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SEPARATION OF FULLERENES WITH NOVEL STATIONARY PHASES IN MICROCOLUMN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Newly designed bonded phases have been synthesized and evaluated for the separation of fullerene molecules. Further investigations about the retention behaviour of fullerenes with these phases have been carried out systematically.

In this study we describe the separation of fullerenes with novel bonded stationary phases in microcolumn high performance liquid chromatography (micro-HPLC) and also propose the basic separation mechanism of those molecules obtained from the preliminary chromatographic observations. The results indicate that the octadecyldiphenyl bonded silica phases, which have been synthesized from octadecyldiphenylchlorosilane as the silanization reagent, possess a better retentivity for fullerenes than the octadecyldimethylsilica phase (i.e. monomeric ODS phase) having a similar surface coverage value.

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INTRODUCTION

Many reports about the chromatographic separation of C_{60} and C_{70} fullerenes have been published [1-3] because the isolation or collection of a specific fullerene is one of the first series in characterization. More recently, the separation of so-called higher-fullerenes, such as C_{76}, C_{78}, C_{82} and C_{84} etc., has become an important subject for chromatographers [4-5]. The molecular features of C_{60} and C_{70} are dissolved enough to be distinguished easily, however, there are only slight size and shape differences among higher-fullerene molecules having similar or identical carbon atom numbers in their chemical structures. Therefore, the liquid chromatographic separation of fullerene molecules with molecular size and shape recognition capabilities of bonded phases can be considered as the most powerful separation techniques for these purposes, and it is important to investigate the separation mechanism of fullerenes based on the analysis of their retention behaviors with various bonded phases in HPLC.

To investigate the retention behaviour of fullerenes with chemically bonded silica phases, we have introduced various stationary phases such as, commercially available ODS phases [5-8], multi-legged phases [9], multi-methoxy phases [6,10] and multi-phenyl phases [11], in the last few years. This is due to the fact that fullerene molecules can be seen as the very large polycyclic aromatic hydrocarbons (PAHs) and, therefore, each fullerene molecule is regarded as a good sample to study the retention mechanism in HPLC. On the other hand, if the chromatographic separation mechanism of fullerenes with the stationary phases is elucidated, a newly designed stationary phase which can effectively separate them will be developed by using the concept based on that mechanism.

In this study we introduced three alkyldiphenyl bonded silica phases having both alkyl-chains and two phenyl-rings in their chemical structures, and studied the

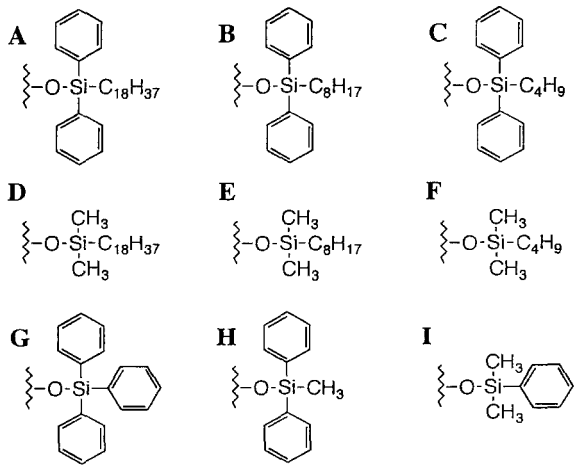

FIGURE 1 Structures of the chemically bonded phases used in this study. (A)C18Diph, (B)C8Diph, (C)C4Diph, (D)C18, (E)C8, (F)C4, (G)Triph, (H)Diph and (I)Monoph.

chromatographic retention behaviors of C_{60} and C_{70} fullerenes with these stationary phases in HPLC. Systematic analysis of the retention behaviors was also carried out by comparison of the results with alkyldiphenyl phases to those of corresponding alkyldimethyl phases and some phenyl-derivatized bonded phases described earlier [11].

EXPERIMENTAL

Bonded Phase Synthesis

Chemical structures of bonded phases investigated were shown in Figure 1. These bonded phases were synthesized in a similar manner as described previously [10,12], and the basic characteristics of these phases are summarized in Table 1.

Table 1 The characteristics of bonded phases.

	bonded phase	pore size (Å)	carbon content (%)	surface coverage (μmol/m^2)	ligand interval (Å)
A	C_{18}Diph	120	8.48	1.54	10.4
B	C_8Diph	120	5.89	1.56	10.3
C	C_4Diph	120	4.15	1.35	11.1
D	C_{18}	120	9.08	2.52	8.1
E	C_8	120	5.08	2.68	7.9
F	C_4	120	1.73	1.50	10.5
D	C_{18} (Develosil ODS-5)*	100	20	3.31	7.1
D	C_{18} (Develosil ODS-N-5)*	100	16	2.47	8.2
D	C_{18} (Develosil ODS-P-5)*	100	11	1.57	10.3
G	Triph 70 Å	70	11.2	1.14	12.1
H	Diph 70 Å	70	13.7	2.20	8.7
I	Monoph 70 Å	70	10.8	3.40	7.0
G	Triph 150 Å	150	4.79	1.19	11.8
H	Diph 150 Å	150	6.11	2.16	8.8
I	Monoph 150 Å	150	5.35	3.81	6.6

*commercially available ODS phases (Nomura Chemical, Seto, Japan).

Three commercially available ODS phases, Develosil ODS-5, Develosil ODS-N-5 and Develosil ODS-P-5, were also used for comparison. These phases were obtained from Nomura Chemicals (Seto, Japan) as gifts.

Chromatographic Measurements.

The bonded phases were packed using a slurry method into a fused-silica capillary (Tokyo Chemical Industries, Tokyo, Japan) of 0.53 mmi.d. x 200 mm length. The microcolumn HPLC system consisted of a microfeeder MF-2 pump (Azuma Electric, Tokyo, Japan), a Rheodyne 7520 injector (Cotati, CA, USA) with a 0.2 μL injection volume and a Uvidec 100-III UV detector (Jasco, Tokyo, Japan) set at 320 nm. The mobile phase was guaranteed reagent grade n-hexane (Kishida Chemical, Osaka, Japan). The typical flow-rate was 4 μL/min., and the mixture of toluene and cyclohexane was used as the sample solvent. The chromatographic measurements were done at least three times. The column temperature was controlled using a modified Hewlett-Packard Model 5820-II Gas Chromatographic oven (Yokogawa Analytical Systems, Musashino, Tokyo, Japan).

RESULTS and DISCUSSION

Figure 2 shows the chromatograms for the separation of C_{60} and C_{70} fullerenes with three alkyldiphenyl bonded silica phases under the same conditions. The retentivity of the C_{18}Diph bonded phase is larger than those of the C_8Diph and C_4Diph phases. This suggests the contribution of the long alkyl chains in the bonded phase to the fullerenes retention, because of almost comparable surface coverage values of these three phases shown in Table 1. In order to make this behaviour clear, the separation of C_{60} and C_{70} was carried out with various bonded phases shown in Figure 1. These retention data have also been summarized in Table 2. Develosil ODS-5 has the best separation performance

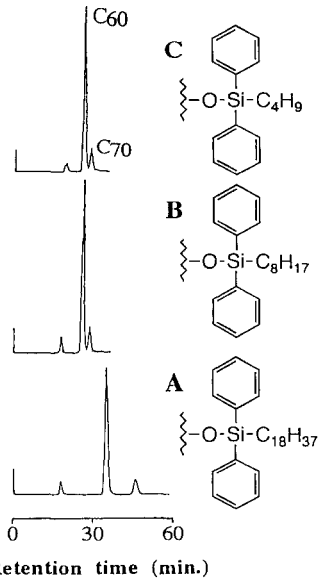

FIGURE 2 Chromatograms for the separation of C60 and C70 with three alkyldiphenyl bonded phases. (A)C18Diph, (B)C8Diph and (C)C4Diph; mobile phase, n-hexane; column temperature, 30 °C.

among various ODS phases investigated previously [7], similarly, the C_{18}Diph phase gives the best separation for fullerenes as can be seen from these data.

By comparison of the retention data with C_{18}Diph and those with Develosil ODS-P-5, it can be said that two phenyl-rings in the bonded phase structure contribute significantly to the retention value of fullerene molecules. Similar trends are also observed by comparing the data of C_4Diph phase with those of C_4 phase. In spite of the lower surface coverage value of C_{18}Diph phase to C_{18} phase, the C_{18}Diph phase possesses the stronger retentivity toward fullerene molecules than the C_{18} phase. The results also indicate the contribution of the phenyl-rings to the retention of fullerenes and these results have a good agreement with the data

Table 2 Retention data for the separation of C_{60} and C_{70} with various bonded phases. Mobile phase, n-hexane; column temperature, 30 ℃.

	bonded phase	capacity factor (k')		separation factor
		C_{60}	C_{70}	α (k' C_{70}/k' C_{60})
A	C_{18}Diph	1.02	1.67	1.64
B	C_8Diph	0.42	0.57	1.36
C	C_4Diph	0.41	0.56	1.37
D	C_{18}	0.52	0.84	1.63
E	C_8	0.086	0.124	1.44
F	C_4	0.061	0.087	1.43
D	C_{18} (Develosil ODS-5)*	0.87	1.60	1.84
D	C_{18} (Develosil ODS-N-5)*	0.81	1.32	1.64
D	C_{18} (Develosil ODS-P-5)*	0.44	0.64	1.45
G	Triph 70 Å	2.28	3.59	1.58
H	Diph 70 Å	1.85	2.89	1.56
I	Monoph 70 Å	0.86	1.24	1.44
G	Triph 150 Å	0.45	0.63	1.39
H	Diph 150 Å	0.45	0.64	1.41
I	Monoph 150 Å	0.27	0.37	1.36

*commercially available ODS phases (Nomura Chemical, Seto, Japan).

obtained with Triph, Diph and Monoph phases [11], where a smaller retentivity of Monoph phases than Triph and Diph phases having two phenyl-rings in their structures has been observed for the separation of fullerenes.

It can also be said from the comparison of the separation factors with C18 (120 Å pore size, 9.08 C%) and those with Develosil ODS-N-5 (100 Å pore size,

16 C%), where both phases have very similar surface coverage values, that the capacity factors for the fullerenes with these two bonded phases are different according to the difference of the silica gel properties.

On the other hand, the effect of alkyl chain length can be seen among C_{18}Diph, C_8Diph and C_4Diph, and corresponding alkyldimethyl phases. With the C_{18}Diph phase, larger capacity factors for fullerenes and better separation factors for fullerenes were found in comparison to those observed with C_8Diph and C_4Diph phases. This observation indicates that the length of alkyl chains in the bonded phases contribute to the retention of fullerenes, and that C_8 and C_4 chains are too short to interact effectively with fullerene molecules. These results have a good agreement with the trends seen in our previous investigation [7].

Table 3 shows the retention data for the separation of C_{60} and C_{70} with the C_{18}Diph phase at various column temperatures, and the corresponding van't Hoff plots are shown in Figure 3. For both C_{60} and C_{70}, linear van't Hoff plots are obtained over the temperature range investigated (from 0 °C to 80 °C), however, the separation factor between these fullerenes decreases more slowly than Develosil ODS-P-5 when the column temperatures are elevated. As the Develosil ODS-P-5 phase possesses an almost identical surface coverage value as the C_{18}Diph phase. This phenomenon can be explained by the uniformity of the ligand interval (about 10.4 Å). That is to say, C_{18}Diph phase can show better uniformity of the ligand interval than that of the ODS-P-5 in order to interact effectively with fullerenes.

Although further consideration about the retention mechanism of fullerenes with chemically bonded stationary phases is needed, it can be concluded from the above systematic evaluations that:

1) two phenyl-rings at the bottom part of the bonded phase contribute to effective interaction between the bonded phase ligands and fullerene solutes;

Table 3 Retention data for the separation of C_{60} and C_{70} with C_{18}Diph at various column temperatures. Mobile phase, n-hexane.

column tmperature (℃)	capacity factor (k')		separation factor α (k' C_{70}/k' C_{60})	
	C_{60}	C_{70}		
0	1.24	2.13	1.72	[1.54]*
20	1.09	1.81	1.66	[1.44]*
30	1.02	1.67	1.64	
40	0.971	1.58	1.63	[1.42]*
50	0.916	1.48	1.62	
60	0.852	1.38	1.62	[1.38]*
70	0.827	1.32	1.60	
80	0.767	1.23	1.60	

*the data with Develosil ODS-P-5 in the previous study [8].

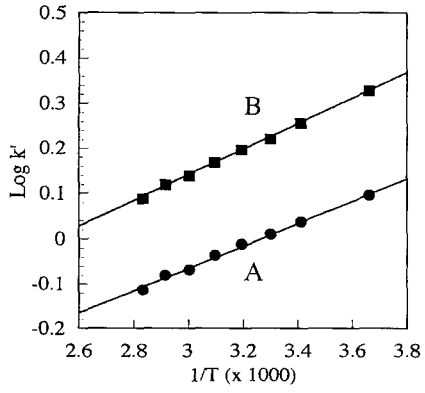

FIGURE 3 van't Hoff plots for C60 and C70 fullerenes with the C18Diph phase. (A)C60 and (B)C70; mobile phase, n-hexane.

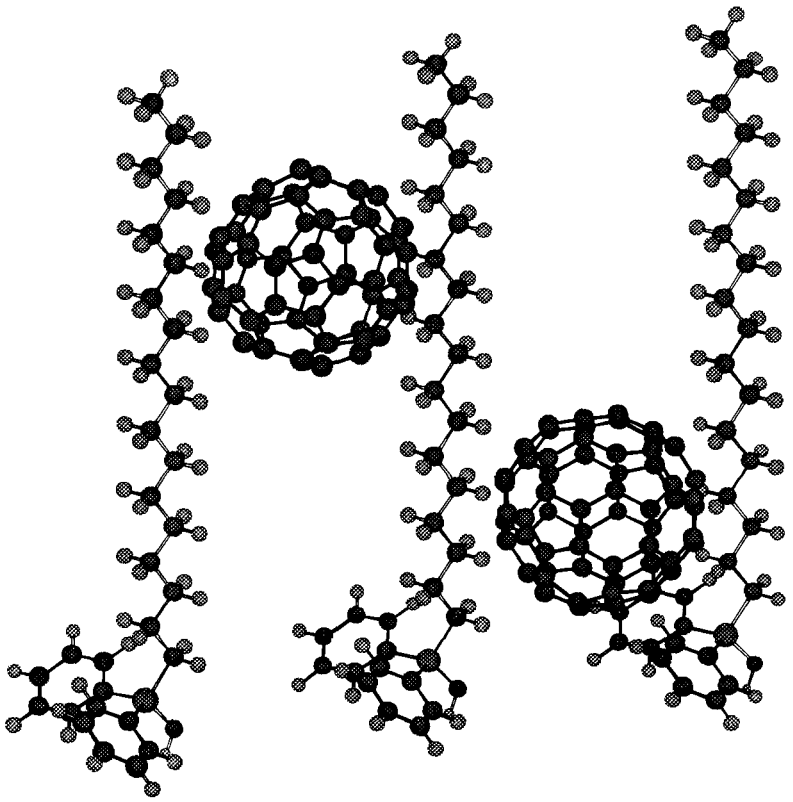

FIGURE 4 Schematic diagram of the interaction between C60 and the C18Diph bonded phase. The model was depicted by Chem3D Plus software (Cambridge Scientific Computing Inc., MA, USA). For convenience by the software, the bonded phase was drawn with hydroxyl group (-OH) as the residual functional substituent bonded to the silicon atom in the silanization reagent.

2) the phenyl rings bonded to the silicon atom induce the uniformity of the interval of alkyl chains, and therefore, the good retentivity can be obtained (the interaction model between C_{60} and C_{18}Diph phase based on this concept is shown in Figure 4), because the phenyl rings work as the spacer of bonded phase ligands on the silica support;

3) there is a possibility that a critical chain length exists to interact effectively with fullerenes, though it seems longer is better.

Some additional studies are currently undergoing in our laboratory in order to elucidate the separation mechanism of fullerenes using other novel stationary phases such as liquid-crystal bonded phases [13,14].

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THE LIQUID CHROMATOGRAPHIC SEPARATION OF METABOLITES OF BENZO[a]PYRENE WITH γ-CYCLODEXTRIN AS A MOBILE PHASE ADDITIVE

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ABSTRACT

The effects of γ-cyclodextrin as a mobile phase additive on the retention characteristics of fourteen metabolites of benzo[a]pyrene were investigated with reversed-phase liquid chromatography. It was possible to separate dihydrodiols, diones, and monohydroxyl-benzo[a]pyrenes as individual classes of metabolites. Also, with γ-cyclodextrin in the mobile phase, monohydroxyl-benzo[a]pyrene isomers that were very difficult to separate were readily separated. For example, 6-hydroxyl-benzo- [a]pyrene was separated from 12-hydroxyl-benzo[a]pyrene and 9-hydroxyl-benzo[a]pyrene was separated from 2-hydroxyl-benzo- [a]pyrene. The four tetrols were readily separated with shorter retention times using 4.0 mM of γ-cyclodextrin in a methanol-water (55:45) mobile phase. Overall, the results showed that γ-cyclodextrin was more effective in separating the metabolites than was β-cyclodextrin.

INTRODUCTION

Benzo[a]pyrene (B[a]P) is a common environmental pollutant, and it is considered to be a carcinogen (1-4). The microsomal enzyme complex, aryl

hydrocarbon hydroxylase (AHH), and metabolically related enzymes convert B[a]P to epoxides, phenols, dihydrodiols, diones, and water-soluble conjugates (1-3). It has been shown that reversed-phase high-performance liquid chromatography (HPLC) is a sensitive and rapid technique for the isolation and separation of B[a]P metabolites (4-14). As an example, Selkirk et al. (7) analyzed the metabolites of benzo[a]pyrene from human liver microsomes and human lymphocytes using HPLC. In 1979, Tjessum and Stegeman (15) used an alkaline gradient system to improve the separation of monohydroxyl-B[a]P isomers using a highly alkaline eluent consisting of a series of alkylamines in a linear gradient mode. This procedure, however, did not separate other B[a]P metabolites such as, tetrols, diones, and dihydrodiols. Also, the high pH may have damaged the column. Elnenaey and Schoor (6) developed a method to separate twelve isomeric monohydroxyl-B[a]P by using HPLC with fluorescence detection and various sequences of methanol-water gradients. They showed that with this approach many isomers had almost identical retention times (6). Recently, Rozbeh and Hurtubise (5) reported a systematic method for optimizing binary and ternary mobile phases for the separation of a complex mixture of fourteen metabolites of benzo[a]pyrene using HPLC.

The use of cyclodextrins in chromatography is important, and it has been discussed in several books and reviews (16-18) Cyclodextrins (CD) are tours-shaped molecules with α-1,4 linkage of glucopyranose units. The interior of the cyclodextrin cavity is fairly nonpolar due to the glucosidic oxygen and hydrogen atoms in the cavity (18). The most frequently studied cyclodextrins are α-, β-,

and γ-CD, which have different cavity diameters (18). Cyclodextrins molecules can form complexes with several types of compounds. For example, Zukowski et al. (19) investigated the use of mobile phases containing α-CD or β-CD for the separation of disubstituted benzene derivatives with a C_{18} column. Their results indicated that only β-CD imposes a distinct selectivity toward ortho, meta, and para isomers with reversed-phase systems, which resulted in a complete separation of the isomers (19). Also, the effects of different concentrations of β-CD on the retention and selectivity of various aromatic isomers have been investigated (20).

Frequently, γ-CD is used in chromatography as a complexing reagent for large molecules. For example, γ-CD chemically bonded to silica gel is a highly selective stationary phase for the HPLC separation of C_{60} and C_{70} fullerenes (21). Hurtubise and co-worker (22) showed that polycyclic aromatic hydrocarbons, nitrogen heterocycles, and hydroxyl aromatics of different sizes will interact with β-CD to different extents, and this permits the separation of some of these compounds from their isomers. In a previous paper (23), we described the chromatographic separation of fourteen metabolites of benzo[a]pyrene in reversed-phase HPLC with β-CD as a mobile phase additive. Woodberry et al. (24) demonstrated that a bonded γ-CD column with methanol-water mobile phase is capable of separating benzo[a]pyrene-trans-7,8-dihydrodiol from benzo[a]pyrene-trans-9,10-dihydrodiol with better selectivity than β-CD bonded phases. γ-Cyclodextrin has not been used as extensively as a mobile phase modifier because of its expense. However, Roussel and Favrou (25-26) described the chromatographic separation of eight enantiomers using β- or γ-CD as a mobile

phase modifier. They concluded that γ-CD shows better selectivity toward the enantiomers, while weaker complexes were formed with β-CD.

The aim of this paper is to demonstrate the practical significance of γ-CD as a mobile phase additive in the chromatographic separation of a complex mixture of fourteen metabolites of benzo[a]pyrene. Also, the work reported here considers the chromatographic behavior of different pairs of metabolites in methanol-water mobile phases modified by γ-CD. Also, comparisons were made between γ-CD versus β-CD for the retention characteristics of pairs of metabolites, and a complex mixture of fourteen metabolites. Moreover, the effects of the cavity size of β-CD versus γ-CD on the selective separation of some of the metabolites were emphasized.

EXPERIMENTAL

Apparatus

HPLC was performed with a Waters liquid chromatograph equipped with model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a U6K injector, a dual channel free-standing model 440 UV detector set at 254 nm, and a linear 1200 dual channel, 5.0 V recorder (Linear Instruments Co. Concord, CA). A model FIAtron heating block (Oconomowoc, WI, U.S.A.) constant temperature control system was used to keep the temperature of the column at 25 ± 0.1°C. Separations were carried out with a 5-μm Baker-bond C_{18} (250 mm x 4.6 mm i.d.) from J.T.Baker (Pillipsburg, NJ, U.S.A.).

Reagents

Methanol (MeOH) and water were HPLC grade and were purchased from J.T.Baker Inc. (Pillipsburg, NJ). The γ-CD samples were obtained from American Maize-Products Company (Amaizo, Hammond, IN). The benzo[a]pyrene (B[a]P) metabolites were purchased from the National Cancer Institute (NCI) repository at Midwest Research Institute (MRI, Kansas City, MO).

Procedures

The mobile phases were prepared as follows. Methanol and water were prefiltered through a membrane 0.45 μm filter (Millipore type HA). An accurate amount of γ-CD, which was vacuum dried at 75° C for 8 hr, was dissolved in an appropriate amount of water. Then, a specific quantity of methanol was added to the γ-CD solution. After complete dissolution of the γ-CD at room temperature, an amount of MeOH:H_2O, which was the same composition used to dissolve the γ-CD, was added to the volumetric flask to bring the volume to 1000 ml. Solutions of 0.1 mg/ml for individual metabolites and 0.01 mg/ml for the mixture of fourteen standards were prepared in methanol. To assure stability, the solutions were stored under nitrogen gas at -15°C and in the dark. The amount of the sample injected into the liquid chromatograph was 3-4 μl for individual metabolites and 10 μl for the mixture of fourteen compounds. All chromatograms were obtained at 25°C. The flow rate was 1.0 ml/min. The void volume was determined by injection of a methanol solution of potassium nitrite. The capacity factors were calculated from the expression, $k' = (t_R - t_o)/t_o$, where t_R is the

retention time of the solute and t_o is the retention time of potassium nitrite. Triplicate injections for the metabolites of benzo[a]pyrene were carried out using a mobile phase without and with γ-CD. The values of k′ in Table 1 and Table 2 are the averages from these injections.

RESULTS AND DISCUSSION

Mobile Phases and γ-CD Concentrations

A preliminary investigation with nine different concentrations of methanol-water indicated that by decreasing the methanol content of the mobile phase the k′ values increased for each individual metabolites. Therefore, lower concentrations of methanol resulted in larger elution times for the metabolites, which is undesirable. Thus, two different compositions of binary mobile phases were employed in this investigation to determine the effects of γ-CD on the retention characteristics of the fourteen metabolites. The methanol-water ($MeOH:H_2O$, V:V) mobile phases used were 75:25 and 80:20 with different concentrations of γ-CD. The maximum analytical concentration of γ-CD in $MeOH:H_2O$ (75:25) was 3.5 mM and for the 80:20 composition it was 2.5 mM.

The Effects of Mobile Phase Additives on the Retention Characteristics of the Metabolites

It is known that the hydrophobic interactions and dispersion forces operating between the bonded alkyl moiety of the stationary phase and the nonpolar part a molecule play an important role in determining the retention

characteristics of the sample in HPLC (27). Since hydrophobic interactions are affected by several factors such as the chain length and the amount of bonded alkyl moieties in the stationary phase, as well as the type and content of the organic modifier in the mobile phase, the addition of cyclodextrin to the mobile phase is expected to cause changes in the capacity factors of the solutes. It is also known that both the molecular size and geometry of benzo[a]pyrene are such that benzo[a]pyrene will not fit entirely into the cavity of a single β-CD molecule (24,28,29). Also, Patony and Warner (29) showed that a benzo[a]pyrene complex exhibits a significant induced ellipticity in the presence of γ-CD, while α-CD or β-CD did not produce a major induced circular dichroism signal. β-CD is comprised of seven glucose units as opposed to the eight glucose units of γ-CD (16-18). Consequently, the interior cavity of γ-CD is larger than the cavity of β-CD (18). Also, two factors that are important for inclusion of a solute into a cyclodextrin cavity are the size and geometry of the compound. γ-CD can accommodate larger molecules than β-CD, and this would lead to size selectivity with the two cyclodextrins. An earlier investigation from this laboratory showed that twelve metabolites from a complex mixture of fourteen compounds can be separated by an optimum binary mobile phase of acetonitrile-water (ACN:H_2O) (65:35) and an optimum ternary mobile phase containing acetonitrile-methanol-water (ACN:MeOH:H_2O) (17:50:33) (5). However, with methanol-water (81.75:18.25) two pairs of isomers, namely, 2-OH-B[a]P and 9-OH-B[a]P, and tetrol I-2 and tetrol II-2 could not be resolved into their individual pairs. With other MeOH:H_2O binary mobile phase compositions, two pairs of monohydroxyl-

TABLE 1

The k'-Values of the Metabolites of Benzo[a]pyrene for Methanol-Water (80:20) with Different Concentrations (mM) of γ-CD

		γ-CD (mM)				
No.	Compound[a]	0.0	1.0	1.5	2.0	2.5
1	Tetrol I-1	0.55	0.49	0.44	0.41	0.38
2	Tetrol II-1	0.65	0.53	0.49	0.48	0.45
3	Tetrol I-2	0.66	0.62	0.55	0.52	0.50
4	Tetrol II-2	0.74	0.76	0.71	0.67	0.62
5	B[a]P-t-9,10-dihydrodiol	0.66	0.62	0.59	0.57	0.51
6	B[a]P-t-7,8-dihydrodiol	2.31	2.17	2.15	2.05	1.95
7	B[a]P-1,6-dione	7.6	7.15	6.45	6.02	5.66
8	B[a]P-3,6-dione	8.95	8.4	7.62	7.2	6.74
9	12-OH-B[a]P	9.96	8.6	8.05	7.45	6.89
10	9-OH-B[a]P	12.7	10.4	9.96	9.46	9.05
11	2-OH-B[a]P	12.7	11.7	11.4	10.5	9.95
12	7-OH-B[a]P	14.5	13.2	12.6	11.8	10.8
13	3-OH-B[a]P	18.8	15.0	14.4	12.7	11.0
14	6-OH-B[a]P	10.8	9.26	8.72	8.31	7.36

[a] 1. Benzo[a]pyrene-r-7,t-8,9,c-10-tetrahydrotetrol (I-1)
2. Benzo[a]pyrene-r-7,t-8,c-9,t-10-tetrahydrotetrol (II-1)
3. Benzo[a]pyrene-r-7,t-8,9,10-tetrahydrotetrol (I-2)
4. Benzo[a]pyrene-r-7,t-8,c-9,10-tetrahydrotetrol (II-2)
5. Benzo[a]pyrene-trans-9,10-dihydrodiol
6. Benzo[a]pyrene-trans-7,8-dihydrodiol
7. Benzo[a]pyrene-1,6-dione
8. Benzo[a]pyrene-3,6-dione
9. 12-Hydroxybenzo[a]pyrene
10. 9-Hydroxybenzo[a]pyrene
11. 2-Hydroxybenzo[a]pyrene
12. 7-Hydroxybenzo[a]pyrene
13. 3-Hydroxybenzo[a]pyrene
14. 6-Hydroxybenzo[a]pyrene

The structures of the compounds are given in Ref. 5.

B[a]P isomers, 12-OH-B[a]P and 6-OH-B[a]P, and 2-OH-B[a]P and 9-OH-B[a]P were the most difficult metabolites to separate (5).

In a follow-up paper, Rozbeh and Hurtubise (23) employed several concentrations of β-CD as a mobile phase additive to improve the separation of the two most difficult to separate pairs of isomers. Substantial improvements were obtained for the separation of a mixture of fourteen metabolites with MeOH:H_2O (65:35), ACN:H_2O (65:35), and ACN:MeOH:H_2O (50:17:33) with β-CD. The isomers, 6-OH-B[a]P and 12-OH-B[a]P, were separated with a relatively high concentration of β-CD in the methanol-water mobile phase (23). However, one pair of metabolites, 2-OH-B[a]P and 9-OH-B[a]P, could not be resolved with β-CD in methanol-water(23). Also, large capacity factors for monohydroxyl-B[a]P and low solubility of β-CD, at lower concentrations of methanol were a problem (23). Therefore, in this work, the effects of different concentrations of γ-CD in the mobile phase on the retention of the metabolites were examined.

Table 1 shows the effects of different concentrations of γ-CD on the capacity factors of fourteen metabolites of benzo[a]pyrene with MeOH:H_2O (80:20). The k′ values of the metabolites for MeOH:H_2O (75:25) with various concentrations of γ-CD are shown in Table 2. Comparison of the data from the separation of benzo[a]pyrene metabolites with β-CD in MeOH:H_2O mobile phases (23) with results from this investigation indicated that the capacity factors of all fourteen metabolites decreased to a greater extent in the presence of γ-CD compared to β-CD. For example, the k′ value of 3-OH-B[a]P changed from 18.8 with no γ-CD in the mobile phase to 11.0 with 2.5 mM γ-CD in MeOH:H_2O

TABLE 2

The k'-Values of the Metabolites of Benzo[a]pyrene for Methanol-Water (75:25) with Different Concentrations (mM) of γ-CD

	γ-CD (mM)					
Solute[a]	0.0	1.0	1.5	2.0	3.0	3.5
Tetrol I-1	0.61	0.58	0.56	0.49	0.43	0.40
Tetrol II-1	0.80	0.66	0.62	0.59	0.58	0.56
Tetrol I-2	0.84	0.73	0.69	0.61	0.60	0.57
Tetrol II-2	1.08	0.94	0.92	0.83	0.77	0.74
B[a]P-t-9,10-dihydrodiol	0.95	0.85	0.75	0.74	0.68	0.58
B[a]P-t-7,8-dihydrodiol	3.94	3.68	3.22	3.06	2.80	2.5
B[a]P-1,6-dione	9.1	8.9	8.6	8.12	7.62	7.3
B[a]P-3,6-dione	11.0	10.7	10.2	9.64	9.5	8.57
12-OH-B[a]P	11.9	10.7	10.2	9.9	9.03	8.90
9-OH-B[a]P	14.7	13.4	13.0	11.9	11.3	10.7
2-OH-B[a]P	15.1	14.7	14.1	13.5	12.9	12.3
7-OH-B[a]P	20.9	19.0	17.9	16.6	15.7	14.8
3-OH-B[a]P	23.2	21.2	20.3	19.6	19.1	18.0
6-OH-B[a]P	11.9	11.4	11.1	11.0	9.74	9.50

[a] See the footnote of Table 1 for the full names of the compounds.
The structures of the compounds are given in Ref. 5.

(80:20) (Table 1). In contrast, the k′ value of 3-OH-B[a]P decreased from 24.0 with no β-CD to only 22.0 with 3.5 mM β-CD in the MeOH:H_2O (70:30) (23). These results clearly indicate that the larger decrease in the k' values of the metabolites caused by the addition of γ-CD in the mobile phase, resulted from the weakening of the hydrophobic interaction between the solutes and stationary phase.

Also, the ability of γ-CD to form a stronger complex with a given metabolite compared to β-CD was apparent. In addition, the higher solubility of γ-CD compare to β-CD permitted complex formation to occur more readily compared to β-CD (17-18). It is logical that β-CD exhibited a smaller effect on the retention times of the metabolites because of its cavity size (~7.8 Å). This would permit only partial inclusion complex formation with the nonpolar part of the B[a]P molecule (30). In contrast, the larger cavity of the γ-CD permits greater interaction with the B[a]P isomers.

Separation of Structural Isomers

The separation of isomers in HPLC is of great importance. In this work, separate mixtures of four tetrols, two dihydrodiols, two diones, 12-OH-B[a]P and 6-OH-B[a]P, 9-OH-B[a]P and 2-OH-B[a]P, and 7-OH-B[a]P and 3-OH-B[a]P were prepared. In previous work, a mixture of four tetrols could be separated with MeOH:H_2O (55:45) with 4.0-5.0 mM of β-CD (23). In this work, a similar investigation for the tetrols with different concentrations of γ-CD in the mobile phase was undertaken. A larger reduction in the k′ values of the four tetrols was obtained with γ-CD compare to β-CD (23). The decrease in the retention times of tetrols with 4.0 mM of γ-CD in MeOH:H_2O (55:45) resulted in the sharpening of the bands and a reasonably good separation of the four stereoisomers. Therefore, it was concluded that MeOH:H_2O (55:45) with 4.0 mM γ-CD was a better mobile phase for the separation of this class of metabolites for the β-CD and γ-CD mobile phases investigated.

TABLE 3

Selectivity Factors (α) for Some Pairs of Benzo[a]pyrene Metabolites in MeOH:H_2O (70:30) Without and With 3.0 mM β-CD

	α-Values[b]	
Pairs of Metabolites[a]	0.0	3.0
B[a]P-t-9,10-dihydrodiol and B[a]P-t-7,8-dihydrodiol	4.38	4.73
B[a]P-1,6-dione and B[a]P-3,6-dione	1.21	1.12
12-OH-B[a]P and 6-OH-B[a]P	1.00	1.05
9-OH-B[a]P and 2-OH-B[a]P	1.01	1.02
7-OH-B[a]P and 3-OH-B[a]P	1.05	1.15

[a] See the footnote of Table 1 for the full names of the metabolites.

[b] α-values were calculated from the equation $\alpha = k_2'/k_1'$.

TABLE 4

Selectivity Factors (α) for Some Pairs of Benzo[a]pyrene Metabolites in MeOH:H_2O (75:25) Without and With 3.0 mM γ-CD

	α-Values[b]	
Pairs of Metabolites[a]	0.0	3.0
B[a]P-t-9,10-dihydrodiol and B[a]P-t-7,8-dihydrodiol	4.15	4.11
B[a]P-1,6-dione and B[a]P-3,6-dione	1.20	1.25
12-OH-B[a]P and 6-OH-B[a]P	1.00	1.08
9-OH-B[a]P and 2-OH-B[a]P	1.02	1.14
7-OH-B[a]P and 3-OH-B[a]P	1.11	1.22

[a] See the footnote of Table 1 for the full names of the metabolites.

[b] α-values were calculated from the equation $\alpha = k_2'/k_1'$.

The data in Tables 3 and 4 compare the selectivity factors (α) for several pairs of metabolites obtained with MeOH:H_2O (70:30) containing 3.0 mM β-CD and MeOH:H_2O (75:25) with 3.0 mM γ-CD. These mobile phases were compared because with MeOH:H_2O (70:30) 3.0 mM of β-CD can be dissolved, which is the same as the concentration of γ-CD in MeOH:H_2O (75:25) (23). With larger concentrations of methanol, the solubility of β-CD decreases (18). For example, with MeOH:H_2O (75:25), only 2.5 mM of β-CD completely dissolved in the mobile phase. The capacity factors were reduced to a larger degree, and the selectivity increased for the two diones with γ-CD in the mobile phase compare to β-CD (compare Table 3 and 4). For example, the α-values for the two diones increased from 1.12 with 3.0 mM of β-CD to 1.25 with 3.0 mM γ-CD in methanol-water mobile phases (Table 3 and Table 4). The two diones can be separated with γ-CD or β-CD in the methanol-water mobile phases, but with γ-CD shorter retention times are obtained. It is shown, that in all but one case, the α-values for all pairs of metabolites increased with γ-CD in methanol-water mobile phases.

A substantial decrease in the capacity factors of monohydroxyl-B[a]P metabolites was obtained with γ-CD versus β-CD in methanol-water mobile phases (Table 1 and Table 2, see reference 23). A mixture of 6-OH-B[a]P and 12-OH-B[a]P showed only one peak with MeOH:H_2O (75:25) (Table 2) (Figure 1). However, the two components were resolved with γ-CD in MeOH:H_2O (Figure 2). For example, the α-value of 12-OH-B[a]P and 6-OH-B[a]P increased from 1.0 without γ-CD to 1.08 with 3.0 mM γ-CD in MeOH:H_2O (75:25) (Table 4). The

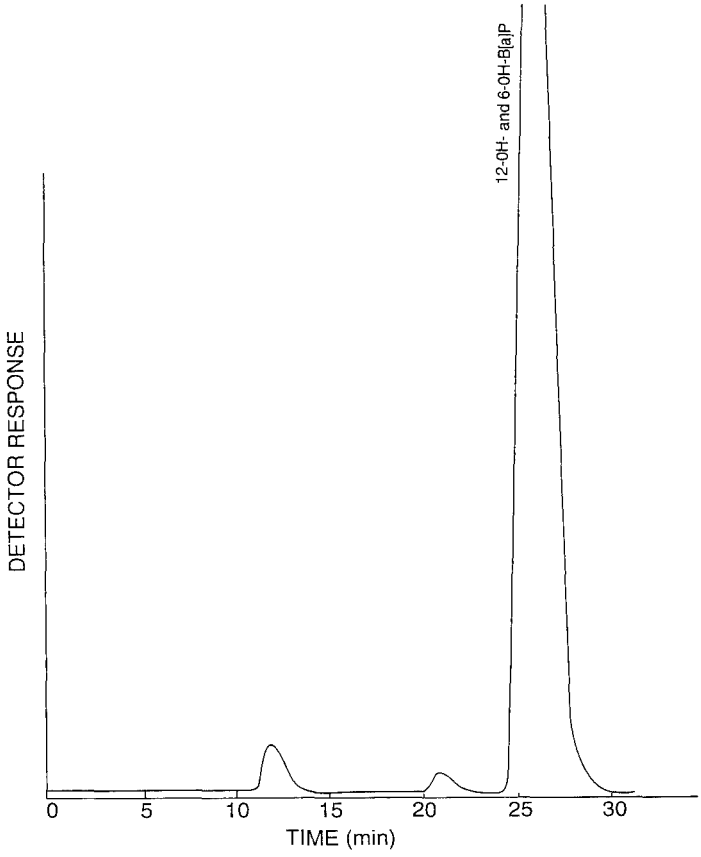

FIGURE 1. Chromatogram of 6-OH-B[a]P and 12-OH-B[a]P obtained with MeOH:H_2O (75:25). The full names of the compounds are given in the footnote of Table 1. The smaller peaks in the chromatogram are due to impurities.

addition of γ-CD significantly reduced the k′ values of these two metabolites and improved the efficiency of separation compared to the mobile phase without cyclodextrin (Table 2). It was reported earlier that a relatively high concentration of β-CD was also capable of separating these two metabolites. For example, the

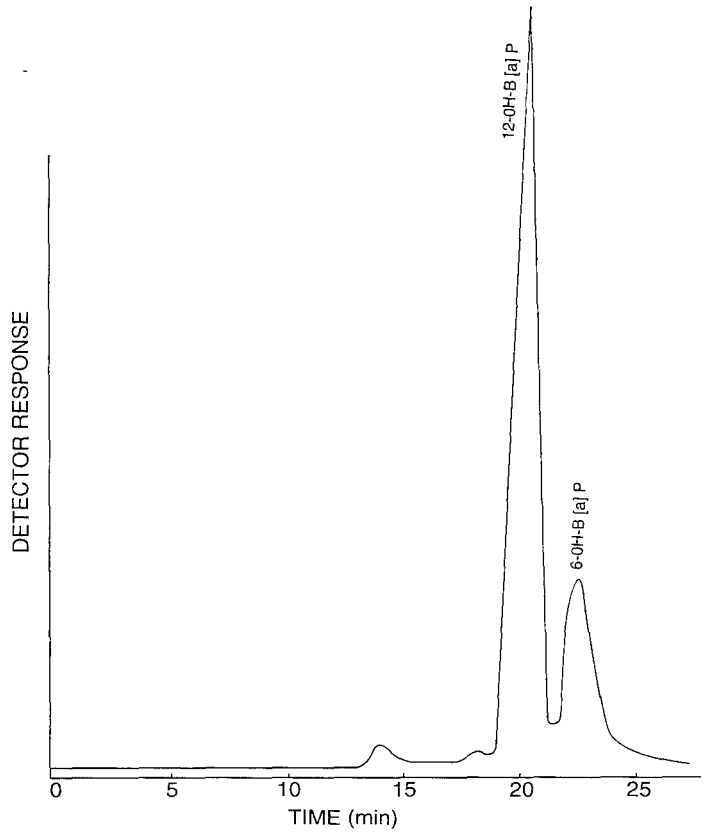

FIGURE 2. Chromatogram of 6-OH-B[a]P and 12-OH-B[a]P obtained in MeOH:H_2O (75:25) with 3.5 mM γ-CD. The full names of the compounds are given in the footnote of Table 1. The smaller peaks in the chromatogram are due to impurities.

capacity factors of 12-OH-B[a]P and 6-OH-B[a]P with 3.5 mM of β-CD in MeOH:H_2O (70:30) were 16.4 and 17.2, respectively (23). However, as Table 2 shows, the capacity factors are much smaller with 3.5 mM γ-CD, which illustrates one of the advantages of using γ-CD for these compounds.

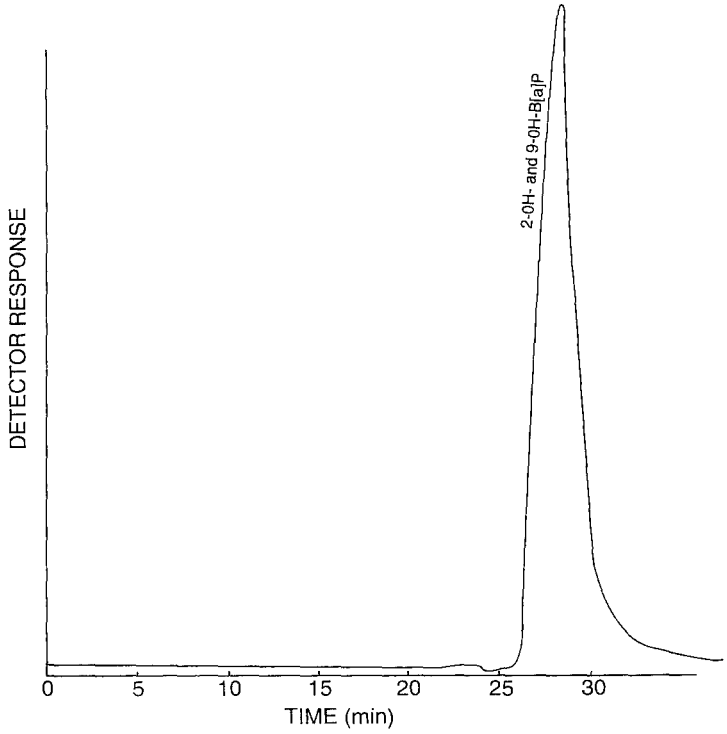

FIGURE 3. Chromatogram of 2-OH-B[a]P and 9-OH-B[a]P obtained with MeOH:H_2O (80:20). The full names of the compounds are given in the footnote of Table 1.

Figures 3 and 4 shows the chromatograms for 2-OH-B[a]P from 9-OH-B[a]P in methanol-water (80:20) with and without γ-CD. In the absence of γ-CD, the chromatographic bands of the two metabolites overlapped severely and no separation was achieved (Figure 3). With β-CD in the mobile phase, no separation was acquired with methanol-water for these two isomers (23). In contrast, with γ-CD in the mobile phase, the two metabolites were easily separated and baseline

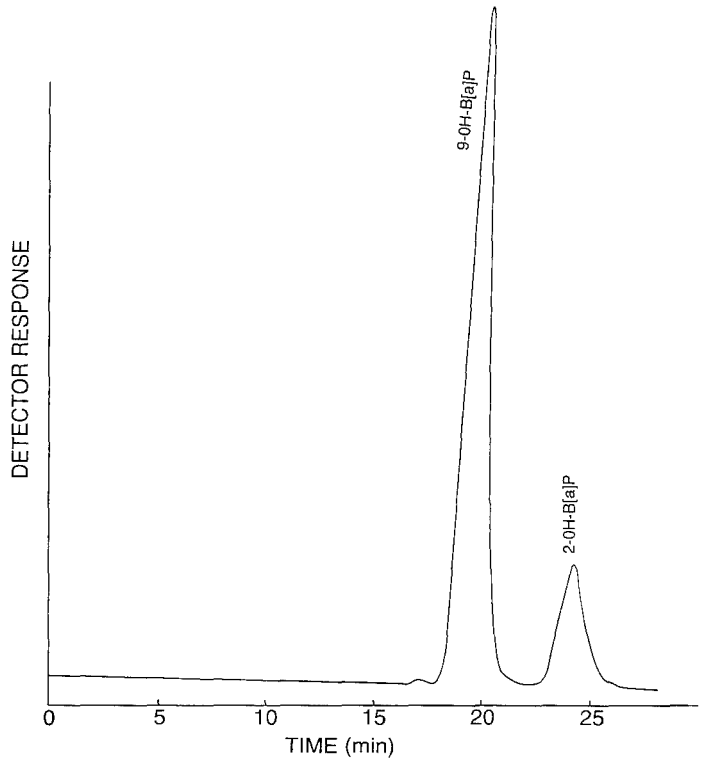

FIGURE 4. Chromatogram of 2-OH-B[a]P and 9-OH-B[a]P obtained in MeOH:H_2O (80:20) with 1.7 mM γ-CD. The full names of the compounds are given in the footnote of Table 1.

resolution was obtained (Figure 4, Table 1). Similar results were obtained in MeOH:H_2O (75:25) with γ-CD in the mobile phase for 2-OH-B[a]P and 9-OH-B[a]P (Table 2). This was due mainly to the greater interaction between γ-CD and 9-OH-B[a]P, which resulted in a larger decrease in the k′ values for this metabolite. The 2-OH-B[a]P and 9-OH-B[a]P isomers were the most difficult

pairs of metabolites to separate with methanol-water mobile phases (5,23). A previous investigation indicated that the optimization procedure with MeOH:H_2O mobile phases resulted in the separation of all pairs of metabolites, except for the 2-OH-B[a]P from 9-OH-B[a]P (5). Also, addition of β-CD as a mobile phase modifier resulted in a very good separation of twelve of the fourteen metabolites, except for the 2-OH-B[a]P from 9-OH-B[a]P (23).

Effects of γ-CD Concentration on the Separation of a Complex Mixture of Metabolites

As the data in Tables 1 and 2 show, the tetrols and B[a]P-t-9,10-dihydrodiol have very small k′ values, and it was not possible to separate these compounds completely. However, a mixture of the four tetrols was completely separated with good baseline resolution in MeOH:H_2O (55:45) with 4.0 mM of γ-CD. Thus, for investigating a complex mixture of the metabolites with the mobile phase systems in Table 1 and 2, it was decided to omit the tetrols in this mixture. With γ-CD in methanol-water, the retention times of all the metabolites of B[a]P decreased substantially compare to β-CD (Table 1 and Table 2, see reference 23). Figure 5 shows a chromatogram of a mixture of ten metabolites of benzo[a]pyrene. The chromatogram indicates a successful separation of the ten B[a]P metabolites is possible with MeOH:H_2O using γ-CD as a mobile phase modifier. Three classes of compounds were completely separated in MeOH:H_2O (80:20) with 1.7 mM of γ-CD (Figure 5). These were dihydrodiols, diones, and monohydroxyl-B[a]P. If the tetrols were present in the mixture, they would have

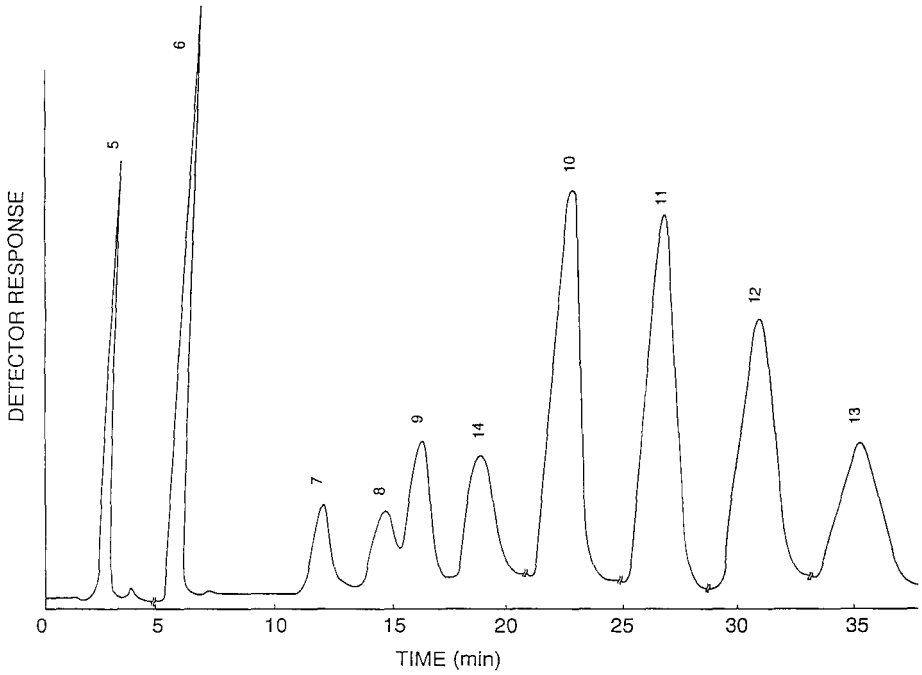

FIGURE 5. Chromatogram of ten metabolites of B[a]P obtained with MeOH:H_2O (80:20) with 1.7 mM γ-CD. The full names of the compounds are given in the footnote of Table 1.

essentially the same retention as B[a]P-t-9,10-dihydrodiol and would have co-chromatographed with tetrol I-2. As discussed earlier, without γ-CD in the mobile phase, there was overlap between 2-OH-B[a]P and 9-OH-B[a]P, and the presence of β-CD in methanol-water system didn't affect the separation of this pair of metabolites (23). Figure 5 shows that the six monohydroxyl-B[a]P metabolites can be readily separated. Comparison of Figure 5 and the chromatogram of fourteen metabolites separated with MeOH:H_2O (65:35) with 4.0 mM of β-CD

from a previous investigation (23), showed that in the region of tetrols and B[a]P-t-9,10-dihydrodiol, β-CD was more effective than γ-CD in separating the tetrols and the dihydrodiol. However, for the separation of dihydrodiols, diones, and monohydroxyl-B[a]P metabolites, MeOH:H_2O (80:20) with γ-CD resulted in a good compound-class separation, band sharpening, shorter retention times, and better separation of the ten metabolites than with β-CD in the mobile phase (23).

CONCLUSIONS

It can be concluded that a good separation of a mixture of three classes of metabolites, namely, dihydrodiols, diones, monohydroxyl-B[a]P metabolites is possible using methanol-water with γ-CD as a mobile phase additive. Mobile phases containing γ-CD showed better selectivity toward the monohydroxyl-B[a]P isomers compare to an optimum binary methanol-water mobile phase (5) or methanol-water with β-CD (23). The capacity factors for the meta- bolites decreased to a larger extend with γ-CD compare to β-CD and a desirable capacity factor range of $1< k'< 15$ was obtained for the metabolites with MeOH:H_2O (80:20) and γ-CD. Also, the selectivity factors for monohydroxyl-B[a]P metabolites increased with γ-CD compared to β-CD in methanol-water mobile phases. Isomers that were difficult to separate, 6-OH- and 12-OH-B[a]P, and 9-OH- and 2-OH-B[a]P, were separated with good resolution with γ-CD in methanol-water. With γ-CD, the retention behavior of the 2-OH-B[a]P and 9-OH-B[a]P was remarkably different than with methanol-water or methanol-water with β-CD. A good separation for the four tetrols can be obtained with 4.0 mM of

γ-CD in MeOH:H_2O (55:45). The elution order of the fourteen metabolites didn't change by addition of γ-CD. Earlier work showed that the elution order of some of the metabolites changed with β-CD in the mobile phase. Also, γ-CD, as a mobile phase additive, was shown to be more effective than was β-CD for the separation of the most difficult to separate pairs of monohydroxyl-B[a]P metabolites.

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2,5-DIHYDROXYBENZOHYDRAZIDE AS ELECTROACTIVE LABELING REAGENT FOR ALIPHATIC ALDEHYDES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL AND ULTRAVIOLET DETECTION

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ABSTRACT

A method was developed for the analysis of aldehydes by high performance liquid chromatography coupled with electrochemical and ultraviolet detection. The electrochemical oxidation of carbonyl compounds derivatized with 2,5-dihydroxybenzohydrazide (2,5-DHBH) was investigated at porous graphite electrodes. The compounds were separated on an Adsorbosphere column with a methanol-acetonitrile-phosphate buffer eluent and detected at graphite electrodes set at an oxidation potential of +0.3 V. The influence of the mobile phase buffer concentration and pH on the detector response was also studied. The electrochemical detection was compared with conventional UV detection and was found to achieve enhanced specificity and sensitivity (S/N=3). The

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derivatization was shown to be quantitative and the response linear between 1-15 ng/ml. The method is rapid, reproducible and the detection limit is 130 fmol for an injection volume of 5 µl with cyclohexanecarboxaldehyde as internal standard (I.S.). This technique is applicable to the assay of carbonyl compounds in flavor chemistry.

INTRODUCTION

The determination of carbonyl compounds in analytical chemistry is becoming always more important for the monitoring of formation reactions or the transformation of aldehydes [1] and for the study of the fractions of the volatile compounds responsible for the organolectic characteristics of natural aromas and flavoring agents [2,3].

The analysis of microquantities of compounds implicated in these mechanisms allows the study of the trend of oxidative degradation phenomena, change in flavor, racemization, and to carry out controls on the genuineness in the field of essential oils and aromas [4].

The normal techniques for the determination of carbonyl compounds are GC [5], Headspace CGC [6], and GC coupled with MS and FTIR [7]. Furthermore, the technique of derivatization for spectrophotometric [8] and spectroflurometric HPLC analysis was used [9,10]. We also proposed a sensitive and specific method for the evaluation of aldehydes, octanal and decanal, which are responsible for the organolectic characteristics of essential citric oils and flavoring agents. To this end we established a new reaction of derivatization of these two aldehydes and cyclohexane-carboxaldehyde, as internal standard (I.S.), with 2,5-dihydroxybenzo-hydrazide (2,5-DHBH) to form electroactive hydrazone measurable by

HPLC with electrochemical detection (ED). This method, moreover, could be applied to the analysis of cosmetic products [11], topical formulations [12] and medical bactericides [13] containing octanal and decanal.

EXPERIMENTAL

Apparatus

The HPLC apparatus comprised two Model 510 pumps, a Model 712 WISP auto-injector and a Model 490E absorbance detector (Waters Assoc., Milford, MA, USA) set at 257 nm and 0.05 absorbance units full scale. The UV detector was connected in series with the electrochemical detector (Model 5100A Coulochem; ESA, Bedford, MA, USA) which consisted of a control module and an analytical cell (Model 5010) containing two in-line porous graphite coulometric electrodes.

The analysis was performed in the oxidative mode. The ED sensitivity range and response time were set at 100 nA and 10 s, respectively. Signals from the detectors were converted to chromatographic traces and integrated by an APC IV computer system (NEC, Boxborough, MA, USA) using Maxima 820 software (Waters Assoc., Milford, MA, USA).

All melting points are uncorrected. Mass spectra were obtained on a model Kratos MS 25 RF. IR spectra were recorded on a Perkin-Elmer 1600 Fourier transformed spectrometer as KBr disks. Elemental analysis for C, H, N were obtained on a Carlo Erba 1106 analyzer (Milan, Italy). UV absorption spectra were recorded on a Uvikon 860 (Kontron, Zurich, Switzerland) spectrometer in CH_3CN/MeOH 9:1 solution. Analytical thin

layer chromatography (TLC) was performed on Merck 60 F_{-254} silica gel plates.

Chemicals

2,5-dihydroxybenzoic acid, cyclohexanecarboxaldehyde, octanal, decanal and KH_2PO_4 were obtained from Fluka (Buchs, Switzerland). HPLC-grade methanol and acetonitrile were from Carlo Erba (Milan, Italy). Milli-Q water (Millipore, Bedford, MA, USA) was used. Other chemicals used were of reagent grade or better.

Chromatographic conditions

Separations were performed on a 3 μm Adsorbosphere column (100x4.6 mm i.d.; Alltech, Deerfield, IL, USA) fitted with a guard column (Hypersyl ODS RP-18, 5 μm particles, 4x4 mm i.d.; Policonsult, Rome, Italy) and eluted, isocratically, with acetonitrile-0.05 M phosphate buffer-methanol (39/39/22, v/v/v) adjusted to pH 7.0 with KOH. The mobile phase was filtered through GS-type filters (0.22 mm, Millipore, Bedford, MA, USA) and on-line degassed with a Model ERC-3311 solvent degasser (Erma, Tokyo, Japan). Chromatography was performed at ambient temperature, at a flow-rate of 1.0 ml/min. Peak areas were quantified by the internal standard.

Synthesis

2,5-dihydroxybenzohydrazide (2,5-DHBH,**1**) was prepared in a similar manner to that described in literature [14,15].

UV: λ_{max} 327 nm, $\varepsilon = 5{,}233\ M^{-1} \cdot cm^{-1}$.

Cyclohexanecarboxaldehyde hydrazone of 2,5-DHBH (CY-2,5-DHBH, **3a**) 2,5-DHBH (80 mg, 0.48 mmol) and cyclohexanecarboxaldehyde **2a** (90 μl, 0.48 mmol) were dissolved in methanol (5 ml) and acetic acid (1 ml). The mixture was stirred for 1 h at room temperature and then water was added. A precipitate was collected, washed and dried. The solid product was crystallized from methanol-water gave CY-2,5-DHBH as colorless needles (92 mg, 73%) m.p. 137-139°C.

Anal. Calc'd. for $C_{14}H_{18}N_2O_3$ (M.W. 262.31): C, 64.11; H, 6.92; N, 10.68. *Found*: C, 63.97; H, 6.95; N, 10.65. MS (IE 70 eV) *m/e*: 262 (M^+), 152 (M^+-$C_6H_{11}CH$=N), 137 (M^+- $C_6H_{11}CH$=N-NH). IR (KBr) νmax (cm^{-1}): 3450, 3234, 2913, 1625, 1548, 1449. UV: λ_{max} 257 nm, ε_1 = 11,150 $M^{-1}\cdot cm^{-1}$; λ 337 nm, ε_2 = 5,375 $M^{-1}\cdot cm^{-1}$.

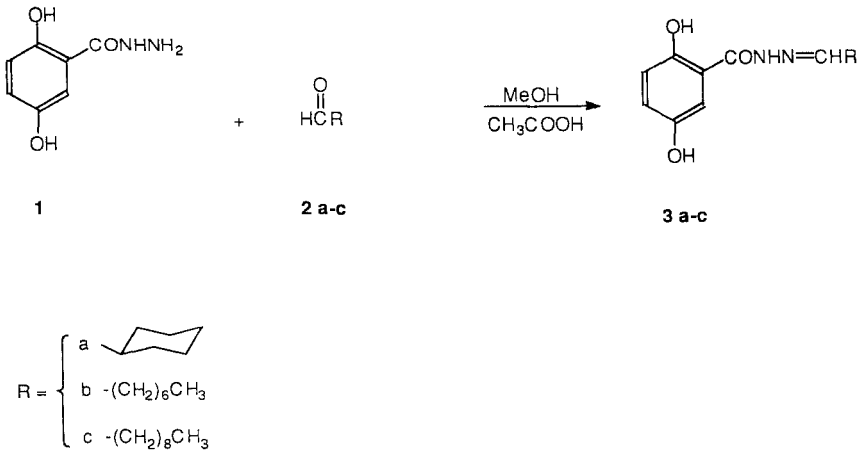
Octanal* and *Decanal hydrazone of 2,5-DHBH (OC-2,5-DHBH and DE-2,5-DHBH respectively) were prepared by the same procedure described above for CY-2,5-DHBH. The elemental analysis and the melting point data for new compounds are described below.

OC-2,5-DHBH (**3b**): White needles (70 mg, 63%) m.p. 144 -145°C.

Anal. Calc'd for $C_{15}H_{22}N_2O_3$ (M.W. 278.35): C, 64.76; H, 7.90; N, 10.06. *Found* : C, 64.58; H, 7.45; N,10.23. MS (IE 70 eV) *m/e*: 278 (M^+), 194 (M^+ -$(CH_2)_5CH_3$), 137 (M^+- $CH_3(CH_2)_6CH$=N-NH). IR (KBr) νmax (cm^{-1}): 3381, 2947, 1637, 1577. UV: λ_{max} 257 nm, ε_1 = 11,924 $M^{-1}\cdot cm^{-1}$; λ 334 nm, ε_2 = 5,691 $M^{-1}\cdot cm^{-1}$.

DE-2,5-DHBH (**3c**): Light pink needles (90 mg, 69%) m.p. 151 -152°C.

Anal. Calc'd for $C_{17}H_{26}N_2O_3$ (M.W. 306.41): C, 66.64; H, 8.55; N, 9.14. *Found:* C, 66.52; H, 8.50; N, 9.53. MS (IE 70 eV) *m/e*: 306 (M^+), 194 (M^+ -$(CH_2)_7CH_3$), 137 (M^+- $CH_3(CH_2)_6CH$=N-NH). IR (KBr) νmax (cm-1): 3197,



Scheme 1: Reaction of aldehydes **2 a-c** with 2,5-DHBH to give hydrazone derivatives **3 a-c**

2924, 1643, 1588. UV: λ_{max} 257 nm, ε_1= 12,258 $M^{-1} \cdot cm^{-1}$; λ 335 nm, ε_2 = 5,411 $M^{-1} \cdot cm^{-1}$.

RESULTS

Scheme 1 illustrates the reaction of aldehydes **2 a-c** with 2,5-DHBH to give hydrazone derivatives **3 a-c.** Experiments were performed to determine optimum derivatization time in order to give maximum conversion of **2 a-c** to their electroactive derivatives. Figure 1 shows the trend of the derivatization procedure which was complete after 60 min with 90% yield and the compounds show the stability in the reaction mixture until 24 h after the optimum. This derivatization procedure

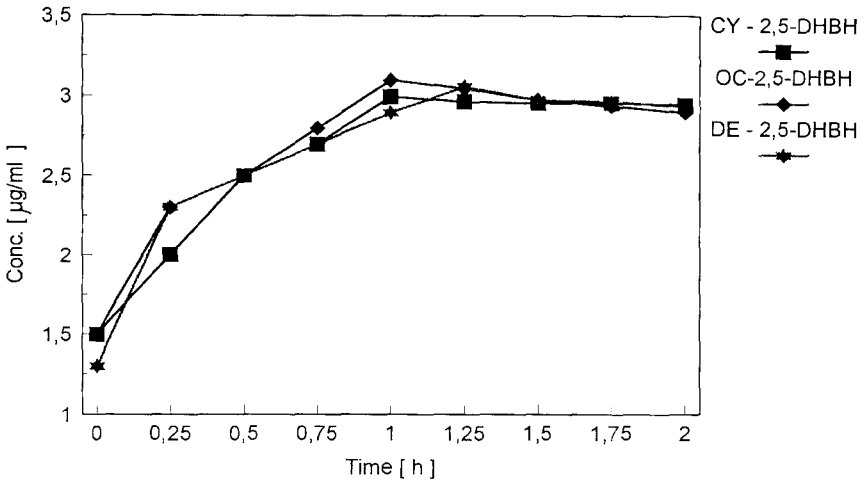


Figure 1: Concentration of CY-2,5-DHBH **3 a-c** *versus* time curve

achieves enhanced sensitivity. The linearity of the method was also determined: five standards containing concentrations of **3 a-c** in the range 1-15 ng/ml. The calibration curve of I.S. showed a correlation coefficient of 0.9997 and the equation of the linear regression was y= 0.0679 + 0.5076x where x was the area ($\cdot 10^6$) under the peak. Typical chromatograms of UV and ED detection of standards are shown in fig. 2 and 3 respectively, the analysis was complete within 10 minutes and the retention times were 1.65, 2.54, 4.23 and 9.01 for 2,5-DHBH, CY-2,5-DHBH, OC-2,5-DHBH and DE-2,5-DHBH; the injected volume and concentrations were 5 µl and 100 pmol/ml, respectively. Detection limit was determined from five runs and was in the range of 100-200 fmol per injection (S/N= 3). These values are significantly lower (at least five times) than those registered for conventional UV detector (fig. 2). The

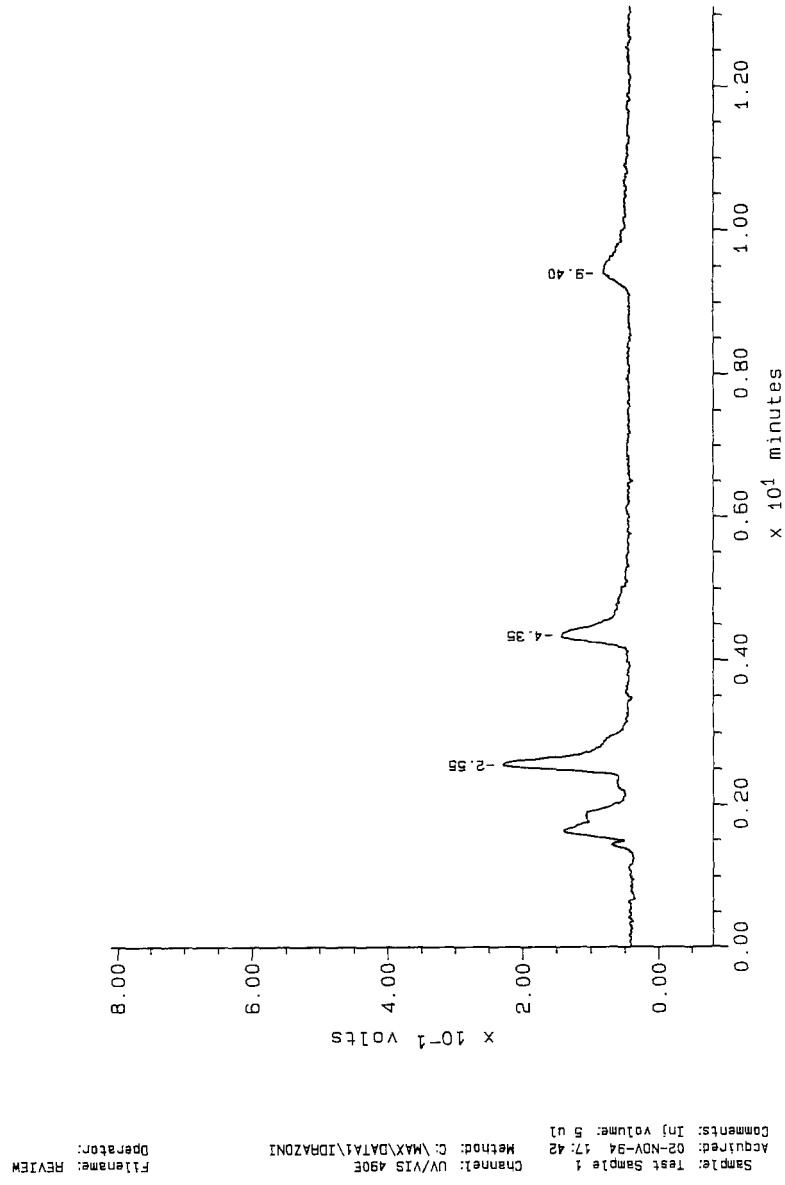


Figure 2: Chromatogram obtained with UV detector fixed at 257 nm by injection of 5 µl of the standard solution of **1** and **3 a-c** (100 pmol/ml)

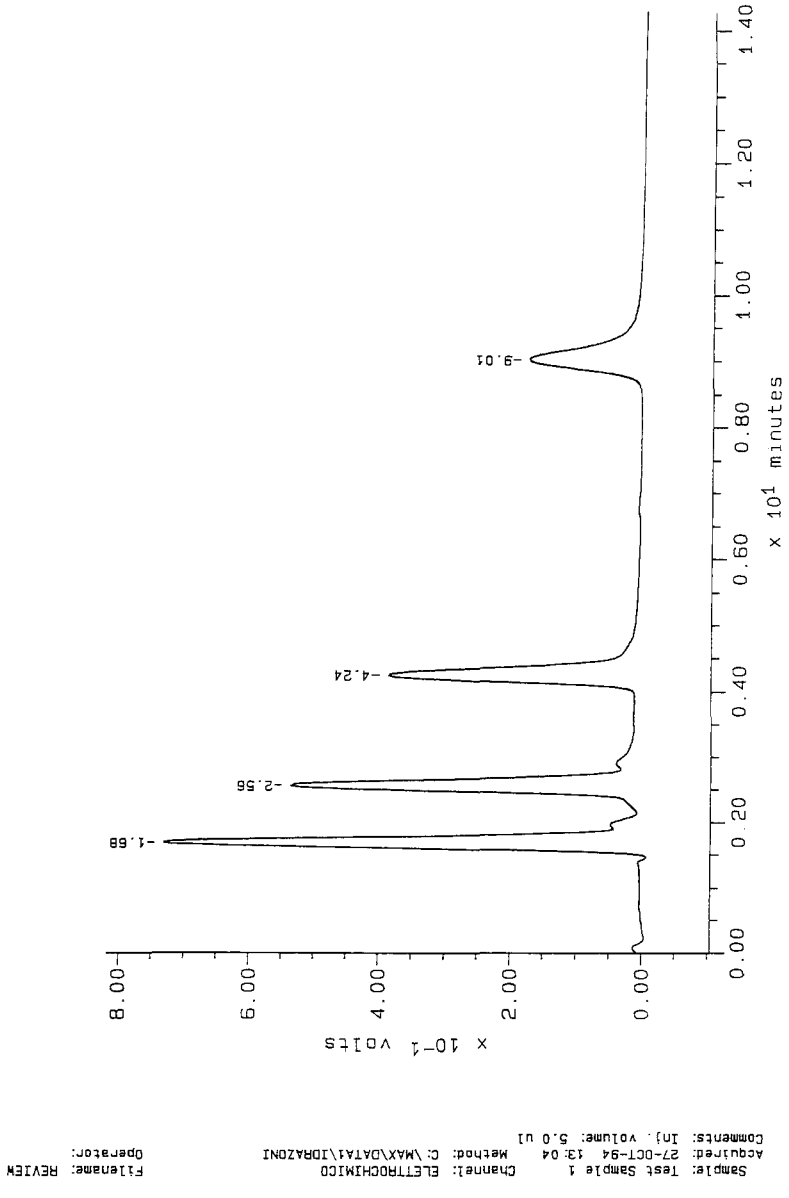


Figure 3: Chromatogram obtained with ED detection obtained by injection of 5 µl of the standard solution of **1** and **3 a-c** (100 pmol/ml)

derivatization was shown to be over 90% and the response linear between 1-15 ng/ml.

DISCUSSION

HPLC coupled with electrochemical detection was used to determine trace amounts of electroactive compounds because of its high sensitivity and selectivity [16]. Moreover, enhanced selectivity is achieved by HPLC-ED in this method without blank interference due to the limited number of substances which can undergo redox reactions under this condition. Several parameters were examined in order to optimize the electrochemical detection of hydrazone derivatives. Preliminary experiments indicated that under the chromatographic conditions reported above, the **3 a-c** derivatives responded at the ED at oxidation potentials lower than +0.8 V. With additional applied potential, no further increase in hydrazone peak heights occurred and a rise in the background current was observed. Electroactive properties of the derivatives **3 a-c** were examined with their hydrodynamic voltammograms (fig. 4), the figure indicates that the best potential is +0.3 V, because a superior potentials there would be an amplified response only for the derivatization reagent (2,5-DHBH), which could mask in the chromatogram the result of the I.S. that has a retention time of 2.54 min.

The ED performance was markedly influenced by the ionic strength but not by the pH of the mobile phase. With increasing concentrations of the phosphate buffer (from 0.01 to 0.05 M), an increase of the hydrazone

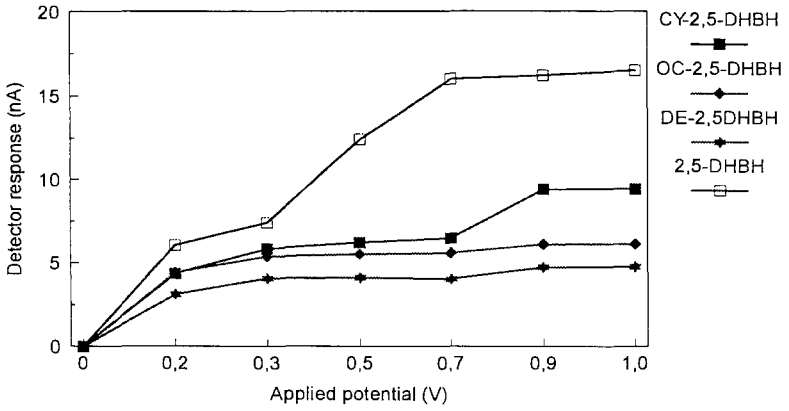
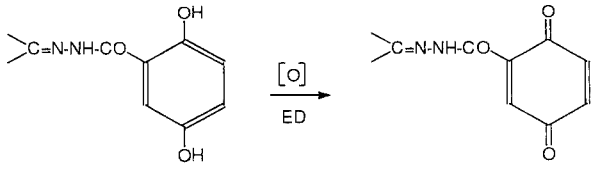


Figure 4: Hydrodynamic voltammograms of the electroactive derivatives **3 a-c**



Scheme 2: General oxydative mechanism of electroactive Schiff bases **3 a-c** at porous graphite electrodes.

electrochemical response was observed. No significant improvement in the detector response was achieved by further increasing the phosphate molarity, which was consequently fixed at 0.05 M and the pH at 7.0.

This method offers the possibility of determining octanal and decanal with a simple reaction of derivatization and formation of electroactive Schiff bases using cyclohexanecarboxaldehyde as internal standard. The chromatographic analysis with UV detection fixed at 257 nm (Fig. 2) of the

standard solution indicates that the method is also applicable to conventional UV detectors but surely for concentrations >100 pmol/ml. The applied potential, as reported in literature [17], selectively oxidizes the hydroquinone derivatives (**3 a-c**) according to the following reaction (Scheme 2). In conclusion, 2,5-dihydroxybenzohydrazide can easily be used as electroactive labeling reagent for aliphatic aldehydes by high performance liquid chromatography with electrochemical detection.

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DIRECT CHROMATOGRAPHIC SEPARATION OF 2-(2-AMINO-1,3-THIAZOL-4-YL)METHYLGLYCINE AND ITS METHYL ESTER ENANTIOMERS USING A CHIRAL CROWN ETHER COLUMN

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ABSTRACT

Direct liquid chromatographic separation of 2-(2-amino-1,3-thiazol-4-yl) methylglycine and its methyl ester enantiomers was achieved using a chiral crown ether column. The separation was strongly influenced by column temperature and the pH of mobile phase. The method can detect the undesired (+)-enantiomer down to a level of 0.5% and is routinely employed to determine the purity of the desired (-)-enantiomer.

INTRODUCTION

(-)-2-(2-Amino-1,3-thiazol-4-yl)methylglycineand its methyl ester are two intermediates in a process to synthesize CI-992, a renin inhibitor with potential antihypertensive activity (1). Chemical resolution produced the desired (-)-enantiomer. Optimization of the resolution and determination of enantiomeric

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purity of the (-)-enantiomer required a chiral method that would allow the separation of the undesired (+)-enantiomer from a large excess of the (-)-enantiomer.

A crown ether-based stationary phase has been utilized for separation of primary amine-containing compounds (2-11). The same stationary phase was used to evaluate the enantiomeric separation of 2-(2-amino-1,3-thiazol-4-yl)methylglycine and its methyl ester. The effects of flow rate, column temperature, organic modifier, and mobile phase pH were also determined.

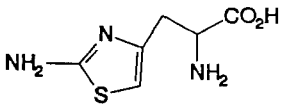
EXPERIMENTAL

Apparatus

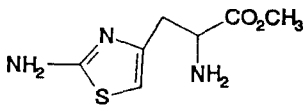
Chromatographic analysis was performed using a Waters 590 pump, a Micromeritics 728 autosampler and a Rheodyne 7010 injector with a 20 μl sampling loop, a Hitachi L-4000 variable wavelength UV detector, and a Hitachi D-2000 Chromato-Integrator. The chiral column is a Crownpak CR(+), (150 x 4.0 mm, 5 micron particle size) from Chiral Technologies, Inc., Exton, PA. A Brinkmann Lauda Model RMS-6 refrigerating circulator was used to control the column temperature. The mobile phase pH was determined using an Orion Model EA 940 expandable ionAnalyzer with a ROSS SURE-FLOW combination pH electrode.

Chemicals

Methanol (HPLC grade) was purchased from EM Science, Gibbstown, NJ. Perchloric acid (70%) was obtained from MCB Manufacturing Chemists, Inc., Cincinnati, OH. Water (HPLC grade) was from a Milli-Q water purification system. 2-(2-Amino-1,3-thiazol-4-yl)methylglycine, (+)-2-(2-amino-1,3-thiazol-4-yl)methylglycine, (-)-2-(2-amino-1,3-thiazol-4-yl)methylglycine, and the corresponding methyl esters were prepared at Parke-Davis Pharmaceutical Research Division, Holland, MI (12).



(A)



(B)

FIGURE 1. Chemical structures of (A) 2-(2-amino-1,3-thiazol-4-yl)methylglycine (abbreviated as acid) and (B) 2-(2-amino-1,3-thiazol-4-yl) methylglycine, methyl ester (abbreviated as ester).

Chromatographic Conditions

The mobile phase was aqueous perchloric acid except for the organic modifier study in which methanol was mixed with aqueous perchloric acid. The UV detection wavelength was set at 210 nm. Sample amount injected for the determination of enantiomeric purity was about 0.12 μmole.

RESULTS AND DISCUSSION

The mechanism for enantioselective retention of various amino acids on the crown ether stationary phase has been detailed (10). 2-(2-Amino-1,3-thiazol-4-yl) methylglycine, a synthetic amino acid, and its methyl ester as shown in Figure 1 each has a free amine adjacent to the chiral center. This structural characteristic makes them candidates for enantiomeric separation using a crown ether HPLC column. It would be desirable to resolve all four enantiomers.

Effect of the pH of Mobile Phase

As shown in Figure 2, the pH of mobile phase has a dramatic effect on the enantiomeric separation. The enantiomeric resolution increases with decrease in mobile phase pH. This is consistent with the earlier observations (3,9). At pH 1.60 or above, the acid was partially resolved and the ester was well resolved. At pH 1.00, all four enantiomers were well resolved.

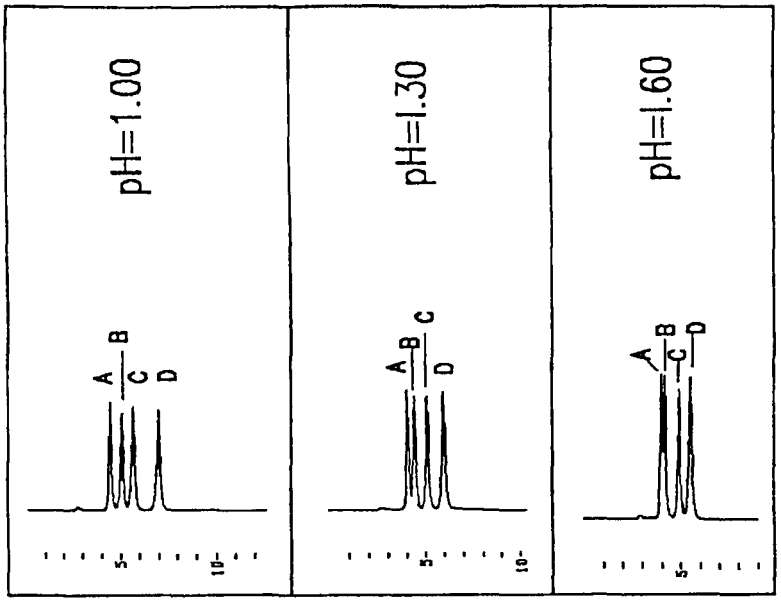


FIGURE 2. Effect of the pH of mobile phase on separation of A=(+)-acid; B=(-)-acid; C=(+)-ester; (D)=(-)-ester. The column temperature was 25°C and flow rate was 0.5 mL/min.

Effect of Column Temperature

The enantiomeric resolution increased as the column temperature decreased for acid and ester as shown in Figure 3. This is mainly attributed to the combined effects of increased chiral recognition and increased retention at lower temperature (10,11). All four enantiomers were resolved at 25°C. Partial separation was obtained for (-)-acid and (+)-ester at 15°C. All four enantiomers were resolved again at 5°C with (+)-ester eluted earlier than (-)-acid.

Effect of Flow Rate

Changes in flow rate from 0.3 mL/min. to 0.7 mL/min. showed little effect on separation and resolution as seen in Figure 4.

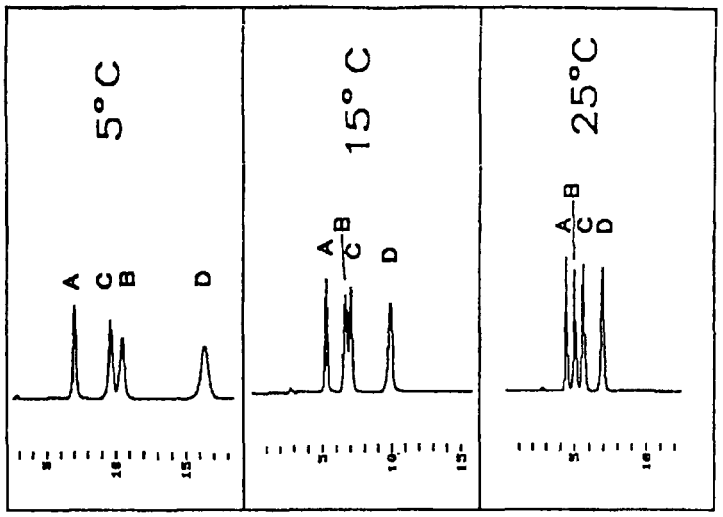


FIGURE 3. Effect of column temperature on separation of A=(+)-acid; B=(-)-acid; C=(+)-ester; D=(-)-ester. The pH of mobile phase was 1.00 and flow rate was 0.5 mL/min.

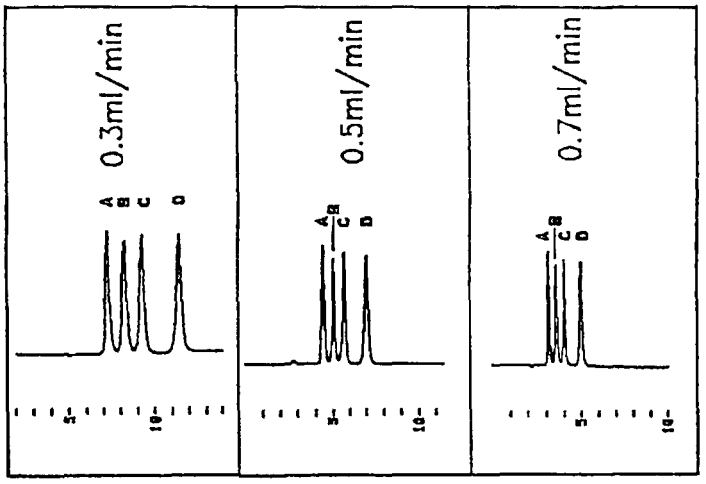


FIGURE 4. Effect of flow rate on separation of A=(+)-acid; B=(-)-acid; C=(+)-ester; D=(-)-ester. The pH of mobile phase was 1.00 and column temperature was 25°C.

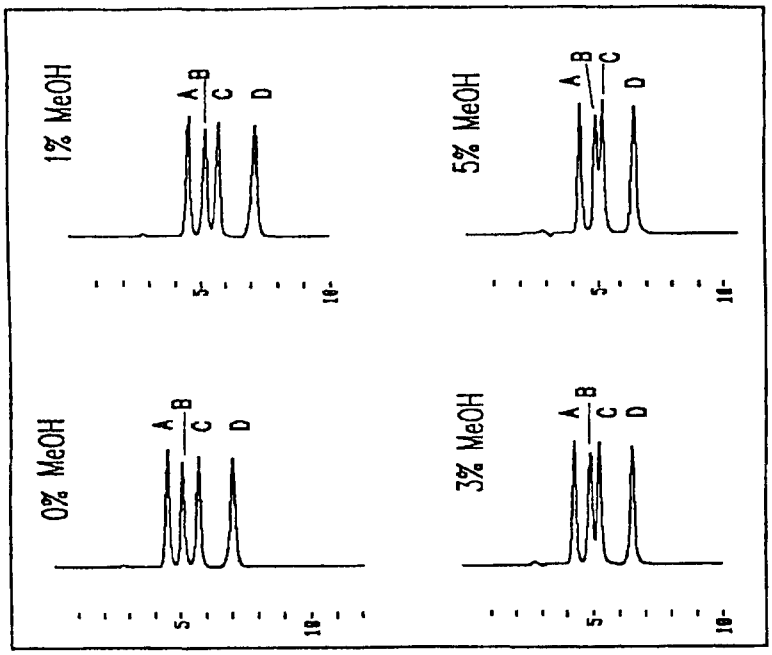


FIGURE 5. Effect of methanol content on separation of A=(+)-acid; B=(-)-acid; C=(+)-ester; D=(-)-ester. The pH of mobile phase was 1.00 and flow rate was 0.5 mL/min.

Effect of Methanol

Compounds with greater hydrophobicity in general have longer retention times on a crown ether stationary phase (10). Methanol has been used as a modifier in mobile phase to reduce retention (6,10). The increase of methanol content in mobile phase from 0 to 5% did not significantly change the enantiomeric resolution for acid and ester as shown in Figure 5. However, the appreciable decrease in retention for ester led to a partial separation between (-)-acid and (+)-ester when methanol content was increased to above 3%.

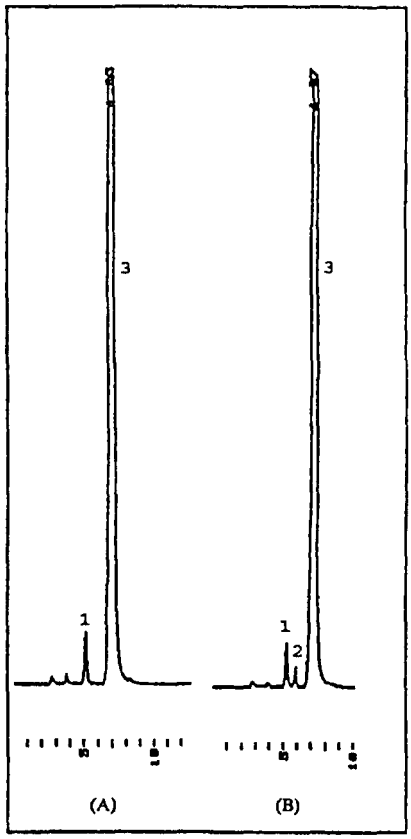


FIGURE 6. (A) Chromatogram of an enantiomerically pure (-)-ester sample (B) chromatogram of the same sample spiked with 0.5% (+)-ester. 1=(-)-acid; 2=(+)-ester; 3=(-)-ester. Mobile phase was an aqueous perchloric acid (pH 1.00), column temperature was 25°C and flow rate was 0.5 mL/min.

Applications

As a result of the study above, the crown ether stationary phase was found to be useful for direct chiral separation of 2-(2-amino-1,3-thiazol-4-yl) methylglycine and 2-(2-amino-1,3,thiazol-4-yl)methylglycine, methyl ester. The chromatogram obtained from an (-)-ester sample using the conditions given in Figure 6(A) indicated that a small amount of (-)-acid as an impurity was well resolved from a large amount of desired (-)-ester. The same sample when spiked with 0.5% (+)-ester as shown in the chromatogram in Figure 6(B) further demonstrated that the method can detect the undesired enantiomer to the level of 0.5%.

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APPLICATION OF ARTIFICIAL NEURAL NETWORKS IN THE OPTIMIZATION OF HPLC MOBILE-PHASE PARAMETERS

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ABSTRACT

The prediction capability of forward feed neural networks was tested c computer generated capacity factors. The capacity factors were simulate from equations reflecting the contribution of mobile phase changes in pH organic modifier concentration, and ion-pair concentration. Simulated dat allows an appropriate experimental design which assures the training of th network does not involve memorization but guarantees the network w generalize. The use of different mathematical forms to calculate th behaviour of capacity factor with changes in pH, methanol concentratior and ion-pair concentration permitted us to explore the capability of neur networks to fit a variety of curves. Each of the independent variables wer studied separately, and then in combination. The effect of variabl transformation played a very important role in effective training of th network. The neural network output equations were used to formulate nonlinear regression problem and the behaviour of this model was compare to the neural network system. When the neural network systems had onl sufficient processing units needed to solve the problem, nonlinear regressio models and neural networks arrived at identical solutions. When the networ

contained excessive neurons, nonlinear regression techniques were unstable, having high intraparameter correlations and showing matrix singularity.

INTRODUCTION

Mobile phase composition, in a reverse-phase ion-pair chromatographic system, plays a very vital role in the resolution of the various components in given sample. Consequently, optimization of the different parameters like - pH, organic modifier concentration, and ion-pair concentration is critical. Usually, the approach toward developing a reliable HPLC method for analysis is more or less intuitive, and is often time-consuming. Some of the crucial aspects in the selection of an appropriate mobile phase include complex nonlinear data manipulations, restrictions on the number of predictor parameters, and necessity for a large volume of data. The complexity of such an approach makes it ideal for the application of versatile data treatment techniques such as neural networks.

The use of artificial neural networks (ANN) to fit complex data is becoming popular in many scientific fields. ANN have been used to predict aqueous solubility of organic compounds [1], analyze quantitative structure-activity relationships [2], analyze NMR data [3], predict protein secondary structure [4], analyze complex pharmacodynamic data [5], and to model nonlinear pharmacokinetic data [6]. The principles of neural networks have been well described in textbooks [7 8], as well as reviewed in journals [9 10]. The process by which neural network is applied to a problem involves selection of input parameters, deciding the desired output, and selection of an appropriate network. The network should be as simple as will

adequately reproduce the training data. We have elected to test the applicability of neural networks using the standard backpropagation algorithm for optimizing the composition of the mobile phase. The effect of pH, organic modifier content and ion-pair concentration on capacity factor was simulated using appropriate mathematical relations.

METHODS

A neural network program based on the popular back-propagation algorithm was employed in our study. All the networks consisted of three layers - input, hidden and output layers. Input neurons do not process the data but, feed it to the neurons in the higher layers. The hidden and the output neurons remap the data in a more classifiable form. This is done by transforming the sum of the products of the weights and the corresponding inputs using a flexible function. Our system used the sigmoid function as the squashing function. The connectivity of parallel processing system depended on the complexity of the relationship under investigation. The chosen network was trained with the generated data which was scaled between 0.05 and 1.00.

The effect of each of the mobile phase parameters was studied individually as well as in combination. In the individual cases, either pH, methanol concentration, or ion-pair concentration served as the input and the capacity factor as the target. In the combination case all three parameters were inputs

with capacity factor as the output. In almost all the cases variable transformation yielded in lower total sum of squares (tss).

Organic Modifier Concentration

Relevant data was generated from an exponential function which reasonably describes the behaviour of capacity factor with changes in the concentration of methanol. The mathematical relation is depicted in eq(1), where $k_1 = 10$,

$$K' = k_1 e^{k_2 * [methanol]} \qquad (1)$$

$k_2 = 0.202$, and methanol concentration ranged from 50 to 100 per cent.

Ion-Pair Concentration

Data was generated from the hyperbolic equation which mimics the effect of the concentration of the ion-pair reagent on the capacity factor, shown in eq(2),

$$K' = \frac{k_1}{1 + \frac{k_2}{[amine]}} \qquad (2)$$

where $k_1 = 18.94$, $k_2 = 29.86$ and the amine concentration was between 0.01and 40.

pH

The dependence of capacity factor on hydrogen ion concentration was described with eq(3), where Ka = 1.4E-8, $k_1 = 0.29$, and $k_2 = 8.34$.

$$K' = k_1 + \frac{k_2 * [H^+]}{1 + \frac{[H^+]}{k_a}} \qquad (3)$$

Combination

The dependence of capacity factor on all of the previous mobile phase components was described by the eq(4). Three amine concentrations (5, 30, 55), twelve pH values from 2 to 8, and ten values of methanol concentrations

$$K' = \frac{\frac{k_1 e^{-k_2 * \ln[methanol]}}{1 + \frac{k_3}{[amine]}} + k_4 e^{-k_4 * \ln[methanol]} * 10^{-pH}}{\frac{10^{-pH}}{k_a}} \qquad (4)$$

were used to generate the training set.

Nonlinear Regression

Nonlinear regression analysis was conducted using SAS[11]. Upon training the data to a particular network, the system output equations were derived to

formulate a nonlinear least squares parameter estimation problem. For example, the output equation of system consisting of one input, no hidden, and one output is shown in eq(5). In eq(5), Wij stands for the weight of the connection

$$Output = \frac{1}{1 + e^{(-W_{ij} * I + b_j)}} \qquad (5)$$

between the neurons i and j, I is the input to the neuron j, and bj stands for the bias associated with neuron j.

RESULTS AND DISCUSSION

Variable transformation is one of the important aspects to be considered in the improvement of the training ability of a neural network. Figure 1a shows the changes in the tss along the course of training with the hydrogen ion concentration as the input and capacity factor as the output variables. As shown (Fig 1a), the tss could not reach a minimum value but instead an oscillation is observed. In order to avoid this problem, we transformed the hydrogen ion concentration to pH, and the behaviour of tss is depicted in Figure 1b, which shows a steady tss value of $\sim$0.04.

The behaviour of the capacity factor to the changes in pH was well emulated by a one input (pH), one hidden, and one output (K') network. Figure 2 shows a good correlation between the neural trained output and the generated

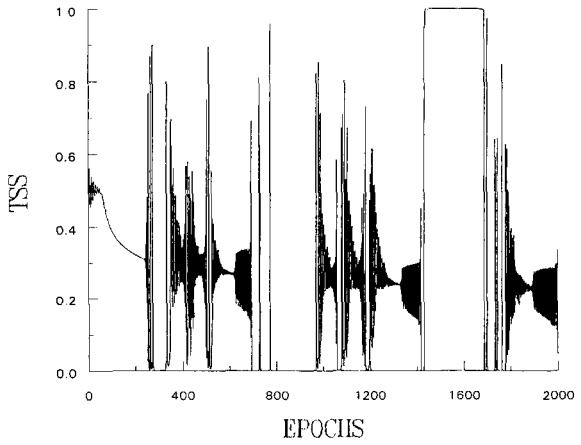

Figure 1a. Plot of the number of epochs and the corresponding tss when hydrogen ion concentration was the input and K was the output.

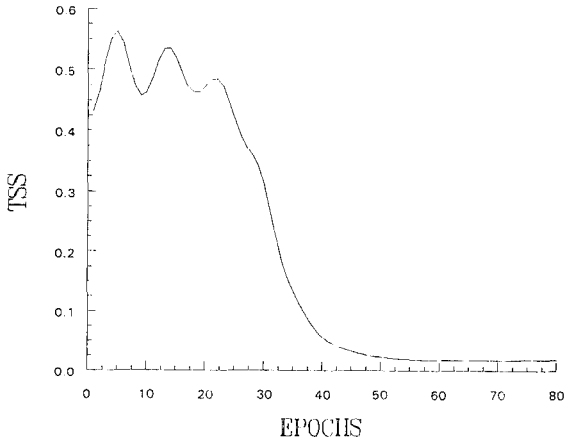

Figure 1b. Plot of the number of epochs and the corresponding tss when pH was the input and K was the target.

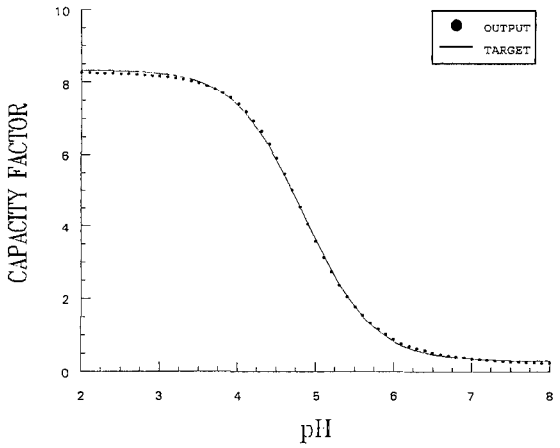

Figure 2. Plot of neural network trained output and calculated capacity factors reflecting the changes in pH of the mobile phase (r=0.9999).

Table 1
SAS Estimates and the Neural Network Derived Parameters Associated with Effect of pH of the Mobile Phase.

PARAMETER	NEURAL NETWORK	SAS
w1	-6.6352	-6.6082
w2	9.1653	9.2044
b1	2.8344	2.8170
b2	-3.3941	-3.400

K' ($r = 0.9999$). As seen in Figure 2, the magnitude of change due to alterations in pH is maximum in the pH 4-6 range, the capacity factor plateaus in the either extremes. Upon successful training, the system output equations were derived and nonlinear regression was applied to estimate the parameters which include the various weights and biases. Table 1 shows the similarity between SAS estimates and the neural network parameters ('wi' denotes the ith weight and 'bi' denotes ith bias).

The exponential curve describing the effect of change in methanol concentration in the mobile phase was emulated with a network consisting of no hidden neurons. The logarithmic transformation of capacity factor resulted in lower tss. The neural network trained output and the calculated capacity factors with methanol concentrations ranging from 50 to 100% are depicted in Figure 3, ($r = 0.9935$). The SAS estimates from the output equations and the neural network parameters are presented in Table 2.

The curvature governing the effect of the ion-pair reagent in the mobile phase on the capacity factor was well reproduced with a neural network system consisting of one input (amine concentration), no hidden neuron and one output (K'). Figure 4 shows the correlation between the neural network output and the calculated K' ($r = 0.9925$). The weights and biases of the neural network and the SAS nonlinear estimates are presented in Table 3.

The complex nature of the combined effects of all three mobile phase variables could be emulated by a three hidden neuron network. Figure 5 depicts

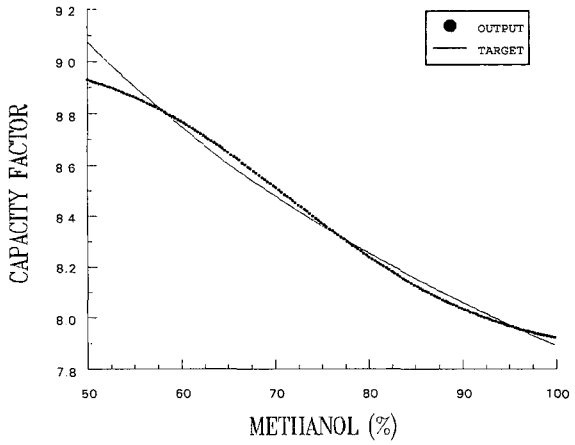

Figure 3. Plot of neural network trained output and calculated capacity factors reflecting the changes in methanol concentration in the mobile phase. (r=0.9935)

Table 2
SAS Estimates and Neural Network Derived Parameters Associated with the Effect of Methanol Concentration in the Mobile Phase.

PARAMETERS	NEURAL NETWORK	SAS
w1	-4.862	-4.862
b1	2.682	2.682

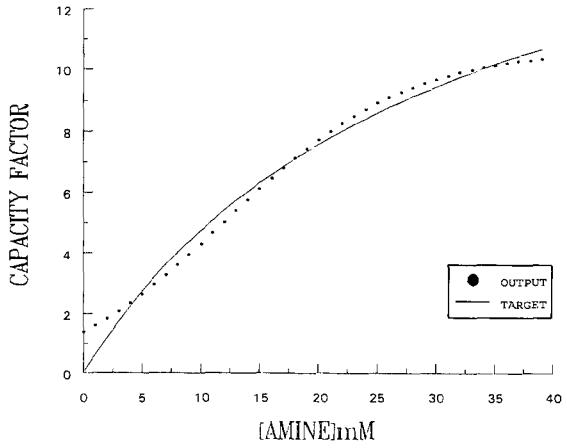

Figure 4. Plot of neural network trained output and calculated capacity factors reflecting the changes in the ion-pair reagent in the mobile phase. (r=0.9925)

the neural network output and the calculated K' (r = 0.9999). Table 4 presents a comparison between the SAS estimates and neural network parameters.

An important aspect that needs to be focussed is the feasibility of developing an ANN system that has potential practical significance. The attributes of such a system would be to generalize the solution with sparse data and robustness toward any noise in the data set. We explored this issue by training the neural network with only few data points and test the system for the other untrained points. The case of combined effects on the capacity factor was considered, which would be a rigorous test for the ability of ANN to recognize the pattern governing the relation between the input and target

Table 3
SAS and Neural Network Derived Parameters Associated with the Effect of Ion-pair Reagent Concentration in the Mobile Phase.

PARAMETER	NEURAL NETWORK	SAS
w1	5.3330	5.3333
b1	-1.8441	-1.8441

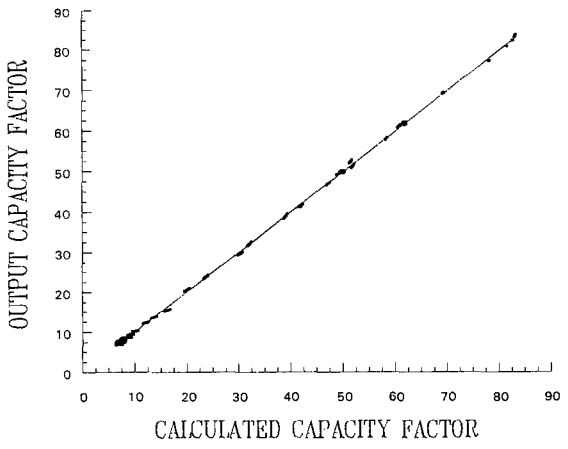

Figure 5. Plot of neural network trained output and calculated capacity factors reflecting the effects of pH, methanol and amine concentrations in the mobile phase (r=0.9999).

Table 4
SAS and Neural Network Derived Parameters Associated with the Combined Effects of pH, Methanol, and Ion-pair Reagent in the Mobile Phase

PARAMETER	NEURAL NETWORK	SAS
w1	-0.6826	-0.6727
w2	-8.7640	-8.7600
w3	-0.0370	-0.0357
w4	10.2680	10.0138
w5	-5.3810	-5.4455
w6	0.0507	0.0465
w7	2.7207	2.7164
w8	1.8429	1.8874
w9	-0.0386	-0.0337
w10	6.4646	6.4559
w11	2.0858	2.0791
w12	2.3476	2.3368
b1	3.89433	3.8900
b2	3.3506	3.4054
b3	-2.8092	-2.8263
b4	-4.2470	-4.2408

variables with minimum information. Neural networks were trained with as low as 8, 12, and 16 points in three different experiments and using the optimized system parameters we tested for 429 points. In order to examine the robustness of the ANN, we also trained the network with data adulterated with 30% error. Neural networks were successful in predicting the capacity factors

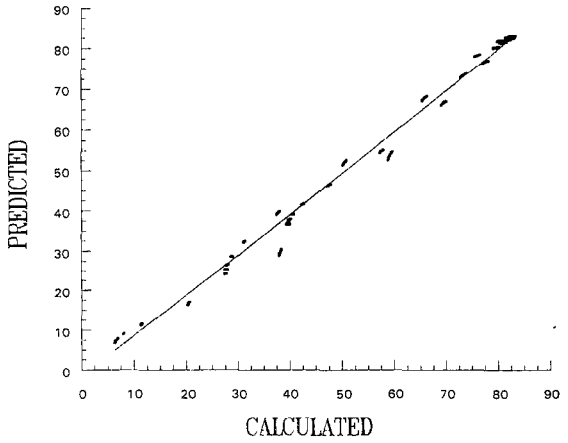

Figure 6. Plot of the neural network predicted and the actual calculated capacity factors when the network was trained with only 16 points and tested for 429 points (r=0.9969).

Table 5
Correlation Coefficients of the Neural Network's Predictions Trained with Sparse Data

NUMBER OF DATA POINTS TRAINED	CORRELATION COEFFICIENT (r)
8	0.9037
8[a]	0.9077
12	0.9354
16	0.9969
16[a]	0.9658

[a] 30% error was included in the training set

accurately. Figure 6 depicts the actual and the predicted capacity factors reflecting the changes in all three mobile phase parameters, when the network was trained with 16 points. This proves the power of ANN, and their potential for practical applications. The summary of results from these tests is shown in Table 5.

CONCLUSIONS

Neural networks prove to be very powerful in elucidating the individual as well as combined effects of the various mobile phase variables considered on the capacity factor of a chromatographic method. It is also shown that neural networks yield similar results as nonlinear regression technique. At the same time neural networks offer greater flexibility and potential than nonlinear regression techniques in that ANN can generalize the pattern even with few data points. Data points fewer than the parameters to be estimated makes the application of the standard nonlinear least squares awkward.

As investigated in this article, parallel distributed processing systems offer a great advantage over the traditional approaches in that model specification is not necessary in neural networks, which is otherwise quite cumbersome. Other important considerations include emulating patterns with sparse data, which economizes the number of experiments and robustness of the technique.

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A COMPARATIVE STUDY OF DIFFERENT SOLID PHASE EXTRACTION PROCEDURES FOR THE ANALYSIS OF ALKALOIDS OF FORENSIC INTEREST IN BIOLOGICAL FLUIDS BY RP-HPLC/DIODE ARRAY

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ABSTRACT

Different Solid Phase Extraction (SPE) procedures are studied for the pretreatment of urine, blood plasma and serum samples, for the analysis of alkaloids of general clinical-toxicological interest. Various SPE cartridges, extraction conditions and solvents were used in the search of the best recovery and clean-up. In the whole study the internal standard was used as a chromatographic standard e.g. it was added in the sample after the end of the extraction. Analysis of the extracts was performed by RP-HPLC with photodiode array detection. Quantitation was performed in four selected wavelengths and comparative results are provided. The extraction reproducibility was also studied employing cartridge-to-cartridge variations. The method was also applied in the analysis of urine samples of heroin and cocaine users.

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INTRODUCTION

Solid phase extraction is established over the last years as a very effective method for sample pretreatment and clean-up. The majority of the recent works related with toxicological analysis report the use of SPE, using a variety of microcolumns and extraction conditions. SPE offers various advantages compared to liquid-liquid extraction such as higher efficiency (therefore higher selectivity and recovery), usage of smaller quantities and solvent volumes, ease and convenience in handling, absence of emulsions, smaller time consumption, and automation options, thus it is the method of choice especially in the clean-up of complex mixtures like biological fluids, feedstuff, foods etc [1].

SPE is widely used in the pretreatment of forensic and clinical samples prior to a chromatographic determination of alkaloids. Both free and conjugated morphine have been extracted from blood on Extrelut silica columns and analysed by GC [2, 3]. Morphine, 6-monoacetylmorphine and codeine were extracted from urine on Clean-Screen Dau columns (silica based cation exchange copolymer) prior to the GC analysis [4]. This extraction column gave better recovery and purification than a C_{18} column or a liquid-liquid extraction procedure for the extraction of 6-monoacetylmorphine from urine [5]. Morphinone and other urinary metabolites of morphine have been extracted from guinea pig urine and bile on C_{18} columns [6]. Morphine and codeine have been extracted from whole blood on a C_{18} extraction column and analysed by HPLC/UV [7]. The analysis of morphine, normorphine, codeine, norcodeine and their glucuronides has been reported by HPLC electrochemical detection following SPE on C_8 cartridge [8]. Morphine and its glucuronides have been extracted from human plasma [9, 10] and neonatal guinea pig plasma [11] with the use of C_{18} cartridges or from blood plasma with the use of Clean Screen bonded silica columns [12]. Morphine and hydromorphine have been analysed in plasma by HPLC combined with coulometric detection after SPE on C_{18} columns

[13]. Similar methods employing silica based C_{18} extraction columns, have been applied for the extraction of codeine and its metabolites from blood plasma [14, 15, 16] and urine [15, 16]. SPE of cocaine and its metabolites has been reported on some types of columns. Extrelut [17], Amberlite XAD-2 [18], and Chem-Elut [19] columns have been used for the pretreatment of urine [17, 18, 19] or whole blood and plasma [17]. Extraction of such basic drugs takes place in alkaline environment (pH 9.0-10.0), so that the alkaloids obtain their non-protonated form and are strongly retained on the reversed phase microcolumn by non-polar interactions. Graphitised carbon columns have also been used for the SPE of basic drugs [20].

A comparison of different extraction (liquid-liquid or SPE) techniques for the analysis of drugs in biological fluids was the purpose of some studies. Octadecylsilane materials proved to give the best overall results for the extraction of ten representative compounds of acidic, basic, amphoteric hydrophobic and hydrophilic classes, but depending on the class of the compound other materials gave very satisfactory results. Basic drugs were best extracted on polystyrene divinyl benzene or cyanopropyl columns; amphoteric compounds could also be extracted on polymers [21]. Morphine was best extracted from urine by a combination of liquid-liquid and SPE on C_{18} columns [22]. Variation in the type of sorbent, results in variations in solute-sorbent interactions and secondary to matrix-sorbent interactions which finally result in variations in the recovery and the clearness of the samples [23]. The same authors reported manufacturer-to-manufacturer and batch-to-batch variations of the examined C_{18} cartridges. Differences also occurred after SPE from aqueous solutions and serum, indicating inclusion of endogens in the extraction process. Batch-to-batch variations can not be avoided, and are attributed to the variation on content and availability of polar groups on silica [24, 25]. The reproducibility of a SPE extraction procedure is critical, since there are numerous factors affecting the extraction mechanism namely: type of extracted compound, type of sample matrix and extraction sorbent, preconditioning procedure, composition, volume and pH value of the eluting

solvent, mass of the sorbent [1]. The presence of all these factors result to a much more versatile and finally more selective system comparing to a liquid-liquid system, but they also result to low reproducibility. The latter is attributed mainly to the sorbent condition which varies not only between the different types but also between different manufacturers, or even lots of the same manufacturer.

In an earlier report, we compared different SPE cartridges for the RP-HPLC of morphine and codeine in biological fluids [26]. This subject is now expanded in the study of the whole extraction procedure for the analysis of a variety of drugs by HPLC diode array detection. The scope of the work was the study of SPE of selected basic drugs for systematic toxicological analysis. The selected compounds of general clinical-forensic interest were analysed and extracted in a variety of SPE cartridges with different procedures and analysed by HPLC-diode array as previously described [27]. The study included the optimisation of the SPE in order to achieve the best recovery and removal of the interfering compounds.

EXPERIMENTAL

Apparatus

The experiments were carried out in a Shimadzu quaternary low pressure gradient system. The solvent lines were mixed in a FCV-9AL mixer and an LC-9A pump was used to deliver the mobile phase to the column which was thermostated in a CTO-6A oven. Introduction of the samples on the column was achieved by a SIL-9A auto sampler and detection was performed on a SPDM 6A photodiode array detector. Chromatograms were stored on the hard disk of a Laptop 286 PC and printed on a Seikosha SP-1900 printer. Data was analysed both on the Laptop 286 PC and on a Vip 386 PC. Degassing of the solvents was achieved by ultrasonication

under vacuum and continuous helium sparging in the solvent flasks through a DGU-2A degassing unit. All the mentioned apparatus were from Shimadzu (Kyoto, Japan). Separation of the alkaloids was made on an Adsorbospher HS C_{18} 5 µm 250 x 4.6 mm I.D. columns obtained from Alltech Associates (Deerfield, IL).

Materials

Morphine, codeine, 6-monoacetylmorphine, diamorphine, nalorphine, cocaine and benzoylecgonine were obtained from Alltech as methanolic solutions at concentrations of 1000 ppm. Papaverine was obtained from the Forensic Medicine and Toxicology Laboratory of the Aristotle University of Thessaloniki. Flufenamic acid was from ELPEN (Athens, Greece). All the stock solutions were prepared by dissolving the appropriate amount in HPLC grade methanol and kept refrigerated.

Ammonium acetate solutions were prepared by dissolving the appropriate quantity of the analytical grade compound, which was obtained from Merck (Darmstadt, Germany), in double-deionised water. Glacial acetic acid was purchased from Merck. The aqueous buffers after their preparation and pH adjustment, were filtered in a glass vacuum solvent filtration apparatus through a 0.2 µm Anodisk 47 mm glass filter obtained from Alltech. All the organic solvents used in this study, were of HPLC grade and obtained from Merck.

The borate buffer pH 9.2 was prepared by mixing 250 ml of 0.025 M sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) and 18 ml of 0.1M sodium hydroxide. Both compounds were analytical reagent grade and obtained from Merck.

The SPE cartridges used were Bond Elut C_{18}, Bond Elut C_8 (Analytichem International, Varian, Harbor City, CA), Alltech C_{18}, Alltech C_8, Alltech Toxiclean (Alltech Associates, Deerfield, IL), Altech C_{18} Rigas (Rigas Labs, Thessaloniki Greece), Bakerbond C_{18} (J.T. Baker, Gross Gerau, Germany), Separcol C_{18} (Anapron spol Sr.O, Bratislava, Slovakia).

Chromatographic conditions

The chromatographic conditions used were reported previously [27]. The mobile phase was a mixture of MeOH-ACN-1.2% CH_3COONH_4, 40:15:45 (v:v:v) as the mobile phase, at a flow rate of 0.8 ml/min. Quantitation was performed in four selected wavelengths 225, 239, 254 and 289 nm. Especially for the quantitation of cocaine and benzoylecgonine an additional wavelength (232 nm) was chosen due to the low absorbance signal at 289 nm.

Extraction of standard solutions

The first step of the extraction optimisation was the extraction of standard methanolic solutions of the analysed compounds. The microcolumns were preconditioned by elution with 3 ml of methanol, 2 x 3 ml of double deionised water and 2 ml of borate buffer (pH 9.2). Then 100 µl of the standard solutions were applied on column. The columns were washed with 2 x 3 ml of water, dried with application of vacuum (15-20 psi) for 10 min and the alkaloids were eluted from the sorbent with 2 ml of a suitable organic solvent. The resulting solutions were evaporated to dryness in a water bath at 45 ^{o}C, under a gentle stream of nitrogen. The residues were reconstituted with 100 µl of a solution of the internal standard. Aliquots of 20 ml of the resulting solutions were analysed on the HPLC.

All the fractions collected after buffer and sample application or washing during the SPE optimisation in all the samples, were tested for the presence of the analysed alkaloids in order to confirm that no loss occurred in these steps.

The use of the internal standard as a chromatographic standard was chosen in order to have the standard in constant concentration. In most of the reported studies the standard is added before the extraction. In such a case the quantitative results are

also affected by the standards extraction recovery and may be misleading. For example if the absolute recovery of both the standard and the analyte is about 50%, the final results will show a recovery of about 100%. The recovery calculated when the addition of the standard is made just prior to the HPLC analysis, is the absolute recovery of the extraction.

Extraction of blood plasma-serum samples.

The extraction cartridges that proved to be most suitable for the SPE of the alkaloids were tested for the pretreatment of blood plasma and serum samples. Samples of 100 μl of plasma or serum were combined in an Eppendorf tube with 100 μl of a standard solution of opium alkaloids. 200 μl of acetonitrile and 100 μl of the borate buffer were added in the mixtures and the tubes were shaken in the vortex for 1 min and centrifuged for 15 min at 4000 rpm. The supernatants were applied on the SPE columns which had previously been conditioned as already reported. The extraction procedure reported for the extraction of standard solutions, was also followed and the final solutions were evaporated to dryness as previously described. The residues were reconstituted with 100 μl of a 2.5 ng/μl solution of flufenamic acid.

The same procedure was applied for the extraction of cocaine and benzoylecgonine from plasma samples. The final solutions obtained from the SPE were evaporated to dryness and the residues were reconstituted with 100 μl of a 5 ng/μl solution of nalorphine (internal standard)

Extraction of urine samples.

Urine samples could be analysed without a protein precipitation step, however for general toxicological analysis it is advisable to apply such a step together with a

dilution of an unknown sample for better protection of the apparatus. Samples of 100 μl of urine were mixed in Eppendorf tubes with 200 μl of acetonitrile, 500 μl of borate buffer and 100 μl of opiate (or cocaine) standard solution. The mixtures were vortexed for 1 min and centrifuged at 2000 rpm for 5 min. The liquid of each tube was applied to the SPE cartridge which had been previously conditioned as already reported. The extraction procedure reported for the extraction of standard solutions, was also followed and the final solutions were evaporated to dryness as previously described. The residues were reconstituted with 100 μl of the internal standard solution. Especially for the Alltech Toxiclean column an additional preconditioning procedure (the one that is suggested by the manufacturer) was also followed. The column was eluted with 2 x 1 ml MeOH, 2 x 1 ml H_2O and 2 x 1 ml aq. 1 mole/lt CH_3COOH. The urine sample was applied on column and the column was eluted with 2 x 1 ml H_2O and let dry with application of vacuum. The compounds were eluted from the column with 2 x 1 ml of MeOH (procedure B) or with 2 x 1 ml MeOH containing 2% NH_4OH (procedure C).

Quantitative study

Quantitative studies were again divided in two sections: study of the major opium alkaloids and study of cocaine and its metabolites. Calibration curves for the determination of opium alkaloids in plasma samples were constructed using the standard addition method. The samples were spiked at eight different concentrations (0.1-15 ng/μl for morphine, 6-MAM, codeine and 0.02-3 ng/μl for papaverine) and after the selected pretreatment they were repeatedly (five times) analysed on the HPLC. In the urine samples the added concentrations were 0.3-15 ng/μl for morphine, 6-MAM, codeine and diamorphine and 0.06-5 ng/μl for papaverine. The concentration of flufenamic acid (internal standard) was fixed at 2.5 ng/μl.

Cocaine and benzoylecgonine were added on plasma in eight different concentrations in the range 0.1-15 ng/μl and on urine in eight different concentrations in the range 0.083-12.499 ng/μl. Nalorphine (internal standard) was present in all samples at 5 ng/μl.

In both analyses the internal standard was used as chromatographic standard e.g. it was added on the sample just prior to the analysis, in order to have a constant concentration. The final reconstitution of the residues of the extraction was always made with the internal standard solution.

RESULTS AND DISCUSSION

Extraction optimisation

The solvents tested for the elution of the alkaloids from the extraction columns were methanol and a (1:1) mixture of dichloromethane-acetone. The recoveries obtained after the extraction in a variety of columns are given in Table I. Other elution solvents tested, were ethanol and acetonitrile which resulted to lower recoveries. Preconditioning of the columns with a higher pH buffer (pH 10) resulted in losses (especially for cocaine) which were attributed to the alkaline hydrolysis of the analysed compounds. As it is clearly seen in Table I methanol resulted to the best overall recovery for the alkaloids. Elution with the mixture of dichloromethane-acetone gave very good results for the non-polar alkaloids like cocaine, papaverine and diamorphine, but poor recovery for the polar compounds like morphine, codeine and benzoylecgonine.

The recoveries obtained for the extraction from spiked plasma samples are presented in Table II. Diamorphine was rapidly hydrolysed in plasma under the tested conditions, therefore no quantitative analysis could be performed. As it is seen in

TABLE 1.

Absolute Recoveries of Various Cartridges for the SPE of Standard Solutions with Concentrations 5 ng/μl for the Opium Alkaloids and 4 ng/μl of Cocaine Alkaloids. Eluting Solvent is Methanol (A), and a (1:1) Mixture of Dichloromethane-Acetone (B). Details in the Experimental Section.

SPE microcolumn	Alkaloid recovery (%)													
	Morphine		6 MAM		Codeine		Diacetylmor-phine		Papaverine		Benzoylec-gonine		Cocaine	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Bond Elut C_{18}	50.2	33.9	72.5	39.7	55.6	26.3	--	--	79.2	68.17	39.93	31.23	30.26	74.21
Bond Elut C_8	45.2	49.5	51.1	55.7	43.9	59.9	--	--	55.3	109.2	80.22	26.25	89.12	77.16
Altech C_{18}	48.8	30.1	66.9	46.3	37.9	32.5	29.3	48.3	56.8	83.4	47.59	19.56	48.71	68.59
Altech C_8	88.3	37.9	87.5	61.7	105.2	45.5	75.9	32.6	104.9	103.1	32.95	18.65	39.75	96.12
Altech Extrelut	90.9	56.1	95.75	54.2	76.9	19.5	72.1	--	106.2	83.6	--	--	--	--
Altech Toxiclean	85.5	45.2	93.4	46.7	71.2	39.3	70.9	--	100.3	80.9	79.56	29.32	68.82	75.25
Altech C_{18} Rigas	42.1	NT	40.6	NT	39.5	NT	39.1	NT	63.1	NT	NT	NT	NT	NT
Bakerbornd C_{18}	42.6	NT	50.2	NT	42.9	NT	--	NT	59.1	NT	41.21	25.62	35.12	67.95
Separcol C_{18}	48.3	NT	42.7	NT	39.4	NT	22.5	NT	76.2	NT	NT	NT	NT	NT

NT= Not tested

TABLE 2.

Absolute Recoveries of Various Cartridges for the SPE of Spiked Plasma Samples with 5 ng/μl of Opium Alkaloids (1 ng/μl for Papaverine) and 4 ng/μl of Cocaine Alkaloids. Elution Solvent is Methanol, Except (*) Where the Elution Solvent is a (1:1) Mixture of Dichloromethane-Acetone. Details in the Experimental Section.

Analyte	Recovery (%)						
	Morphine	6-MAM	Codeine	Diamorphine	Papaverine	Benzoylecgonine	Cocaine
Altech Toxiclean	88.9	79.6	67.5	19.6	79.9	69.36	71.6
(*)	<10	<10	<10	<10	60.4	NT	NT
Bond Elut C_8	90.1	43.5	29.4	12.5	43.2	55.1	78.25
(*)	<10	<10	<10	<10	59.1	NT	NT
Bakerbond C_{18}	<10	<10	<10	55.2	103.2	34.4	<10
(*)	25.4	63.5	48.9	<10	100.1	NT	NT
Altech C_{18}	NT	NT	NT	NT	NT	31.1	<10
Altech C_8	NT	NT	NT	NT	NT	42.8	46.8
Bond Elut C_{18}	NT	NT	NT	NT	NT	36.0	32.1

NT=Not tested

Table II the best results were obtained with the use of the Altech Toxiclean column. The same column achieved a very satisfactory clean-up, so it was chosen for the SPE of the plasma samples for the rest of the study. Elution of the Bakerbond C_{18} column with methanol, gave very low recoveries for the polar opium alkaloids but high for diamorphine and papaverine. On the contrary, relatively higher recoveries were obtained, with the use of the apolar mixture of dichloromethane-acetone as the eluting solvent. This was in contrast with the results obtained in the other cartridges, and it can be attributed to the high coverage of the silica material on the Bakerbond column and the domination of the apolar interactions in retention of the alkaloids on column, in contrast with the other columns (Bond Elut C_8, Altech Toxiclean) where the sorbent material is assumed to be more polar. Precipitation of the proteins of the plasma or

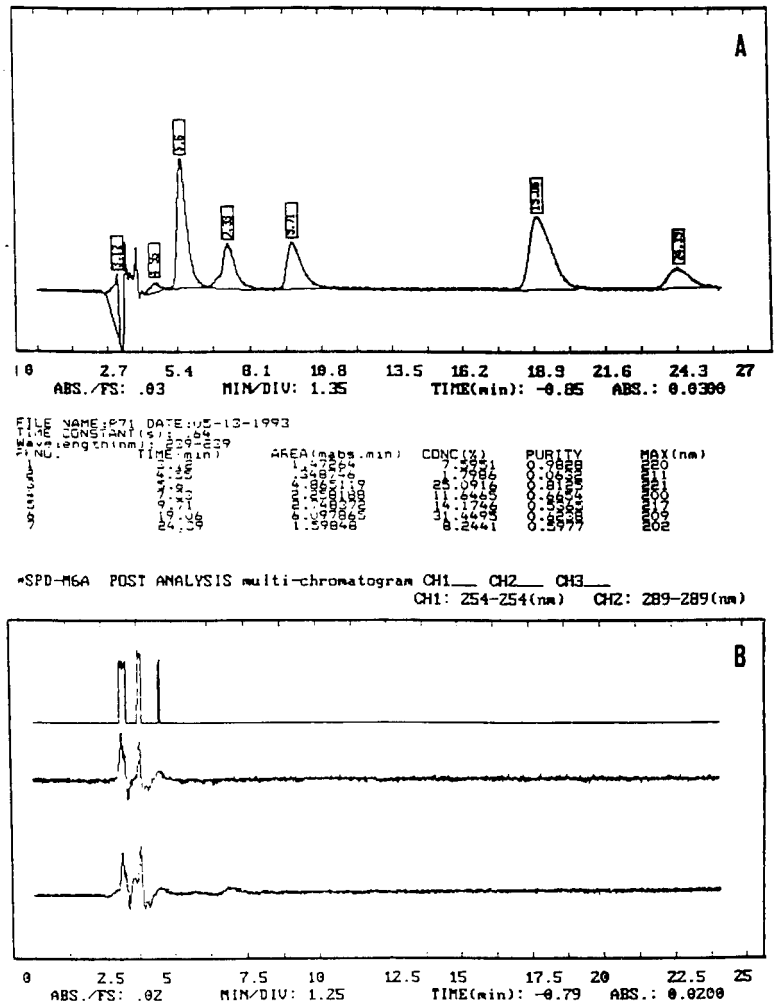

Fig. 1. A: Chromatographic analysis of plasma spiked with morphine (5 ng/µl)=5.6 min, 6-MAM (5 ng/µl)=7.33 min, codeine (5 ng/µl)=9.17 min, papaverine (1 ng/µl)=19.06 min and the internal standard flufenamic acid (2.5 ng/µl)=24.39 min. Quantitation at 239 nm.
B: Chromatographic analysis of the same plasma sample prior to the addition of the alkaloids. Quantitation at 254 and 289 nm.

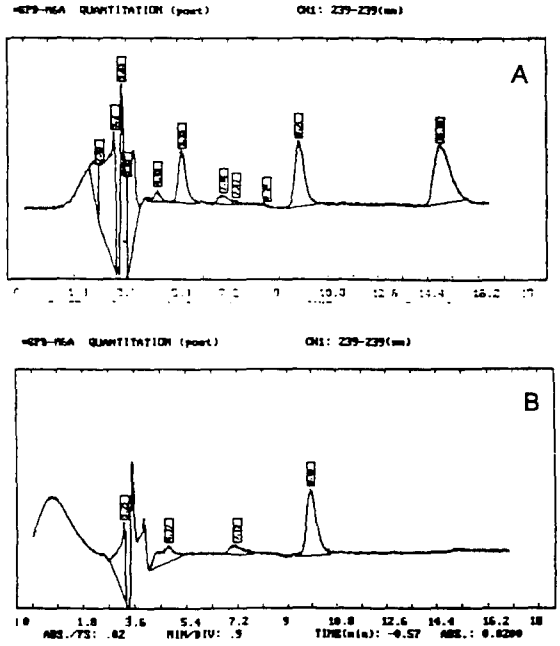

Fig. 2. **(A)** : Chromatographic analysis of plasma spiked with cocaine=14.55 min, benzoylecgonine=5.55 min and the internal standard nalorphine=9.72 min (at concentration 5 ng/μl for all the three compounds).
(B) : Chromatographic analysis of blank plasma with the addition of the internal standard.
Quantitation at 239 nm on both chromatograms.

serum samples was also tried with addition of trichloroacetic acid and it actually resulted in quite "cleaner" samples. Unfortunately the following pH adjustment of the sample, for the SPE was not successful and resulted in low recoveries. A typical chromatogram of a plasma sample spiked with opium alkaloids is given in Fig. 1 A whille Fig. 1 B shows the chromatographic analyis of the same sample prior to the addition of the alkaloids. Fig. 2 shows the chromatographic analysis of a spiked with coca alkaloids (Fig. 2 A) and a blank (Fig. 2 B) plasma sample.

TABLE 3.

Absolute Recoveries of Various Cartridges for the SPE of Urine Spiked with 5 ng/μl of the Opium Alkaloids and 4 ng/μl of Cocaine Alkaloids. Elution Solvnet is Methanol, except Procedure (C) where the Elution Solvent is Methanol with 2% NH_4OH. Details in the Experimental Section.

SPE Cartridge	Alkaloid Recovery (%)						
	Morphine	6 MAM	Codeine	Diacetyl morphine	Papaver ine	Benzoyle cgonine	Coca-ine
Altech Toxiclean (A)	136.9	59.6	70.5	<10	101.2	85.4	84.6
(B)	25.2	<10	<10	<10	35.9	NT	NT
(C)	39.6	42.2	46.5	26.8	129.5	NT	NT
Altech C_{18} Rigas	136.2	39.2	86.5	<10	123	71.4	56.2
Altech C_8	NT	NT	NT	NT	NT	32.6	80.2
Bond Elut C_{18}	139.4	36.9	79.5	36.5	119.3	91.3	78.6
Bond Elut C_8	108.8	72.6	89.5	60.64	112.2	90.1	89.3
Bakerbond C_{18}	51.7	49.6	40.9	21.9	100.0	75.7	79.3

NT=Not tested

The recoveries obtained after the SPE from spiked urine samples are presented in Table III. Bond Elut C_8 proved to be the best fitted column for the pretreatment of urine samples and it was used for the rest of the study. Preconditioning of the Altech Toxiclean column in acidic environment (procedures B and C) did not improve the experimental results. Typical chromatograms of spiked urine and blank samples and urine samples of drug users are given in Fig. 3 for the analysis of opium alkaloids and in Fig. 4 for the analysis of coca alkaloids.

As a general result from the literature [24] but also from the above study and our experience, there should be no judgement of a "bad" or a "good" cartridge. Like in

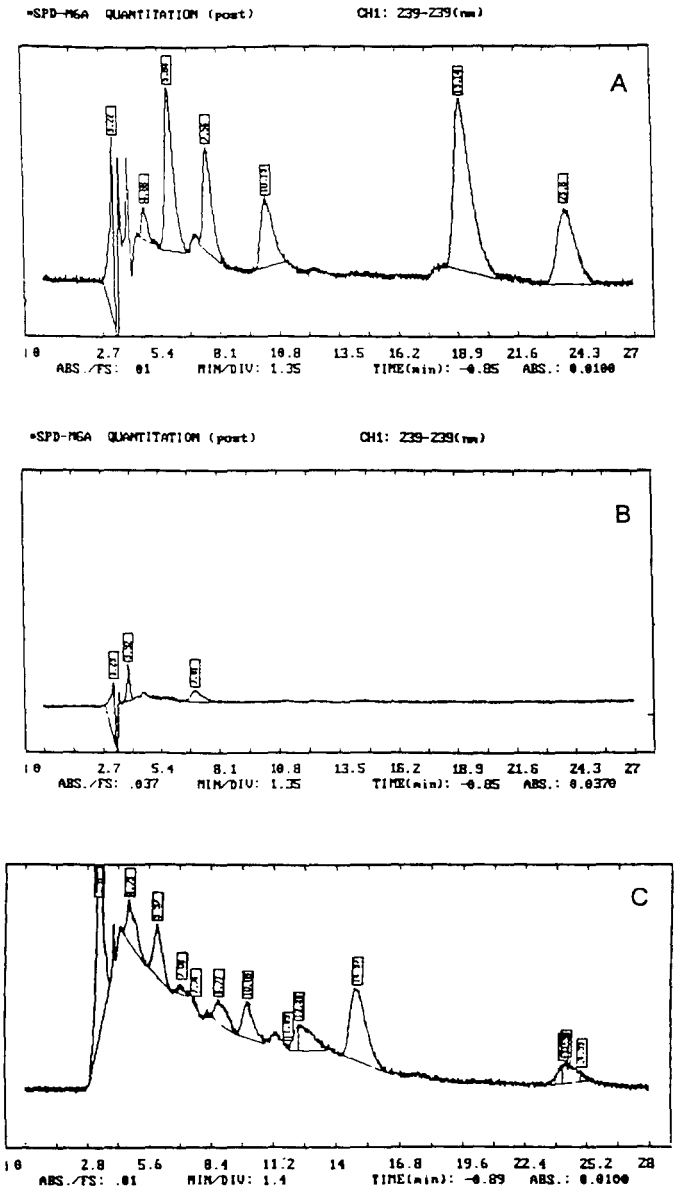

Fig. 3. **A**: Chromatographic analysis of urine spiked with morphine (5 ng/µl)=5.84 min, 6-MAM (5 ng/µl)=7.56 min, codeine (5 ng/µl)=10.19 min, papaverine (1 ng/µl)=19.14 min and the internal standard flufenamic acid (2.5 mg/ml)=25.8 min.
B: Analysis of a blank urine sample.
C: Analysis of urine of a heroine user.
Quantitation at 239 nm.

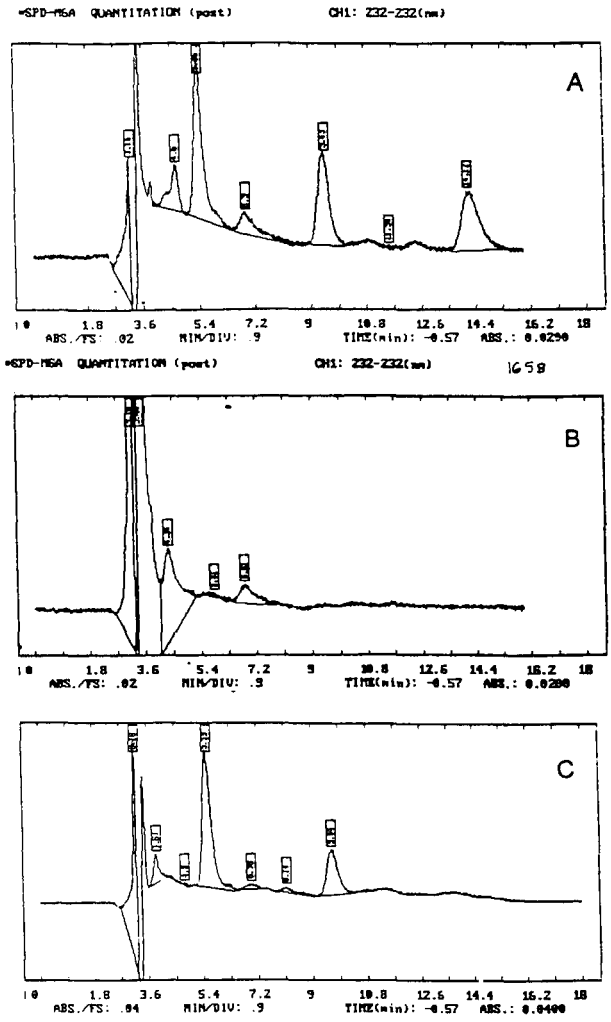

Fig. 4. **(A)** : Chromatographic analysis of urine spiked with cocaine=14.10 min, benzoylecgonine=5.37 min and the internal standard nalorphine=9.41 min(5 ng/μl).
(B) : Chromatographic analysis of blank urine and
(C) : Chromatographic analysis of urine of a cocaine user
Quantitation at 232 nm.

HPLC, some materials are most suitable for a specific application and give very good results for a type of compounds (e.g. polyaromatic compounds) but do not fit for the analysis of other types (alkanes etc.). Multiple interactions take place during the extraction procedure and the presence of the secondary interactions (like ionic interactions of the analyte with the free silanol groups) may prove very useful in the SPE [1, 28].

Quantitation

The simultaneous determination of morphine, 6-MAM and codeine in urine is of great interest for the toxicological analysis since it offers an undoubtful indication of the use of heroine. In the same way the determination of cocaine and its major metabolite benzoylecgonine in urine is the proof of cocaine usage. These two analyses are of the most common tasks of a toxicology laboratory. The linear regression equations obtained after the analysis of spiked plasma samples are presented in Table IV. The best results were obtained with 225 or 239 nm as the detection wavelength. The higher correlation coefficient observed at 254 nm is due to the smaller number of points of the calibration curve at this wavelength. The linear regression equations for the analysis of spiked urine samples are given in Table V.

Reproducibility of the SPE

The reproducibility of the SPE procedure can be evaluated by run-to-run, day-to-day, cartridge-to-cartridge and lot-to lot reproducibilities [1, 26]. The main reason for the different performance of cartridges of the same type is the irreproducibility of the sorbent preparation and packing procedure which according result in variation in the

TABLE 4.

Calibration Curves for the Analysis of Spiked Plasma Samples.

Alkaloid	Wavelength (nm)	Calibration Curve Equation *	Correlation Coefficient (R) *
Morphine	225	Y= - 0.186505 + 0.367174 X	0.99628
	239	Y= 0.11279 + 0.190643 X	0.99964
	254	Y= 0.09163 + 0.362636 X	0.99965
	289	Y= 0.005393 + 0.059263 X	0.99671
Codeine	225	Y= -0.01391 + 0.19836 X	0.99721
	239	Y= -0.00937 + 0.22943 X	0.99847
	254	Y= 0.032505 + 0.244442 X	0.99931
	289	Y= 0.03241 + 0.029747 X	0.99820
6-MAM	225	Y= -0.01391 + 0.1983 X	0.99624
	239	Y= 0.22623 + 0.190643 X	0.99817
	254	Y= 0.04478 + 0.134858 X	0.99935
	289	Y= -0.00236 + 0.02396 X	0.99332
Papaverine	225	Y= 0.040989 + 0.596276 X	0.99375
	239	Y= 0.262112 + 1.52974 X	0.99351
	254	Y= -0.07495 + 1.70751 X	0.99925
	289	Y= 0.004088 + 1.70751 X	0.99991
Benzoylecgonine	225	Y = 0.0010451 + 0.040302 X	0.99861
	232	Y = 0.025065 + 0.051979 X	0.99895
	239	Y = 0.020731 + 0.036586 X	0.99639
	254	Y = 0.035059 + 0.024408 X	0.98224
Cocaine	225	Y = 0.04674 + 0.040147 X	0.99828
	232	Y = 0.004525 + 0.12652 X	0.99884
	239	Y = 0.154942 + 0.069656 X	0.99912
	254	Y = 0.060245 + 0.024408 X	0.98666

TABLE 5.

Calibration Curves for the Analysis of Spiked Urine Samples.

Analyte	Wave-length (nm)	Calibaration Curve Equation	Correlation Coefficient (R)
Morphine	225	Y= 0.216705 + 0.189115 X	0.99854
	239	Y= 0.160075 + 0.176587 X	0.99654
	254	Y= 0.12709 + 0.200581 X	0.99355
	289	Y= 0.03319 + 0.0300719 X	0.99915
Codeine	225	Y=0.149443 + 0.16999 X	0.99594
	239	Y= 0.102895 + 0.12965 X	0.99357
	254	Y= 0.07361 + 0.157462 X	0.99941
	289	Y= 0.020538 + 0.027523 X	0.99630
6-MAM	225	Y= -0.029689 + 0.149111 X	0.99459
	239	Y= 0.11234 + 0.1354113 X	0.99976
	254	Y= 0.108323 + 0.115248 X	0.99959
	289	Y= 0.026214 + 0.020304 X	0.99832
Diamorphine	225	Y= 0.070489 + 0.042835 X	0.99635
	239	Y= 0.07845 + 0.022182 X	0.99712
	254	Y= 0.03645 + 0.023023 X	0.99642
	289	Y= 0.020892 + 0.014042 X	0.99696
Papaverine	225	Y= -0.09392 + 0.420322 X	0.99393
	239	Y= 0.13548 + 1.614382 X	0.99659
	254	Y= 0.133587 + 1.32986 X	0.99695
	289	Y= 0.016933 + 0.088413 X	0.99564
Benzoylec gonine	225	Y = 0.91954 + 0.092464 X	0.99908
	232	Y = 0.21600 + 0.222987 X	0.99932
	239	Y = 0.45642 + 0.24384 X	0.99910
	254	Y = 0.18958 + 0.11288 X	0.99958
	289	Y = 0.07951 + 0.07895 X	0.99509
Cocaine	225	Y = 0.0031656 + 0.07577 X	0.99807
	232	Y = 0.107571 + 0.1876004 X	0.99911
	239	Y = 249856 + 0.223251 X	0.99852
	254	Y = 0.052504 + 0.094247 X	0.99873
	289	Y = -0.02478 + 0.0655867 X	0.99860

TABLE 6.

Reproducibility of SPE of Standard Solutions, Spiked Plasma and Spiked Urine Samples. Sample Concentration 5 ng/μl of morphine, 6-MAM, Codeine and 1 ng/μl for Papaverine in the Plasma Sample. The Urine Sample was also Spiked with 5 ng/μl of Diamoprhine.

Analyte	Altech Toxiclean (plasma)		Bond Elut C_8 (urine)	
	Mean Recovery (%)	RSD (%)	Mean Recovery (%)	RSD (%)
Morphine	88.9	4.64	108.8	6.12
6-MAM	79.6	6.42	72.6	2.98
Codeine	67.5	5.90	89.5	3.87
Diamorphine	--	--	60.64	8.97
Papaverine	79.9	3.78	112.2	4.71

coverage of the hydroxyl groups of the silica base material. Therefore there are differences on the content and the type and activity of polar silanol groups in the sorbent. The reproducibility of the selected SPE procedures was checked as cartridge-to-cartridge reproducibility. Five Altech Toxiclean cartridges of the same batch were used for the repeated SPE of a plasma sample spiked with opium alkaloids. Five Bond Elut C_8 cartridges were used for the analysis of spiked urine samples. The results of this study are given in Table VI. Diamorphine showed the highest variations, phenomenon which was attributed both to the irreproducibility of the quantitation of the chromatographic peak [27] and to the instability of the compound and its possible hydrolysis.

Reusability of the SPE columns

The reusability of the SPE columns was tested by repeating five times the extraction of a spiked with plasma sample on an Altech Toxiclean column and the

TABLE 7.

Recoveries of the Alkaloids from Spiked Plasma and Urine Samples after Extraction on Reused Columns. Plasma Spiked with 5 ng/μl of Morphine, 6-MAM, Codeine and 1 ng/μl of Papaverine. Urine Sample was also Spiked with 5 ng/μl of Diamoprhine.

Analyte	Recovery (%)				
	1st use	2nd use	3rd use	4th use	5th use
Morphine	93.5	94.4	87.1	80.2	72.1
6-MAM	85.3	82.1	77.6	72.3	70.1
Codeine	66.4	68.3	62.1	60.5	58.8
Papaverine	83.4	78.4	73.2	68.9	63.2

extraction of a spiked urine sample on a Bond Elut C_8 column. In both samples an opiate standard solution (5 ng/μl for morphine, 6-MAM and codeine, 1 ng/μl for papaverine) was added and the selected extraction and preconditioning procedure was followed. The regeneration of the columns was made with elution with 3x 1 ml hexane, 5 x 1 ml MeOH and 2 x 1 ml deionised water. Hexane was applied in order to remove the apolar endogenous components that have been retained mostly by hydrophobic interactions and methanol and water in order to remove the polar endogenes that have been retained by ionic interactions and did not elute with the alkaloids. Directly after the regeneration, the borate buffer and the samples were applied on the columns. The results are given in Table VII. As can be seen the recoveries are quite satisfactory for both columns, with a significant loss of performance after each use. The cleanliness of the samples was also satisfactory showing no extra peaks in the late chromatograms. However as it's also recommended in the literature [1], it's not advisable to reuse the SPE columns, especially after the use of a high pH buffer, which may deteriorate the silica based material.

CONCLUSIONS

Different SPE columns and procedures were tested for the pretreatment of blood plasma-serum and urine samples. The type of the sorbent, the preconditioning procedure and the nature of the eluting solvent are of the most important factors in the extraction optimisation. The selected extraction procedure was found reproducible and precise. The method was also applied in the analysis of urine samples of heroin and cocaine users. Detection of the alkaloids should preferably be performed at the low UV region. Reuse of the SPE columns results in decreased extraction performance.

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SEPARATION OF BIOACTIVE QUADRI-TERPENIC ACIDS FROM THE FRUIT OF *LIGUSTRUM LUCIDUM AIT* BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

High-speed countercurrent chromatography (HSCCC) was used for the separation of quadri-terpenic acids from the fruit of *Ligustrum lucidum Ait* using a two-phase solvent system composed of hexane/ethyl acetate/methanol/water (3:6:2:1, v/v). From 250mg of the crude extract, the method yielded 87 mg of oleanolic acid at 91.5% purity and 58 mg of ursolic acid at 93.2% purity in about 2.5 h.

INTRODUCTION

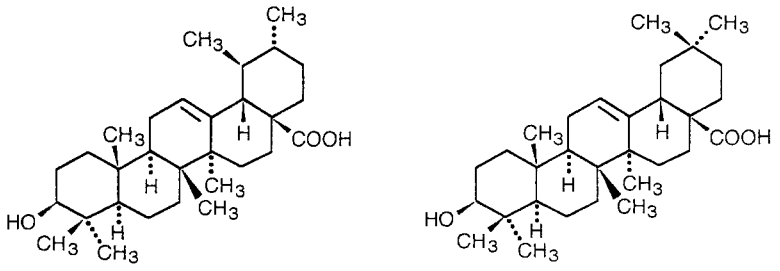
Oleanolic acid and ursolic acid are similar bioactive quadri-terpenic acids (Fig. 1), both being used

as anti-inflammatory and anti-tumor drugs (1). In the earlier studies (2-4), oleanolic acid was considered as a main bioactive component in *Ligustrum lucidum Ait*. Recently, HPLC analysis revealed that the plant extract also contained ursolic acid which often exceeds the amount of oleanolic acid (5). Wu et al. (6) also isolated ursolic acid from the fruit of *Ligustrum lucidum Ait* by column chromatography. In this paper, we conducted a separation of oleanolic acid and ursolic acid from the fruit of *Ligustrum lucidum Ait* by high-speed countercurrent chromatography (HSCCC) (7).

EXPERIMENTAL

Apparatus

HSCCC experiments were performed using a coil planet centrifuge equipped with a multilayer coil column that was designed and fabricated at the Beijing Institute of New Technology Application, Beijing, China. The multilayer coil was prepared by winding a 1.6mm ID PTFE (polytetrafluoroethylene) tube coaxially onto the column holder hub. The total capacity of the column measured 230 ml. The HSCCC centrifuge was equipped with an FMI pump (Zhejiang Instrument Factory, Hangzhou, China), an injection valve and a fraction collector.



Oleanolic acid **Ursolic acid**

FIGURE 1. Chemical structures of oleanolic acid and ursolic acid.

Reagents

All organic solvents were of analytical grade and purchased from Shanghai Chemical Factory, Shanghai, China. Oleanolic and ursolic acids standards were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Analytical grade phosphomolybdic acid was purchased from Beijing Chemical Factory, Beijing, China.

Extraction of Terpenic Acids

The extraction was initiated by degreasing 200g dried powder of *L. lucidum* fruit by petroleum ether followed by extracting twice each with 1000 ml of chloroform for 2 hours at 80°C. The chloroform extract was combined and evaporated to dryness. Then the residue

was dissolved in 50ml of ethanol and decolorized by filtering through active carbon. Ethanol was removed under reduced pressure at 60°C and the residue was in turn dried in vacuum. This procedure yielded 3.2g of light yellow powder which was subsequently subjected to HSCCC separation.

HSCCC Separation

HSCCC experiment was performed with a two-phase solvent system composed of hexane/ethyl acetate/ methanol/water (3:6:2:1, v/v). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated before use. In each separation, the multilayer coil was first entirely filled with the upper stationary phase. The lower mobile phase was pumped into the inlet of the column at a flow rate of 1.5 ml/min, while the apparatus was rotated at 800 rpm. After the mobile phase front emerged and the two phases had established hydrodynamic equilibrium, the sample solution (250 mg of the extract in 10ml of the mobile phase) was injected through the injection valve. The effluent from the outlet of the column was collected into test tubes with a fraction collector. Each fraction was subjected to color reaction analysis using phosphomolybdic acid (8) (Table 1).

TABLE 1
Color Reaction Analysis of HSCCC Fractions

Fraction No.[1]	Retention Time (min)	Color Reaction[2]	Component[3]
1	48	-	
3	54	-	
5	60	-	I
7	66	-	I
9	72	-	
11	78	-	
13	84	+	II
15	90	+	II
17	96	-	
19	102	++	III
21	108	+++	III
23	114	+++	III
25	120	++	III
27	126	-	
29	132	+	IV
31	138	++	IV
33	144	++	IV
35	150	++	IV
37	156	+	IV
39	162	-	

[1] Fraction volume: 4.5 ml/tube
[2] Reaction condition: Each fraction (5μl) on GF254 plate (Merck) was treated with 5% of phosphomolybdic acid in ethanol for 10 minutes at 110°C.
- negative; + light purple; ++ purple; +++ dark purple.
[3] Component I is itself yellow; Components II, III and IV are colorless.

HPLC Analysis

The crude extract and the HSCCC fractions were analyzed by HPLC using Shimadzu HPLC equipment (Shimadzu Corporation, Kyoto, Japan) consisting of an LC-10AD pump, an SPD-10A UV-VIS detector, a manual injector and a C-

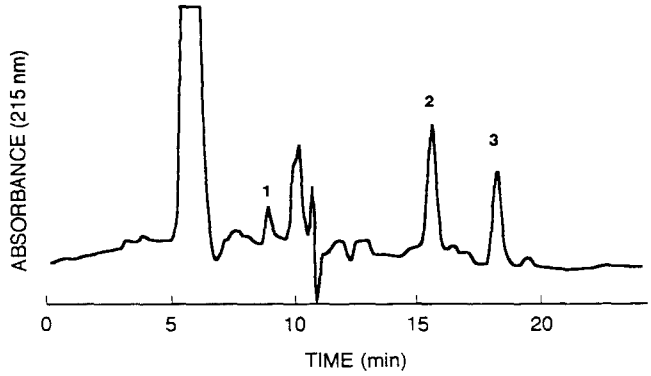


FIGURE 2. HPLC analysis of the crude extract of *L. lucidum* fruit. Mobile phase: methanol-water (9:1, v/v); flow-rate: 0.5 ml/min; detection 215 nm. Peak 1: unknown compound; peak 2: oleanolic acid; and peak 3: ursolic acid.

R10A recording processor. The analyses were performed with Shim-pack CLC-ODS-C_{18} column, 15 X 0.60cm ID (Shimadzu). The mobile phase composed of methanol-water (9:1, v/v) was isocratically eluted at a flow-rate of 0.5 ml/min and the effluent was monitored at 215 nm.

RESULTS AND DISCUSSION

HPLC analysis (Fig. 2) of the crude extract from the fruit of *Ligustrum lucidum Ait* showed that it contained oleanolic acid (peak 2) and ursolic acid (peak 3) by comparison with authentic samples. A 250mg amount of the crude extract was separated by HSCCC. Tests with

phosphomolybdic acid of the CCC fractions revealed the colors shown in Table 1. Component I (which was itself yellow) failed to give a positive test while the other three components (themselves colorless) gave a purple color characteristic of quadri-terpenic acids (8). Component III (corresponding peak 2 in HPLC) and Component IV (corresponding to peak 3 in HPLC) were oleanolic acid (87 mg, 35% in weight, 91.5% pure by HPLC) and ursolic acid (58 mg, 23% in weight, 93.2% pure by HPLC), respectively. Recrystallization from methanol produced 41 mg of oleanolic acid that was 98.5% pure (HPLC) and 39 mg of ursolic acid, 97% pure (HPLC). Fractions containing component II (corresponding to peak 1 in HPLC) produced 7 mg of an unknown compound. On the basis of its short retention time in both HPLC and CCC together with a positive color reaction, this compound appears to be a polar derivative relating to the above products.

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ANALYTICAL PROCEDURE FOR DETERMINATION OF S-ADENOSYLMETHIONINE, S-ADENOSYL-HOMOCYSTEINE, AND S-ADENOSYLETHIONINE IN SAME ISOCRATIC HPLC RUN, WITH A PROCEDURE FOR PREPARATION AND ANALYSIS OF THE ANALOG S-ADENOSYLHOMOCYSTEINE SULFOXIDE

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ABSTRACT

Virtually all methyltransferase enzymes are regulated largely by the relative levels of S-adenosylmethionine (SAM) to its metabolic product, S-adenosylhomocysteine (SAH). Ethionine is the hepatocarcinogenic antimetabolite of methionine, and has been found to produce hypomethylation of hepatic DNA when fed to rats in acute doses. The hypomethylation apparently results from the accumulation in the liver of S-adenosylethionine (SAE), the sulfur activation product of ethionine, which is a competitive inhibitor of DNA methylase. Researchers seeking to measure SAM and SAH levels by HPLC in the past have experienced numerous analytical problems because of their separation characteristics. Previous methods have either required two separate HPLC runs or used gradient elution to measure the two compounds. The method outlined here, is an accurate and precise method, that measures SAM and SAH as well as SAE in a single isocratic HPLC run. S-Adenosyl-l-homocysteine sulfoxide

(SAHO), the sulfoxide of SAH is known to be formed by spontaneous oxidation of SAH during sample preparation and storage. We have prepared the SAHO using a 4 hour process. Since SAHO is not readily available commercially, the present method could be very beneficial to researchers who need to verify whether SAH oxidation has occurred in analytical samples, or whether oxidation of the SAH has occurred in tissues.

INTRODUCTION

S-adenosylmethionine (SAM), the substrate of DNA methylase, is a major methyl group donor used in all tissues by all methyltransferase enzymes except the enzymes that synthesize methionine. These enzymes are regulated largely by the relative levels of SAM to its metabolic product, S-adenosylhomocysteine (SAH), a DNA methylase inhibitor (1). A decrease in the SAM/SAH ratio, either by reduction in SAM or by increase in SAH, can inhibit the activity of methyltransferase enzymes (2). Studies with rats and mice fed either amino acid-defined diets lacking methionine and choline or choline-devoid diets containing proteins with low methionine content (3-8), have shown decreased levels of hepatic SAM (9,10) and suppressed level of hepatic DNA methylation (14). These methyl-deficient diets have also caused increased hepatic levels of SAH, further decreasing SAM availability (10). Further, the chronic administration of these methyl-deficient diets has been shown to produce liver cancer in male F344 rats and B6C3F1 mice (3-8). Studies have shown that in rats the hepatic contents of SAM and SAH are more dramatically altered by chronic dietary methyl deprivation than are SAM and SAH levels in other tissues (10). Such results indicate that levels of DNA methylation are subject to dietary manipulation and support the postulated relationship between methyl deficiency and hepatocarcinogenicity (5,6 and 15-19).

In eukaryotic cells, the extent and patterns of DNA methylation appear to regulate the expression of genetic information (20-22). Any circumstance that causes a decrease in the SAM/SAH ratio results in inhibition of DNA methylation. Thus, it has been suggested that resulting alterations in gene expression might induce tumor formation.

Physiological methyl insufficiency may also play a causative role, at least in part, in a variety of toxic and pathologic endpoints produced by a multiplicity of circumstances. Recently, there has been increasing interest in this area of research, as evidenced by the literature and reports at scientific meetings. Thus, convenient methodologies for monitoring such studies are necessary.

Ethionine is the hepatocarcinogenic antimetabolite of methionine and has been found to produce hypomethylation of hepatic DNA when fed to rats in acute doses (11). The hypomethylation apparently results from the accumulation in the liver of S-adenosylethionine (SAE), the sulfur activation product of ethionine, which is a competitive inhibitor of DNA methylase (11-13). Thus, ethionine is a convenient material for use in hypomethylation studies. Researchers are often interested in determining SAM and SAH and sometimes SAE in the same sample. Therefore, it would be most convenient to determine the levels of all of the compounds in the same HPLC run.

Researchers seeking to measure SAM and SAH levels by HPLC are aware that the compounds are rather unique and cause special analytical problems because of their separation characteristics. For example, HPLC retention times are greatly affected by small pH changes. Molloy *et al.* (23) have reported an HPLC method that measured SAM and SAH in the same run, but their method used a gradient. The method outlined here accomplishes the same results using only one buffer and can be used to measure SAE as well. The stable conditions of isocratic chromatography have the advantages of being more convenient and efficient as well as giving more accurate quantification than a gradient, since the baseline of a chromatogram from a gradient usually drifts, mechanical errors such as pump variations are eliminated, and the HPLC does not have to return to initial conditions and equilibrate between each sample.

S-Adenosyl-l-homocysteine sulfoxide (SAHO), the sulfoxide of SAH, is known to be formed by spontaneous oxidation of SAH during sample preparation and storage. Duerre *et al.* (24) developed a method for preparation and characterization of SAHO from SAH. The method they reported required a 10

to 12 hour waiting period, and the addition of crystalline beef liver catalase. Their characterization used paper chromatography and ultraviolet light absorption. We have prepared the SAHO using a 4 hour process, without addition of catalase. Since SAHO is not readily available commercially, the present method could be very beneficial to researchers who need to verify whether SAH oxidation has occurred in analytical samples, or whether oxidation of the SAH has occurred in tissues.

MATERIALS AND METHODS

Extraction Procedure

Approximately 0.7 g (0.4-1.0 g) of tissue was weighed immediately after sacrifice, diced, and transferred to a 10 ml culture tube. The SAM, SAH and SAE were extracted from the tissue using the method of Shivapurkar and Poirier (10), with modifications. A volume of 0.1 M sodium acetate, pH 6.0, (ice cold) equal to two times the weight of the tissue was added. The resulting solution can be stored in ice up to 1 hour while other samples are being prepared. The samples were then homogenized with a Tissuemizer (Tekmar, Cincinnati, OH) at setting of 45 for 30 (3 x 10) seconds (samples must be kept cold). The protein was precipitated using 40% (w/v) trichloroacetic acid (a volume 1.5 times the original weight of the tissue). The solution was mixed well on vortex and placed in ice for 30 minutes. To remove the precipitated protein, the tubes were spun in a CRU-5000 centrifuge (International Equipment Co., Needham Heights, MA), maintained at 5°C, at 1000 x g for 10 minutes. The supernatant containing SAM, SAH, and SAE was decanted into a conical shape glass centrifuge tube and the volume was recorded. If necessary the procedure could be interrupted at this point. Supernatant should be stored at −70°C until assayed and should not be stored more than one week. An equal volume of ice-cold peroxide-free diethyl ether was added (to adjust to pH to 3.3). The ether must be peroxide-free (1 ppm or less), or the SAH will be oxidized rapidly to form SAHO. The tubes were

vortexed for 20 seconds and then were spun in a centrifuge to accomplish separation of the phases. The unwanted top layer was aspirated off, using a pasteur pipette attached to vacuum. Any solid matter at the interface was also removed when possible. The extraction steps were repeated once. The samples were filtered through a millipore filter (0.45 μm). The samples were then ready for HPLC analysis for SAM, SAH and SAE. Samples should be kept cold at all times until analyzed and should be injected onto the HPLC within 24 hours in order to minimize decomposition of the SAM and SAH content.

HPLC Analysis

Twenty (20) μl (or an appropriate volume) of the extracted samples were injected onto a Hewlett-Packard 1090 HPLC (Wilmington, DE), equipped with a Beckman Ultrasphere ODS, 4.5 mm x 25 cm (5μm particle size) column (Fullerton, CA) maintained at 25.5°C using a column heater, a pre-column filter (#C-751, ChromTech, Apple Valley, MN), and a Hewlett-Packard photodiode array detector operated at 260 nm. The mobile phase was 50 mM NaH_2PO_4, 10 mM Heptanesulfonic acid (sodium salt, 1-hydrate, HPLC grade, Eastman Kodak, Rochester, NY), 20% methanol adjusted to pH 4.38 with phosphoric acid, with a flow rate of 0.9 ml/min. The mobile phase was degassed for at least 1 hour before the pumps were started. The column was equilibrated for 1 hour before injections were started.

Tissue concentration calculation

External analytical standards of SAM (#102407, Boehringer Mannheim, Indianapolis, IN), SAH (#102393, Boehringer Mannheim, Indianapolis, IN), and SAE (#A-2758, Sigma, St. Louis, MO) were dissolved in 0.001 N HCl. A series of concentrations of each compound was injected onto the HPLC and concentration response curves were constructed. Standards of SAM, SAH, and SAE were injected at concentration ranges of 1-10 ng/μl.

The following calculation was used to determine the concentration of SAM, SAH or SAE in tissue samples:

$$\frac{\mu g}{g \text{ tissue}} = \frac{\text{Area of peak}}{\text{slope}} \times \frac{\text{total vol. (ml)}}{\text{sample wt (g)}}$$

Preparation of S-adenosyl-l-homocysteine sulfoxide (SAHO)

Using the analytical method outlined above, we discovered that over a period of one week the SAH in both the standards and samples may be completely converted to another product by the presence of even a minute quantity of a strong oxidizing substance, such as peroxide (>1ppm). The HPLC peak for the SAH gradually diminished as a new peak appeared at an earlier retention time and increased nearly proportional in area. The new product was identified to be SAHO using Mass Spectoral Analysis by Fast Atom Bombardment (FAB) and NMR.

Duerre *et al.* (24) reported a procedure for preparation of the SAHO in 1970; we have developed a faster and simpler procedure to produce the chemical in pure form. SAH was oxidized with hydrogen peroxide (H_2O_2) (#H 341-500 Fisher, Pittsburgh, PA) to produce SAHO. SAH standard, 20 mg, was placed in a test tube with 1 ml of methanol. The SAH did not dissolve readily in methanol, leaving some of the solid material on the bottom of the test tube. A 300 μl volume of 50% H_2O_2 was added, and the tube was vortexed, and sonicated briefly. As the H_2O_2 reacted, the SAH was dissolved. After the solution was allowed to stand at room temperature for $3^1/_2$ hours, a 5 μl aliquot was diluted with 200 μl of water and injected onto the HPLC to confirm that all of the SAH had been converted to the new product. When the SAH peak had disappeared, the resulting product was precipitated using 5 ml of cold (-20°C) absolute ethanol (it was necessary that the ethanol be very dry or the product which is very soluble in water would redissolve). The test tube was allowed to stand in the freezer for about 30 minutes and spun in a CRU-5000 Centrifuge at 1000 x g for 10 minutes

at 5°C. The ethanol which contained the H_2O_2 was decanted off. The product in the bottom of the test tube was rinsed with an additional 5 ml of cold absolute ethanol, vortexed and centrifuged. The ethanol was poured off and the precipitate was dried in the Speed Vac (Savant, Farmingdale, NY). The final product, which was a dry white solid, was stored in the freezer over a desiccant. The yield was about 90% by weight. A small amount of the material was dissolved in water for injection onto the HPLC. Injections were made onto HPLC using two different mobile phases to obtain two separate chromatograms. One of the mobile phases was the one described above, used to separate SAM, SAH and SAE. A single peak with a retention time of 3.4-3.6 minutes (Figure 1A) was obtained, compared to a 5.4-5.6 minutes (Figure 1B) retention time for SAH. The second mobile phase used was 15% methanol in water which also showed a single peak, this time with a retention time of 4.6 minutes, compared to 8.1 minutes for SAH. The identity of the product (SAHO) and the SAH were confirmed by NMR and FAB.

RESULTS AND DISCUSSION

We have developed a precise and accurate HPLC method for measuring SAM, SAH, and SAE in the same run. The advantage of the new method was that we could measure all three compounds in only 15 minutes (Figure 2). The retention times were as follows:

Compound	Standards	Samples
SAH	~ 5.5 min.	~ 5.5 min.
SAM*	~ 9.5 min.	~ 10.5 min.
SAE*	~ 10.5 min.	~ 11 min.

*The differences in retention times between samples and neat standards were due to pH differences of the material injected. Standards taken through the reaction process, have the same RT as the samples. (Figures 2 A-D).

We compared our previous method of measuring SAH on the HPLC, using 0.05M KH_2PO_4 in 8% methanol (14), with an approximate retention time

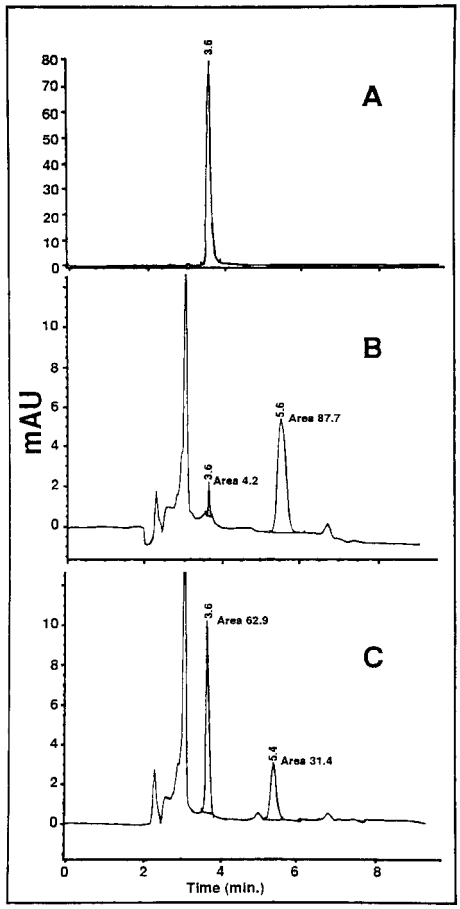

FIGURE 1

HPLC of: A. Purified oxidation product of SAH, identified to be SAHO by NMR and FAB, B. Standard SAH (3.0 ng/μl) treated as a sample using ether containing over 1 ppm. peroxide, and C. Sample B. run again 7 days later. SAH peak has diminished, and SAHO peak has grown proportionally over a period of time.

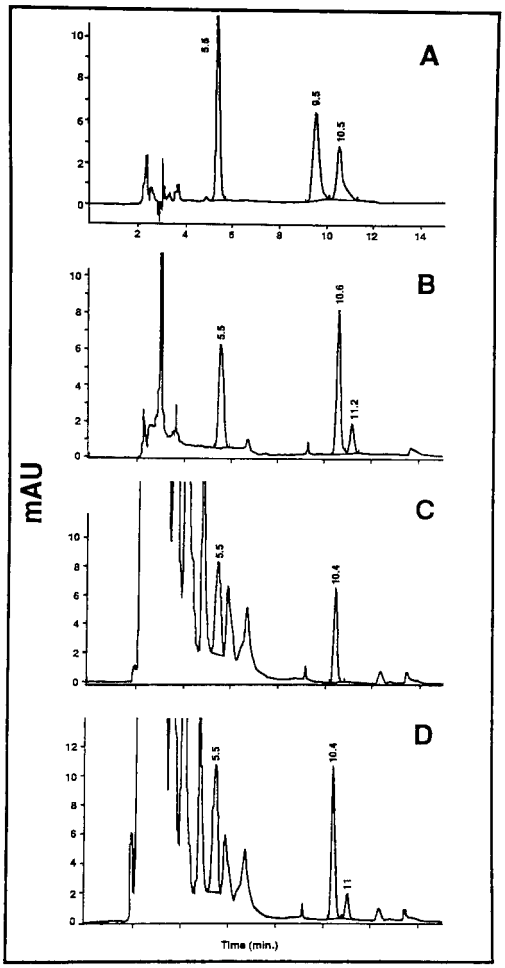

FIGURE 2

Isocratic separation using the present HPLC method of: A. Standards of 3 ng/μl SAH (5.4 min.), SAM (9.5 min.) and 1.5 ng/μl SAE (10.5 min.) in 1 x 10^{-4} N HCl, B. Standards of 3 ng/μl SAH (5.5 min.), SAM (10.6 min.) and 0.75 ng/μl SAE (11.2 min.), processed as samples, C. Rat liver sample, SAH (5.5 min.) and SAM (10.4 min.). (Normal rat liver does not contain SAE.), IID. Rat liver sample with standards of 1.5 ng/μl SAH (5.5 min.), SAM (10.4 min.) and 0.75 ng/μl SAE (11.0) added.

of 8.5 minutes and a total run time of 25 minutes with our new method. Both methods measured the SAH accurately and precisely when we ran the same sample by the two different methods.

Using the present method we measured the amount of SAM and SAH in rat liver tissue (Figure 2C). We ran the same extract sample again after small amounts of the standards SAM, SAH and SAE had been added (Figure 2D). The size of the SAM and SAH peaks increased proportionately to the amount of standard added, and the SAE peak appeared. Since normal rat liver does not contain SAE, it will only be found in the liver of treated rats. We have used this method in our laboratory to measure SAM and SAH in various tissue, including liver, spleen, brain, kidneys and in whole rat embryos. We have been able to measure amounts as small as 2.5 μg per gram of tissue, and have found that SAH was not present at detectable levels in some tissue, such as some rat brain samples. This lack of SAH in the rat brain tissue was confirmed by running the samples again using our previous method of measuring SAH.

Experiments have shown accurate pH and ionic concentrations of the mobile phase to be very important. Higher pH gave shorter retention times while higher concentration of NaH_2PO_4 moved SAM and SAE closer to the SAH. Lower methanol content moved SAM and SAE together. These facts are useful if one of the peaks needs to be moved to avoid interfering peaks.

Using the HPLC method describe here, we have shown that samples should be analyzed quickly to avoid oxidation of SAH to SAHO. We have described a procedure for preparing SAHO from SAH in just four hours with a yield of approximately 90% by weight. Samples of the product and the SAH were analyzed by NMR. The hydrogens on the carbons adjacent to the sulfur atom showed the greatest shift, as would be expected if the sulfur was bonded to an oxygen atom. The NMR indicated nearly equal amounts of 2 very similar products, possibly the two stereoisomers of the sulfur atom being made asymmetric by oxidation. A sample was subjected to FAB to determine whether there was 1 or 2 oxygens attached to the sulfur atom. The molecular weight of

the product was found to be 400, which is the molecular weight of the SAHO ($C_{14}H_{20}N_6O_6S$). The product was determined to be 99% pure. The melting point of the solid material was 185-187°, where it decomposed to a brown product. This agrees with reports in the literature which states the SAHO decomposes at 185-188° (25).

Experiments were also performed to determine whether it would be possible to identify SAHO if it were present in a liver sample using the HPLC method described above. We were able to find a peak in liver samples with a retention time that corresponded with SAHO. The peak area increased in size when SAHO was added to the sample. It was also determined that when SAH was added to a sample that had been extracted with ether containing >1 ppm peroxide, and aliquots injected onto the HPLC over a period of 2 days, the SAH peak decreased and the SAHO peak increased. This indicated the importance of analyzing the prepared samples as soon as possible, since strong oxidants in the samples may cause errors in measuring the SAH. When samples extracted with ether containing >1ppm peroxide were stored in the refrigerator for more than 2 months, the SAH peak completely disappeared.

As researchers become more and more interested in studying DNA methylation, it is important that methods are available to accurately measure the various components used in the methylation process. The method described above, is an accurate and precise method, that has the advantages of measuring three compounds SAM, SAH and SAE.

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HPLC SEPARATION OF TWO NOVEL DIKETOPIPERAZINE ISOMERS ON POLYVINYL ALCOHOL FUNCTIONALIZED SILICA GEL

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ABSTRACT

An HPLC method is described for the analytical separation of two novel diketopiperazine isomers isolated from a fungal culture, *Tolypocladium* sp. (SCF-0729). Separation was achieved on a high-performance polyvinyl alcohol silica gel column with a 1-chlorobutane: methanol gradient system. Selectivity, retention and resolution obtained with this support were all highly reproducible. Direct scale up of analytical methodology allowed for the preparative separation of this isomeric pair.

INTRODUCTION

Adsorption chromatography employing native silica gel often provides significant capabilities in the resolution of isomeric mixtures.[1-4] Modifications to the silica (or alumina) backbone such as silver ion impregnation (argentation) can further enhance the separation of isomeric

pairs.[5-6] This capability results from differential pi-pi interactions of olefinic containing compounds with the immobilized metal ion. Despite this capability, significant difficulties arise when labile mixtures, sensitive to moisture and acid, need to be resolved. Recent developments in silica gel surface modifications have expanded the capabilities for isomeric separations. In the case of polyvinyl alcohol (PVA) functionalization, spherical silica particles are coated with mono-molecular vinyl alcohol followed by polymerization of the vinyl alcohol. This process accesses both external and internal surfaces of the support resulting in a phase devoid of acidic silanols.[7] This alcoholic support surface has proven to be a highly selective and stable normal phase matrix for the otherwise difficult separation of mixtures.[8]

In the course of searching for bioactive substances from fungal fermentations, we have isolated Sch 54794 and Sch 54796 from the fermentation of *Tolypocladium* sp (SCF-0729).[9] These diketopiperazines were part of an isomeric mixture that decomposed on silica gel. We wish to describe the baseline separation of this isomeric mixture on polyvinyl alcohol coated silica gel with 1-chlorobutane:methanol as the mobile phase.

EXPERIMENTAL

Materials and Methods

HPLC grade 1-chlorobutane and methanol were obtained from Fluka Chemic AG (Buchs, Switzerland) and Fisher Scientific (Pittsburgh, PA, USA), respectively.

Chromatographic Apparatus

Analytical separations were performed on an integrated Hewlett Packard (Palo Alto, CA) HP 1090 Series II Liquid Chromatographic System comprising a PV-5 ternary solvent delivery module, a 250 µl auto injector, and a photodiode array detector equipped with a standard 8 µl flow cell of 6 mm path length. A YMC Inc. (Wilmington, North Carolina) PVA-Sil 120 Å, 5 µm (4.6 mm x 15 cm) column was employed which was preceeded by a guard column (4.0 mm x 2.3 cm) containing the same stationary phase. A linear gradient of 2-10% methanol in 1-chlorobutane over 20 minutes was utilized to optimize separation and resolution. Samples were dissolved prior to injection in 5% methanol/1-chlorobutane to obtain 1 mg/ml solutions. Analytical flow rate was 1 ml/min and U.V. detection was either at 220 or 275 nm.

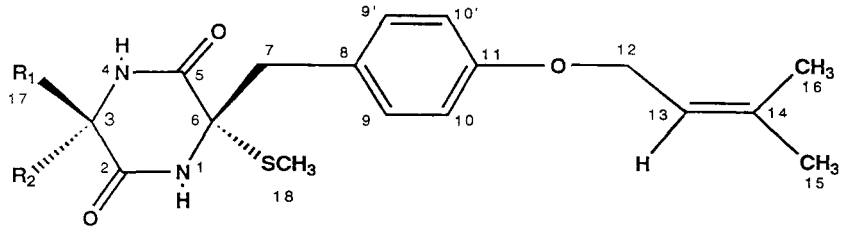
Semi-preparative separations were performed on a system comprised of the following components: a Waters 600 multi-solvent delivery module (Waters Chromatography Division, Milford, MA) equipped with a Rheodyne Model 7125 manual injector (Rainin Instrument Co., Woburn, MA) that contained a 2 ml sample loop. Sample components were detected with a Waters Model 481 variable wavelength UV detector set at 275 nm. Signals were plotted on a Waters Model 740 data module and monitored at an attenuation of 1024. Chromatographic support was a YMC, Inc., PVA-Sil 120 Å, 5 µm column (20 x 250 mm) that was preceded by a 30 x 10 mm guard column. Mobile phase conditions were identical to that described under analytical except flow rate was 8 ml/min. The semi-preparative column received from YMC Inc. was shipped in 0.5% sodium azide. Because PVA-Sil media is primarily used

as a size exclusion support for biomolecular separations, a test mixture of thyroglobulin, albumin, β-lactoglobulin and cytochrome C was separated with 0.2 M NaCl/0.1 M sodium phosphate buffer (pH 7). Before normal phase chromatography could be performed, the column was thoroughly washed with the five bed volumes (300 ml) of water, followed by (300 ml) of methanol before equilibration with mobile phase.

Preparative reverse phase chromatography was performed with instrumentation as described in the semi-preparative section. A YMC Inc. C-18 column 120 Å, 15 um/irregular media (30 x 500 mm) was preceded by a (30 x 50 mm) guard column. The *cis/trans* diketopiperazine mixture was enriched, but co-eluted under a 20 minute linear gradient of 80-90% methanol in water at 20 ml/min.

RESULTS AND DISCUSSION

As shown in Figure 1, Sch 54794 (**1**) and Sch 54796 (**2**) are *cis/trans* diketopiperazine isomers that were obtained from the fermentation of *Tolypocladium* sp. (SCF-0729). Purification of the isomeric pair involved ethyl acetate extraction of the fermentation broth (8L). This yielded 3 g of an oily residue that was dissolved in methanol: methylene chloride (1:1). After removal of insoluble material, the bioactive soluble portion was concentrated *in vacuo* and then precipitated with hexane. The precipitate (1.8 gm) was further purified on a C-18 preparative column. This yielded 500 mg of the *cis/trans* isomeric mixture which could not be resolved utilizing silica, neutral or basic alumina, nor by HP-20P or LH-20, chromatographic approaches.



Sch 54794 (1) : $R_1 = H$ $R_2 = SCH_3$
Sch 54796 (2) : $R_1 = SCH_3$ $R_2 = H$

FIGURE 1: Structure of diketopiperazines

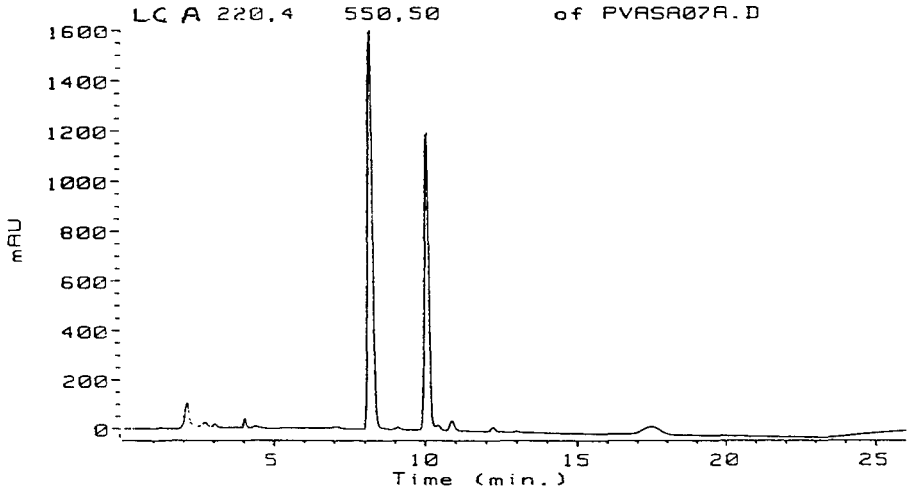


FIGURE 2: Analytical separation of *cis/trans* mixture (50 µg)
(see Experimental section for details)

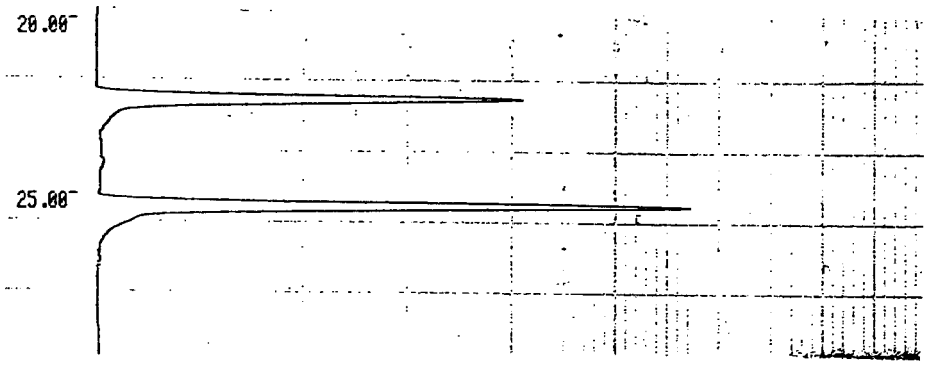


FIGURE 3: Semi-preparative separation of diketopiperazine *cis/trans* mixture (see Experimental section)

Figure 2 illustrates the excellent separation of this mixture on PVA-Sil. Retention times for the pure *cis* isomer (**1**) and pure *trans* isomer (**2**) were 8.2 and 10.1 minutes respectively; void volume (Vo) = 2.2 mL, efficiency N=35,315 plates calculated for the *trans* isomer. Multiple chromatographic analyses were performed on this mixture and yielded highly consistent results. (standard retention time error < 0.10 min for 10 replicate runs)

The ratio of *cis/trans* components for the isomeric mixture was subsequently shown to vary when the culture was refermented. A nearly complete reversal of abundance (1:2) was encountered when a subsequent fermentation was evaluated.

Figure 3 represents the purification of each isomer by direct scale-up under semi-preparative conditions. With 40 mg of the mixture from C_{18}, 10 mg of pure *cis* isomer t_{R1} = 22.3 min. and 20 mg of pure *trans* isomer t_{R2} = 25.4 min. were obtained.

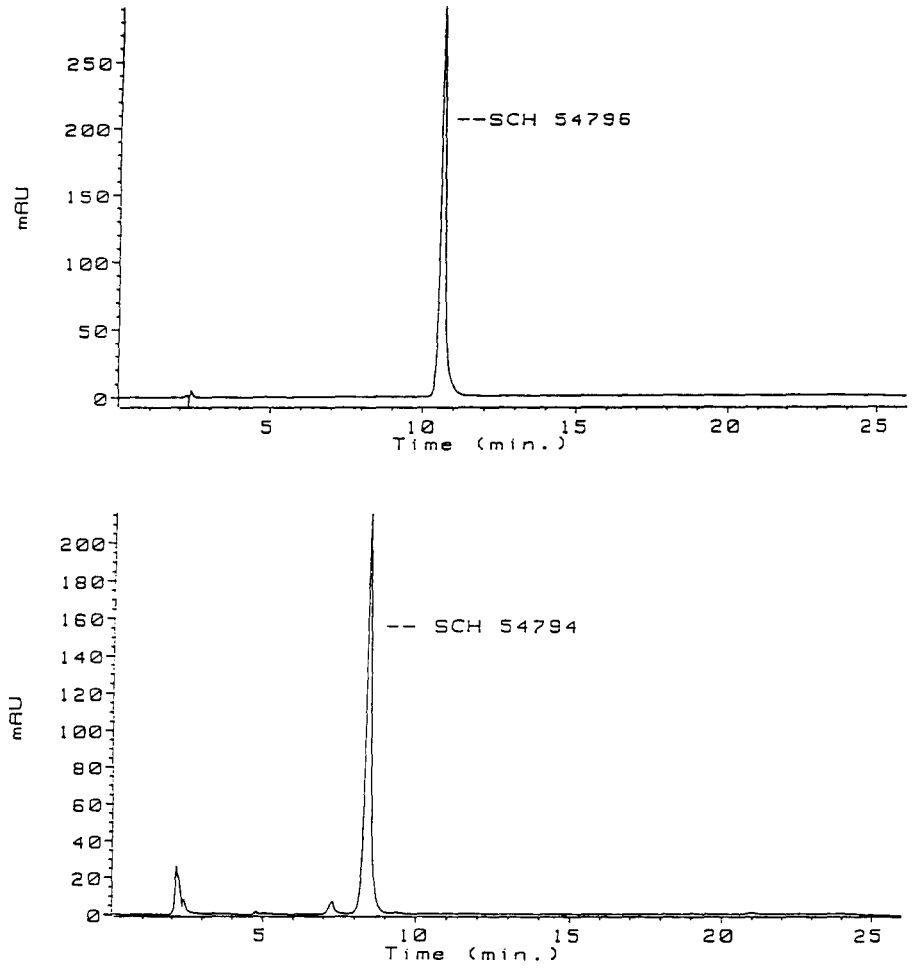


FIGURE 4: Purified *cis* and *trans* diketopiperazines (5µg each)

Analytical re-evaluation of fractions generated (see Figure 4) confirmed the complete separation of *cis* and *trans* diketopiperazine isomers.

CONCLUSION

Polyvinyl alcohol functionalized silica gel has been demonstrated to provide unique capabilities for the isomeric separation of diketopiperazines from *Tolypocladium* sp. (SCF-0729). This rigid and stable stationary phase is well suited for the separation of this acid labile mixture. The uniformity of the polyvinyl alcohol surface combined with its lack of non-specific adsorption yielded high sample recoveries. Selectivity, retention and resolution obtained with this phase were all highly reproducible. Direct scale up of analytical methodology allowed for the baseline preparative separation of this difficult isomeric mixture. Future applications of this chemically modified silica support are expected.

ACKNOWLEDGMENTS

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SIMULTANEOUS QUANTITATION OF AMPHETAMINE AND 4'-HYDROXYAMPHETAMINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A relatively simple HPLC procedure used to detect and quantify amphetamine and 4'-hydroxyamphetamine in rat urine has been developed. Following Bond Elut® solid phase extraction, the two analytes – amphetamine and 4'-hydroxy-amphetamine, and the two internal standards – methamphetamine and 4'-hydroxy-methamphetamine were separated by HPLC using a phenyl column and detected by UV at 215 nm. The limit of quantitation for amphetamine and 4'-hydroxy-amphetamine was 0.92 and 0.81 μg/ml, respectively. Based on replicated analysis of controls at 1.6, 6.5, and 16.0 μg/ml , the method is accurate (94 – 103% of target), and precise (% CVs of 1.3 – 5.2). The linear range of the assay is suitable for the quantitation of both analytes at urine concentrations that commonly result from administration of a single dose of amphetamine to the rat.

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INTRODUCTION

A quantitative method to simultaneously determine the concentrations of amphetamine and 4'-hydroxyamphetamine would be beneficial for *in vivo* studies on the oxidative metabolism of amphetamine. Simultaneous determination of amphetamine and 4'-hydroxyamphetamine has been achieved using gas chromatography/mass spectrometry (1, 2). High performance liquid chromatography (HPLC) offers an alternative approach to analysis where analyte derivatization is not desirable. Although a number of HPLC methods have been developed to detect amphetamines (3), the only methods described for simultaneous measurement of the parent amphetamine and 4'-hydroxyamphetamine have required either radioactive drug (4) which is not commercially available, or specialized chiral columns with derivatization (5). The ability to perform relatively simple HPLC analysis, without derivatization, in a single step would be highly desirable.

The rat, when compared to other species, is an excellent model for studying *in vivo* 4-hydroxylation of amphetamine since rats predominantly utilize the 4-hydroxylation pathway as observed by Axelrod (6). Conjugation of amphetamine metabolites was characterized by Dring and coworkers (7) who found that 4'-hydroxyamphetamine was conjugated by glucuronidation, and benzoic acid was conjugated by sulfation. It is, therefore, necessary to hydrolyze rat urine in order to quantify the amount of 4'-hydroxyamphetamine formed in any *in vivo* studies.

Reverse-phase HPLC is a suitable approach to achieve the separation of nonderivatized 4'-hydroxyamphetamine and amphetamine because 4'-hydroxyamphetamine is water soluble and amphetamine is readily soluble in acids. HPLC with ultraviolet (UV) detection, however, was not widely considered by most investigators because of amphetamine's poor UV absorption characteristics. The derivatization requirement of GC/MS detection for the quantification of 4'-hydroxyamphetamine and/or amphetamine prompted Farrell and Jefferies (8) to suggest that an HPLC method maybe more attractive. Subsequent success in the

use of silica-based reverse-phase HPLC-UV to separate drugs of forensic interest including amphetamine and 4'-hydroxyamphetamine (3, 9-12) suggest this alternative approach to detect amphetamine and 4'-hydroxyamphetamine.

Preliminary studies in our laboratory demonstrated chromatographic conditions that could be used for UV detection of amphetamine and 4'-hydroxyamphetamine standards (13), but did not address matrix differences which may arise from hydrolysis of urine samples. The combination of solid phase extraction (SPE) by Bond Elut® C18 columns and reverse phase HPLC with a phenyl column resulted in a satisfactory analytical method.

MATERIALS

4'-Hydroxyamphetamine bromide and 4'-hydroxymethamphetamine were generously provided by Dr. Anthony S. Murabito of SmithKline Beecham (Philadelphia, PA). ß-Glucuronidase (Type H-1), *d*-amphetamine sulfate, and *d*-methamphetamine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Bond-Elut® C18 SPE columns were purchased from Varian (Harbor City, CA). All solvents were of HPLC grade from Burdick and Jackson (Muskegon, MI). All other reagents were of reagent grade or better.

Rat urine was collected from male Sprague Dawley (SD) rats purchased from Sasco, Inc. (Omaha, NE). Following acclimatization to environmentally controlled animal housing facilities, these animals were kept in individual Nalgene (Rochester, NY) metabolic cages for urine collection. Food and water were available *ad libitum*.

The spectra of the analytes in the appropriate solvent were obtained using a Varian (Walnut Creek, CA) Cary 2200 UV-VIS scanning spectrophotometer.

The separation and detection of amphetamines was achieved by a Varian (Walnut Creek, CA) HPLC system, which consisted of a VISTA 5500 pump, UV200 scanning detector, and VISTA 402 Data Integrator. It was also equipped with a Rheodyne (Cotati, CA) injector with a 10 µl loop, a Microsorb® (Rainin, Emeryville, CA) phenyl reverse phase column, and a C18 column from Alltech

(Deerfield, IL), both columns had the respective Alltech (Deerfield, IL) pre-column cartridge system attached.

METHODS

Urine Hydrolysis and Solid Phase Extraction

Urine hydrolysis was performed as described by Yamamoto and coworkers (14) to account for the total amount of amphetamine metabolites excreted. Briefly, 0.5 ml of rat urine and 0.5 ml (1000 units) of ß-glucuronidase with sulfatase activity in 0.1 M acetate (pH 5.0) were added to screw cap test tubes and incubated at a 37° C overnight. The tubes were stored at 4° C until extraction.

The SPE procedure described by Shimosato (15) was modified to elute both amphetamine and 4'-hydroxyamphetamine. The Bond Elut® C18 columns were conditioned by successively washing with 1 ml each of methanol and 50 mM potassium phosphate (pH 11). While the columns were being conditioned, the following was added to the hydrolyzed urines: 0.5 g sodium chloride, 0.5 ml 50 mM potassium phosphate (pH 11), and internal standards (4'-hydroxymeth-amphetamine, 1.3 µg, and methamphetamine, 3.1 µg). The mixtures' pHs were adjusted to 11 using ammonium hydroxide. The solutions were thoroughly mixed and loaded onto the conditioned columns. After the urinary solutions had passed through, the columns were washed in succession with 1 ml each of 50 mM potassium phosphate (pH 11), freshly prepared 30% methanol, and acetonitrile. Successive elution with 1 ml each of freshly prepared 2% glacial acetic acid in acetonitrile for the amphetamines, and 2% hydrochloric acid in acetonitrile for the hydroxy-metabolites was performed. The eluates were pooled and dried under a stream of air at room temperature and stored dry at 4° C until ready for analysis.

Analytes Extraction at various Loading and Washing pHs.

To examine the effect of pH on the loading and washing steps, 0.5 ml aliquots of 50 mM potassium phosphate solutions (pH 8.5 to 13.0) were added to

preparations of 0.5 ml spiked (4'-hydroxyamphetamine and amphetamine) and hydrolyzed urines prior to adding sodium chloride and internal standards. Potassium phosphate (50 mM) solutions at respective pHs were used to condition and wash the SPE columns. The pH of all other steps was not changed.

HPLC

Analysis was performed using the HPLC system described above. An injection volume of 25 µl was delivered to the 10 µl injector loop to achieve complete loop loading. Detection was at 215 nm, with a sensitivity of 0.005 AU/mV. The mobile phase was 50 mM potassium phosphate (pH 3), methanol, and acetonitrile (85:10:5), at a flow rate of 1 ml/min. The run time was 18.5 minutes.

Analyte concentration estimation

Standards were drug-free rat urine spiked with 4'-hydroxyamphetamine (0.8, 1.6, 3.3, 4.9, 6.5, 8.1, 16.3, and 32.6 µg/ml) and amphetamine (0.9, 1.8, 3.7, 5.5, 7.3, 9.2, 18.3, and 36.7 µg/ml). These concentrations are for free drugs and do not reflect the actual weighed amount of the respective salt. Standards were processed through the hydrolysis and extraction procedures described above, including addition of the internal standards. The standard curve was constructed based on the peak area ratios of analyte to internal standard (4'-hydroxy-amphetamine/4'-hydroxymethamphetamine; amphetamine/methamphetamine), with the curves equation calculated by the least-squares method. Peak area ratios for urine sample data were determined, and the respective analyte concentrations were calculated from the standard curve equation.

Validation of Method

The precision and accuracy, limit of quantitation (LoQ) and recovery of this analytical method were determined in accordance with established procedures (16). The linearity of the standard curves was determined by calculating the coefficient of linear regression (Pearson product-moment correlation coefficient, r). Within-

and between-run precision and accuracy of control reference solutions (QC) at three concentrations and low standards for determination of LoQ were performed in three runs: all samples were in replicates of five in one run, and replicates of three in the other two runs. The former was used for within-run determinations, and the means of all three runs were used for between-run determinations. Precision was defined by the relative standard deviation (RSD), where RSD = (standard deviation ÷ mean) x 100%. Accuracy was calculated by dividing the mean analyte concentrations determined from standard curve by the weighed-in analyte concentrations, and multiplying the result by 100%.

Analyte recovery was determined at three 4'-hydroxyamphetamine (1.6, 6.5, and 16.3 μg/ml) and amphetamine (1.8, 7.3, and 18.3 μg/ml) concentrations. Ten urine samples per concentration were aliquoted; five tubes were prepared and extracted normally (internal calibrators). The remaining five tubes (external calibrators) went through the hydrolysis and extraction steps with amphetamine and 4'-hydroxyamphetamine added just before the dry down step. The internal standards were added before extraction as described above. Percentage recovery was calculated by dividing the mean of internal calibrator ratios by the mean of the external calibrator ratios and multiplying by 100%.

RESULTS AND DISCUSSION

Solid Phase Extraction

Liquid to liquid (L/L) extraction has been the method of choice for extracting amphetamine and 4'-hydroxyamphetamine from biological samples over the years. Preliminary data, however, revealed L/L extraction of hydrolyzed rat urine was insufficient for obtaining usable HPLC chromatograms due to high background; up to 50 peaks were seen in a chromatogram. The alternative approach of using back extraction which would partition the analytes into the appropriate phase under acidic or basic conditions did not resolve the problem of numerous background

peaks. SPE technology offered another approach. When Bond Elut® C18 columns were used, the resulting chromatograms had only five major peaks, very few minor peaks, and the highest recovery of analytes.

The pKa of amphetamine is 9.9, and that of 4'-hydroxyamphetamine is 10.7 (17). The SPE column loading and washing pHs can affect the recovery of these analytes. SPE column recovery was determined as the peak area achieved from the eluate relative to the peak area of unextracted material. The concentrations of 4'-hydroxyamphetamine and amphetamine used were 6.5 and 7.3 μg/ml, respectively. At pH 8.5 – 11, the recoveries of 4'-hydroxyamphetamine and 4'-hydroxymethamphetamine were between 20 and 30% for both compounds, whereas those of amphetamine and methamphetamine were greater than 90%, at pH 8.5 to 11.5. At higher pH, 12 and 13, the recovery of all four compounds suffers, decreasing to between 3 and 12% for the hydroxylated amphetamines and between 70 to 85% for amphetamine and methamphetamine (Figure 1). This study demonstrated that maximum recovery from the SPE columns can be achieved when loading and washing pHs of 10-11 were used.

Determination of the percentage recovery of 4'-hydroxyamphetamine over the entire extraction procedure (i.e., using internal and external calibrators) resulted in the recoveries of 31, 24, and 24% for the three concentrations (1.6, 5.3, and 16.3 μg/ml) of QC samples used. For the three concentrations of amphetamine (1.8, 7.3, and 18.3 μg/ml), the current procedure yields 93, 93, and 96% recovery, respectively. There was excellent agreement between recoveries determined during pH optimization experiments (see above) and this subsequent recovery determination experiment.

UV Spectra

The spectra (200 to 300 nm) of amphetamine, methamphetamine, 4'-hydroxy-amphetamine and 4'-hydroxymethamphetamine in the HPLC mobile phase are shown in Figure 2. The UV spectra for amphetamine, methamphetamine, and 4'-hydroxyamphetamine in HPLC mobile phase are essentially the same as those

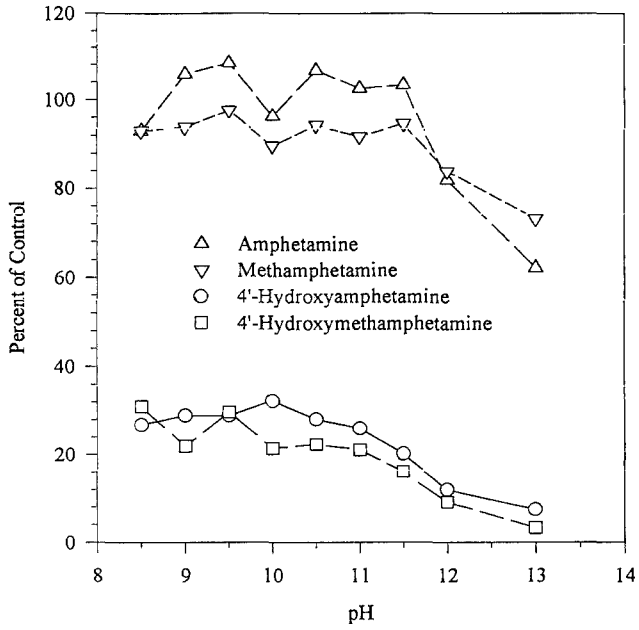

FIGURE 1. Recovery of analytes from SPE columns when the loading and washing pHs vary (see method section).

determined previously in 0.1 N HCl (18). The listed λ_{max} values for 4'-hydroxyamphetamine in 0.1 N HCl are 220 and 274. In HPLC mobile phase they were 230 and 274 for both 4'-hydroxyamphetamine and 4'-hydroxymethamphetamine. Those for both amphetamine and methamphetamine are 252, 257, and 263 nm in 0.1 N HCl and in HPLC mobile phase. Using detection wavelengths at any of these λ_{max} would only allow detection of a single set of analytes, i.e., either the hydroxylated amphetamines, or the set of amphetamine and methamphetamine. At 215 nm significant UV absorption is exhibited by all four analytes (Figure 2). Although it is not the ideal wavelength for selective detection of the individual analyte, it is optimal when detection of all four analytes simultaneously is important.

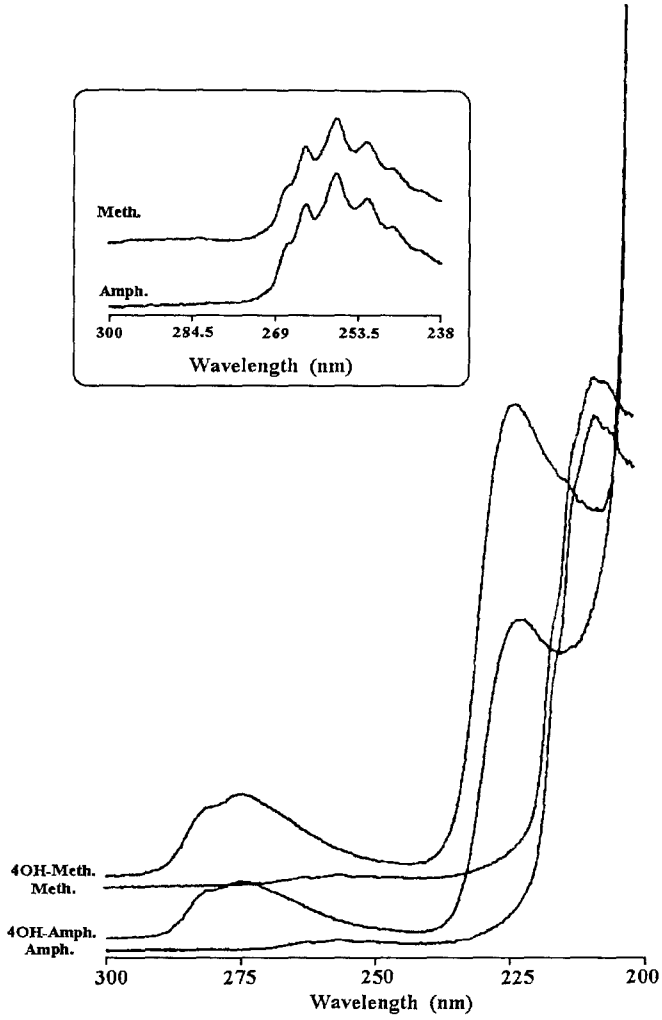

FIGURE 2. UV absorption spectra of 4'-hydroxyamphetamine (4OH-Amph), 4'-hydroxymethamphetamine (4OH-Meth), amphetamine (Amph), and methamphetamine (Meth). HPLC mobile phase (50 mM potassium phosphate:methanol:acetonitrile; 85:10:5) was used as solvent. Analyte concentrations were 7.5 µg/ml, and the wavelengths scanned were between 200 and 300 nm. The inset spectra were 100 µg/ml of amphetamine and methamphetamine in the solvent as above, and the wavelengths scanned were between 238 and 300 nm.

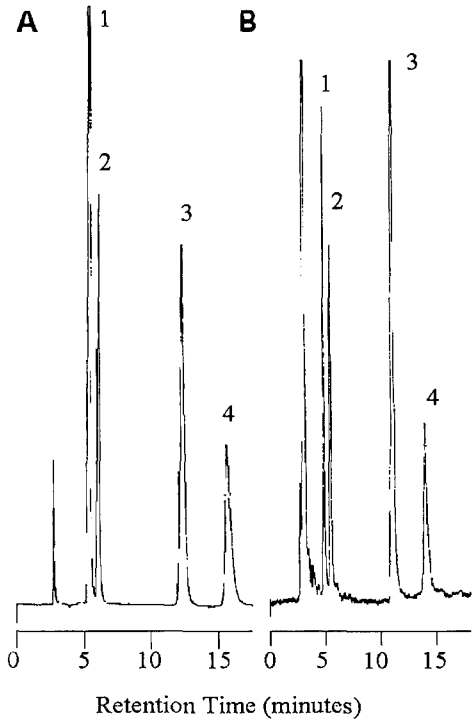

FIGURE 3. High performance liquid chromatograms of unextracted amphetamine standards (Figure 3A), and urine extract from female SD rat (Figure 3B). Peaks labeled 1, 2, 3, and 4 are 4'-hydroxyamphetamine, 4'-hydroxymethamphetamine (internal standard), amphetamine, and methamphetamine (internal standard), respectively. The standards in 3A contained 16.3, 2.6, 18.3, and 6.2 μg/ml, respectively.

Chromatography

When HPLC was used to separate the four analytes from hydrolyzed urine using C8 or C18 columns, background material interfered with the detection of the hydroxylated compounds, and this could not be overcome by changes in flow rate or mobile phase composition. Using a phenyl column, chromatography with fairly low background interference was obtained. By increasing the phosphate

concentration of mobile phase from 25 mM to 50 mM, the 4-hydroxylated analytes were adequately separated from both the solvent front and background interference as shown for unextracted amphetamine standards and an urine extract from a SD rat (Figure 3). Changing the flow rate to higher or lower than 1 ml/min did not markedly improve the shape or separation of peaks. Retention times for the amphetamines were 5.3, 6.0, 12.2, and 15.6 minutes for 4'-hydroxyamphetamine, 4'-hydroxymethamphetamine, amphetamine, and methamphetamine, respectively.

Method Validation

The coefficients of linear regression of the standard curves for both 4'-hydroxyamphetamine and amphetamine were consistently greater than 0.98. Tables 1 and 2 summarize the precision and accuracy of analysis for 4'-hydroxyamphetamine and amphetamine. During the process of determining the LoQ, 4'-hydroxyamphetamine at 0.4 and 0.6 μg/ml and amphetamine at 0.5 and 0.7 μg/ml were also evaluated; however, their peaks signals were too weak for integration. The precision and accuracy values calculated for LoQs of 4'-hydroxyamphetamine at 0.8 μg/ml and amphetamine at 0.9 μg/ml were within the recommended 20% limits (16). Using the rejection limit of 15% (16), the precision and accuracy values of the QCs for between- and within-run were acceptable. The between- and within-run precisions of QCs for 4'-hydroxyamphetamine remained below 5% RSD, and the RSDs for amphetamine were within 7%.

This HPLC separation and detection method demonstrated adequate linearity, precision, and accuracy to detect amphetamine and 4'-hydroxyamphetamine in *in vivo* experimental samples (Tables 1 and 2). The LoQ of the method is approximately 1 μg/ml, which is sufficient for *in vivo* studies where animals were dosed with mg quantities of amphetamine. In the rat, a dose of amphetamine excreted in the urine over 12 to 24 hours was approximately 20% parent

TABLE 1

Summary Table of 4'-Hydroxyamphetamine Precision and Accuracy.

Samples	Weighed-in concentrations (µg/ml)	Determined concentrations (Mean ± SD)	Relative Standard Deviation (RSD)	Accuracy (%)
	Between-runs			
LoQ	0.81	0.82 ± 0.03	3.50	101.0
QC1	1.63	1.68 ± 0.07	4.42	102.8
QC2	6.51	6.53 ± 0.08	1.27	100.4
QC3	16.29	16.03 ± 0.59	3.70	98.4
	Within-run			
LoQ	0.81	0.79 ± 0.10	12.26	98.0
QC1	1.63	1.60 ± 0.05	3.31	98.3
QC2	6.51	6.44 ± 0.34	5.23	99.0
QC3	16.29	15.38 ± 0.38	2.46	94.4

Note: The means ± SD are presented in the table. A total of three separate analytical runs were done, with n=3 for the first two and n=5 for the third run. Between-run values were calculated using the mean for each run. The within-run values were calculated from the last analytical run (n=5). See method section for equations.

TABLE 2

Summary Table of Amphetamine Precision and Accuracy.

Samples	Weighed-in concentrations (μg/ml)	Determined concentrations (Mean ± SD)	Relative Standard Deviation (RSD)	Accuracy (%)
	Between-runs			
LoQ	0.92	0.90 ± 0.18	19.44	97.9
QC1	1.83	1.86 ± 0.08	4.55	101.7
QC2	7.34	7.49 ± 0.06	0.75	102.1
QC3	18.34	18.70 ± 0.84	4.51	101.7
	Within-run			
LoQ	0.92	0.77 ± 0.03	4.34	84.1
QC1	1.83	1.77 ± 0.03	1.85	96.6
QC2	7.34	7.55 ± 0.28	3.74	102.9
QC3	18.34	18.97 ± 1.30	6.87	103.4

Note: The means ± SD are presented in the table. A total of three separate analytical runs were done, with n=3 for the first two and n=5 for the third run. Between-run values were calculated using the mean for each run. The within-run values were calculated from the last analytical run (n=5). See method section for equations.

compound and 40% 4'-hydroxyamphetamine (7). If a 200 g rat was given 1 mg of amphetamine (5 mg/kg) and excreted 10 ml of urine over this time, that should translate to 20 μg/ml amphetamine and 40 μg/ml 4'-hydroxyamphetamine. These concentrations are well within the LoQ of this method.

ACKNOWLEDGMENTS

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A RAPID HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE MEASUREMENT OF DICLOFENAC IN HUMAN PLASMA

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ABSTRACT

A high performance liquid chromatographic method is described for the determination of diclofenac in human plasma, using naproxen as the internal standard. This method is simple, rapid and cost-effective. Zinc sulphate (5%) and methanol were used for extraction. Prepared samples were analysed on a nucleosil C18 column using a 35:65 acetonitrile-water phosphate buffered mobile phase (pH 2.8) and ultraviolet detection at 280 nm. The assay was linear in the range 30 ng/ml to 2 μg/ml, with recovery of extraction ranging from 82 to 97% and a detection limit of 30 ng/ml.

INTRODUCTION

Diclofenac sodium ([o-(2,6-dichloroanilino)-phenyl] acetate) is a non-steroidal anti-inflammatory drug prescribed commonly for acute and chronic musculo-skeletal pain, including arthritis, and more recently for the

treatment of post-operative pain. Diclofenac is 99% bound to serum protein, particularly albumin. Only 60% of oral diclofenac reaches the systemic circulation, mainly due to first pass metabolism, and the elimination half-life is approximately 1.3 ± 0.3 h [1].

The pharmacokinetics of diclofenac sodium are well documented, with maximum plasma concentrations (C_{max}) and time to C_{max} (T_{max}) depending upon the formulation and dose investigated, eg Voltaren-Retard (R) 100 mg 654 ± 329 ng/ml after 6.4 h, sucralfate-covered tablets 50 mg 773 ± 80 ng/ml after approximately 1 h, Diclogesic 100 mg 536 ± 63 ng/ml after 4.1 ± 0.9 h, Voltaren 75 mg 1400 ng/ml after 2.6 h [2-5].

High Performance Liquid Chromatography (HPLC) is the preferred method of analysis for the measurement of diclofenac, with preparation of samples being more rapid than Gas Chromatography (GC) [6-7]. Although Schumacher *et al* described a method using Thin Layer Chromatography (TLC), this lacks sensitivity and Battista *et al* commented on the absence of useful colour reagents for detection on TLC plates [8-9]. Many of the HPLC methods reported for the measurement of diclofenac involve evaporation and reconstitution as part of the sample preparation [5,10-19]. The method described here requires no evaporation stage and therefore allows a more rapid turnover of samples. Other methods not involving evaporation have been described [20-23]. One of these [20] not only has a limit of detection that is higher than this procedure (400 compared to 30 ng/ml) but also involves protein precipitation using perchloric acid which

attacks diclofenac and is therefore best avoided [23]. The method described by Wiese and Hermansson [21] involves a post-column UV photoreaction transformation of diclofenac into a fluorescent derivative, and that by Brunner and Luders [22] involves automation. Moncrieff [23] also described fluorimetric detection of diclofenac, using heat (85°C) to denature the samples. Linearity was reported from 40 ng/ml (compared to 30 ng/ml for this method) and baseline separation was not achieved for the diclofenac peak at 5.92 minutes.

The procedure described here is a simple, rapid and relatively inexpensive method for the measurement of diclofenac, eg extraction is liquid-liquid and does not require the use of solid-phase cartridges. It provides baseline separation of diclofenac with no inherent background interference at the retention times of interest in blank plasma samples.

MATERIALS

Zinc sulphate heptahydrate (ACS grade), diclofenac sodium and naproxen ((s)-6-methoxy-(-methyl-2-naphthaleneacetic acid)) were purchased from Sigma Chemical Company (Poole, UK). Sodium lauryl sulphate (AR grade), sodium dihydrogen orthophosphate (AR grade), acetonitrile (HPLC grade), orthophosphoric acid (AR grade) and methanol (HPLC grade) were purchased from Fisons Scientific Equipment (Loughborough, UK). Water used for analytical applications was purified using a Purite Select Analyst HP system (Thame, UK).

Apparatus

The HPLC system consisted of an SP8800 pump, SP8780 autosampler, SpectraChrom 100 variable wavelength detector and SP4400 Chromjet integrator (Thermo Separation Products, Stone, UK) and a C_{18} Nucleosil, 25 cm x 4.6 mm I.D., 5 μm particle size, reversed-phase column (Jones Chromatography Ltd, Hengoed, UK).

Chromatographic Conditions

The mobile phase was comprised of 1 mM sodium lauryl sulphate, 10 mM sodium dihydrogen orthophosphate and acetonitrile-water (35:65 v/v). Orthophosphoric acid was used to adjust the pH to 2.8. A 0.45 μm Gelman Sciences membrane was used to filter the mobile phase before use. The flow rate of the pump was 1.5 ml/min and the temperature ambient (range 27-30°C). The UV detector was set at a wavelength of 280 nm and 0.01 a.u.f.s.. Naproxen was used as the internal standard.

METHODS

Calibration

Fresh stock solutions of diclofenac and naproxen (1 mg/ml in water and 50% acetonitrile mobile phase respectively) were made on a weekly basis and stored at 4°C until use. Freshly made calibration standards were

prepared on a daily basis when required in the following concentrations, diclofenac 30 ng/ml to 2 μg/ml and naproxen 1 μg/ml.

Sample Extraction and Preparation

Blood samples were collected in 5 ml lithium heparin tubes and centrifuged at 2000g, 27°C for 10 min. The plasma was decanted off and either used immediately or stored at -70°C until use. Disposable polypropylene test tubes were used for all subsequent work.

Naproxen (1 μg/ml) was used to spike 1 ml of plasma. After the addition of 100 μl of 5% zinc sulphate (w/v in water) the sample was vortex mixed for 2 min. Methanol (3 ml) was added before vortex mixing for a further 2 min. Aqueous buffer (0.44 ml) was then added. This buffer consisted of 100 mM sodium dihydrogen orthophosphate and 10 mM sodium lauryl sulphate. Orthophosphoric acid was used to adjust the pH to 2.8 before the solution was filtered with a Gelman Sciences 0.45 μm filter. The sample was then finally vortex mixed for a further 1 min. The sample was centrifuged at 2000g, 27°C for 10 min and the supernatant decanted off, 100 μl of which was injected onto the HPLC column.

Extraction Efficiency

Three different concentrations of diclofenac (0.1, 1 or 2 μg/ml) and 1 μg/ml naproxen (n=6 for each concentration) was used to spike drug-free

plasma which was then taken through the extraction procedure. The extraction efficiency was then determined by comparing the results to a series of non-extracted aqueous standards.

Reproducibility

Aliquots of plasma (1 ml) were spiked with either 0.05, 0.2 or 1 μg/ml diclofenac (n=10 for each concentration) and naproxen 1 μg/ml and analysed during one working day in order to determine intra-assay reproducibility. Inter-assay reproducibility was investigated using pooled plasma (diclofenac 1 μg/ml and naproxen 1 μg/ml) as a quality control. This was analysed over an eight week period, being stored at -70°C until use with a single 1 ml aliquot used per sample run.

Light Sensitivity

To determine whether the storage conditions and/or the length of time before analysis was critical, twenty 1 ml aliquots of drug-free plasma were dispensed into colourless test tubes and spiked with diclofenac 1 μg/ml and naproxen 1 μg/ml. Ten of these were kept under darkened conditions while the other ten were left under normal laboratory conditions (ambient temperature, in daylight but out of direct sunlight) for between 15 min and 6 h.

Stability

Sample stability at ambient temperature was investigated over a 20 h period. The extract from pooled plasma, which had been spiked to a final concentration of either 0.05, 0.2 or 1 μg/ml diclofenac and 1 μg/ml naproxen, was analysed every hour for 20 h using an autosampler.

Measurement of Diclofenac Following 50 mg Oral Dose

After taking a baseline blood sample, a male volunteer received oral diclofenac 50 mg. Blood samples (5 ml) were collected via an intravenous cannula at 15, 30, 45, 60, 70, 80, 90, 105, 120, 150, 180, 240, 300, 360 min after administration. The blood samples were centrifuged and analysed immediately.

RESULTS

Measurement of Diclofenac

Figure 1 shows chromatograms of peaks for diclofenac (II) and naproxen (I) from a) aqueous standard, diclofenac 1 μg/ml and naproxen 1 μg/ml, b) plasma blank, c) plasma sample spiked with diclofenac 1 μg/ml and naproxen 1 μg/ml and d) plasma 80 min after oral administration of diclofenac 50 mg plus naproxen 1 μg/ml. Retention times for naproxen and diclofenac were approximately 5.8 and 10.6 min respectively.

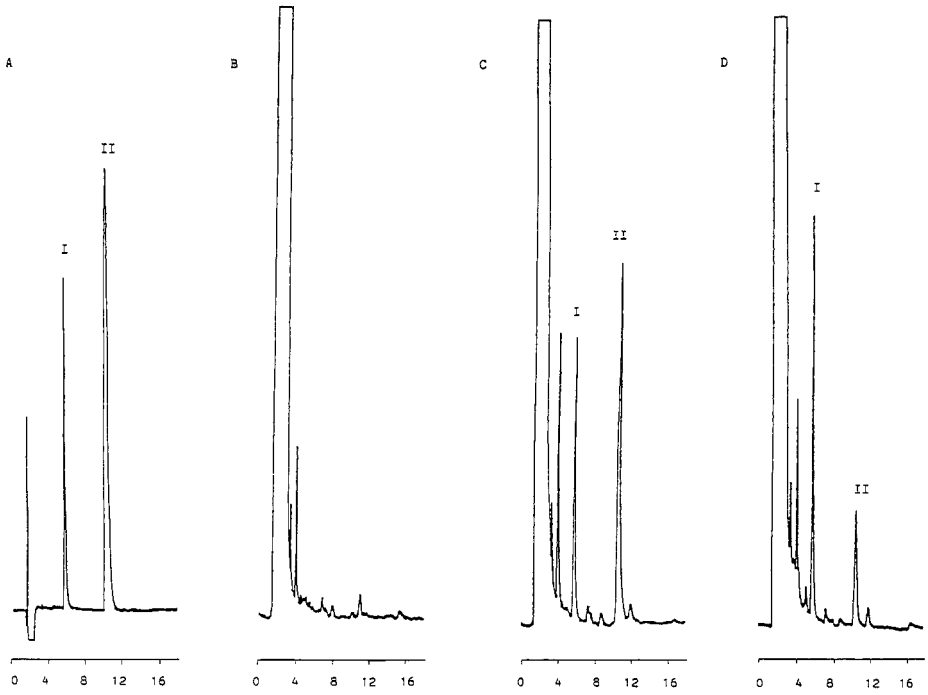

FIGURE 1. Typical chromatograms of the recovery of diclofenac (II) and naproxen (I) from aqueous standard diclofenac 1 μg/ml naproxen 1 μg/ml (A), plasma blank (B), plasma spiked with diclofenac 1 μg/ml naproxen 1 μg/ml (C) and plasma 80 minutes after an oral dose of 50 mg diclofenac (D).

Calibration

Figure 2 shows the linear correlation obtained between diclofenac concentration and peak-height ratio over the range 30 ng/ml to 2 μg/ml (n=6 for each of the 8 concentration points). The regression equation was $y = 1.32x - 0.014$ and the correlation coefficient 0.999. The standard

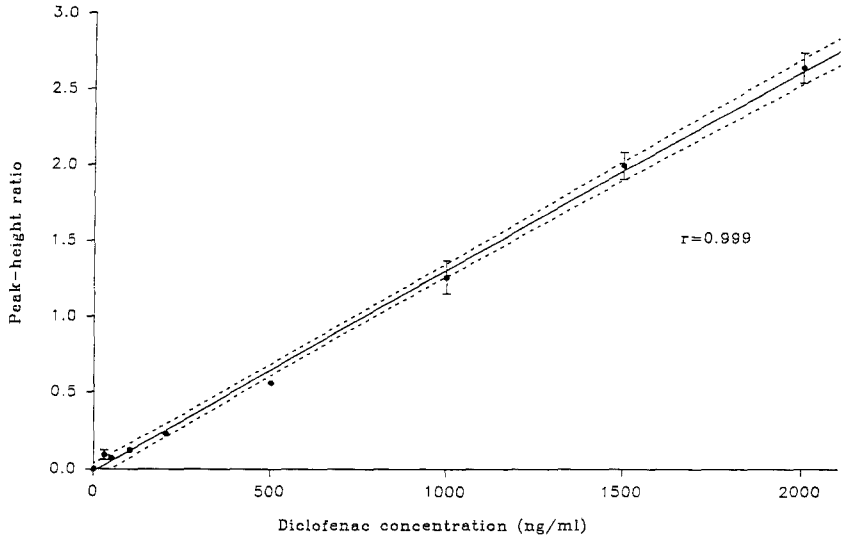

FIGURE 2. Calibration curve of diclofenac in human plasma.

TABLE 1. Recovery of Extraction of Diclofenac and Naproxen (Internal Standard)

			Naproxen 1μg/ml	
Diclofenac concentration (μg/ml)	Recovery (mean ± SD) (%)	CV (%)	Recovery (mean ± SD) (%)	CV (%)
0.1	97.2 ± 6.78	7.00	89.7 ± 1.08	1.20
1.0	83.7 ± 0.98	1.17	89.7 ± 1.75	1.95
2.0	82.0 ± 1.06	1.29	91.7 ± 2.57	2.80

n=6 for each concentration
CV=coefficient of variation

deviation for the slope and intercept were 0.021 and 0.023 respectively. The limit of detection was 30 ng/ml (signal-to-noise ratio of 3:1).

Extraction Efficiency

The recovery of extraction of diclofenac from human plasma was between 82 and 97% over the range of concentrations 0.1 to 2.0 μg/ml (Table 1). The coefficient of variation was 7% or less for all three concentration levels of diclofenac studied.

Reproducibility

Intra-assay reproducibility for the three concentrations of diclofenac investigated is shown in Table 2 (n=10 for each concentration). The coefficient of variation for intra-assay reproducibility ranged from 2.4 to 11.1%. Inter-assay reproducibility, determined by analysis of the quality control plasma samples over an 8 week period, had a coefficient of variation of 4.4%.

Light Sensitivity

Using the statistical package Minitab release 10 for Windows, neither the length of time before analysis (over the range 15 min to 6 h) nor exposure to light had a statistically significant effect upon the amount of diclofenac measured in spiked plasma samples ($p = 0.214$ and 0.063 respectively).

TABLE 2. Diclofenac Reproducibility and Stability Over a 20 Hour Period

Diclofenac concentation (ng/ml)	Intra-assay reproducibility (n=10) (peak-height ratio drug/IS)			Diclofenac concentration of pooled extract (ng/ml)	
	mean	SD	CV (%)	mean	SD
50	0.09	0.01	11.1	56	5.7
200	0.22	0.01	4.5	177	10.1
1000	1.25	0.03	2.4	989	8.0

CV=coefficient of variation

Stability

The diclofenac content of pooled plasma was stable over the 20 h period investigated. Table 2 shows the mean and standard deviations for the three concentrations investigated.

Measurement of Diclofenac Following 50 mg Oral Dose

Plasma levels of diclofenac 50 mg after oral administration are shown in Figure 3. Using the procedure described, plasma levels of diclofenac were still measurable 6 hours after oral administration, ie approximately 4 to 5 elimination half-lives.

DISCUSSION

Using the described procedure, concentrations as low as 30 ng/ml of diclofenac (signal-to-noise ratio of 3:1) can be measured, with no inherent background interference in blank plasma samples. The calibration curve was

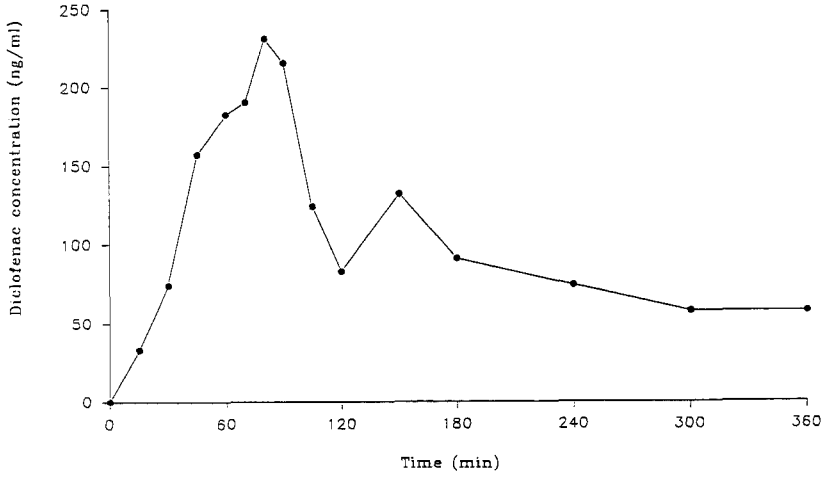

FIGURE 3. Plasma levels of diclofenac in a volunteer after a 50 mg oral dose.

linear between 30 ng/ml and 2 μg/ml (correlation coefficient 0.999). Recovery of diclofenac from human plasma was good for a variety of concentrations, eg for 0.1 and 2.0 μg/ml recovery was 97 and 82% respectively. This procedure was reproducible, both in terms of within-day and day-to-day reproducibility. Coefficient of variation for intra- and inter-assay reproducibility were 2.4-11.1 and 4.4% respectively.

Diclofenac (in plasma) was not effected by exposure to light, over the six hour period investigated. Once taken through the extraction procedure, diclofenac (in extract) was stable over twenty hours. Consequently a batch of samples could be prepared and extracted simultaneously and then analysed overnight using an autosampler.

ERRATUM

Our procedure has been used to analyse blood taken from a volunteer following an oral dose of diclofenac 50 mg up to six hours post-administration and could be used for clinical research.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FREE AMINO ACIDS IN SHRIMP

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ABSTRACT

A reverse phase high performance liquid chromatographic method for the analysis of total free amino acids in shrimp, utilizing pre column fluorescence derivatization is described. The primary amino acids were treated with o-phthalaldehyde (OPA). The reaction products were separated on a Microsorb Short-ones 3-μm reversed-phase column with gradient elution development. 15-OPA amino acids were separated in 21 min. Secondary amino acids were reacted with 4-chloro-7-nitrobenzofurazan (NBD). The separation was carried out on a Lichrosorb RP-C18, 5μm column. Wild and cultured Mexican shrimp species (Penaeus vannamei) were analyzed. Free amino acid contents of glycine, alanine and proline were higher than those of other amino acids. Total Free amino acid content was significantly higher in cultured than wild shrimp. The OPA-retention time could be measured within ±0.1 relative standard deviation and the relative peak areas, based on the internal standard calculation methodology, were within ±3% or less. The coefficient of variation of 18 kinds of amino acid samples were about 10%, the detection limit was 100 fmol.

INTRODUCTION

Dissolved free amino acids (DFAAs) are important nutrients in marine food webs. DFAAs concentrations are most often studied by high pressure liquid chromatography of fluorescent derivatives with O-phthalaldehyde (OPA) (1).

Amino acid analysis is an important technique which finds many applications in analysis of sea foods, and related fields. HPLC has been used for the determination of free amino acids in uterine fluid and blood plasma samples of four species of viviparous sharks (2); HPLC-OPA free amino acid determination in seawater (3); determination of free amino acid composition in muscle and hemolymph of prawn (4) and quantitative distribution of free amino acids in the white and red muscle of tuna (5). However, only one report has appeared describing the use of HPLC techniques for the quantitative of free amino acid analysis in shrimp (6), and others have described the quantitative determination of free amino acids in seafood but using an amino acid analyzer (7).

The purpose of this article was to report the separation, base line resolution, and quantitation of the OPA and NBD amino acid derivatives in shrimp. This methodology can be utilized in determining free amino acids as important contributors to quality flavor in seafood; osmoregulators in crustaceans and environmental factors during growth.

MATERIALS AND METHODS

Sample Preparation

Extracts for analyses were prepared by blending the shrimp tissue in a waring blendor homogenizer at a ratio of 1.0 g shrimp to 2.0 ml of trichloroacetic acid (TCA). The extracts were centrifuged and part of the supernatant analyzed (7).

Primary Amino Acids

OPA derivatizing solution

To 10 mg OPA dissolved in 250 µl of methanol, 37.5 µl 30% Brij 35, 25 µl of 2-mercaptoethanol and 3 ml of 0.5 M potassium borate buffer pH=10.4 were added. This solution was diluted to 10 ml with borate buffer, and mixed well. It was then stored under refrigeration in the dark and allowed to stand for 24 h before use. The preparation was made one day prior to use.

Sample derivatizing preparation

A sample of TCA extract was diluted with 5 ml of sodium citrate buffer pH=2.2, filtered (whatman 934-AH) and diluted with alfa-aminobutiric acid (2.5µM/ml) as an internal standard (IS) as follows:

100µl TCA extract + 40µl IS --> 1 ml

Sample derivatization.- Immediately prior to loading the injection loop, a combination of 0.5 ml of OPA solution and 0.5 ml of sample or amino acid standard solution containing IS was prepared in a small test tube and mixed. It was injected within 2 min.

Secondary Amino Acids

Preparation of the NBD derivatives

Equal volumes of TCA extract sample, 0.4 M borate buffer, and the NBD solution (2 mg/ml in methanol) were combined and the mixture was heated for 5 min at 60°C in a closed screw capped vial. The reaction was stopped by cooling the mixture to 0°C. 10µl were injected onto the column. The quantification was made using an external standard.

Chromatographic Equipment

The instrument used consisted of a Varian Model 5000 microprocessor controlled high performance liquid chromatograph coupled to a Fluorichrom fluorescence detector equipped with a deuterium lamp. The detector was connected

to a Model Vista 401 data collection system (Varian Associates, Inc. USA). The OPA and NBD derivatives were detected with the monochromator set at 330 nm and 418 nm cut-off filter. The sensitivity was set at 1 µA full scale. The sample was introduced with a Rheodyne Model 7120 valve (Berkeley, CA, USA) equipped with a 10 µl loop.

Primary amino acid separations were carried out on a 10cm x 4.6 mm I.D. Microsorb Short-ones diameter spherical HPLC column packed with 3µm Reversed-phase C-18 octadecyl dimethylsilane particles (Rainin Instrument Co. Inc., Emeryville, CA, USA) connected to a pre-column (3 cm x 4.6 mm I.D.) packed with the same material.

A Lichrosorb RP-C18, 5µm, (30 cm x 4.6 mm ID) column was used for determination and separation of secondary amino acids (Alltech/Applied Science, USA).

Statistical Analysis

The data were analyzed by descriptive statistics and one way ANOVA method to test for significant differences among shrimp samples, followed by testing of specific mean differences using Tukey's multiple comparison procedure (8).

RESULTS AND DISCUSSION

The excitation and emission wavelengths for OPA-amino acids are 340 nm, 455 nm and 220 nm, 370 nm for NBD-iminoacids, respectively.

Solvent composition, buffer concentration, and pH are major factors affecting reverse phase chromatography.

Fully automated HPLC equipment allowed unattended determination of amino acid profile within 21 min. Precise control of operating conditions and temperature (23°C) are essential to achieve maximum resolution. The

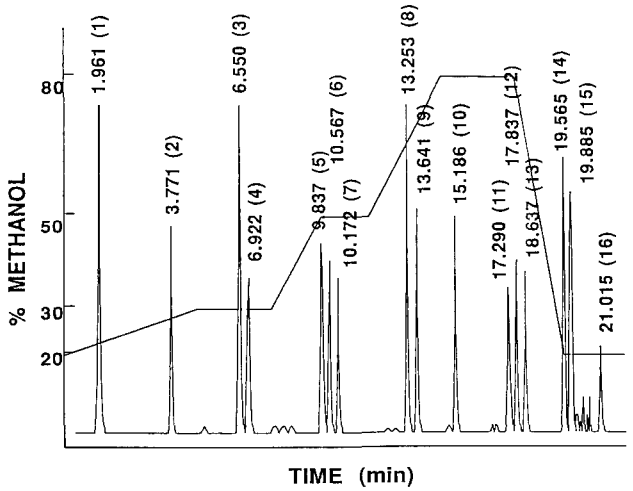

FIGURE 1. Separation of OPA-amino acid standard. Operating conditions: Column Microsorb Short-ones. Flow rate 1.2 ml/min. Solvent A: 0.1 M sodium acetate buffer + 1% tetrahydrofuran, pH=6.2. Solvent B: Methanol. Sample loop 10µl. Exitation at 330 nm, emission filter 418 nm. Peaks: 1=aspartic acid, 2=glutamic acid, 3=serine, 4=histidine, 5=glycine, 6=threonine, 7=arginine, 8=alanine, 9=tyrosine, 10=alfa-aminobutiric acid (IS), 11=methionine, 12=valine, 13=phenylalanine, 14=isoleucine, 15=leucine, 16=lysine.

reproducibility of the derivatization method and HPLC technique was checked using two samples. The results indicated that the coefficient of variability was less than 10%. The method did not yield higher or lower results and relative standard deviation systematically.

Figure 1 shows a representative chromatogram of the standard OPA free amino acids and a profile of the multistep mobile phase gradient. Linear relationships between peak areas and concentrations for each amino acid are shown. The gradient was composed of a 7-step solvent program which increased the separation efficiency of amino acids.

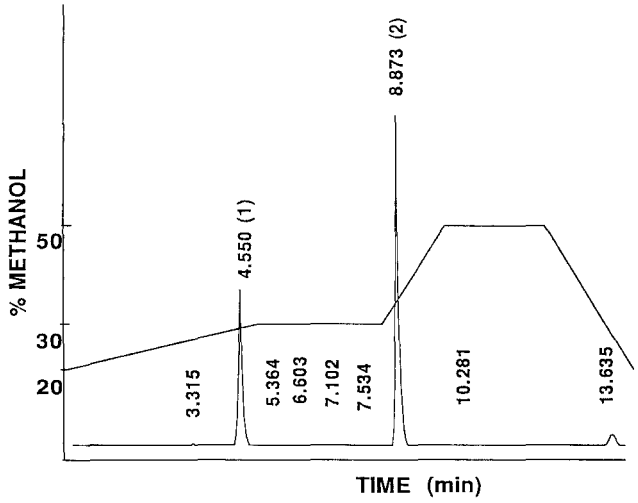

FIGURE 2. Separation of NBD-amino acid standard. Operating conditions: Column Lichrosorb RP-C18. Flow rate 1.2 ml/min. Solvent A: 0.1 M sodium acetate buffer + 1% tetrahydrofuran, pH=6.2. Solvent B: Methanol. Sample loop 10µl. Exitation at 220 nm, emission filter 370 nm. Peaks: 1=hydroxyproline, 2=proline.

Figure 2 shows a typical chromatogram of standard NBD iminoacids and its elution gradient. The elution gradient was the first part of OPA-aminoacids. The solvent programming was performed automatically with a microprocessor unit. The last OPA-amino acid eluting under these conditions was lysine, which has a retention time of 21 min. The NBD amino acids were eluted in 9 min.

Although shrimp samples contain components which might interfere with amino acid analysis, no extra peaks were observed on the chromatogram. Presumably, the specificity of the fluorometric procedure and selectivity of the solvent system eliminated this type of interference.

Precision was examined by reacting and analyzing five replicate standard amino acid mixtures and comparing peak areas for the respective mixtures. Coefficients of variation ranged from 10% for lysine to 3% for the rest of the

amino acids, at injection levels of 0.5 µmol in 10 µl. The OPA-retention time could be measured within ±0.1 relative standard deviation and the relative peak areas, based on the internal standard calculation methodology were within ±3 or less. The coefficient of variation of 18 kinds of amino acid samples was about 10%, the detection limit was 100 fmol (9, 10). This result is in agreement to that reported by Wang, et al., (3), but their final time of analysis was 40 min.

The correlation coefficients for concentrations versus response was >0.998 for all derivatives, except for lysine (r=0.991).

A typical separation of OPA- and NBD-amino acids can be seen in figure 3 and 4. The Table I shows the individual content of free amino acids in wild and cultured shrimp.

The results agree with Simpson, et al., (11) and McCoid et al.(7). However, this method of analysis was effective in separating serine and threonine; also, threonine was present in trace concentrations (Fig 3, Tr=10.501). On the other hand, fluorometry is known to be about one hundred times more sensitive than colorimetry (12), and the classical amino acid analyzers were based in ion-exchange operated in post column derivatization mode and utilized ninhydrin.

The separation of components in the raw extract of shrimp was: glycine (22.18-37.88%); arginine (22.28-32.67%) and proline (12.57-29.15%), which were present in the highest concentration. This result agrees with Takada et al., (1988) for free amino acid contents in eighteen species of imported frozen shrimps. These data represent approximately 27.55%, 26.36% and 20.81% of the total amino acid content, respectively, and comprised 72.58-76.87% of free amino acid pool. Aspartic acid content was the lowest and only had a mean of 3.07 mg%. It was followed by phenylalanine and hydroxyproline.

Cultured shrimp had higher concentration of the total free amino acid concentration than wild shrimp.

Wing et al., (1990) constructed an automated continued monitoring system for DFAAc, and stated that "DFAAc concentrations are most often studied by HPLC of fluorescent derivatives with OPA. This procedure is laborious, requires

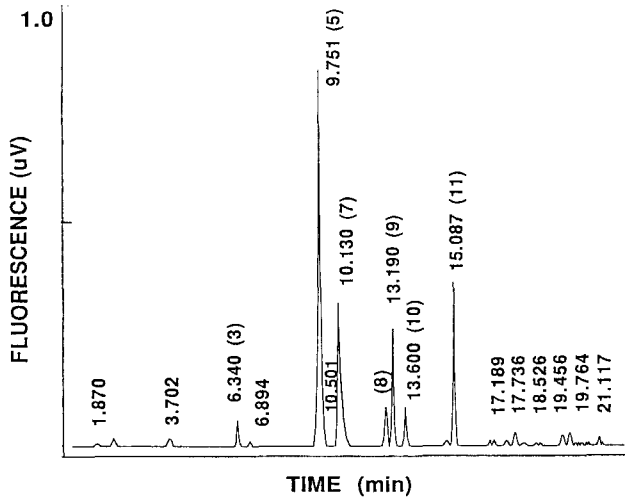

FIGURE 3. Amino acid analysis of wild shrimp. Operating conditions same as in Fig. 1. OPA-aminoacids;3=serine, 5=glycine, 7=arginine, 8=taurine, 9=alanine, 10=tyrosine, 11=alfa-aminobutiric acid (IS).

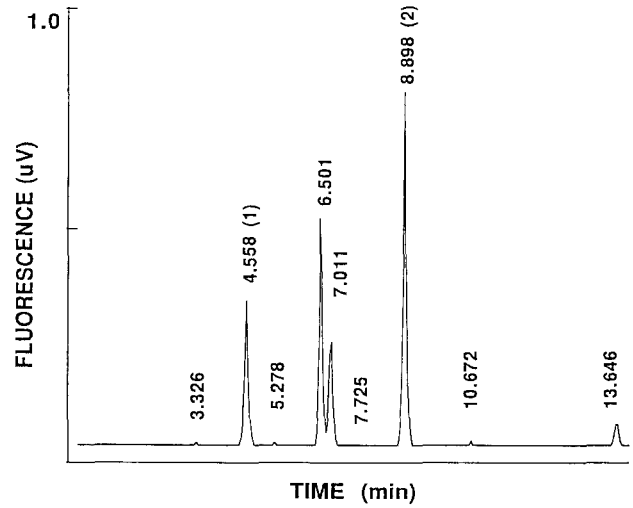

FIGURE 4. Amino acid analysis of wild shrimp. Operating conditions same as in Fig. 2. NBD-aminoacids. Peaks: 1= hydroxyproline, 2= proline.

TABLE 1

Free Amino Acids in White Srimp (P. Vannamei) Wild and Cultured 1[1]

Free Amino Acids	Wild[2]	Cultured 1[2]	Cultured 2[2]
		OPA-Amino Acids	
Aspartic	2.43 ± 0.29 a	2.72 ± 0.13 a	4.05 ± 0.24 b
Glutamic	19.99 ± 1.38 a	19.54 ± 1.64 a	20.51 ± 1.28 a
Serine	20.86 ± 1.69 a	10.41 ± 0.39 b	19.54 ± 1.75 a
Histidine	68.27 ± 20.27 a	138.23 ± 22.42 b	101.10 ± 20.37 b
Glycine	367.55 ± 10.66 a	373.15 ± 22.11 a	575.14 ± 36.79 b
Arginine	439.13 ± 21.87 a	429.61 ± 34.14 a	389.84 ± 37.01 a
Taurine	71.51 ± 4.27 a	22.54 ± 1.94 b	88.57 ± 7.99 c
Alanine	73.23 ± 3.87 a	94.48 ± 8.07 b	82.42 ± 8.81 ab
Thirosine	20.24 ± 3.62 a	24.84 ± 2.04 a	27.74 ± 3.35 a
Methionine	12.13 ± 0.78 a	5.56 ± 0.43 b	11.94 ± 1.03 a
Valine	18.91 ± 1.44 a	18.68 ± 0.52 a	22.93 ± 1.15 b
Phenylalanine	8.23 ± 0.86 a	4.76 ± 0.61 b	10.18 ± 0.41 a
Isoleucine	12.45 ± 0.78 a	8.92 ± 0.71 b	14.80 ± 0.96 a
Leusine	18.53 ± 1.48 a	13.52 ± 1.38 b	20.51 ± 1.87 a
Lysine	21.74 ± 1.24 a	17.49 ± 2.04 a	20.40 ± 2.84 a
		NBD-Amino Acids	
Proline	168.94 ± 12.61 a	490.29 ± 54.24 b	334.66 ± 31.67 c
Hydroxyproline	11.92 ± 1.27 a	7.23 ± 0.61 b	5.16 ± 0.56 c
Total Free Amino Acids	1344.14 a	1681.97 b	1749.40 c

[1] mg/100g.
[2] a,b,c = significative differences ($P<0.05$).

extensive sample handling, and yields results hours or days after sampling". However, with our method, seawater can be analyzed directly and results can be obtained in 21 min for OPA-DFAAc or 9 min for NBD-DFAAc.

The described method has been used in our laboratory to assess types and amounts of amino acids in regional foods, fish meal, and complex food samples.

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SIMULTANEOUS HPLC QUANTIFICATION OF TWO DERMATOTOXINS, 5-METHOXYPSORALEN AND FALCARINOL, IN HEALTHY CELERY

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ABSTRACT

A method for the simultaneous quantification and separation of the dermatotoxins, falcarinol and 5-methoxypsoralen (5-MOP), in uninfected, commercially available celery is reported. Four methods of plant extraction were compared to determine the most efficient method. The analyses of crude celery extracts employed a Spectra-Physics 8800 series high performance liquid chromatograph equipped with a forward optical scanning detector. The compounds were resolved on an Alltech C-18 5 mm (25 cm x 4.6 mm ID) column preceded by a C-18 guard column. The dermatotoxins were eluted with a linear gradient of 50% methanol and water to 100% methanol in 30 minutes. The concentration of 5-MOP and falcarinol found in commercially available celery was 0.08-0.24 μg/g and 0.9-20 μg/g, respectively.

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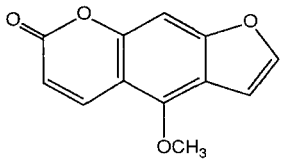
INTRODUCTION

Several genera among the Araliaceae and Apiaceae families (which include many common vegetable and spices in *Hedera*, *Schefflera*, *Panax*, *Apium*, *Falcaria*, *Daucus*, *Oenanthe*, and others) have been shown to contain the polyacetylene, falcarinol (Figure 1) (1). This compound was demonstrated by Gafner *et. al.* (2), and Hansen and Boll (3) to be a potent skin sensitizer; a 0.5% topical application of falcarinol elicited severe bullous reactions in sensitized animals. The amount needed for the elicitation of this delayed hypersensitivity reaction is 0.2 μg/8 mm^2 area (2). Ultraviolet light was not required for the sensitization reaction to occur. The presence of such a potent contact allergen in a wide variety of plants leads us to believe that falcarinol sensitizations are more common than generally assumed.

Dermatitis cases due to falcarinol from English Ivy, Algerian Ivy, and Common Ivy tend to be the most generally reported (2, 3). However, no cases of falcarinol dermatitis have been attributed to celery. The majority of dermatitis cases to celery and parsnip handlers and processors are linked to the linear furanocoumarins (psoralens), which are present in these plants (5). The psoralens are potent skin sensitizers in the presence of ultraviolet light. The amount required to elicit a photosensitivity response by the linear furanocoumarin 5-MOP is 0.1 μg/8 mm^2 skin area (6). It was once believed that psoralens were only found in celery infected with the fungus *Sclerotinia sclerotiorum*; however, this was disproved when Beier *et al.* (7) and Innocenti *et al.* (8) detected psoralens in fresh uninfected celery. A study by Picardo *et al.* (9) reported that photosensitivity was not involved in one case of celery dermatitis. We suggest that this case and many cases of other non-photosensitive dermatitis common to handlers of celery may be attributable to falcarinol

The linear furanocoumarins have been shown to be both potent photosensitizing agents and to have several applications in medicine (10, 11). These compounds also exhibit phototoxicity during therapy and are suspected of being carcinogenic (10, 12).

FALCARINOL



5-METHOXYPSORALEN

FIGURE 1. Structures of dermatotoxins found in celery.

Due to the widespread occurrence of the linear furanocoumarin and polyacetylene compounds (Figure 1) in many human foods, it is important to know the amounts of toxins present in the ingested plants. Both classes of compounds have been demonstrated to be present in celery (1, 5). However, the simultaneous quantification of both classes of dermatotoxins in the same plant has not been performed previously. 5-Methoxypsoralen, the most common furanocoumarin in celery, was chosen as the a representative of the linear furanocoumarins. This study reports quantities comparable to the levels of 5-MOP found by Beier *et. al.* (7) and also determines the concentration of falcarinol in uninfected, commercially available celery.

METHODS

Chemicals: 5-Methoxypsoralen used as a standard was purchased from Sigma Chemical Company (St. Louis, MO). Falcarinol was isolated from English Ivy (*Hedera helix*) by repeated column chromatography and the purity of the sample was established by high performance liquid chromatography to be >95%. Analytical spectroscopy, nuclear magnetic resonance (NMR) and mass spectrometry (MS), confirmed the sample to

be falcarinol. All solvents used were HPLC grade and water was double distilled through a Corning Mega-pure glass distillation system (Corning, Parkersburg, WV).

Plant material: Celery cultivar Tall Utah 5270 R grown and packed in California was bought at a local market in the original carton that had been packed in a cold shipment container. All of the celery selected looked healthy, fresh, and had no signs of any disease. The leaves and bases of each celery bunch were trimmed and discarded. The stalks were cut, weighed, and extracted according to the following methods. Method 1: the extraction and work-up of frozen plant material was extracted according to the procedure of Beier *et. al.* (7). Briefly, frozen plant material was thawed, weighed and extracted with ethyl acetate. Then the crude extract was loaded onto a C-18 SepPak, and the cartridge was then eluted with 60% acetonitrile in water. The eluant was then analyzed for content of dermatotoxins. Method 2: frozen plant material was thawed and 5-g samples (6-8 replicates of each variety) were diluted with 15 ml dichloromethane and homogenized with a Brinkman Polytron homogenizer (3 x 15 ml). After homogenization, the dichloromethane extracts were taken to dryness by rotary evaporation. and the residue was dissolved in 1 ml methanol and sonicated for 1 minute. The solution was filtered through a 0.2 μm Metricel membrane filter. Method 3: 5-gram samples of fresh stalks replaced frozen material in Method 2. Method 4: dried 5-g samples were extracted with 15 ml of methanol (MEOH) using the Brinkman polytron (3X). The methanol extract was concentrated and 5 ml of water was added. The aqueous mixture was then extracted with 5 ml of ethyl ether (3X) and evaporated to dryness. The residue was then resuspended in 200 μl and filtered through a 0.2 μm Metricel membrane filter.

High Performance Liquid Chromatography: Falcarinol and 5-MOP were resolved using a Spectra-Physics 8800 system with forward optical scanning detector on an Alltech C-18 reverse phase resin column. An econosphere C-18 reverse phase column (250 mm x 4.6 mm i.d., 5mm particle size) was eluted with 50% HPLC grade Methanol and 50% double distilled water followed by a linear gradient to 100% methanol in 30 minutes. After an isocratic period of 5 minutes at 100% MeOH, a

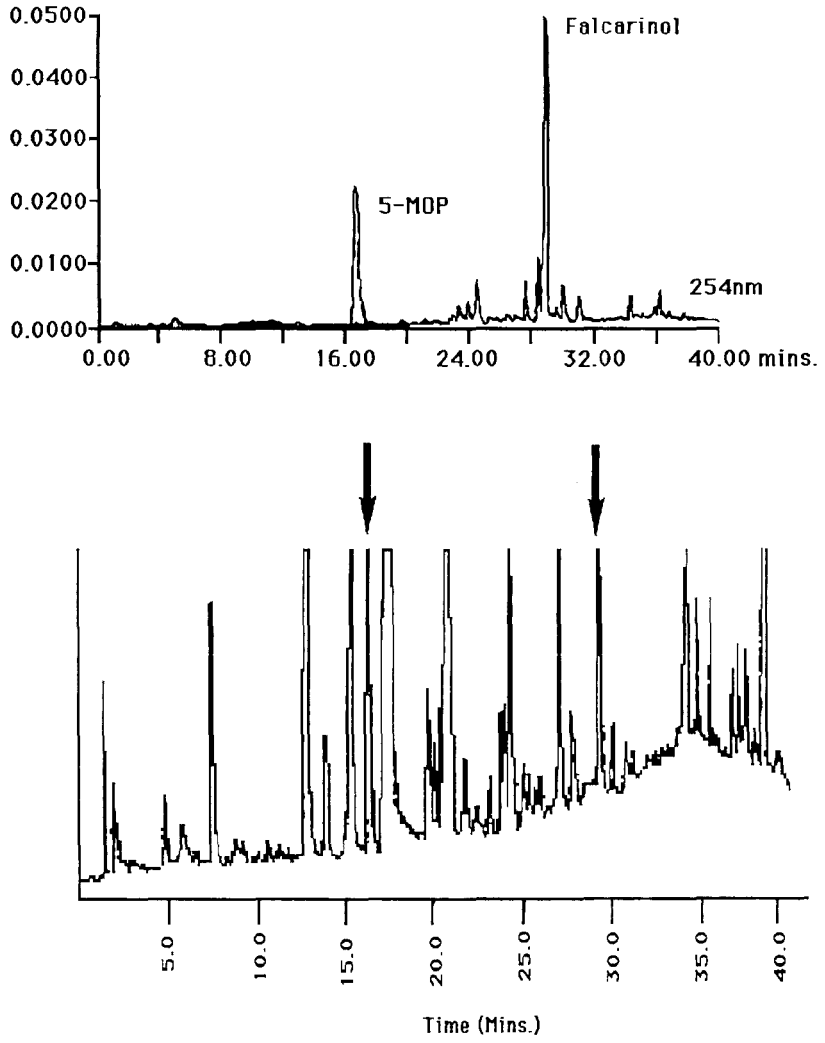


FIGURE 2. HPLC chromatograph of **A**) standards (falcarinol and 5-MOP) and **B**) of a celery extract. Arrows denote retention time of 5-MOP (16.23 min) and falcarinol (29.3 min). The absorption maxima and secondary maxima of falcarinol and 5-MOP obtained from the forward optical scanning detector of the celery samples were identical to the standards in **A**.

second linear gradient was used to return to initial conditions (50% MeOH). A flow rate of 1 ml/min was used. The UV absorbance was monitored at 254 nm. Previously isolated falcarinol and 5-MOP were used as standards in the HPLC analysis (Fig. 2). Ten µl samples were injected into the HPLC. The limit of detection for falcarinol and 5-MOP was 0.05 µg and 0.008 µg, respectively.

RESULTS AND DISCUSSION

Four methods for the extraction of celery were employed to quantitate the amounts of falcarinol, a polyacetylene, and 5-MOP, a furanocoumarin, in commercially available celery. The levels of 5-MOP in commercially available celery varied from 0.08 to 0.24 ppm whereas falcarinol was present at higher concentrations of 0.9-20 ppm (Table 1). No significant difference was observed in the amounts of dermatotoxins extracted by the different methods or material (i.e. frozen, fresh or dried). Co-injection of the falcarinol and 5-MOP standards with a plant sample confirmed the occurrence of both compounds. A representative HPLC chromatograph can be seen in Figure 2.

Although both compounds were found in the celery plant, falcarinol was not identified until Method 1 (Beier *et al.*'s method) was

TABLE 1. Content of 5-methoxypsoralen and falcarinol in healthy, commercially available celery (Utah 5260-R).

METHOD	5-MOP[1]	FALCARINOL
1	0.11 ± 0.09	(2.8 ± 1.1)[2]
2	0.09 ± 0.09	2.9 ± 1.0
3	0.12 ± 0.08	3.1 ± 1.5
4	0.10 ± 0.03	2.7 ± 1.8

[1]All values are reported in parts per million (ppm) and values represent the mean ± S.E. for groups of 6-8 replicates.
[2]Value was obtained only after rinsing C18 SEP-PAK with 100% methanol (See discussion).

modified (See Table 1-Method 1). Brier *et al.* employed a procedure that rinsed a C_{18} SEP-PAK cartridge with 60% acetonitrile (8 ml) in order to elute the furanocoumarins (7). We determined that this procedure was not sufficient to remove falcarinol from the SEP-PAK cartridge. In our workup of the sample, the SEP-PAK was washed with 100% methanol after rinsing with 60% acetonitrile. The levels of falcarinol and 5-MOP were then quantitated as described in the Methods Section.

The standard curve for the furanocoumarin and polyacetylene were generated over a concentration range of 0.008-3.0 ppm and 0.05-40 ppm, respectively. The curves for each compound had correlation coefficients of >0.992 in each case.

Our study indicates that both falcarinol and 5-MOP are present in uninfected, commercially available celery. The novel extraction procedures and analyses described in this report are rapid methods for the simultaneous quantification of both dermatotoxins. No significant differences in the levels of dermatotoxins were observed with the different extraction techniques. The amount required to elicit a photosensitivity response by the linear furanocoumarin 5-MOP is 0.1 μg/8 mm^2 skin area (6). For falcarinol, the amount needed for the elicitation of a delayed hypersensitivity reaction is 0.2 μg/8 mm^2 area (2). In the uninfected celery plant, the quantity of falcarinol is 10-fold greater than 5-MOP. Therefore, it appears likely that the polyacetylenic dermatotoxin, falcarinol, contributes to more celery dermatitis cases than has been previously reported.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF GINSENOSIDES USING PHOTOREDUCTION FLUORESCENCE DETECTION

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ABSTRACT

A high performance liquid chromatographic method using photoreduction fluorescence detection was described for the analysis of ginsenosides. Ginsenosides were separated on an amino column using acetonitrile and aqueous 2-*tert*-butylanthraquinone(t-BAQ) solution. Column effluent was passed through a 45cm-PTFE capillary tube coiled around a 10W-UV lamp to reduce t-BAQ to a highly fluorescent dihydroxy anthracene derivative which was detected by a fluorescence detector. The detection limit for the ginsenoside Rg_1 by this method was found to be about 130ng. This method is less influenced by other UV-absorbing compounds compared to the conventional HPLC-UV detection method.

INTRODUCTION

Ginseng has been widely used as a tonic and restorative agent in Oriental cultures. Ginsenosides are known to be the major constituents of ginseng. Many research papers describe analytical methods of ginsenosides. They include gravimetry, colorimetry, TLC-densitometry, radioimmunoassay, GC and HPLC methods. Among them GC and HPLC methods are commonly adapted for the quantitative analysis of ginsenosides (1-4).

Two advantages of GC are good sensitivity and resolution, however it requires a time-consuming sample preparation step involving hydrolysis and trimethylsilylation(4). The major disadvantage of current GC method is that the individual gisenosides are not quantitated, i.e. only two broad group, panaxadiols and panaxatriols, can be analyzed. Bombardelli et. al. reported direct trimethylsilylation of intact ginsenosides and subsequent GC analysis using harsh GC condition(5).

HPLC methods afford simple sample preparation, however they lack the sensitivity of flame ionization detection(FID) as in GC. Ginseng saponin is a poor chromophore and consequently UV detection is limited to short wavelengths, typically 203 nm. However, many compounds absorb at this wavelengths and interfere with the analysis and the detection sensitivity is not good. The refractive index(RI) detector is much less sensitive.

Post-column photochemical reactions have recently been applied successfully to LC detection, since these methods have demonstrated the selectivity and sensitivity needed for modern analyses(12-15). Photochemical reactions have many advantages over normal thermal post-column reactions (6-8). Since photochemical reactions proceed via

free radical intermediates, the reaction rates are very fast (9-14). Photoreactions require photons instead of chemical reagents, which makes the reaction device more simple and minimizes band broadening. Furthermore, these methods have good selectivity because only a narrow range of analytes can undergo photoreaction (15-17).

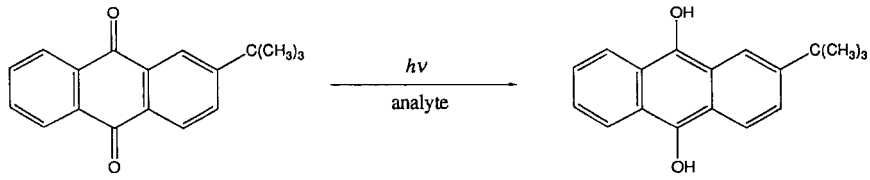
In photoreduction detection, a photoreductive compound is usually added to the HPLC eluent. t-BAQ was used in this study, the photoreduction process is illustrated in SCHEME. t-BAQ itself is very weakly fluorescent but is converted to a highly fluorescent 2-tert-butyldihydroxyanthracene upon irradiation by UV light in the presence of proton-donating substrates.

Birks and Gandelman applied this method to the analysis of monosaccharides and cardiac glycosides (18,19). They used a 10m-knitted PTFE tubing coiled around the fluorescence black lamp and used anthraquinone-2,6-disulfonate (AQDS) or t-BAQ as the photoreactive additive. Their reported detection limits for monosaccharide and cardiac glycoside were about 80ng and 2ng respectively. In this paper, we report the application of this method to the analysis of ginseng saponins.

EXPERIMENTAL METHODS

Materials

2-*tert*-butylanthraquinone (FW 264.32, mp 98-100°C) was purchased from Aldrich and recrystallized from acetonitrile. HPLC grade water was prepared by using a Milli-Q system(Millipore). Acetonitrile (Merck, Germany) was of HPLC grade. Other chemicals were of analytical reagent grade. White ginseng and notoginseng was purchased from the local herbal drugs



2-*tert* -butylanthraquinone 2-*tert* -butyldihydroxyanthracene

SCHEME. Mechanism of photoreduction fluorescence detection.

market in Seoul and ginseng saponin standards were obtained from Korea Ginseng and Tobacco Research Institute.

Instruments

An SLC-100 pump (Samsung, Korea) equipped with a 50μl loop injector (model 7125, Rheodyne, USA), Hitachi F-1050 fluorescence detector(excitation 400nm, emission 525nm), and Hitachi L-4200 UV detector was used. Chromatographic data was processed by a Shimadzu C-R4A Chromapac integrator. For the analysis of ginseng saponins, a Lichrosorb NH_2 column (250mm x 4mm i.d., 10μm, Merck) was used and the eluent was acetonitrile/water (80/20) which contained a photoreactive additive. Figure 1 illustrates the overall photoreaction-HPLC system.

Photochemical reactor

A 10W-UV lamp was purchased at the local electronics market(2.5cm x 32cm, cylinder type, Sam-Gong Co., Korea). The 45cm PTFE capillary tube(0.3mm i.d. x 1.5 mm o.d., Alltech associates) was coiled around the lamp and was wrapped with aluminium foil to increase the photon flux of the lamp by reflection. The reactor was purged with nitrogen to remove oxygen. The photochemical reactor is shown in Figure 2.

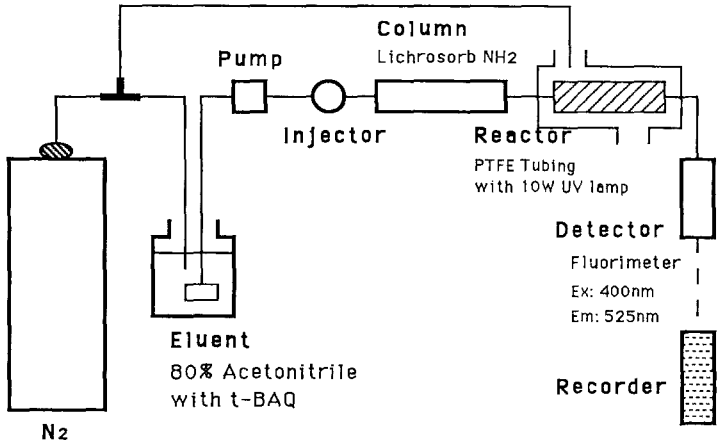


FIGURE 1. Scheme of photoreduction fluorescence detection system.

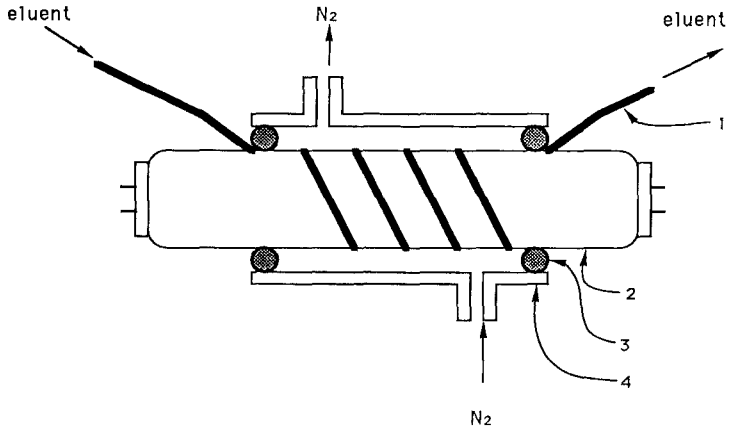


FIGURE 2. Schematic presentation of the photochemical reactor.
1. 45cm PTFE coil; 2. 10W UV lamp; 3. O-ring
4. Polyvinyl Chloride(PVC) pipe.

Analysis of ginsenosides

White ginseng(3g) was extracted with hot methanol. The dried methanol extract was suspended in water and extracted with diethylether. The aqueous layer was extracted with water-saturated n-butanol(3 x 50 ml). Combined butanol extracts were dried under reduced pressure. The residue was dissolved in 10 ml of water, passed through a 0.45 μm membrane filter and subjected to HPLC analysis. Notoginseng radix was treated by the same method. The peaks were identified by co-injection with authentic samples.

RESULTS AND DISCUSSION

Optimization of analytical conditions

Many factors affect the photoreaction. They include concentration of the photoreactive additive, irradiation time, photoreaction temperature, content of water in the effluent, and length of reactor coil. Among these concerns the concentration of photoreactive additive and irradiation time were the major factors which affect the S/N ratio. In order to optimize the photoreactive additive concentration, the concentration of t-BAQ was changed from 7.5×10^{-5}M to 7.5×10^{-4}M and added to the eluent of acetonitrile/water(80/20). The signals of ginsenoside Rg_1 or glucose was compared under these conditions. Figure 3 shows the effect of t-BAQ concentration on the signal intensity. The S/N ratio of ginsenoside Rg_1 increased with increased concentration of t-BAQ up to 4.0×10^{-4}M, however over this concentration the S/N ratio decreased. In fact, signal intensities of ginsenoside Rg_1 was increased even over 4.0×10^{-4} M , but in proportion

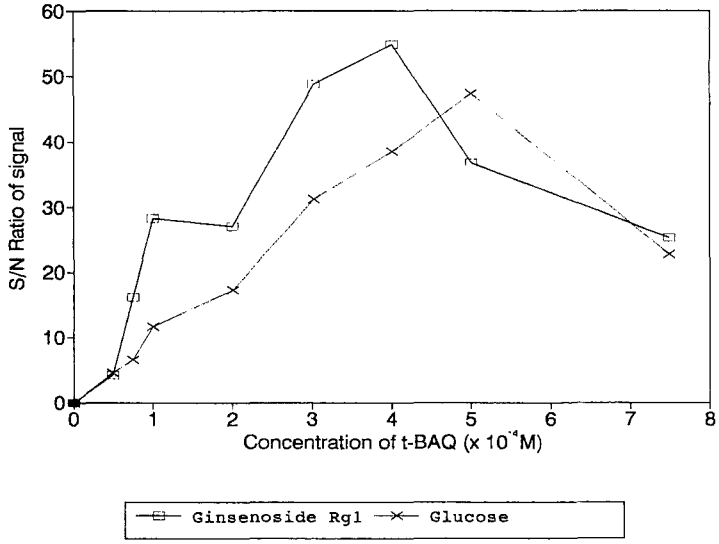


FIGURE 3. Effect of the concentration of t-BAQ on the peak response. conditions : 10W UV lamp with 45cm PTFE tubing; column : Lichrosorb NH_2 column (250mm x 4mm i.d., 10μm); eluent : acetonitrile/water (80/20) with various concentration of t-BAQ; flow-rate : 1ml/min; reaction time : 4sec; detection : fluorescence (excitation : 400nm, emmition : 525nm).

to the concentration of t-BAQ, the fluorescence background signal intensity was also increased. Consequently, the optimal concentration of t-BAQ passing the reactor was found to be 3.0×10^{-4}M. Fluorescence background and noise were reduced with purified and immediately prepared eluent.

The optimal irradiation time was checked by varying the flow rate of eluent or the length of reactor coil. The irradiation time was calculated from the flow rate of the eluent and the internal volume of the capillary PTFE reaction coil. The internal volume of the reaction coil was calculated from the differance of retention time of salicylic acid with and without the reaction

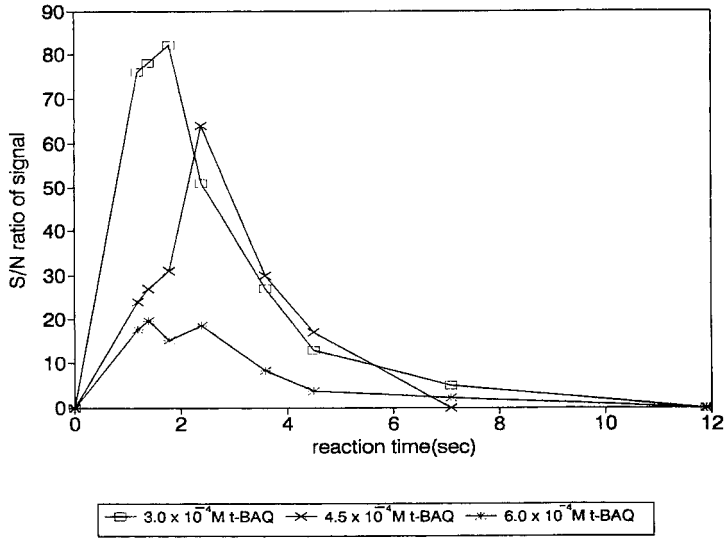


FIGURE 4. Effect of the photoreaction time to the signal-to-noise ratio of ginsenoside Rg_1 (conditions are same as in FIGURE 3).

coil. FIGURE 4 shows the effect of photoreaction time on the signal-to-noise ratio of ginsenoside Rg_1. The highest S/N ratio was observed at 2 sec of reaction time with a $3.0x10^{-4}$ M t-BAQ solution. Both the S/N ratio and peak area were reduced with longer reaction times due to the degradation of the dihydroxyanthracene compound(18-19).

Atmospheric oxygen can penetrate the PTFE capillary resulting in noisy signals. Nitrogen purging of the reactor was very important as Birks et. al. reported (18,19). A Nitrogen purge also acts as a cooling flow of the reactor.

The percent water in the eluent also affected the signal response. An increased signal was observed using a low % water eluent. Also the amino

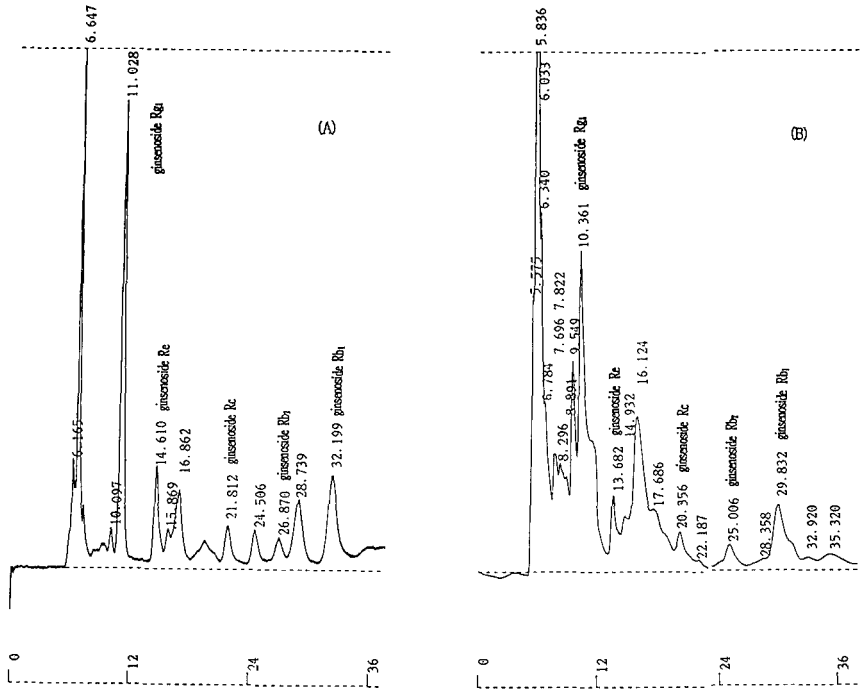


FIGURE 5. Chromatogram of white ginseng : photoreaction (A) and UV (B) detection.
conditions of (A) : 10W UV lamp with 45cm PTFE tubing; column : Lichrosorb NH_2 (250mm x 4mm i.d., 10μm); eluent : 80% acetonitrile with 3.03 x 10^{-4}M t-BAQ; detection : fluorescence (excitation : 400nm, emmition : 525nm).
conditions of (B) : column : Lichrosorb NH2 (250mm x 4mm i.d., 10μm); eluent : 80% acetonitrile; flow-rate : 0.7ml/min; detection : UV 203nm.

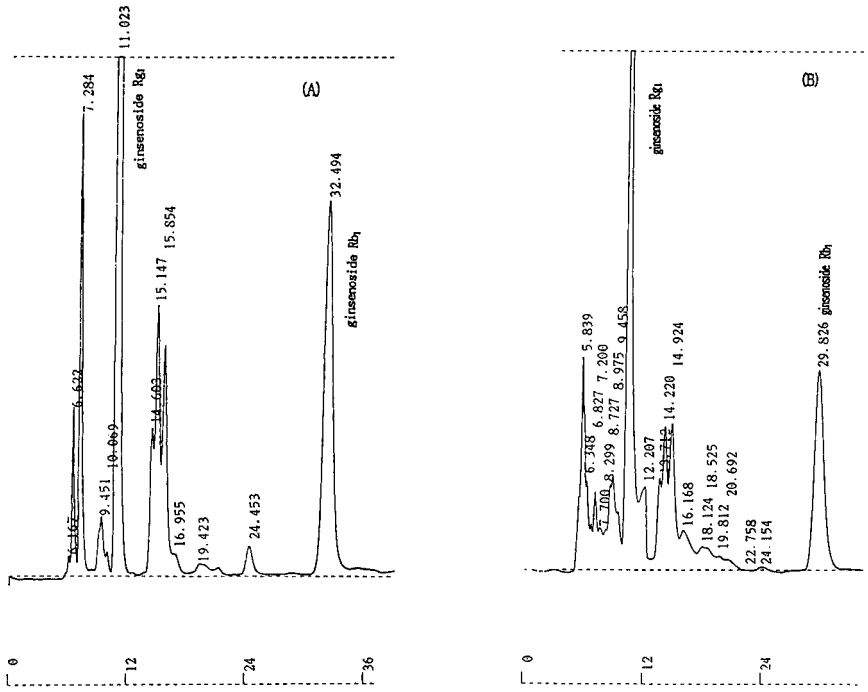


FIGURE 6. Chromatogram of Notoginseng root in photoreaction (A) and UV (B). Conditions are same as in FIGURE 5.

column was more efficient than an ODS column since the former can separate ginsenosides with a low-water percent eluent.

Calibration curve, detection limit and reproducibility

The linearity of response was examined with ginsenoside Rg_1. The correlation coefficient of the calibration curve of ginsenoside Rg_1 and Rb_1 was 0.9993 in the range of 1 - 100 μg. The detection limit(S/N=3) of ginsenoside Rg_1 was 125ng which was almost same as UV detection.

Detection limits of ginsenoside Rb_1, Rb_2, Rc, Rd, Re and Rg_2 were 500ng, 100ng, 250ng, 1000ng, 125ng and 1000ng, respectively. The sensitivity of the photoreaction fluorescence detection method was better than the RI detection method and similar to the UV detection method. However this method shows good selectivity for the ginsenosides.

RSD(relative standard deviation) of peak areas for the repeated injections of ginsenoside Rg_1 was ca. 2.3%(n=12).

Chromatography

Figures 5 and 6 represent the chromatograms of white ginseng and notoginseng root. Compared to UV detection, the PRF method shows good selectivity for ginsenosides. UV-absorbing impurities are not detected by using PRF. Most notables are the peaks which interfere with the signals for ginsenoside Rg_1 and ginsenoside Re when UV detection is employed. These impurity peaks are greatly attenuated or absent when PRF is the method of dection.

ACKNOWLEDGEMENT

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LIQUID CHROMATOGRAPHY CALENDAR

1995

MAY 28 - JUNE 2: HPLC'95, 19th International Symposium on Column Liquid Chromatography, Convention Center, Innsbruck, Austria. Contact: HPLC'95 Secretariat, Tyrol Congress, Marktgraben 2, A-6020 Innsbruck, Austria.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

1996

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

1997

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

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

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.

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

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

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

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

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

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10. Material that cannot be typed, such as Greek symbols, script letters and structural formulae, should be drawn carefully with dark black India ink. Do not use any other color ink.

Additional Typing Instructions

1. The manuscript must be prepared on good quality **white bond paper**, measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). The typing area of the first page, including the title and authors, should be 5½ inches wide by 7 inches high (14 cm x 18 cm). The typing area of all other pages should be no more than 5½ inches wide by 8½ inches high (14 cm x 21.6 cm).

2. The **title, abstract, tables and references** are typed single-spaced. All other text should be typed 1½-line spaced or double line spaced.

3. It is essential to use **dark black** typewriter or printer ribbon so that clean, clear, **solid characters** are produced. Characters produced with a dot/matrix printer are not acceptable, even if they are "near letter quality" or "letter quality." Erasure marks, smudges, hand-drawn corrections and creases are not acceptable.

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

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

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

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

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