)	3.083
V_c (L/kg)	2.389
$V_{d(area)}$ (L/kg)	3.437
$V_{d(ss)}$ (L/kg)	3.681
Cl_B (ml/h/kg)	0.7727
AUC_{iv} (mg·h/L)	7.765
$AUMC_{iv}$ (mg/L)	36.984
MRT_{iv} (h)	4.763

Abbriviations: K_{10}-first-order elimination rate constant; K_{12}, K_{21} are the first-order rate constants describing distribution between central (plasma) and peripheral compartment (tissues); $t_{1/2\alpha}$ is distribution half-life after iv; $t_{1/2ß}$ is elimination half-life after iv; V_c = volume of the central compartment; $V_{d(area)}$ is apparent volume distribution calculated using AUC; Vd(ss) is apparant volume of distribution at stead state; Cl_B is body clearance of the drug.

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DETERMINATION OF OXOLINIC ACID IN SEAWATER, MARINE SEDIMENT, AND JAPANESE OYSTER *(CRASSOSTREA GIGAS)* BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method was developed to detect oxolinic acid in seawater, marine sediment, and japanese oyster (*Crassostrea gigas*). Seawater was analysed after filtration and centrifugation. Sediment and oyster tissues were respectively analysed after liquid-phase and solid-phase extraction. Linearity and precision were checked over the concentration range 0.05-2.50 µg/mL or µg/g. Limits of detection and determination were respectively 0.01 and 0.04 µg/mL or µg/g. Recoveries of oxolinic acid were 102.1% from seawater, 68.1% from sediment and 88.3% from oyster. Oxolinic acid concentrations in seawater, sediment and oyster spiked at 0.50 µg/mL or µg/g were stable at - 20°C for 60 days.

INTRODUCTION

The quinolone oxolinic acid (5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo[4,5-g]quinoline-7-carboxylic acid) is a commonly used antibiotic for treatment of infectious diseases in fish farming. The drug is administered to fish as medicated pelleted feed at a dosage of 10-20 mg/kg body weight for 8-10 days.

Since fish suffering from bacterial diseases usually show reduced food intake and oxolinic acid is poorly absorbed through the intestinal tract of fish (1), oxolinic acid may subsequently pass into the environment of the fish farm. On one hand, fragments of the medicated food pellets could be taken up by filter-feeders such as shellfish, or reach sediment (2). Oxolinic acid may also be released from pellets in a dissolved form which can be absorbed by organisms directly from the water. On another hand, much of the drug could pass through the fish and enter the environment in an unchanged and active form via faeces (3). The drug-containing faeces from the farmed fish could, either be eaten by the wild fauna, or reach sediment.

The development of fish farming in ancient salt-marshes along the French Atlantic coastline may be responsible for the pollution of seawater, marine sediment and shellfish by oxytetracycline (4, 5, 6). The current knowledge of the environmental impacts of oxolinic acid used in marine aquaculture is merely poor. Thus, it is necessary to develop adequate methods for kinetic and residues studies of oxolinic acid in seawater, sediment and edible shellfish.

The commonly used microbiological assays for determining oxolinic acid are less sensitive and less specific than the

chromatographic methods (7). Several methods using high-performance liquid chromatography (HPLC) with octyl or octadecyl silane columns for analysing oxolinic acid in fish plasma and tissues have been described (1, 8, 9, 10, 11, 12, 13, 14, 15). Only two optimized methods in fish tissues have been adapted to the determination of oxolinic acid in sediment (16) and blue mussel (17).

The purpose of the present work was to develop simple, rapid and accurate HPLC methods for analysing oxolinic acid in seawater, marine sediment and japanese oyster (*Crassostrea gigas*). The need for these methods was the ability to study occurence, persistence and metabolism of oxolinic acid in seawater, sediment and japanese oyster, via experiments carried out both *in situ* and under controled tank conditions.

MATERIALS AND METHODS

Chemicals

Acetonitrile (BDH Chemicals, Toronto, Canada) and methanol (Carlo-Erba, Milano, Italy) were of HPLC-grade. Chloroform, orthophosphoric acid, potassium dihydrogenphosphate, hydrochloric acid and sodium hydroxide (Merck, Darmstadt, Germany) were analytical-grade reagents. Ethyl acetate (Janssen, Geel, Belgium) was of analytical-grade. The water used in buffers and eluents was distilled and purified with an Elgastat Spectrum RO2 (Elga Ltd, Buckinghamshire, England). Oxolinic acid was purchased as a pure standard from Sigma (St. Louis, MO, U.S.A.).

Apparatus

The HPLC system consisted of a chromatograph Varian 5000 equipped with a Valco injection valve, a Vari-Chrom UV 50 variable-wavelength absorbance detector (Varian, Palo Alto, CA, USA) and a Merck D-2500 integrator (Darmstadt, Germany). The data were handled with a computer Deskpro 386/s Mod 40 3.5 (Compaq, Houston, TX, USA) equipped with the HPLC Manager Software System (Merck). The analytical column, a 5 μm LiChroSpher 100 RP-18E, 125 X 4.6 mm I.D. (Merck), was equipped with a 5 μm LiChroSpher 100 RP-18E guard column, 4 X 4.6 mm I.D.

Chromatographic Conditions

The mobile phase consisted of acetonitrile and 0.02 M orthophosphoric acid solution (24:76 v/v), pH 2.3. The mixture was filtered with a Millipore HPLC solvent filtration system (Millipore, Bedford, MA, USA) and Whatman 47 mm, 0.20 μm nylon filters (Maidstone, England) and then sonicated for 15 min.

The chromatographic experiments were performed at ambient temperature. The operating flow rate was 1.0 mL/min and the UV detector was set at 262 nm and 0.01 a.u.f.s. The sample volume injected on the column was 50 μL. The guard column was removed at intervals of 150 to 200 sample injections.

The new columns were conditioned prior to use by flushing with acetonitrile and water 75:25 v/v (2h), 50:50 v/v (2h), 40:60 v/v (2h), 30:70 v/v (2h) and mobile phase (5h) at a flow rate of 0.2 mL/min. Moreover, the columns were reconditioned for 2 h after each day of

operation with acetonitrile and water (24:76 v/v) at a flow rate of 0.2 mL/min.

Standard Solutions

A stock solution of oxolinic acid was prepared in 0.03 M aqueous sodium hydroxide at a concentration of 1 mg/mL and was stable for one month when stored at 4°C. Working standard solutions were prepared by dilution in water immediatly before use. All these solutions were stored in dark.

Extraction Procedures

A 2-mL volume of a seawater sample was filtered through a Minisart NML 26 mm, 0.45 µm cellulose acetate membrane (Sartorius, Göttingen, Germany). A 1.5-mL volume of the filtrate was transferred to a polypropylene tube and centrifugated at 10,000 g for 5 min at 4°C in a Jouan Model MR 1822 centrifuge (Jouan, Saint Herblain, France).

A 1 g-sediment sample was extracted three times with 4 mL of 0.2 M sodium hydroxide. After homogenization for 5 min (Heidolph, Bioblock Scientific, Illkirsch, France) and centrifugation at 10,000 g for 10 min at 4°C (Jouan Model MR 1822 centrifuge), the supernatants were combined. The supernatants, into which 2.5 mL of 1 M hydrochloric acid were added, were extracted with a mixture of 2 mL of chloroform and 2 mL of ethyl acetate. After homogenization for 5 min (Heidolph) and centrifugation at 10,000 g for 10 min at 4°C (Jouan Model MR 1822 centrifuge), the organic phase was transferred in a 5-mL vial and evaporated to dryness under nitrogen stream at 35°C. Prior to analysis, extract was dissolved in 0.5 mL of mobile phase.

Oyster tissues were homogenized using a high-speed blender (Ultra-turrax, Bioblock). A 1 g-homogenate was transferred to a polypropylene tube (8 mL) and extracted three times with 4, 4 and 2 mL of a phosphate buffer (0.1 M aqueous dihydrogenphosphate adjusted with 1 M sodium hydroxide to pH 7.0). After homogenization for 5 min (Heidolph) and centrifugation at 10,000 g for 10 min at 4°C (Jouan Model MR 1822 centrifuge), the combined supernatants were concentrated by passing through a 3 mL-octadecyl solid-phase extraction cartridge (Bond Elut, Analytichem International, Harbor City, CA, USA). Before use, cartridge was activated with methanol (3 mL) and phosphate buffer (3 mL). After the sample had been passed, cartridge was flushed with 3 mL of water and oxolinic acid was eluted with 3 mL of methanol:1 M orthophosphoric acid (90:10 v/v). The eluate was evaporated to dryness under nitrogen at 35°C and reconstituted to 0.5 mL in mobile phase. Prior to analysis, the sample was centrifugated at 10,000g for 10 min at 4°C (Jouan Model MR 1822 centrifuge).

All the aforementioned steps of the extraction procedures were conducted in subdued light.

Method of Validation

An overall validation of the method was performed using seawater, sediment and japanese oyster. A daily calibration curve was obtained by spiking samples at six levels of oxolinic acid (0.05, 0.10, 0.25, 0.50, 1.00 and 2.50 μg/mL or μg/g) and analysing two replicates for three consecutive days. The precision of the method was checked by spiking samples at a concentration of 0.50 μg/mL or μg/g with oxolinic

acid and analysing five replicates for two consecutive days. The extraction recoveries of oxolinic acid were determined for three consecutive days by comparing peak heights obtained by chromatographing spiked and extracted samples with peak heights obtained by chromatographing the pure drug standard. A 60 days-study of stability was performed by analysing samples spiked at a concentration of 0.50 μg/mL or μg/g with oxolinic acid and stored at - 20°C.

RESULTS AND DISCUSSION

Chromatographic Conditions

Reversed phase-HPLC has often been used for determining quinolones antibiotics in fish plasma and tissues and gave good performance in terms of plate numbers (11). In our experiment, a LiChroSpher 100-RP 18E prepacked column was used as a reversed phase column in order to separate, with high efficiency, oxolinic acid in seawater, marine sediment and oyster tissues. The importance of using "endcapped" materials which contain a negligible proportion of accessible hydroxyl groups had yet been demonstrated.

All the previous workers using reversed-phase systems added an organic modifier to the predominantly aqueous eluent. Comparisons of the three modifiers - methanol, tetrahydrofuran and acetonitrile - indicated that more symmetrical peaks were given by acetonitrile (data not shown). Different aqueous buffers - disodium hydrogenphosphate, potassium dihydrogenphosphate and dipotassium hydrogenphosphate

- were tested in order to optimize the chromatographic conditions. When using them, baseline drift and severe peak tailing were consistently noted, and the buffer precipitated inside the tubing that fed organic solvent from the pump to the column. When using 0.02 M orthophosphoric acid solution as aqueous eluent, the chromatograms were free of interfering peaks. Neither tailing nor baseline drift were evident and no precipitation occured inside the tubing.

Different proportions of acetonitrile and 0.02 M orthophosphoric acid solution, giving a scale of pH from 2.0 to 5.0, were tested. The highest efficiency was obtained with acetonitrile and 0.02 M orthophosphoric acid solution (24:76 v/v), pH 2.3. Lower proportions of acetonitrile and higher pH values of the mobile phase gave tailing peaks or lower capacity factors and showed lower correlation between peak height and drug concentration (data not shown).

Under the operating conditions, the capacity and asymmetry factors were respectively 3.8 and 1.3. Ending each day of operation, recycling with acetonitrile and water (24:76 v/v), pH 7.4 through the HPLC column for 2h allowed the column life to increase, because reversed phases are unstable at low pH values. Under these conditions, there was no loss of bonded layer and column was stable for a long period.

Oxolinic acid was eluted in 6.5 min. No changes in retention times were noted with continual column use. No additionnal peaks, possibly resulting from impurities or degradation products, that could interfere with the oxolinic acid peaks, were noted on the chromatograms. The samples did not contain these related compounds

in significant amount for the HPLC assay. Typical chromatograms are shown in Figure 1.

After 150-200 injections, precolumn was saturated with seawater, sediment and shellfish tissues components and gave a drifting baseline. Analytical column was used for more than 1,500 injections of samples without any change in its performances. The final number of theoretical plates was 2,500 compared with the initial value of 2,700.

Extractions and Recoveries

The mean recovery of oxolinic acid from spiked seawater was 102.1% [relative standard deviation (RSD) = 4.5%, number of samples (n) = 47] over the concentration range 0.05-2.50 μg/mL (Table 1). Thus, prior to analysis, a simple clean-up procedure of seawater samples was enough for obtaining a high recovery.

The highest recoveries of oxolinic acid from sediment were achieved with three extraction cycles, each with 4 mL of 0.2 M sodium hydroxide, followed by another extraction with a mixture of chloroform and ethyl acetate at an acid pH. The mean recovery of oxolinic acid from spiked sediment was 68.1% [RSD = 1.7%, n = 30] over the concentration range 0.10-2.50 μg/g (Table 1). This mean recovery was similar to the one obtained by Björklund *et al.* (16) applying their method described for fish muscle tissues to sediment (mean = 70.9%, RSD = 5.1%). The relatively low recovery obtained could be explained by the formation of complexes between the drug and some components of the sediment, such as divalent cations (Mg^{2+}, Fe^{2+}, Cu^{2+}). Formation of such complexes could modify hydrophilic and

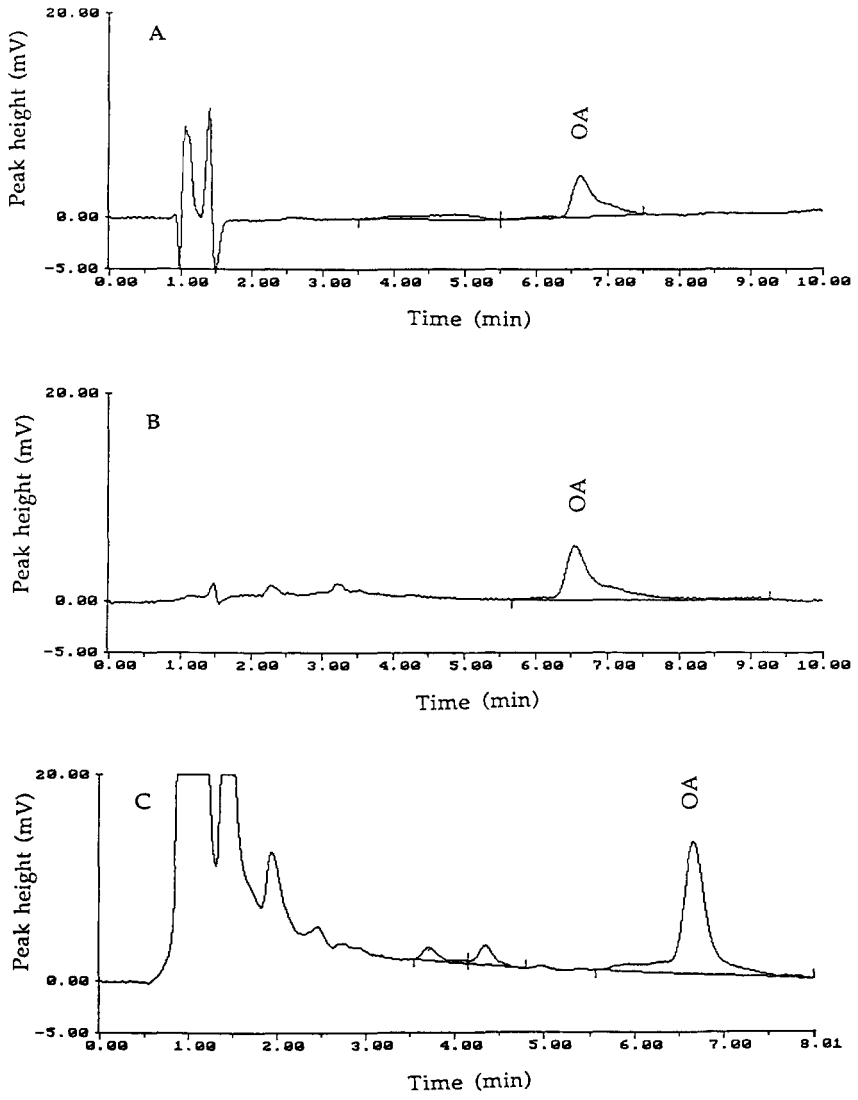

FIGURE 1

HPLC Chromatograms **A**, Seawater Sample Containing Oxolinic Acid (OA) at a Concentration of 0.50 µg/mL; **B**, Sediment Sample Containing OA at a Concentration of 0.40 µg/g; **C**, *Crassostrea gigas* Sample Containing OA at a Concentration of 1.40 µg/g. Conditions: Mobile Phase, Acetonitrile and 0.02 M Orthophosphoric Acid Solution, pH 2.3 (24:76 v/v); Column 125 X 4 mm, C_{18} (5 µm); Flow Rate, 1.0 mL/min; Wavelength, 262 nm; Detector Sensitivity 0.01 a.u.f.s.; Injection Volume, 50 µL.

TABLE 1

Recoveries of Oxolinic Acid from Seawater, Sediment and Japanese Oyster Tissues Spiked with Various Amounts of Oxolinic Acid. Mean in % (Coefficient of Variation in %) [Number of Samples].

	Seawater	Sediment	Oyster
0.05 μg	98.3 (9.0) [6]		86.3 (3.5) [6]
0.10 μg	102.5 (7.7) [6]	68.5 (3.0) [6]	87.9 (2.3) [6]
0.25 μg	104.6 (4.2) [9]	68.9 (1.7) [6]	88.8 (4.2) [6]
0.50 μg	102.7 (3.5) [10]	67.8 (1.7) [6]	87.2 (1.7) [6]
1.00 μg	103.6 (0.7) [8]	68.2 (1.1) [6]	88.5 (2.1) [6]
2.50 μg	100.9 (2.4) [8]	68.5 (1.0) [6]	90.9 (0.6) [6]

hydrophobic properties of free oxolinic acid. Thus, they could not be quantitatively extracted and consequently detected by the extraction and HPLC method developed for free oxolinic acid.

In a preliminary study, recoveries of oxolinic acid from spiked oyster tissues were compared by using the liquid-phase extraction procedure above described for sediment and the solid-phase extraction procedure for fish tissues described by Björklund (11). Since better recoveries were obtained with the second procedure, the method of Björklund (11) was optimized in order to adapt it to oyster tissues. Octyl

and octadecyl solid-phase extraction cartridges from Analytichem were compared. The effects of the nature and volume of solvents for conditioning, washing and elution of the columns were also tested. The best recovery was obtained with octadecyl solid-phase extraction cartridges, methanol and phosphate buffer as conditioning solvents, water as washing solvent and a mixture of methanol and 1 M orthophosphoric acid as elution solvent (data not shown). The carrying out of an elution gradient showed the optimal pourcentage of methanol in the elution solvent to be 70%. Since the evaporation to dryness should be time and money-saving, an elution solvent containing 90% of methanol was choosen. Under these conditions, the mean recovery of oxolinic acid from oyster was 88.3% [RSD = 2.4%, n = 36], over the concentration range 0.05-2.50 μg/g (Table 1). This mean recovery was near-by the one obtained by Samuelsen *et al.* (17) by applying their method described for fish muscle tissue to blue mussel (85.4-93.5%).

Linearity, Sensitivity, Precision and Stability

A statistical test of linearity was performed for each calibration curve separately, using a weighted analysis of variance ANOVA (18, 19). All the calibration curves showed linearity in the range examined (0.05-2.50 μg/mL or μg/g) at the 0.05 level (Table 2). A good correlation was obtained between concentrations and peak heights : all the correlation coefficients were between 0.970 and 0.999 (Table 2).

The limits of detection and determination of oxolinic acid in seawater, sediment and oyster tissues were respectively 0.01 and

TABLE 2

Linearity and Regression Data for the Calibration Graphs Obtained from Seawater, Sediment and Japanese Oyster Spiked with Oxolinic Acid from 0.05 to 2.50 μg/mL or μg/g
y = ax + b; y = Peak Height (μV) ; x = Oxolinic Acid Concentration (μg/mL or μg/g) ; a = Slope ; b = Intercept

		Seawater	Sediment	Oyster
Slope	Day 1	$1.173\ 10^{-4}$	$9.513\ 10^{-5}$	$6.193\ 10^{-5}$
	Day 2	$1.139\ 10^{-4}$	$9.114\ 10^{-5}$	$5.970\ 10^{-5}$
	Day 3	$1.261\ 10^{-4}$	$9.258\ 10^{-5}$	$6.086\ 10^{-5}$
Intercept	Day 1	0.002	0.037	- 0.125
	Day 2	0.012	0.014	- 0.080
	Day 3	0.007	0.008	- 0.081
Correlation Coefficient	Day 1	0.999	0.992	0.981
	Day 2	0.999	0.970	0.980
	Day 3	0.998	0.998	0.980
F Linearity	Day 1	15600.94*	1755.69*	306.93*
	Day 2	12121.68*	325.55*	541.07*
	Day 3	5606.87*	7253.07*	295.05*

* No Significative Difference Between the Calculated Slope and 0 at the 0.001 Level.

0.04 μg/mL or μg/g (precision ±10%). This concentration of 0.04 μg/mL or μg/g was accepted as the limit of determination because a t-test with four degrees of freedom showed this is significantly different from 0 and the mean response was greater than three standard deviations (18, 19). The expression of the later experimental results will be subordinated to these two limits (Figure 2).

The relative standard deviations of within-day precision for samples spiked with oxolinic acid at 0.500 μg/mL or μg/g were between

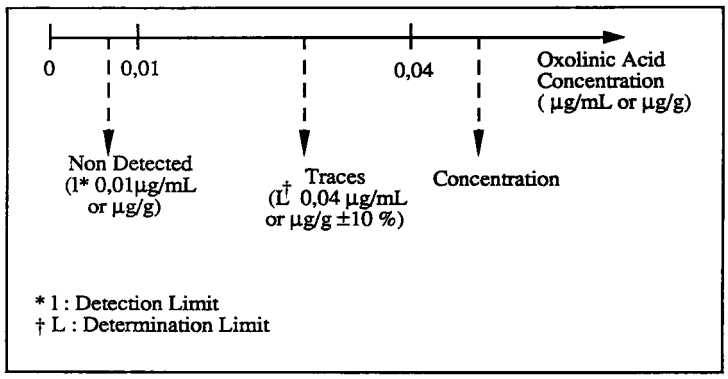

FIGURE 2

Limits of Detection and Determination : Consequences for the Expression of the Oxolinic Acid Concentration.

TABLE 3

Precision Data Obtained from Seawater, Sediment and Japanese Oyster Tissues Spiked with Oxolinic Acid at 0.50 µg/mL or µg/g

	Mean (µg/mL or µg/g)	Standard Deviation (µg/mL or µg/g)	Variation Coefficient (%)	F Scheffé
Seawater				
Day 1	0.509	0008	1.58	0.202*
Day 2	0.508	0.006	1.25	
Sediment				
Day 1	0.339	0.007	2.00	0.095*
Day 2	0.340	0.006	1.83	
Oyster				
Day 1	0.400	0.014	3.14	0.020*
Day 2	0.442	0.015	3.36	

* No Significative Difference Between Means at the Level 95 %.

1.58 and 3.14% (Table 3). The relative standard deviations of between-day precision for samples spiked with oxolinic acid at 0.500 μg/mL or μg/g were between 1.25 and 3.36% (Table 3). An analysis of variance (ANOVA) showed there was no significant difference between the within- and between-day precision at the 0.05 level.

A study of the stability of oxolinic acid in seawater, sediment and oyster samples spiked at a concentration of 0.500 μg/mL or μg/g and stored at - 20°C was performed. All the recoveries of oxolinic acid from the samples stored at - 20°C fall within the average limits calculated using the recoveries results from the validation (18, 19). Moreover, there was no significant decrease of the oxolinic acid recoveries from samples maintained at - 20°C during 60 days.

CONCLUSION

The described methods provide a selective, reliable and precise mean for the rapid determination of oxolinic acid in seawater, marine sediment and japanese oyster. They do not require time-consuming, complex extraction or derivatization techniques. An analyst familiar with the methods could easily process fifteen samples a day. They are suitable for pharmacokinetics and residues studies on oxolinic acid in seawater, sediment and japanese oyster.

ACKNOWLEDGEMENTS

We thank Ms. Michaëlle Larhantec and Christelle Moriceau for their technical assistance.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 17(4), 947-948 (1994)

THE BOOK CORNER

PRACTICAL CAPILLARY ELECTROPHORESIS, R. Weinberger, Academic Press, Inc., New York, 312 pages, 1993. Price: $69.95.

High performance capillary electrophoresis is a powerful analytical technique which offers high resolution, speed and ease of operation and requires small sample size. It is an efficient and excellent micro-analytical technique. Recently, the application of HPCE has enjoyed a phenominal growth, as evidenced from the manuscripts published in the scientific literature and presented at local and international meetings. Although the theory of CE is complex, it can be simplified and presented in a facile way that the reader can understand. Dr. Weinberger has done just that. He has taken a complex topic and presented it in a relatively short book which covers all aspects of CE in an easy-to-understand way. This book, which is reasonably priced, is intended to be a textbook. Of course, there is an advantage to a single-author book which provides continuity of the subject but, on the other hand, might lack the depth and comprehensive discussion of individual topics of an edited book. Practical capillary electrophoresis is a good starting point for those interested in learning about CE. The material is clearly presented in a concise format, too concise in certain cases that it lacks the required depth to be a reference for the seasoned separation scientist. However, as mentioned earlier, it is a good reference for the novice. This book ranks as one of the better published books on CE. It is recommended for the analytical chemist and biochemist and those

interested in the separation of small ions, large biomolecules and neutral and chiral compounds.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 17(4), 949 (1994)

MEETING ANNOUNCEMENT

INTERNATIONAL SYMPOSIUM ON POLYMER ANALYSIS AND CHARACTERIZATION (ISPAC-7)

May 23 - 25, 1994

Les Diablerets, Switzerland

The Seventh International Symposium on Polymer Analysis and Characterization (ISPAC-7) will be a three-day meeting which will include poster sessions, invited lectures and round-table discussions & information exchanges on recent advances in polymer characterization approaches, techniques and applications.

ISPAC-7 will focus on molecular characterization in relation to properties of polymeric systems. Topics will include aqueous polymeric systems (water-soluble polymers, interacting systems, and physical and chemical gels), and heterogeneous polymeric materials (blends, composites, multilayered materials). In addition, an introductory course on polyelectrolytes and the characterization of water-soluble polymers will be held on Sunday, May 22nd, preceding the Symposium.

Further information may be obtained from ISPAC Registration, 815 Don Gaspar, Santa Fe, NM 87501, USA, or from Dr. M. Rinaudo, CERMAV-CNRS, B. P. 53X, 38041 Grenoble Cedex France.

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(4), 951-956 (1994)

LIQUID CHROMATOGRAPHY CALENDAR

1994

JANUARY 16 - 20: 19th IUPAC Symposium on the Chemistry of Natural Products, Karachi, Pakistan. Contact: Prof. Atta-Ur-Rahman, Director H. E. J. Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan.

FEBRUARY 2 - 3: AOAC Southeast Section Meeting, Ramada Hotel & Convention Center, Atlanta, GA. Contact: Doug Hite, Technical Services, P. O. Box 40627, Melrose Station, Nashville, TN 37204, USA.

FEBRUARY 28 - MARCH 4: PittCon'94: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd, Pittsburgh, PA 15235-9962, USA

MARCH 22 - 24: PrepTech '94, A New Conference on Industrial Bioseparations, Meadowlands Hilton Hotel, S3ecaucus, New Jersey. Contact: Symposium Manager, PrepTech '94, ISC, Inc., 30 Controls Drive, Shelton, CT 06484, USA.

APRIL 10 - 15: 207th ACS National Meeting, San Diego, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036, USA.

APRIL 19 - 22: Rubber Division ACS, 145th Spring Technical Meeting, Palmer House Hotel, Chicago, Illinois. Contact: C. Morrison, Rubber Division, P.O. Box 499, Akron, OH 44309-0499, USA.

MAY 8 - 13: HPLC '94: Eighteenth International Symposium on High Performance Liquid Chromatography, Minneapolis Convention Center, Minneapolis, Minnesota. Contact: Mrs. Janet Cunningham, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, USA.

MAY 23 - 25: International Symposium on Polymer Analysis and Characterization (ISPAC-7), Les Diablerets, Switzerland. Contact: Howard G. Barth, ISPAC Chairman, DuPont Company, Central Research & Development, P. O. Box 80228, Wilmington, DE 19880-0228, USA or Dr. M. Rinaudo, CERMAV-CNRS, B. P. 53X, 38041 Grenoble Cedex France.

JUNE 1 - 3: Joint 26th Central Regional/27th Great Lakes Regional Meeting, ACS, Kalamazoo and Huron Valley Sections. Contact: H. Griffin, Univ of Michigan, Ann Arbor, MI 48109, USA.

JUNE 1 - 3: International Symposium on Hormone & Veterinary Drug Residue Analysis, Congress Centre Oud Sint-Jan, Brugge, Belgium. Contact: Prof. C. Van Peteghem, University of Ghent, L:aboratory of Food Analysis, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 5 - 7: VIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques & Applications in Chromatography and Capillary Electrophoresis, Congress Centre Oud Sint-Jan, Brugge, Belgium. Contact: Prof. Dr. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Dept. of Pharmaceutical Analysis, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 12 - 15: 1994 Prep Symposium & Exhibit, sponsored by the Washington Chromatography Discussion Group, at the Georgetown University Conference Center by Marriott, Washington, DC. Contact: Janet E. Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JUNE 13 - 15: Fourth International Symposium on Field-Flow Fractionation (FFF'94), Lund, Sweden. Contact: Dr. Agneta Sjogren, The Swedish Chemical Society, Wallingatan 24, 2 tr, S-111 24 Stockholm, Sweden.

JUNE 19 - 23: 24th National Medicinal Chem. Symposium, Little America Hotel & Towers, Salt Lake City, Utah. Contact: M. A. Jensen & A. D. Broom, Dept. of Medicinal Chem, 308 Skaggs Hall, Univ of Utah, Salt Lake City, UT 84112, USA.

JUNE 19 - 24: 20th International Symposium on Chromatography, Bournemouth International Centre, Bournemouth, UK. Contact: Mrs. Jennifer A. Challis, The Chromatography Society, Suite 4, Clarendon Chambers, 32 Clarendon Street, Nottingham, NG1 5JD, UK.

JUNE 24 - 27: BOC Priestly Conference, Bucknell University, Lewisburg, PA, co-sponsored with the Royal Soc of Chem. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

JULY 31 - AUGUST 5: American Society of Pharmacognosy Annual Meeting, International Research Congress on Natural Products, joint meeting with the Assoc. Français pour l'Enseignement et la Recherche en Pharmacognosie, and the Gesellschaft fur Arzneipflanzenforschung, and the Phytochemical Society of Europe, Halifax, Nova Scotia, Canada. Contact: D. J. Slatkin, P. O. Box 13145, Pittsburgh, PA 15243, USA.

AUGUST 21 - 26: 208th ACS National Meeting, Washington, DC. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 5 - 7: 25th International Symposium on Essential Oils, Grasse, France. Contact: Assoc. des Ingenieurs et Techniques de la Parfumerie (A.I.T.P.), 48 ave. Riou-Blanquet, 06130 Grasse, France.

OCTOBER 16 - 19: 46th Southeastern regional Meeting, ACS, Birmingham, Alabama. Contact: L. Kispert, Chem Dept, Univ of Alabama, Box 870336, Tuscaloosa, AL 35115, USA.

NOVEMBER 2 - 5: 29th Midwestern Regional Meeting, ACS, Kansas City, Kansas. Contact: M. Wickham-St. Germain, Midwest Res. Inst, 425 Volker Blvd, Kansas City, MO 64110, USA.

NOVEMBER 13 - 16: 50th Southwest Regional Meeting, ACS, Fort Worth, Texas. Contact: H. C. Kelly, Texas Christian Univ, Chem Dept, Ft. Worth, TX 76129, USA.

DECEMBER 4 - 6: IBEX'94, International Biotechnology Expo & Scientific Conference, Moscone Center, San Francisco, California. Contact: Cartlidge & Associates, Inc., 1070 Sixth Avenue, Suite 307, Belmont, CA 94002, USA.

1995

MARCH 6 - 10: PittCon'95: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, Louisiana. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

APRIL 2 - 7: 209th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

MAY 31 - JUNE 2: 27th Central regional Meeting, ACS, Akron Section. Contact: J. Visintainer, Goodyear Research, D415A, 142 Goodyear Blvd, Akron, OH 44236, USA.

JUNE 15 - 17: 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.

AUGUST 20 - 25: 210th ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, 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 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 5 - 7: 30th Midwestern Regional Meeting, ACS, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr, Miami, OK 74354, 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 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.

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.

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

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.

1998

MARCH 29 - APRIL 3: 215th ACS National Meeting, St. Louis, Missouri. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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

1999

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.

2001

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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