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SEPARATION OF LOW DENSITY AND VERY LOW DENSITY LIPOPROTEINS FROM HUMAN SERUM BY HYDROXYAPATITE CHROMATOGRAPHY

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ABSTRACT

The separation of human serum lipoproteins were studied by hydroxyapatite chromatograhy with gradient or stepwise elution using potassium phosphate (KPi) buffers at pH 7.4.

The low-density (LDL) and very low-density (VLDL) lipoproteins were separated from human serum on Tiselius-type hydroxyapatite (Bio-Gel HTP DNA grade) column (25 x 1.0 cm) by four stepwise elutions with 75, 200, 300 and 650 mM KPi buffers. The fractions eluted by 300 and 650 mM KPi contained 4.49 mg LDL and 0.68 mg VLDL, respectively. High-density (HDL) lipoprotein was eluted by 75 mM KPi together with the serum proteins such as albumin, globulin *etc*..

INTRODUCTION

The chromatographic separations of lipoproteins into three main classes, high-density (HDL), low-density (LDL) and very low-

density (VLDL) lipoproteins, have been reported using several types of column packing materials such as Bio-Gel [1, 2], Superose [3] and TSK GEL [4-6].

In previous studies, human serum HDL, LDL and VLDL were separated using several types of commercially available hydroxyapatites [7]. The lipoprotein fractions collected by ultracentrifugation method [8] were loaded on the columns packed with some hydroxyapatites and separated into three major classes lipoproteins (HDL, LDL and VLDL) by means of stepwise elutions with several concentrations of phosphate buffers at various pH values. The retention of the lipoproteins on the hydroxyapatite packings depended both upon cations, for example, sodium, potassium and ammonium ions in the phosphate buffers, and the pH value of the mobile phases. Pre-packed hydroxyapatite, which consists of microporous spherical beads used for high-performance liquid chromatography could not separate LDL from VLDL. This suggested that both the particle size and the crystal form of the hydroxyapatite play an important role in the retention and the separation of the human serum lipoproteins.

The best separation into three main classes (HDL, LDL and VLDL) of human serum lipoproteins was performed on Tiselius-type hydroxyapatite (Bio-Gel HTP DNA grade), which consists of hexagonal prisms with a wide range of crystalline sizes (10-170 μm) packed in a column (10 x 1.0 cm I.D.) by three stepwise elutions with potassium phosphate (KPi) buffers at pH 7.4. It requires about 5 h to elute the VLDL, which was retained the longest on the

column, with 650 mM KPi buffer. In practice, however, it reqires about 40 h to prepare the lipoprotein samples for the chromatography by the ultracentrifugation. Then it took about 45 h for purification of VLDL from human serum. The rapid purification and isolation of the lipoproteins from human serum has been essential for quite some time.

In this work, we have studied the separation and fractionation of the lipoproteins from human serum on hydroxyapatite columns without prior procedures such as ultracentrifugation. The chromatographic separations of lipoproteins from the serum proteins, such as albumin, α−, γ-globulins in human serum are investigated using four types of hydroxyapatites by gradient and stepwise elution with KPi buffers at pH 7.4.

EXPERIMENTAL

Materials

HA-Ultrogel (particle size 60-180 μm), a microcrystal hydroxapatite coated with 4% (w/v) cross-linked agarose (IBF Parmindustrie Villeneuve-La-Garenne, France), Nihon Chemical hydroxyapatite (particle size 50-100 μm), a powder crystal (Nihon Chemical Co. Ltd., Tokyo, Japan), Bio-Gel HT (crystal size 10-250 μm), and Bio-Gel HTP DNA grade (crystal size 10-170 μm), both of which are hexagonal prisms of hydroxyapatite (Bio-Rad Labs, Richmond, CA, U.S.A.) were commercially available. Lyphogel, polyacrylamide gel granules (Gelman Sciences, Ann Arbor, MI,

U.S.A.) and Spectrapor 3 membranes (Spectrum Medical Industries, Los Angeles, CA, U.S.A.) were used for concentration and dialysis of the eluted fractions. Other reagents were of analytical-reagent grade.

Instruments

Human sera were prepared from blood using a Kokusan Type H103N centrifuge (Kokusan Enshinki, Tokyo, Japan). An LKB 2120 Varioperpex II peristaltic pump (LKB, Bromma, Sweden) was used to elute the serum lipoproteins. A Soma Model S-310A UV detector (Soma Optics, Tokyo, Japan) and a JASCO Model 820-FP fluorescence spectrophotometric detector (Japan Spectroscopic, Tokyo, Japan) were used for detecting the absorbance of the eluate at 280 nm and for monitoring the light scattering of the eluate at 580 nm, respectively. The electric conductivity of the eluate was detected with a Bio-Rad Model 1710 Gradient Monitor (Bio-Rad Labs, Richmond CA, U.S.A.) . The lipoproteins and the serum proteins in the fractions were identified and characterized using a disk electrophoresis aparatus (Atto, Tokyo, Japan) and Bio-Rad Mini Protean II slab electrophoresis apparatus (Bio-Rad Labs).

Preparation of human serum from peripheral blood

Human blood (*ca.* 10 ml) was collected from normolipidemic males by venipuncture after 12-16 h of fasting. The blood was allowed to stand for 2-3 h at room temperature until agglutination was completed. The serum was collected after centrifugation at 1000 *g* at 15°C for 15 min.

Hydroxyapatite chromatography of human serum

Gradient elution

Four kinds of hydroxyapatite were suspended and swelled with 1 mM KPi (pH 7.4) and slurry-packed in columns (10 x 1.0 cm I.D.), (25 x 1.0 cm I.D.). After the columns were thoroughly equilibrated with 1 mM KPi, 0.3-1.5 ml of the human serum was loaded, then eluted with a linear gradient of KPi (1-700 mM) at pH 7.4. The flow-rate was 12.0 ml/h .

Stepwise elution

After swelling in starting KPi buffer, four types of hydroxyapatite were packed into columns (10 x 1.0 cm I.D.), (25 x 1.0 cm I.D.) and thoroughly equilibrated with the starting buffers. Human serum (0.3-2.0 ml) was loaded, then eluted stepwise with several concentrations (25-650 mM) of KPi (pH 7.4). The flow-rate was 12.0 ml/h. The amount of the proteins in the fraction was determined by the modified Lowry method [9].

Characterization of human lipoproteins and serum proteins by polyacrylamide gel electrophoresis

The lipoproteins in the eluates were characterized by polyacrylamide gel disk electrophoresis (disk PAGE), modified from the method of Frings *et al.* [10]. The eluates (*ca.* 5-10 ml) at various KPi were placed in dialysis bags (molecular mass cut-off values 3500), which were immersed in aqueous 30%(w/v) polyethylene glycol (PEG) 8000. After 5-6h of dialysis, the eluates

were concentrated to 0.1-0.2 ml. If necessary, two or three granules of Lyphogel were added to the concentrates, which were concentrated further. Disk PAGE was performed in a 3.1% (w/v) separation gel and in 2.5% (w/v) sample gel in glass tubes (10 x 0.5 cm I.D. gel bed).

A 30 µl aliquot of concentrated eluate was mixed with 15 µl of 0.25%(w/v) Sudan black B in 30%(w/v) ethanol, and 450 µl of the sample gel solution was added. The mixture was placed on the polymerized gel, and allowed to stand under a daylight fluorescent lamp for *ca.* 30 min. When photopolymerization was complete, the gel tubes were inserted into the electrophoretic cell. Bromophenol blue, 0.01%(w/v), was added to the upper running buffer as a marker. The electrophoresis was completed in about 1 h, at which time the marker had migrated 5 mm from the end of the tube at 3 mA per gel.

Serum proteins in the fractions were also characterized by SDS polyacrylamide slab gel electrophoresis (SDS-PAGE), according to the method of Laemmli [11]. Gels containing 3% (w/v) (stacking gel) and 10% (w/v) (separation gel) acrylamide were prepared from a stock solution of 30% (w/v) acrylamide and 0.8% (w/v) N, N´-methylene-bis acrylamide. The 5.5 x 10 cm separation gel and the 1.0 x 10 cm stacking gel were prepared between glass plates (gel is 0.75 mm thick). A 5 µl-volume of concentrated eluate was mixed with 95 µl of sample solution [a mixture of 0.025 M Tris (hydroxymethyl) aminomethane, 2% (w/v) sodium dodecyl sulphate (SDS), 5%(w/v) 2-mercaptoethanol, 4%(w/v) glycerol and

0.01%(w/v) bromophenol blue (BPB)] and 10-20 μl of it was loaded onto the stacking gel. Electrophoresis proceeded at a current of 10 mA until the BPB marker reached the stacking gel. Thereafter, the current of the apparatus was increased to 20 mA and the electrophoresis continued untile the BPB marker reached the bottom of the separation gel. The migrated proteins were stained for 5 min at room temperature with the staining solution composed of 0.25% (w/v) Coomassie brilliant blue, 50% (v/v) methanol and 10% (v/v) acetic acid. The gel was destained by washing in a mixture of 7.5% (v/v) acetic acid and 2.5% (v/v) methanol.

RESULTS AND DISCUSSION

Hydroxyapatite chromatography of human serum lipoproteins

The chromatograms of the human serum lipoproteins eluted with a gradient from four kinds of hydroxyapatite columns (10 x 1.0 cm I.D.) are shown in Fig. 1. The gradient elution proceeded at a flow-rate of 12 ml/h of KPi buffer at pH 7.4. The absorbance of the column eluate was monitored at 280 nm, which corresponds to the absorption maximum of lipoproteins. Using HA-Ultrogel coated by agarose, LDL and VLDL were eluted with KPi buffer not exceeding a concentration of 50 mM (Fig. 1A). It appears that these LDL and VLDL passed through the column, because they do not penetrate the surface cross-linked 4% (w/v) agarose layer of the hydroxyapatite. Another LDL-(VLDL) fraction was eluted at 250 to 500 mM KPi concentration. These retained LDL and VLDL have smaller

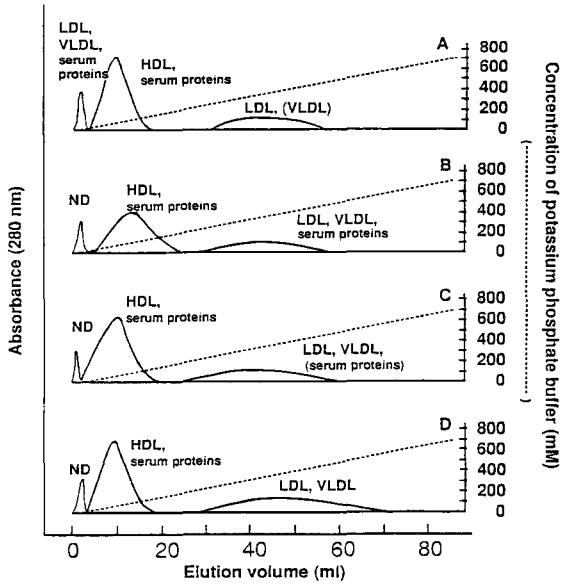

Fig. 1 Chromatograms of human serum HDL, LDL and VLDL on four kinds of hydroxyapatite by gradient elution. ND=not detectable. Columns: HA-Ultrogel (A); Nihon Chemical (B); Bio-Rad HT (C) and Bio-Rad HTP DNA grade (D) hydroxyapatite (10 x 1.0 cm I.D.); eluents: 1-700 mM potassium phosphate (KPi) buffer (pH 7.4); flow-rate: 12.0 ml/h; sample: 0.3 ml human serum.

particles size than those eluted with 50 mM KPi because they penetrated the surface cross-linked agarose and interacted with the core hydroxyapatite. The HDL was eluted with 50 to 200 mM KPi and separated from the LDL-VLDL fractions. However, some serum proteins, such as albumin, α-, γ-globulins were contaminated in the first and second peaks. It showed that a part of LDL and VLDL in the human serum were separated from serum proteins using HA-Ultrogel. This hydroxyapatite is coated by cross-linked

agarose, and provides two separation mechanisms of the lipoproteins. One is the gel permeation mode of the surface agarose gel and the other is adsorption of the lipoproteins to the core hydroxyapatite.

We used three other kinds of hydroxyapatites which are not modified on the surface and have only the adsorptive separation mode. Fig. 1B and C shows the elution patterns of the three main classes of lipoproteins and serum proteins eluted with a gradient from Nihon Chemical and Bio-Gel HT hydroxyapatites, respectively. In the both chromatograms, the HDL and the serum proteins were eluted at concentrations ranging from 50 to 200 mM KPi and the LDL-VLDL fractions eluted at the concentrations above 200 mM together with serum proteins. It has become apparent that HDL and LDL-VLDL fractions could not be separated from serum proteins by these two types of hydroxyapatites with gradient elution.

The hydroxyapatite which best separated the HDL, LDL-VLDL fractions by stepwise elution in the previous study [7], Bio-Gel HTP DNA grade was also used to separate the lipoproteins from human serum with gradient elution (Fig. 1D). HDL was eluted at the 100-150 mM KPi at pH 7.4 together with the serum proteins and the LDL-VLDL fractions were eluted at 250 to 600 mM KPi. The LDL-VLDL fractions include no serum proteins which confirmed by SDS-PAGE. The LDL-VLDL fractions were separated from the HDL-serum protein fractions. However, it can be seen that the LDL and VLDL were not separated each other by a gradient elution from 250 mM to 650 mM KPi.

For the purpose of separating the LDL from VLDL, the combination of the gradient and the stepwise elution were attempted (Fig. 2) using Bio-Gel HTP DNA grade hydroxyapatite. The absorbance of the eluate was monitored at 280 nm and the light-scattering intensity at right angles caused by the lipoprotein particles was also monitored with a fluorescence detector at 580 nm. In order to load a large amount of human serum, we used a longer column (25 x 1.0 cm I.D.). Human serum (1.5 ml) was loaded onto the column and eluted by the gradient elution of the KPi concentration from 1 to 200 mM. After the concentration of KPi buffer reached at 200mM, the concentration of KPi was increased to 300 and 650 mM with stepwise, respectively. Five peaks were detected on this chromatography. The first peak contained serum proteins which eluted at 1 mM KPi (pH 7.4). As the concentration of the KPi gradient increased from 1 to 200 mM, the HDL-serum proteins fractions eluted at about 150 mM. After these serum proteins eluted, a sharp peak of LDL-(VLDL) was eluted immediately upon increasing the KPi concentration to 300 mM. Increasing the concentration to 650 mM resulted in elution of the VLDL fraction. LDL-(VLDL) fractions can be separated from human serum protein using the combination of the gradient and the stepwise elutions, and the VLDL fraction was purified from human serum within 11 h.

It is considered that gradient elution is useful to determine the optimum the KPi concentration to elute the three main classes of lipoproteins from hydroxyapatite. LDL-VLDL fractions can be separated from human serum proteins, such as albumin, α-, γ-

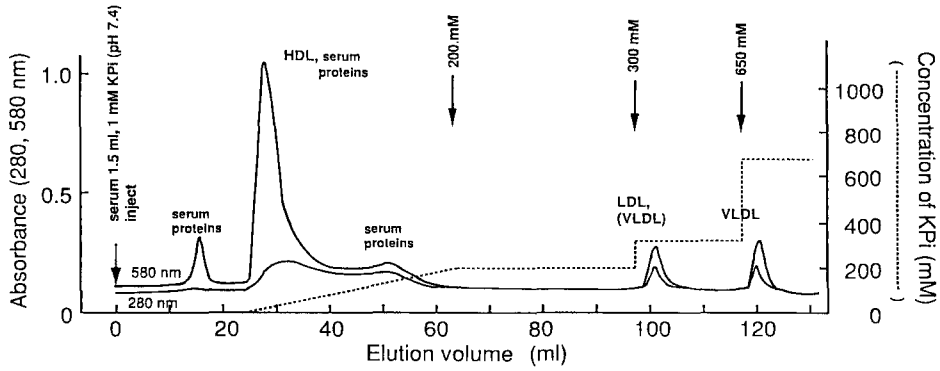

Fig. 2 Elution profile of human lipoproteins by a combination of gradient and stepwise elution. Columns: Bio-Gel HTP DNA grade (25 x 1.0 cm I.D.); eluents: 1-200 mM for gradient and 300, 650 mM KPi (pH 7.4) for stepwise elution; flow-rate: 12.0 ml/h; sample: 1.5 ml human serum.

globulins etc., using Bio-Gel HTP DNA grade. The combination of gradient and stepwise elution allows the separation of the LDL-VLDL and VLDL fractions from human serum proteins.

In general, proteins have been separated by hydroxyapatite chromatography with a stepwise elution of the KPi concentration. This elution mode is favorable for collecting small fractions, because it results in sharp protein peaks which are retained longer on the column. Human serum was eluted from hydroxyapatite with KPi at pH 7.4. The optimum concentrations of KPi for stepwise elution were obtained from the results of the gradient elution.

To separate a large amount of LDL and VLDL from the human serum, we used Bio-Gel HTP DNA grade column (25 x 1.0 cm I.D.) and carried out the four stepwise elutions. A 2 ml-volume

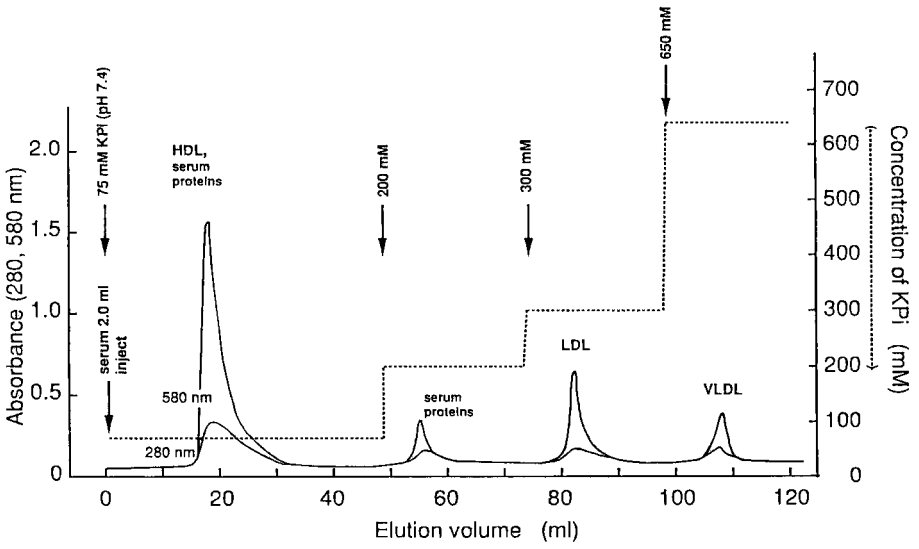

Fig. 3 Stepwise elution profile of human serum lipoproteins. Column: Bio-Gel HTP DNA grade (25 x 1.0 cm I.D.); eluents: 25, 200, 300 and 650 mM KPi (pH 7.4); flow-rate: 12.0 cm/h; sample: 2.0 ml human serum.

of human serum was loaded on the column and eluted with 75, 200, 300 and 650 mM KPi at pH 7.4. Fig. 3 shows the elution profile of human serum and four peaks were detected. The first one contained HDL and serum proteins (fr. 1). Further, the serum proteins were eluted from the column by a KPi concentration of 200 mM (fr. 2). The fractions eluted at 300 mM KPi were mainly LDL (fr. 3) and VLDL was eluted at 650 mM KPi (fr. 4). The frs. 3 and 4 were not contaminated with serum proteins. The amount of the lipoproteins in the both fractions were 4.49 mg (2.25 mg/ml serum) for LDL and 0.68 mg (0.34 mg/ml serum) for VLDL, respectively. The

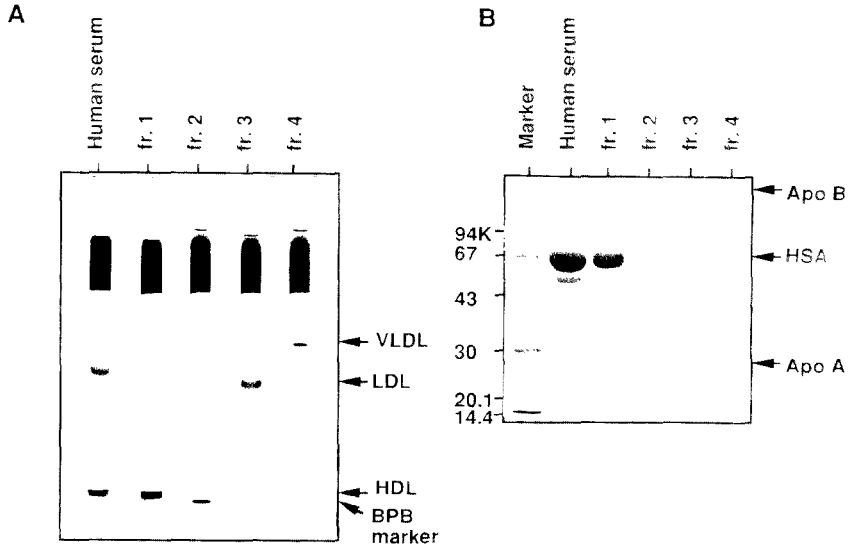

Fig. 4 3% polyacrylamide disk (A) and 12% SDS polyacrylamide (B) slub gel electrophoretic profiles of the fractions collected from hydroxyapatite chromatography of human serum.

lipoproteins and the serum proteins in the fractions were identified by the electrophoresis. Disk PAGE patterns of the fractions are shown in Fig. 4A. The lipid moiety of the lipoproteins was stained by Sudan black B. The fractions eluted by 75, 300 and 650 mM KPi (frs. 1, 3 and 4), corresponding to center cuts of the first, third and forth peaks in the chromatogram contained HDL, LDL and VLDL, respectively. The second peak may represent serum protein, because the fraction showed no lipid staining (Fig. 4A). The serum proteins contained in the fraction were identified by the slub SDS PAGE

patterns with Coomasie briliant blue protein staining (Fig. 4B). The SDS PAGE analysis revealed that the frs. 1 and 2 were contained the most of the serum proteins. The frs. 3 and 4 show no protein band except for the apoprotein B in LDL and VLDL. It was shown that the LDL and VLDL were separated from the proteins in the human serum by hydroxyapatite chromatography of Bio-Gel HTP DNA grade column (25 x 1.0 cm I.D.) within 10 h.

The hydroxyapatite developed by Tiselius *et al.* [12] for chromatographiy is a crystallized form of calcium phosphate. It has been suggested [13] that the crystal size is an effective criterion of the binding strength of hydroxyapatite packing.

It is considered that the separation of the HDL from the serum proteins is difficult on several types of hydroxyapatites. It will be necessary to use materials that provide another separation mode of chromatography to separate HDL from serum proteins.

REFERENCES

1 T. Sata, D. L. Estrich, P. P. S. Wood and L. W. Kinsell, *J. Lipid Res.*, **11**, 331 (1970).

2 L. L. Rudel, J. A. Lee, M. D. Morris and J. M. Felts, *Biochem. J.*, **139**, 89 (1974).

3 P. M. Clifton, A. M. Mackinnon and P. J. Barter, *J. Chromatogr.*, **414**, 25 (1987).

4 I. Hara, M. Okazaki and Y. Ohno, *J. Biochem.*, **87**, 1863 (1980).

5 M. Okazaki, Y. Ohno and I. Hara, *J. Biochem.*, **89**, 879 (1981).

6 M. Okazaki, K. Shiraishi, Y. Ohno and I. Hara, *J. Chromatogr.*, **223**, 285 (1981).

7 U. Matsumoto, H. Nakayama, Y. Shibusawa and T. Niimura, *J. Chromatogr.*, **566**, 67 (1991).

8 L. L. Rudel, C. A. Marzetta and F. L. Johnson, *Methods. Enzymol.*, **129**, 45 (1986)

9 M. A. K. Markwell, L. M. Haas, L. L. Bieber and N. E. Tolbert *Anal. Biochem,.* **87**, 206 (1978).

10 C. S. Frings, L. B. Foster and P. S. Cohen, *Clin. Chem.*, **17**, 111 (1971).

11 U. K. Laemmli, *Nature*, **227**, 680 (1970).

12 A,. Tiselius, S. Hjertén and O. Levin, *Arch. Biochem. Biophys.*, **65**, 132 (1956).

13 M. Spencer and M. Grynpas, *J. Chromatogr.*, **166**, 423 (1978).

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SEPARATION BY PERMEATION EFFECT IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC STUDIES OF SELECTED DITHIA[3.3]CYCLOPHANES AND THIA[3.2]CYCLOPHANENES

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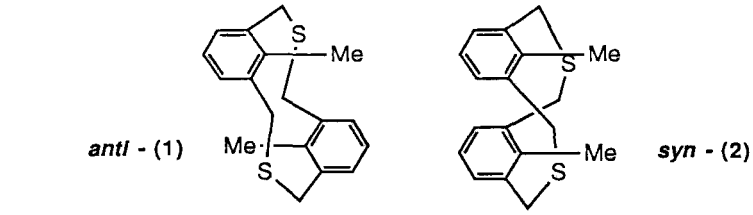
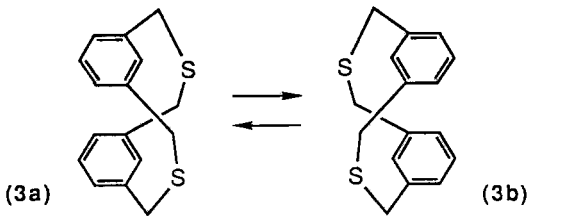
ABSTRACT

The retention order of a series of conformationally mobile dithia[3.3]cyclophanes **3** - **7** was investigated by means of reversed-phase high performance liquid chromatography. By means of the "slot model", the conformational behavior of these dithiacyclophanes, which governs the extent of their interactions with the bonded phase, correlates well with the observed retention order. Another series of conformationally rigid bridge-annelated thia[3.2]cyclo-phanenes **8** - **14** were also similarly investigated. The *anti* conformation allows the whole annelated cyclophanene molecule to slide readily into the "slot" resulting in stronger interactions between the cyclophanene and the bonded phase. The *syn* conformation however has the cyclophane and polycyclic benzenoid moieties held almost perpendicular so that each moiety could not possibly permeate deep into the "slot" independently. Based on the rigid stereochemistries of **8** - **14**, the "slot model" again explains the observed retention order.

* Author to whom correspondence should be addressed.





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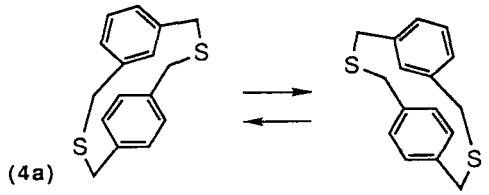

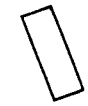

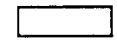

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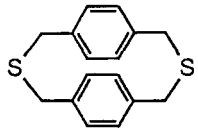

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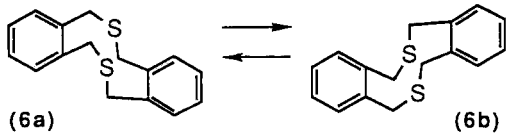

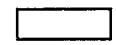

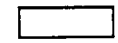

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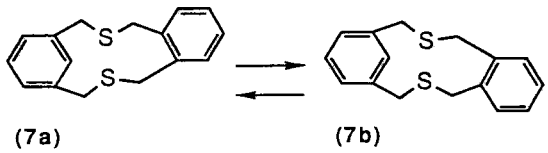

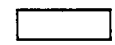

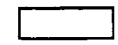

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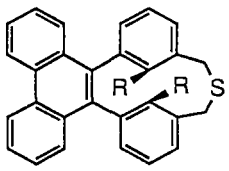
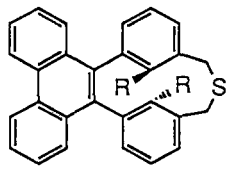


syn

(8) R = H
(9) R = CH_3



anti

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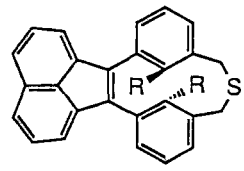
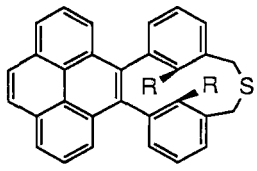


anti

(11) R = H
(12) R = CH_3



syn

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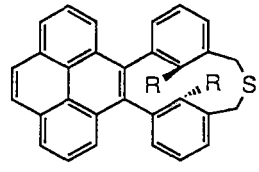
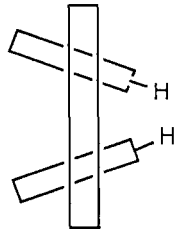


anti

(14) R = CH_3



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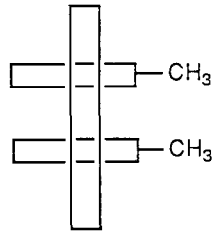
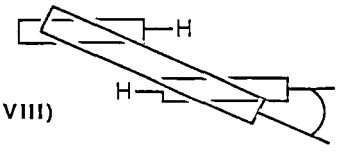
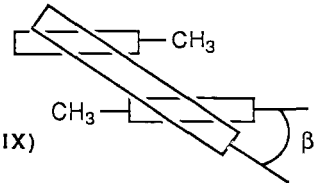


(VII)



(VIII)



(IX)

INTRODUCTION

The syntheses, physicochemical properties and stereochemical aspects of cyclophanes have been of particular interest over the last three decades.[1] Whereas the conformational behavior of mobile cyclophanes could be studied[2] readily by dynamic ^{1}H NMR spectroscopy, some rigid conformers of cyclophanes such as **1**[3] and **2**[3] could in fact be separated. Literature on chromatographic studies of cyclophanes is however limited. Chromatographic separation[4] of a series of rigid naphthalenophanes and optical resolution[5] of selected metacyclophanes have been achieved by high performance liquid chromatography (HPLC). Data on thin layer chromatography (TLC)[6] and HPLC[7] separation of some dithia[3.3]cyclo-phanes have also been reported. There is however no detailed description on the correlation of the retention order of these cyclophanes with their structural properties. There is extensive literature on the dependence of retention of polycyclic aromatic compounds (PACs) on various factors in HPLC studies. Since cyclophanes form a unique family of novel aromatic compounds, we wish to report results from the HPLC studies on the series of dithia[3.3]cyclophanes **3**,[3,8] **4**,[9] **5**,[10] **6**,[11] **7**,[12] and thia[3.2]cyclophanenes **8**,[13] **9**,[14] **10**,[15] **11**-**14**,[16] and correlate their retention behavior with their structural features.

EXPERIMENTAL

Dithiacyclophanes **3** - **7** and thiacyclophanenes **8** - **10** were prepared according to reported procedures.[3-15] Syntheses[16] of compounds **11** - **14** have been achieved in our laboratory and will be published elsewhere.

Chromatographic studies were performed on a Shimadzu LC-6A Binary Gradient Liquid Chromatograph equipped with a Shimadzu SPD-6AV UV-Vis Spectrophotometric (254 nm) Detector and a Shimadzu SCL-6A System Controller. A CHROMPACK reversed-phase C_{18} column (25 cm x 4.9 mm I.D) was used for chromatographic separations. HPLC grade acetonitrile was

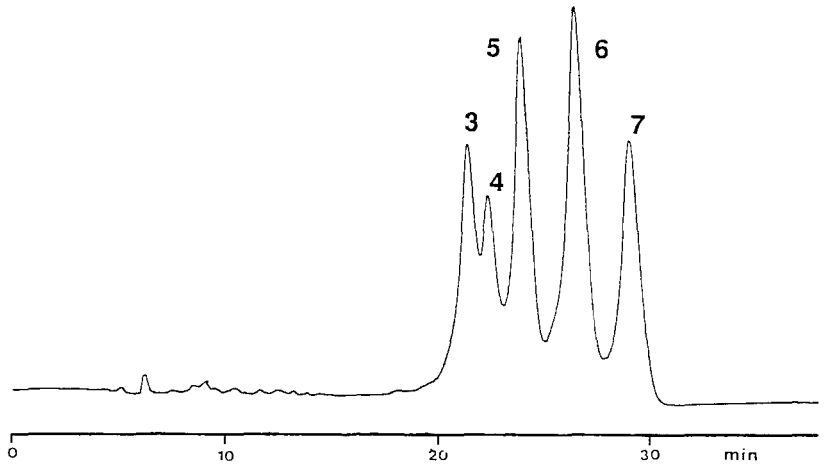


Figure 1. High-performance liquid chromatograms of a mixture of **3** - **7**.

purchased from Fisher Scientific. Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

Isocratic chromatographic conditions were employed using 60% (Figure 1) or 80% (Figure 2) acetonitrile in water with 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min at a maximum pressure of 500 kgf/cm^2.

RESULTS AND DISCUSSION

*Dithiacyclophanes **3** - **7**.*—Reversed-phase HPLC studies of the cyclophane systems **3** - **7** were attempted using various solvent systems. The best resolution of these compounds was achieved using a mixture of acetonitrile and water. Further improvement was obtained with the addition of 1% of trifluoroacetic acid.[17] The order of ease of elution in our work was shown to be **3** > **4** > **5** > **6** > **7** (Figure 1). This however does not correspond to a reversed order to that observed in the TLC studies[6] of the same series of dithiacyclophanes. The conformational behavior of the dithiacyclophanes **3**,[8b]

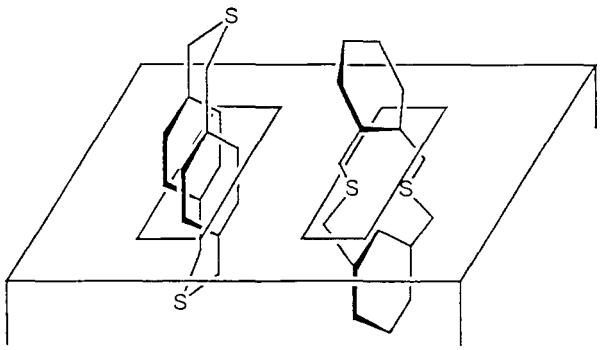


Figure 2. Representation of the slot model of retention based on the permeation effect of the cyclophanes.

4,[9] **6**[11] and **7**[12] at room temperature are known to involve different ring flipping processes as shown. The diagrammatic representations of the averaged relative positions of benzene rings could then be depicted as **I**, **II**, **IV** and **V** respectively. For dithiacyclophane **5**,[10] inversion processes of the bridges are possible but free rotation of the benzene rings is considered unlikely. Thus the two benzene rings in **5** are held more or less parallel to each other as represented by **III**.

The bonded phase of the reversed-phase column is an orderly structure made up of sheets of octadecyl-methyl polysiloxane projecting out of the derivatised silica packing. The retention of any of the dithiacyclophanes will depend on the interaction between the cyclophane and the bonded phase. Each polysiloxane moiety however is large enough to hinder the movement and arrangement of the other moieties and thus a slotted structure[13] is formed (Figure 2). The better the cyclophane fits into the "gaps" or "slots" between the polysiloxane sheets, the stronger the interaction between the cyclophane and the bonded phase and thus the higher the retention time of the cyclophane on the column. The dithiacyclophanes **3** (**I**) and **4** (**II**) have the benzene rings held at an angle and thus would be unable to fit deep into the slots. The more perpendicular

stereochemistry in **II** would allow the 1,3-bridged ring to permeate better into the slots consistent with its observed longer retention time (Figure 1). The conformationally more rigid dithiacyclophane **5** have the benzene rings held parallel and both rings could somewhat squeeze into the slots (Figure 2) thus increasing the interaction between **5** and the bonded phase. The dithiacyclophane **6** exhibits a conformational equilibrium between "chair" conformers **6a** ⇌ **6b** (represented by **IV**) while **7** displays a pendulum behavior between **7a** ⇌ **7b** with averaged linear positions of the benzene rings as shown in **V**. Both **IV** and **V** are expected to permeate most deeply into the slots of the bonded phase (Figure 2) among the five dithiacyclophanes studied, with **V** being the most ideal molecule to slide readily in between the polysiloxane sheets. The above argument based on the permeation effect clearly correlates well the observed order of retention with the conformational behavior of the respective dithiacyclophanes.

*Thiacyclophanenes **8** - **12**.* As discussed earlier, the interactions between dithiacyclophanes **3**, **4**, **6** and **7** with the bond phase may be affected by their conformational mobilities. In order to investigate whether the above slot model could be applied to other cyclophane systems, HPLC studies of conformationally rigid thiacyclophanenes **8** - **12** were carried out under similar conditions. With reference to *anti*-**1** and *syn*-**2**, thiacyclophanenes **8**[13] (**VI**), **9**[14] (**VII**) and **13**[16] (**VII**) were found to exist in rigid syn conformation whereas anti conformers of **10**[15] (**IX**), **11**[16] (**VIII**), **12**[16] (**IX**) and **14**[16] (**IX**) have also been isolated.

The increasing retention order of the thiacyclophanenes studied (Figures 3a - 3c) was found to be **8** < **9** < **11** < **12** < **10**,**13** < **14**. Earlier reports[19,20] on the reversed-phased HPLC studies of PACs show an increasing order of acenaphthylene < phenanthrene < pyrene. Our results (Figure 3a - 3c) indicate a random order if the retention behavior of these compounds depends solely on the nature of the respective PAC moieties. The phenanthrocyclophanenes **8** and **9** were eluted faster but **10** slower than the acenaphthylenocyclophanes **11** and **12** (Figure 3c); whereas phenanthrocyclophane **10** and pyrenocyclophane **13**

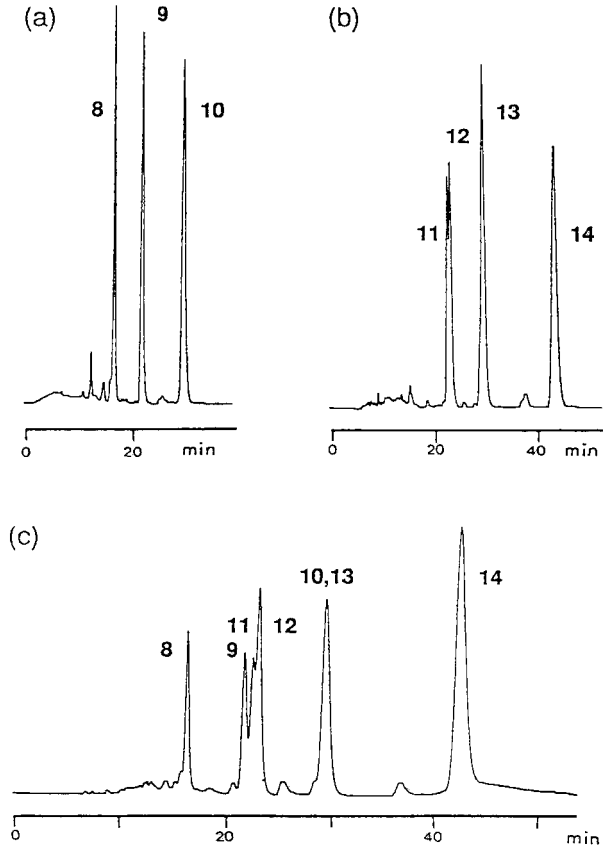


Figure 3. High-performance liquid chromatograms of (a) a mixture of **8** - **10**; (b) a mixture of **11**- **14**; and (c) a mixture of **8** - **14**.

happen to have identical retention times (Figure 3c). The retention order of these cyclophanenes clearly depends also on the stereochemistry of the cyclophane moieties to a significant extent. The presence of methyl groups in **9**, **10**, **12** and **14** will also indirectly affect the overall results (see later discussion).

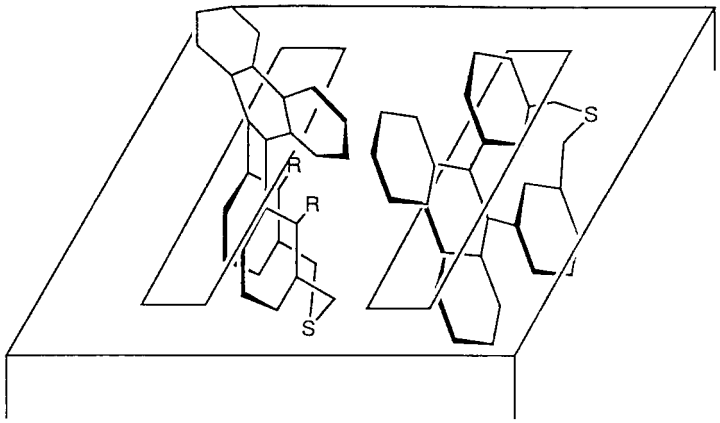


Figure 4. Representation of the slot model of retention based on the permeation effect of the *syn* and *anti* cyclophanenes.

The stereochemistry of the three anti cyclophanenes **10**, **12** and **14** could in general be represented by **IX**. The PAC and cyclophane moieties in each case could interact with the bonded phase independently or more likely the whole annelated cyclophanene molecule could slide readily into the slots (Figure 4). The cyclophane moiety is common among the three compounds and thus their retention order of **12** < **10** < **14** follows that observed for acenaphthylene < phenanthrene < pyrene. Going from **12** (**IX**) to **11** (**VIII**), the small spatial requirement of the two "internal" hydrogen atoms in **11** is expected to result in an outward slide of the two stepped benzene rings leading to a smaller angle β as shown in **VIII**. Such a sliding phenomenon has been described for other related cyclophane systems.[21] Our results (Figure 3b) however show that **11** was eluted before **12** indicating that the smaller angle β in **11** (**IX**) does not increase appreciably its permeability compared with that of **12**. On the other hand the stronger interaction between the two "toluene" units in **12** and the bonded phase could account for its slightly longer retention time.

A comparison of the relative retention order of each pair of syn and anti isomers, namely **9** and **13** against **10** and **14** respectively (Figures 3a and 3b), shows that the syn isomer is always eluted significantly faster than the anti isomer. The two syn cyclophanenes **9** and **13**, as represented by **VII**, have the PAC and cyclophane moieties held almost perpendicularly. The two moieties thus could not possibly permeate deep into the slots independently (Figure 4). The large difference in the retention time of each pair of syn and anti isomers therefore further supports the fact that the stepped stereochemistry of the anti isomers allows the whole molecule to slide readily in between the polysiloxane sheets thus significantly increasing their interaction with the bonded phase.

Of the two cyclophanenes **9** and **13** having the same stereochemistry as represented by **VII**, their retention order of **9** < **13** follows that expected of phenanthrene < pyrene. Going from **9** (**VII**) to **8** (**VI**), the small spatial requirement of the "internal" protons is again expected to result in a change in the molecular geometry of the cyclophane moeity as indicated. The tilting of the two rings is encouraged to minimize their π-π interaction. With the phenanthrene moiety common in both **8** and **9**, their retention order of **8** < **9** depends solely on the nature of their respective cyclophane moieties which resemble those of **3** (**I**) and **5** (**III**) discussed earlier. In addition the "toluene" units in **9** are also expected to interact more strongly with the bonded phase as described in the comparison of **11** and **12**.

CONCLUSION

Our results have demonstrated that the respective retention orders of related dithia[3.3]cyclophanes and another series of bridge-annelated thia[3.2]cyclophanenes could be correlated qualitatively to the conformational behavior of the cyclophane systems based on the permeation effect in a slot model. We believe that the results observed in this work would be valuable in future related investigations on analytical and conformational studies of cyclophanes by HPLC.

ACKNOWLEDGEMENT

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REFERENCES

1. (a) Cyclophanes, edited by P. M. Keehn and S. M. Rosenfeld, Vol. I and II. Academic Press, New York (1983); (b) Cyclophanes I, edited by F. Vögtle, Top. Curr. Chem. 113, 1-182 (1983); (c) Cyclophanes II, edited by F. Vögtle, Top. Curr. Chem. 115, 1-150 (1985).

2. (a) R. H. Mitchell, Adv. in Theoret. Interesting Molec. 1, 135 (1989); (b) Y.-H. Lai, Heterocycles 16, 1739 (1981).

3. R. H. Mitchell and V. Boekelheide, J. Am. Chem. Soc. 96, 1547 (1974).

4. A. Deege, B. Heinecke and G. Schomburg, Chromatographia 15, 65 (1982).

5. K. Meurer, A. Aigner and F. Vögtle, J. Inclusion Phenom. 3, 51 (1985).

6. (a) P. Kuś, J. Chromatogr. 436, 338 (1988); (b) P. Kuś, J. Chromatogr. 442, 378 (1988).

7. J. Śliwiok, P. Kuś and M. Sajewicz, J. Chromatogr. 472, 314 (1989).

8. (a) T. Sato, M. Wakabayashi, K. Hata and M. Kainosho, Tetrahedron 27, 2737 (1971); (b) W. Anker, G. W. Bushnell and R. H. Mitchell, Can. J. Chem. 57, 3080 (1979).

9. (a) F. Vögtle, Chem. Ber. 102, 3077 (1969); (b) V. Boekelheide, P. H. Anderson and T. A. Hylton, J. Am. Chem. Soc. 96, 1558 (1964).

10. (a) J. Bruhin and W. Jenny, Tetrahedron Lett., 1215 (1973); (b) F. Vögtle, Chem. Ztg. 94, 313 (1970).

11. (a) D. W. Allen, P. N. Braunton, J. T. Millar and J. C. Tebby, J. Chem. Soc. C, 3454 (1971); (b) Y.-H. Lai and M. Nakamura, J. Org. Chem. 53, 2360 (1988); (c) T. Okajima, Z.-H. Wang, Y. Fukazawa, Tetrahedron Lett. 30, 1551 (1989).

12. F. Vögtle, Tetrahedron 25, 3231 (1969).

13. Y.-H. Lai and S.-M. Lee, J. Org. Chem. 53, 4472 (1988).

14. Y.-H. Lai and P. Chen, J. Org. Chem. 54, 4586 (1989).

15. Y.-H. Lai, J. Am. Chem. Soc. 107, 6678 (1985).

16. Y.-H. Lai, P. Chen and T.-G. Peck, unpublished results.

17. J. L. Meek and Z. L. Rossetti, J. Chromatogr. 211, 15 (1981).

18. S. A. Wise and L. C. Sander, J. High Resolut. Chromatogr. Chromatogr. Commun. 8, 248 (1985).

19. S. A. Wise, W. J. Bonnett and W. E. May, in Polynuclear Aromatic Hydrocarbons: Chemistry and Biological Effects; A. Bjørseth and A. J. Dennis, eds., Battelle Press, Columbus, Ohio (1980).

20. Y.-H. Lai, S.-G. Ang and H.-C. Li, J. Chromatogr. 606, 251 (1992).

21. R. H. Mitchell, J. S. H. Yan and T. W. Dingle, J. Am. Chem. Soc. 104, 2551 (1982).

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RETENTION OF ANIONS AS A FUNCTION OF MOBILE PHASE pH IN ION-INTERACTION RP-HPLC

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ABSTRACT

Retention of typical anions as a function of pH of the mobile phase in reversed-phase ion-interaction chromatography was studied.

The different behaviours observed for anions of acids characterized by different pK_a values were discussed, in comparison with literature results obtained in reversed-phase and in reversed-phase ion-pair chromatography.

INTRODUCTION

Many separation methods in reversed-phase HPLC chromatography make use of ion-pair mechanisms, in order to improve the chromatographic response (1,2). These technique are generally reported as reversed-phase ion-pair or ion-interaction chromatography and make use of a reversed-phase stationary-phase. In most of these methods the ion-pairing agent is added to the water-organic mobile phase to form an ion-pair with the analyte, which due to its increased lipophilic properties is

retained onto the reversed-phase column, without necessarily implying a modification of its surface.

Less used is the technique employed in our laboratories (3-5), in which the mobile phase is an aqueous solution of the interaction reagent. We propose that the methods which employ a mobile phase containing an organic component are called, as usually, ion-pair chromatography and the methods in which the mobile phase is an aqueous solution are refered as ion-interaction chromatography, in consideration that ionic-interaction mechanisms here predominate, with respect to adsorption mechanisms. The mechanism model in fact which characterizes ion-interaction chromatography assumes that the interaction reagent (which represents the mobile phase), when flowing under isocratic conditions, induces a dynamic modification of the surface of the reversed-phase stationary phase (6). The hydrophobic cation of the interaction reagent (for example $CH_3(CH_2)_nNH_3^+$ alkylammmonium) is adsorbed onto the $C_{18}H_{37}$-bonded apolar surface of the stationary phase, forming a primary positively-charged ion layer. In a second step, the counter-anion A^- is bound as well through electrostatic forces , to form an oppositely-charged diffuse layer. The alkylammonium ion and the counter-ion A^- give likely rise to an ion-pair which is adsorbed onto the stationary phase and at the same time participates to the equilibrium with its ions flowing in the mobile phase:

$$(CH_3(CH_2)_nNH_3^+ \, . \, A^-)_{adsorbed} \; ===== \; CH_3(CH_2)_nNH_3^+ \; + \; A^- \quad (1)$$

Due to the electrical double-layer adsorbed onto it, the surface of the original C-18 stationary phase has been modified into a new stationary phase characterized by anionic and cationic retention properties. The new column obtained, which might be called "catanionic" (in analogy with the catanodic current- potential curve) is in fact able to retain both anionic and cationic analytes, through the formation of ion-pairs respectively with the alkylammonium ion and its anion A-.

Of course, these processes can take place in pH conditions in which the species involved are present as ionized form, i.e. anions are dissociated and amines protonated. The acidic dissociation constants of the components of the interaction reagent as well as of the analytes must therefore be taken into account when planning pH working conditions.

The methods already developed in this laboratory for the separation of anions, amines, amides and imides (3-5) have been performed at pH 6.4, at which many anions are dissociated and many N-containing organic bases are protonated. Retention was shown to depend on the alkyl chain length and on the concentration of the interaction reagent and these effects have been investigated (5). In the present paper we study the effect induced on the retention of typical anions by varied pH conditions of the mobile phase.

Two effects can be expected. The first is directly connected with the molar fraction of the dissociated form of the analyte as a function of the acidic dissociation constant and the second effect could come from a pH-dependence of the composition of the moiety adsorbed onto the stationary phase surface.

Literature reports investigations concerning the effect induced on retention of anions by pH variations of the mobile phase (1,2,7-17). Generally, the capacity factor depends on the acidic dissociation constant of the acid, but different behaviours are observed in reversed-phase chromatography and in reversed-phase ion-pair chromatography (1,2,8,14-17). In this paper the dependence of retention on pH is investigated in reversed-phase ion-interaction chromatography, in which, as mentioned, the mobile phase is an aqueous solution, in the total absence of organic modifier .

MATERIALS AND METHODS

Apparatus

Analyses were carried out with a Merck-Hitachi Lichrograph chromatograph Model L-6200, equipped with a two-channel Merck-Hitachi model D-2500 Chromato-integrator, interfaced with a UV-vis detector model L-4200 and a L-3720 conductivity detector with temperature control, of the same firm.

A Metrohom 654 pH-meter equipped with a combined glass-calomel electrode was employed for pH measurements and a Hitachi mod.150-20 spectrophotometer for absorbance measurements.

Chemicals and Reagents

Ultrapure water from Millipore Milli-Q was used for the preparation of all solutions. Sodium nitrite, sodium nitrate, sodium bromide, sodium iodide, ascorbic acid and methanol were Merck analytical grade reagents. Octylamine, sodium azide and sodium bromate were Fluka analytical grade chemicals. Potassium thiocyanate was C.Erba analytical grade chemical.

Chromatographic conditions

A 5 μm ODS-2 Spherisorb Phase Separation column fully end-capped 250.0 x 4.6 mm with a carbon load of 12% (0.5 mM/g) and a 5 μm Merck Polyspher RP-18 250.0 x 4.0 mm were used , together with a 150.0 x 4.6 mm Lichrospher RP-18 , 5 μm guard pre-column.

The solutions to be used as mobile phase were prepared by adding to the amount of octylamine weighed to prepare a 5.0 mM solution the required amount of ortho-phosphoric acid up to get the desired pH value. The solutions prepared at the different pH values contained therefore the same analytical concentration of octylamine (5.0 mM) and different analytical concentrations of the acid. With this procedure the presence in the mobile phase of any other component different from octylamine and o-phosphoric acid is avoided.

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline signal was obtained; a minimum of 1 hour was necessary. This procedure was always followed when a new mobile phase was used. After use, the column was washed and regenerated by flowing a 50/50 v/v water/methanol mixture (0.5 ml/min for 1 hour). No particular degradation of the column was observed with pH variations.

RESULTS AND DISCUSSION

Experiments with polymer-based reversed-phase C-18 stationary phase.

In order to explore the widest pH range , a C-18 reversed-phase polymeric (styrene-divinylbenzene)-based stationary phase (Merck

Polyspher) whose working conditions range between pH 0 and 14 was firstly employed. The same procedures of dynamic functionalization as described for the silica-based reversed-phase columns have been employed. Several experiments under different conditions have been performed. The use of interaction reagents formed by amines characterized by different structure and chain length (such as tetrabutylammonium, aethylammonium,aethanolammonium,octylammonium,dodecylammonium) was experimented as well as of different anions, namely o-phosphate, salicylate and tartrate, in concentrations ranging between 0.5 mM and 5.0 mM. In order to allow column wettability, mobile phases containing methanol or acetonitrile in concentrations ranging between 5 and 50 % were also used. Unfortunately, in no way it was possible to obtain an appreciable and reproducible dynamic coating of this column. We cannot explain the negative result. The most relevant difference between the two stationary phases can be individuated in the lack of sylanolic residua groups in the polymer-based column. But a series of experiments previously performed for different silica-based reversed-phase stationary phases (18,19) had shown that the best results in dynamic coating were obtained with fully-endcapped stationary phases. The unsuccess in obtaining a stable dynamic functionalization of the polymeric stationary phase surface might likely be correlated to the per cent carbon load which is generally lower in polymer-based stationary-phase with respect to the silica-based ones.

Study of anions pH-dependence with a silica-based reversed--phase C-18 stationary phase.

The pH-dependence of anions retention was therefore investigated in the range imposed by a prudent use of a silica-based -C18 column and experiments in the pH range between pH 2.5 and 8.0 were performed for some typical anions. Octylamine o-phosphate was the interaction reagent and conductometric and spectrophotometric detections were comparatively employed. The dead time, evaluated through the injection of $NaNO_3$ solutions and conductometric detection of the unretained Na^+, resulted to be practically pH-independent, so that the obtained retention times at different pH values could be directly compared.

As expected, the retention of some analytes could be obtained only at pH values at which ionized forms are present. So for example at pH

8.0, the retention of phenol (pKa=10.0) (20) was obtained , which is instead precluded at lower pH values.

The following anions were studied, which all can be separated at different pH values, are characterized by different chemical properties and non null molar absorptivity at 230 nm: nitrate, bromide, bromate, iodide, tiocyanate, nitrite, hydrazoate and ascorbate.

As mentioned, two effects can be played on the retention of anions by pH variations of the mobile-phase.One arises from the acidic dissociation equilibria of the analytes and a second effect could be originated by the pH-dependence of the moiety adsorbed onto the surface of the stationary phase.

Figure 1 shows the behaviour, as a function of pH, of the capacity factors K' for anions of weak acids as nitrite (pK_a = 3.14) , hydrazoate (pK_a = 4.74) and ascorbate (pK_{1a}= 4.14, pK_{2a}= 11.57). All the plots show a maximum for intermediate pH values and could be fitted (r^2 always > 0.84) by a polynomial function of the type : $y=ax^3+bx^2+cx+d$ (y are the capacity factor, x the pH values and the coefficients a ,b,c and d for each regression are reported in the caption of figure 1). The shapes obtained are very similar to the theoretical plots predicted by Snyder in reversed-phase ion-pair chromatography (21), even if the maximum of the function corresponds to different α (fraction of the dissociated species) values.

The increase in retention observed for lower pH values is explained through the increasing concentration of the dissociated species when pH increases. For pH values > about 4, even if the molar fraction of the dissociated species is still increasing, retention decreases. This result could be ascribed to modifications induced at these pH values on the moiety adsorbed onto the surface of the stationary phase, which prevail on the effect due to the dissociation equilibria of the analytes. Other effects can furthermore intervene at higher pH values in decreasing retention, such as : a) a possible lower functionalization extent of the stationary phase , b) a lower attraction electrostatic effect exerted towards the anions by the positevely-charged first electrical layer adsorbed onto the surface (as pH increases, the molar fraction of protonated octylamine decreases) and c) a competition effect of OH^- anions for higher pH values (21).

The hypothesis that at higher pH values, retention of anions depends on the effects that pH plays on the modified stationary phase more than on

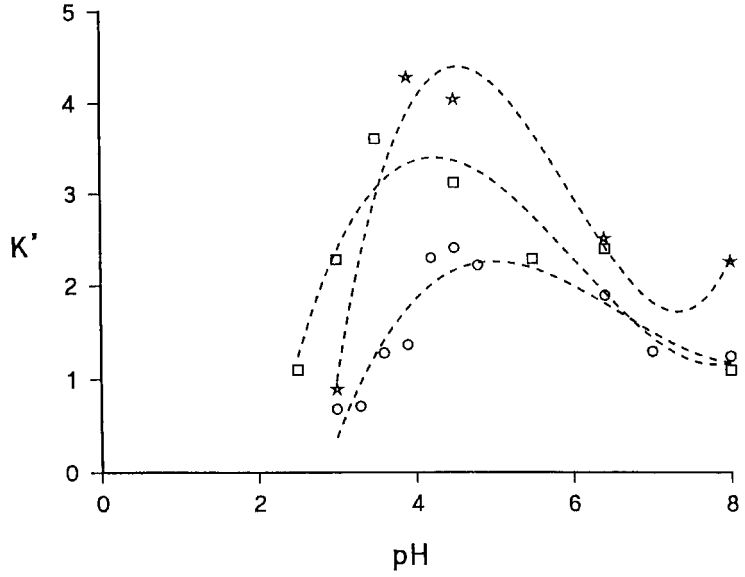

Figure 1.
Capacity factor K' (Y) as a function of pH (X).

✯ ascorbate	$Y = 0.25\ X^3 - 4.45\ X^2 + 24.97\ X - 40.62$	$r^2 = 0.9625$
□ nitrite	$Y = 0.10\ X^3 - 1.80\ X^2 + 9.94\ X - 13.88$	$r^2 = 0.8403$
○ hydrazoate	$Y = 0.07\ X^3 - 1.36\ X^2 + 8.47\ X - 14.63$	$r^2 = 0.8535$

the analyte dissociation, can find a confirm in the retention behaviour shown by anions of strong acids (figure 2), for which the unexpected retention decrease observed with pH increase is ascribable only to a pH-dependence of the moiety adsorbed onto the stationary phase.

Taking on the other hand into account that the adsorbed moiety depends on the mobile phase composition, a calculation was performed of the distribution, in the pH range between 2.0 and 11.0, of all the chemical species involved in the mobile phase. Acid-base equilibria of octylamine and o-phosphoric acid (20,22) were considered as well as ion-pair equilibria. Octylamine is reported (23) to form with o-phosphate five

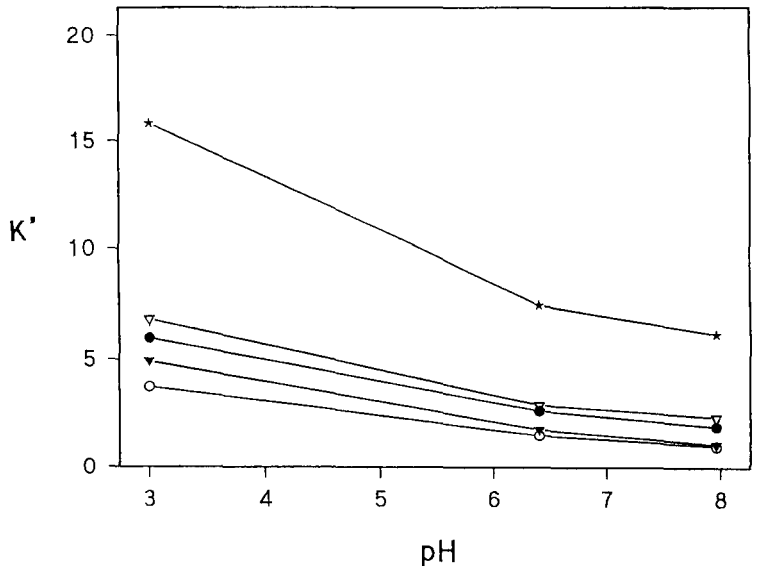

Figure 2.
Capacity factor K' as a function of pH.
★ thiocyanate, ▽ iodide, ● nitrate, ▼ bromate, ○ bromide.

differently-protonated ion-pair species (two of which present electroneutrality) containing octylamine to phosphate molar ratios equal to 1 and 2 . Table I reports the input data of: a) the analytical concentrations of octylamine and of ortho-phosphoric acid and b) the formation constants used (20,22,23) expressed according to the general equilibrium:

$$n\ OctNH_2 + p\ PO_4^{3-} + q\ H^+ ==== H_q(PO_4)_p(Oct\ NH_2)_n^{(3p-q)-} \quad (2)$$

and table II the results obtained. In the pH range investigated octylamine, in agreement with its dissociation constant (20), is predominantly present (>98%) under its protonated form, the remaining portion being distributed, as a function of pH, among the five ion-pair species formed with phosphate. To the two electrically-neutral ion-pairs, for their less

TABLE I

Input data for the calculation of species distribution of table II

a) analytical concentrations

pH	*Octylamine*	*o-phosphoric acid*
2.00	5.00 mM	38.0 mM
3.00	5.00 mM	5.26 mM
3.98	5.00 mM	4.58 mM
6.01	5.00 mM	4.00 mM
6.39	5.00 mM	2.94 mM
7.00	5.00 mM	2.19 mM
7.96	5.00 mM	2.00 mM
10.00	5.00 mM	1.85 mM
11.00	5.00 mM	0.24 mM

b) formation constants logarithms (logK) according to the equilibrium:

$nOctNH_2 + p\ PO_4^{3-} + qH^+ \ ========== \ H_q(PO_4)_p(OctNH_2)_n^{(3p-q)-}$

species	*logK*
H_3PO_4	22.00
$H_2PO_4^-$	19.88
HPO_4^{2-}	12.67
$OctNH_2$	10.65
$OctNH_3^+.H_2PO_4^-$	31.09
$(OctNH_3^+)_2.HPO_4^{2-}$	35.57
$OctNH_3^+.HPO_4^{2-}$	24.19
$(OctNH_3^+)_2.PO_4^{3-}$	24.28
$OctNH_3^+.PO_4^{3-}$	11.98

energetical content, the higher probability to be adsorbed onto the surface of the stationary phase must be assigned. From the data of table II, it can be observed that the sum of the concentrations of these species is decreasing as pH increases. Moreover, according to Stahlberg (24-26), Liu and Cantwell (27), different concentrations of the adsorbed moiety induce onto the stationary phase different surface potentials Ψ_o and, as a consequence, different capacity factors (25).

TABLE II.

Calculation of species distribution (molar concentration), as a function of pH, in solutions containing octylamine and o-phosphoric acid.
Input data of analytical concentration and formation constants in table I.

	pH								
	2.00	3.00	3.98	6.01	6.39	7.00	7.96	10.00	11.00
$OctNH_2$	$1.1.10^{-11}$	$1.1.10^{-10}$	$1.05.10^{-9}$	$1.13.10^{-7}$	$2.71.10^{-7}$	$1.11.10^{-6}$	$1.00.10^{-5}$	$6.91.10^{-4}$	$3.34.10^{-3}$
H_3PO_4	$2.14.10^{-2}$	$6.03.10^{-4}$	$6.12.10^{-5}$	$4.76.10^{-7}$	$1.34.10^{-7}$	$1.74.10^{-8}$	$4.2.10^{-10}$	$3.2.10^{-16}$	$1.2.10^{-19}$
$OctNH_3^+.H_2PO_4^-$	$2.79.10^{-4}$	$8.17.10^{-5}$	$7.93.10^{-5}$	$6.60.10^{-5}$	$4.43.10^{-5}$	$2.37.10^{-5}$	$5.22.10^{-6}$	$2.8.10^{-10}$	$5.1.10^{-13}$
$(OctNH_3^+)_2.HPO_4^{2-}$	$<10.^{-30}$	$2.7.10^{-10}$	$6.5.10^{-30}$	$6.1.10^{-16}$	$3.67.10^{-7}$	$2.4.10^{-10}$	$1.58.10^{-6}$	$6.11.10^{-4}$	$8.11.10^{-5}$
$H_2PO_4^-$	$1.63.10^{-2}$	$4.57.10^{-3}$	$4.44.10^{-3}$	$3.69.10^{-3}$	$2.50.10^{-3}$	$1.32.10^{-3}$	$2.92.10^{-4}$	$2.47.10^{-8}$	$9.5.10^{-11}$
$OctNH_3^+.HPO_4^{2-}$	$3.51.10^{-9}$	$1.03.10^{-7}$	$9.53.10^{-8}$	$8.50.10^{-6}$	$1.39.10^{-5}$	$2.99.10^{-5}$	$6.00.10^{-5}$	$3.48.10^{-7}$	$6.47.10^{-9}$
$(OctNH_3^+)_2.PO_4^{3-}$	$<10.^{-30}$	$1.4.10^{-18}$	$6.3.10^{-27}$	$6.1.10^{-17}$	$4.6.10^{-12}$	$7.1.10^{-13}$	$7.4.10^{-10}$	$9.63.10^{-8}$	$3.32.10^{-8}$
HPO_4^-	$1.00.10^{-7}$	$2.82.10^{-6}$	$2.61.10^{-6}$	$2.33.10^{-4}$	$3.78.10^{-4}$	$8.15.10^{-4}$	$1.64.10^{-3}$	$1.52.10^{-5}$	$5.84.10^{-7}$
$OctNH_3^+.PO_4^{3-}$	$2.1.10^{-19}$	$6.3.10^{-17}$	$5.6.10^{-16}$	$5.4.10^{-11}$	$2.1.10^{-10}$	$1.8.10^{-10}$	$3.37.10^{-9}$	$2.15.10^{-9}$	$4.0.10^{-10}$
PO_4^{3-}	$2.1.10^{-18}$	$6.0.10^{-17}$	$5.3.10^{-15}$	$5.1.10^{-12}$	$2.0.10^{-10}$	$1.75.10^{-9}$	$3.20.10^{-8}$	$3.25.10^{-8}$	$1.25.10^{-8}$
$OctNH_3^+$	$4.72.10^{-3}$	$4.92.10^{-3}$	$4.92.10^{-3}$	$4.92.10^{-3}$	$4.94.10^{-3}$	$4.94.10^{-3}$	$4.92.10^{-3}$	$3.08.10^{-3}$	$1.49.10^{-3}$

On the basis of these considerations , the decrease in retention that we observe for anions of strong acids (figure 2) and of weak acids (figure 1) for the higher pH values could be ascribed to a decreasing number of active sites adsorbed onto the surface of the stationary phase as pH increases as well as to a decrease in the electrical double-layer potential.

It can be useful comparing the results here obtained in reversed-phase ion-interaction chromatography with results reported in literature for reversed-phase chromatography (6-17). In these conditions sigmoidal shapes of K' *vs.* pH are generally obtained, the greater retention corresponding to the lower pH values (6-13). The behaviour is explained (8,9) through the additive property of retention, the experimental retention time observed at the different pH values being the sum of the contributions of the differently-protonated forms of the acid (7) :the undissociated more hydrophobic species is more retained onto a reversed-phase stationary phase than the dissociated one.

On the contrary, in ion-interaction chromatography, the dissociated species is more retained and of consequence the observed retention is increasing with pH, at least at lower pH values and until other effects, as above described, intervene.

The reversed-phase ion-interaction chromatography (an aqueous solution of the interaction reagent being the mobile-phase) and the reversed-phase chromatography (an aqueous-organic mixture as the mobile-phase) can therefore be considered as two extreme situations. Intermediate cases can be identified in ion-pair techniques in which the mobile phase contains the ion-pairing agent and organic modifier. The results obtained with this technique can be explained by mixed mechanisms in which ion interaction or adsorption mechanisms can alternatively predominate, as a function of the experimental conditions like: a) analyte chemical properties, b) organic modifier and c) ion-pairing concentration. When ionic mechanisms prevail parabolic functions of K' vs. pH with lower retention at lower pH values are observed (2,6). When on the contrary reversed-phase partition mechanisms prevail (1), a retention decrease is obtained with pH increasing. In agreement, to mixed mechanisms can be assigned the different behaviours shown, under the same chromatographic conditions (14) by anions characterized by different pK values.

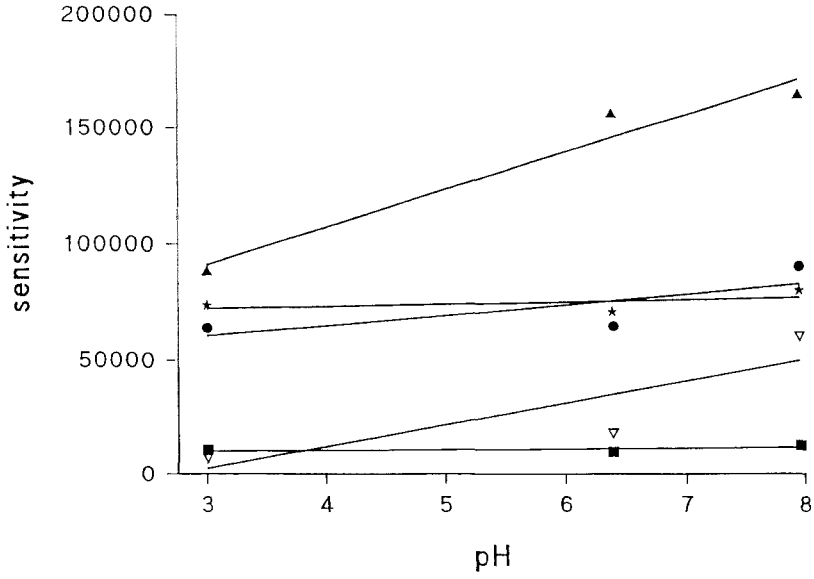

Figure 3.
Sensitivity (peak area for 1.00 ppm) as a function of pH
▲ nitrite, ★ thiocyanate, ● nitrate, ▽ hydrazoate , ■ bromate.

Some considerations can also be made as concerns the sensitivity obtained in the anion separation. Figure 3 shows that the sensitivity (expressed as peak area for concentration of 1.00 ppm) is , as expected, independent on pH for the anions of strong acids while on the contrary increases with pH for the anions of weak acids, as increases the concentration of the dissociated species.

The results here obtained can very usefully assist in the development of separation methods. Some anions in fact show at different pH values comparable sensitivities together with remarkably different retention times. Of consequence resolution ($R_S = (t_2-t_1) /[(1/2 (t_{w1} + t_{w2})]$) (21) can depend on pH, being for example between ascorbate and hydrazoate 0.62 at pH 3.0 , 1.80 at pH 6.39 and 3.00 at pH 8.0. A good choice of the pH value of the mobile-phase can therefore be usefully employed as a confirm criterion in the identification process, to improve resolution or to avoid matrix interference.

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REFERENCES

1. A.Bartha, J. Stahlberg , J.Chromatogr. , 552 : 13-22 (1991)

2. C.P.Terweij-Groen, S.Heemstra, J.C. Kraak, J.Chromatogr. 161 :69-82 (1978)

3. M.C.Gennaro, P.L. Bertolo, J.Chromatogr. 509 : 147-156 (1990)

4. M.C. Gennaro, J.Chromatogr.Sci. , 29 : 410-415 (1991)

5. E.Marengo, M.C. Gennaro, C. Abrigo, Anal.Chem.64 :1885-1893 (1992)

6. B.A. Bidlingmeyer, J.Chromatogr.Sci. 18 : 525-539 (1980)

7. R.M.Lopes Marques, P.J. Shoennmakers, J.Chromatogr. 592 : 157-182 (1992)

8. P.J.Schoenmakers, S. van Molle, C.M.G. Hayes, L.G.M. Uunk, Anal.Chim.Acta 250 : 1-19 (1991)

9. J.A.Lewis, J.W. Dolan, L.R. Snyder, I. Molnar , J.Chromatogr. 592, 197-208 (1992)

10. J.A.Lewis, J.W. Dolan, L.R. Snyder, I. Molnar , J.Chromatogr. 592 :183-195 (1992)

11. F.Szokoli, Zs. Nemeth, J. Inczedy, Chromatographia ,29 :265-268 (1990)

12. B.Bourguignon, F. Marcenac, H.R. Keller , P.F.de Aguiar, D.L.Massart, J.Chromatogr. 628 : 171-189 (1993)

13. S.N.Deming, M.L.H. Turoff, Anal.Chem.50 :546-548 (1978)

14. M.Otto, W.Wegscheider, J.Chromatogr. 258 :11-22 (1983)

15. M.Patthy, T.Balla, P.Aranyi, J.Chromatogr.523: 201-216 (1990)

16. W.A.Bartlett, J. Chromatogr. 493 , 1-14 (1989)

17. A.Zein, M.Baerns, J.Chromatogr.Sci.27 : 249-258 (1989)

18. M.C.Gennaro,C. Abrigo, Fresenius J.Anal.Chem. ,343 : 523-525 (1992)

19. M.C.Gennaro, P.L. Bertolo, A. Cordero, Anal. Chim.Acta 239 : 203-209 (1990)

20. A.E.Martell, R.M. Smith , Critical Stability Constants, Plenum , New York,1976

21. L.R.Snyder, J.J. Kirkland , Introduction to Modern Liquid Chromatography, John Wiley Edit. , New York, Chichester,Brisbane, Toronto, Singapore, 2 ed., (1979)

22. L.G.Sillen, A.E. Martell, Stability Constants of Metal Ions Complexes , Spec. Publ. 17 and 25 , The Chemical Society, London (1964)

23. S.Sammartano, Istituto di Chimica Analitica, Università di Messina, Italia, personal communication

24. J. Stahlberg , J.Chromatogr., 356: 231-245 (1986)

25. J.Stahlberg, Chromatographia ,24 : 820-826 (1987)

26. J.Stahlberg, A. Furangen, Chromatographia ,24 : 783-789 (1987)

27. H.Liu, F.F. Cantwell, Anal.Chem. 63 : 2032-2037 (1991)

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APPLICATION OF NORMAL AND SECOND-ORDER DERIVATIVE SPECTROSCOPY IN IDENTIFYING ORGANIC ACIDS AND SUGAR ACIDS IN LIQUID CHROMATOGRAPHY WITH ON-LINE PHOTODIODE ARRAY DETECTION

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ABSTRACT

The UV-spectra of 16 compounds including organic acids and sugar acids were recorded at pH 2.5 using photodiode array detection in three different chromatographic systems: an ion exchange column with phosphate buffer as eluent (I), a C-8 reversed-phase column with sulphuric acid as eluent (II) and a C-8 reversed-phase column with hydrochloric acid as eluent (III). Differences in the normal spectra in each of the systems were evaluated by calculating match factors between the spectra. Normal spectra and the second-order derivative spectra gave match factor ranges of 603 - 1000 and 0.1 - 999, respectively. The similarity between the normal spectra was very good and excellent between chromatographic systems I and II. On the other hand, the similarity between the second-order derivatives of the spectra was not as good. Thus the normal UV-spectra can be used, together with the retention times, for reliable identification despite of the spectral similarity of some acids. Molecular connectivity indices up to the sixth order were correlated with the retention parameters. The correlation was only moderate in all the systems.

INTRODUCTION

The development of diode-array detectors has been the most important advance in HPLC for the quantitative identification of separated compounds. During the past few years many significant improvements have been made in software and in the engineering of detectors capable of achieving better wavelength resolution and sensitivity (1). With a diode array detector, spectra can be acquired automatically at the peak apex as each peak elutes. The spectra can be compared with those stored in a library (2,3). Such libraries are unfortunately not commercially available and have to be built up by the individual laboratory.

In the case of chemical compounds only those with double bonds and/or phenyl rings usually exhibit UV absorbance. The greater the number of chromophores in the compound the more useful is the UV spectrum for identifying the compound. For example, polycyclic aromatic hydrocarbons (PAH) are very outstanding because of their wide and characteristic UV spectra (2). Self-made UV spectra libraries have been used successfully for identifying many kinds of organic compounds (3,4).

Derivative spectra enhance the differences between spectra by revealing subtle changes in slope that are difficult to observe in the normal spectra (5,6). Second-order derivatives reveal more specific details of the spectra. Peptides especially, show spectral differences in their derivative spectra [2]. However, derivative spectra must be handled with care. Both the standard and unknown spectra should be noise free.

Organic acids are extraordinarily difficult to identify on the basis of their UV spectra because their only chromophore is the carboxyl moiety. The aim of the study was to identify and estimate limitations and potentials of diode array

detection and library matching in identifying organic acids and sugar acids using normal and second derivative spectra. The retention of the compounds was also correlated with the molecular connectivity indices (7-9) in order to determine whether the retentions can be predicted in the systems used.

MATERIALS AND METHODS

Instrumentation

A Hewlett-Packard model 1090 HPLC equipped with an HP 1040A diode array detector was used. An HP data station was used for data storage, comparison and mathematical manipulation of the acquired spectra.

Columns and Chromatographic Procedures

System I: The column was a Bio-Rad Organic Resolution column 30 cm * 8 mm, I.D. The mobile phase was prepared by mixing 0.125 M KH_2PO_4 and 0.125 M K_2HPO_4 (1:1), and then adjusting the pH with phosphoric acid to 2.5. The flow-rate was 0.8 ml/min and the oven temperature 40 C.

System II: A 20 cm * 4.6 mm I.D. column was packed under 500 bar pressure with LiChrosorb C_8 (Merck, Darmstadt), using a slurry technique with acetone as the suspending medium and a 50 ml slurry reservoir. The mobile phase was prepared by adding sulphuric acid into distilled water until the pH was 2.5. The flow-rate was 1 ml/min and the oven temperature was 40 C.

System III: Same column as in system II. The mobile phase was prepared by adding hydrochloric acid into distilled water until the pH was 2.5. The flow-rate was 1 ml/min and the oven temperature was 40 C.

Reagents

Citric acid, maleic acid, succinic acid, formic acid, acetic acid, sulphuric acid, phosphoric acid, potassium dihydrogen phosphate and dipotassium hydrogen phosphate were obtained from Merck (Darmstadt, Germany). Malic acid , malonic acid, tartaric acid, propionic acid , butyric acid, galactonic acid - lactone, glucuronic acid, gluconic acid and gulonic acid - lactone were from Fluka (Buchs, Schwitzerland). Lactic acid was from K&K Laboratories (Cleveland, Ohio). Galacturonic acid was from California corporation for Biochemical Research (Los Angeles, USA). Hydrochloric acid was from Carlo Erba (Milano, Italy). Acetonitrile was of HPLC grade (Merck) and the water was distilled and deionised.

Calculation of the Molecular Connectivity Indices

The molecular connectivity indices for the acids were calculated using the Molconn-X programme (Hall Associates Consulting, Massachusetts, USA) developed by L.H. Hall.

RESULTS AND DISCUSSION

To confirm the identity of a UV spectrum the spectrum must be compared with a set of standard spectra from a spectral library (2). A visual comparison of these spectra usually takes a long time and is not suitable for automated operations. The correlation coefficient for all the corresponding absorbances measured gives the best results (10). With the HP instrument the absorbances can be measured at intervals of 2 nm. Comparison of two spectra is thus based on the correlation coefficient and gives the match factor (coefficient * 1000, range from 0 to 1000). A match of 0 indicates there is no match and 1000 indicates identical spectra. Values greater than 990 generally indicate the spectra are similar. Values between 900-990 indicate there is some similarity, and values below 900 indicate the spectra are different.

The spectra of the organic acids were recorded in three different chromatographic systems. The spectra were recorded at concentrations of the acids that gave maximum absorption in each spectrum stored in a library of 200-800 mAU. The pH was adjusted to a rather low value (2.5) because organic acids have to be analysed liquid chromatographically at a low pH to ensure that the compounds are not in an ionised form. The spectra were recorded from 200 nm to 350 nm even though the only chromophore in the compounds (apart from maleic acid) is the carboxyl moiety which only show absorption at about 200-250 nm in the low UV region.

Repeatability of the Recording Spectra

The repeatability of the recording spectra was tested by first recording the spectrum of each of the compound in each of the systems and then repeating the same procedure. The match factor between the first and second measurement was at least 990.

Differences in Spectra in a Given Chromatographic System

In order to reveal the differences in spectra in a given chromatographic system the UV spectra of the 16 compounds were compared with each other in each of the systems. Thus a total of 120 comparisons was made in each system. Normal spectra had a match factor of 683 - 1000 in system I, 673 - 999 in system II and 603 - 999 in system III. Correspondingly the second-order derivative spectra had a match factor range of 12 - 999 in system I, 0.1 - 995 in system II and 0.1 - 995 in system III. The match factors for each of the acids were compared to all others and the factors thus obtained were averaged in the three systems. The averaged match factors are presented in Table I. The lower the mean match factor is, the more the spectra differ from the spectra of the other acids investigated. Comparison of some of the recorded spectra and their second-order derivatives are presented in Figure 1. The differences in spectra show that, although the carboxyl

TABLE 1

Mean Match Factors for the Normal and Second-Order Derivative Spectra of Each of The Acids Compared to Other Acids in The Three Systems.

Compound	Normal spectra System			Second-order spectra System		
	I	II	III	I	II	III
Lactic acid	926	921	822	794	424	185
Citric acid	916	933	949	833	797	761
Malic acid	920	940	952	844	803	770
Malonic acid	944	956	961	787	735	733
Maleic acid	950	964	947	726	716	670
Succinic acid	928	934	944	679	683	623
Tartaric acid	923	939	952	786	771	721
Formic acid	923	939	952	790	763	743
Acetic acid	928	933	941	618	627	551
Propionic acid	926	934	943	702	704	656
Butyric acid	952	964	944	753	750	721
Galactonic acid -lactone	831	838	791	324	229	222
Glucuronic acid	934	937	946	714	677	677
Gluconic acid	906	906	960	585	528	722
Gulonic acid -lactone	841	846	869	457	424	386
Galacturonic acid	927	927	934	743	717	641

moiety is the only chromophore, the other part of the molecule also affects the spectrum. On the other hand, in many cases the spectrum of a specific acid was almost identical to 1-5 of the other spectra. However, the acids can usually be separated very easily chromatographically and the spectrum is only recorded in order to verify the identity of the compound.

The second order-derivative spectra show greater differences in spectra than the normal spectra (Table I). It could be concluded that second-order derivative spectra are useful in identifying the compounds. However, because the acids absorb

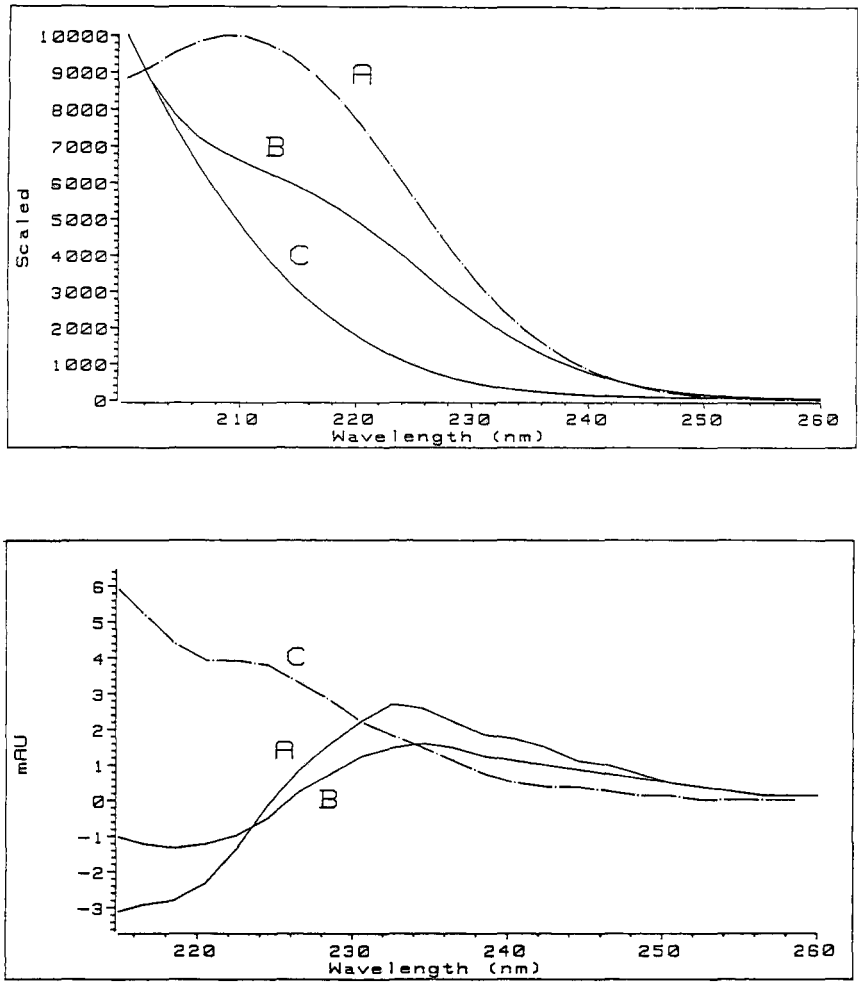

FIGURE 1. Normal spectra (upper) and second order derivative spectra (lower) of citric acid (A), glucuronic acid (B) and lactic acid (C) in chromatographic system I. The match factors in the normal and in the second-order derivative spectra are as follows: A-B: 906, B-C: 857, A-C: 684 and A-B: 941, B-C: 401, A-C: 882, respectively.

TABLE 2

Mean Match Factors for the Normal and Second-Order Derivative Spectra of a Particular Acid Compared to the Same Acid in Another Chromatographic System.

Compound	Normal spectra System			Second-order spectra System		
	I-II	I-III	II-III	I-II	I-III	II-III
Lactic acid	1000	994	995	980	353	369
Citric acid	996	984	993	904	680	855
Malic acid	998	984	996	939	652	775
Malonic acid	993	987	989	966	642	631
Maleic acid	994	997	993	996	632	567
Succinic acid	1000	993	995	995	61	466
Tartaric acid	996	982	997	968	729	767
Formic acid	1000	989	994	991	670	689
Acetic acid	1000	994	994	842	8	178
Propionic acid	1000	993	993	474	691	890
Butyric acid	1000	995	992	994	433	451
Galactonic acid -lactone	996	972	978	769	859	926
Glucuronic acid	998	999	999	942	713	697
Gluconic acid	990	980	980	47	590	614
Gulonic acid -lactone	999	967	978	967	918	948
Galacturonic acid	998	996	998	973	13	348
Mean	997	988	993	859	540	636
S.D.	3	9	7	254	288	226

in the low UV region (200-250 nm), where disturbances caused by the eluent are strongest, and the disturbances multiplies when taking the derivative spectrum, the second-order derivative spectra are not very useful in identifying such compounds.

Influence of the Chromatographic System on the Spectra

As mentioned earlier the spectra obtained with a specific system can be recorded repeatedly. When the main absorption of a compound occurs at a higher

wavelength (> 250 nm), the disturbances caused by the eluent are minimal and the prediction of compound structure by a UV-spectra library search is very reliable. However, in the present study where the only absorbtion occurred in the relatively low UV-region (< 250 nm), the chromatographic system had an effect on the spectra.

The mean differences in the match factors between the spectra obtained in the three different chromatographic systems are presented in Table II. The similarity between the normal spectra is very good and excellent between chromatographic systems I and II. On the other hand, the correspondence between the second-order derivatives of the spectra is not nearly as good. This is probably due to absorption by the ions in the eluent. Thus the second-order spectra are not useful for identifying these acids if the eluent composition is changed.

Comparison of log k' Values with the Molecular Connectivity Indices

The molecular connectivity indices were calculated for the compounds given in Table I. Some of the compounds contained so few atoms that the valence and connectivity indices could be calculated only to the second order. The SAS RSQUARE procedure (11), which performs all possible regressions for dependent variables, and ranks them according to correlation coefficient, was run to obtain regressions of log k' against all two variable combinations of the indices. The best regression equations for the systems I-III are given below:

System I:

$$\log k' = 0.247\ {}^{0}\chi^{v} - 0.296\ {}^{1}\chi + 0.797 \qquad r=0.758 \quad (1)$$

System II:

$$\log k' = 0.514\ {}^{0}\chi^{v} - 0.590\ {}^{1}\chi + 0.0898 \qquad r=0.703 \quad (2)$$

System III:

$$\log k' = 0.473\ {}^{0}\chi^{v} - 0.541\ {}^{1}\chi + 0.0537 \qquad r=0.734 \quad (3)$$

The moderate correlation coefficients indicate that the prediction of retention on the basis of the molecular connectivity indices is not reliable, probably as a result of the relatively large number of oxygen atoms in the compounds studied. Prediction of the retention of oxygen-containing compounds using the molecular connectivity indices on reversed-phase columns has been shown to be difficult (12,13).

CONCLUSIONS

Organic acids are extremely difficult compounds to identify on the basis of their UV spectra owing to the great similarity of their spectra. However, they can be easily separated chromatographically and the UV spectra, together with the retention data, can be used for rather reliable identification. Because of the disturbances in the low UV region (< 250 nm), second order derivative spectra are not useful for identification.

REFERENCES

1. M.V. Pickering, LC·GC Int., 4: 20 (1991).

2. L. Huber, Applications of diode-array detection in HPLC, Hewlett-Packard Co., Publication Number 12-5953-2330, France 1989, p. 90.

3. P. Lehtonen, in Proceedings of the Alko Symposium on Flavour research of alcoholic beverages, ed. by L. Nykänen and P. Lehtonen, Foundation for Biotechnical and Industrial Fermentation Research, 3: 121 (1984).

4. W. Xue, A.K. Samanta, R.M. Carlson, J. Chromatogr. Sci., 28: 477 (1990).

5. D.E.H. Palladino, K.A. Cohen, J. Chromatogr. Sci., 29: 91 (1991).

6. H. Ping-kay, F. Wai-kwong, Analyst, 116: 751 (1991).

7. L.B. Kier, L.H. Hall, Molecular Connectivity in Chemistry and Drug Research, Academic Press, Inc., London, 1976.

8. P. Lehtonen, Chromatographia, 19: 316 (1984).

9. H. Vuorela, P. Lehtonen, R. Hiltunen, J. Liq. Chromatogr., 14: 3181 (1991).

10. K. Tanabe, S. Saeki, Anal. Chem., 47: 118 (1975).

11. SAS Institute, Inc., SAS/STAT Guide for personal computers, SAS Institute, Inc. Cary, USA, p.273.

12. P. Lehtonen, Z. Lebensm. Unters. Forsch., 183: 177 (1986).

13. P. Lehtonen, J. Chromatogr., 398: 143 (1987).

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RESOLUTION OF SYNTHETIC (+)- AND (-)-EPIBATIDINE BY CHIRAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND IDENTIFICATION OF THE NATURAL ENANTIOMER

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ABSTRACT

Methods are described for the separation of the enantiomers (+)- and (-)-epibatidine and (+)- and (-)-N-acetylepibatidine by high performance liquid chromatography using Chiral-AGP and Chiralcel OD chiral stationary phases respectively. Comparison of synthetic material with authentic epibatidine.HCl using retention times and UV spectra allows the unambiguous assignment of the natural product as the (+)- isomer.

INTRODUCTION

Epibatidine, *exo*-2-(6-chloro-3-pyridyl)-7-azabicyclo[2.2.1]heptane (Figure 1), is an alkaloid isolated from the skin of the Ecuadoran poison frog, *Epipedobates*

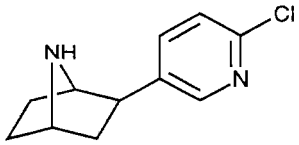

Figure 1. Structure of epibatidine [*exo*-2-(6-chloro-3-pyridyl)-7-azabicyclo[2.2.1]heptane].

tricolor which has been reported by Daly *et al* (1) to possess potent non-opioid analgesic properties. Since the first reported isolation and structural determination, interest has been generated in synthesising sufficient material to confirm the reported biological properties (2,3). In our laboratories the synthesis of (+)- and (-)-epibatidine was carried out since the enantiomer corresponding to the natural product was unknown (3). In this study the development of a chiral high performance liquid chromatographic separation of (+)- and (-)-epibatidine is described. Using this methodology, authentic epibatidine extracted from natural sources could be compared on the basis of retention time and UV spectra. Hence, by identifying which peak corresponds to the (+)- isomer and which to the (-)-isomer, it is possible to assign the enantiomer corresponding to the natural product.

As a further confirmation, separation of (+)- and (-)-N-acetylepibatidine was undertaken. By comparison with authentic material in an analogous fashion and also by spiking of the racemate with authentic N-acetylated material, the enantiomer corresponding to natural epibatidine was unambiguously assigned.

EXPERIMENTAL

Materials

(+)- and (-)-Epibatidine and (+)- and (-)-N-acetylepibatidine were synthesised as previously described (3) with identity and purity confirmed by NMR, MS, HPLC and elemental analysis. Absolute ethanol was obtained from Hayman Limited (Witham, UK). Acetonitrile and hexane were of HPLC grade and

all other reagents were of Analytical Grade, obtained from Fisons (Loughborough, UK). Water was of Millipore MilliQ grade and all solvents were filtered using a glass Millipore system with a 0.45μm filter.

Instrumentation

An HP1090M series high performance liquid chromatograph was used (Hewlett Packard, Avondale, USA). The system comprises an autoinjector, consisting of a Rheodyne 7010 injection valve fitted with a 250 μl loop, an autosampler and a DR-5 solvent delivery system. Detection was by UV at 215nm using a built-in linear photodiode array detector and data was processed using a 79994A PASCAL workstation.

Resolution of (+)- and (-)-epibatidine

Epibatidine was separated on a Chiral-AGP column (Chromtech AB, Sweden) (100 mm x 4.0 mm i.d., 5μm) using a mobile phase of 3% acetonitrile in 10mM dipotassium hydrogen phosphate with 5mM pentane sulphonic acid (PSA), adjusted to pH 7.4 with *ortho*-phosphoric acid. The flow rate was 1ml min^{-1} and all analyses were performed at ambient temperature. Samples were dissolved at approximately 1.0mg ml^{-1} in ethanol and 2μl injection were made corresponding to ca. 1μg of each enantiomer on the column.

Resolution of (+)- and (-)-N-acetylepibatidine

N-acetylepibatidine was separated on a Chiralcel OD column (Diacel Chemical Industries Ltd., Japan) (250 mm x 4.6 mm, 10μm) using a mobile phase of 5% ethanol in hexane (pre-mixed). The flow rate was 2ml min^{-1} and all analyses were performed at ambient temperature. Samples were dissolved at approximately 1.5mg ml^{-1} in ethanol and 5μl injection were made corresponding to ca. 3.5μg of each enantiomer on the column.

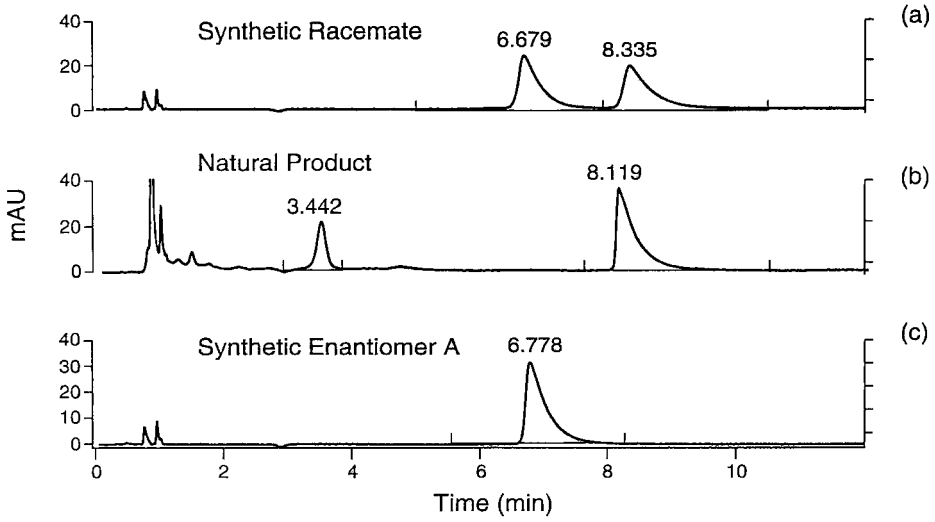

Figure 2. Chromatographic chiral separation using Chiral AGP of underivatised epibatidine corresponding to (a) Synthetic racemic material (b) the natural product and (c) the opposite enantiomer to the natural product.

RESULTS AND DISCUSSION

Initial attempts to resolve epibatidine focused on the application of cyclodextrin and derivatised cyclodextrin bonded CSPs to include the azabicycle within the cyclodextrin cavity, but the basicity of the secondary nitrogen of epibatidine appeared to preclude inclusion hence no resolution was observed. Reports that N-BOC-amino acids, where the basicity of the nitrogen has been removed, could be resolved on hydroxypropyl substituted β-cyclodextrin CSPs (5) led to attempts to resolve the N-BOC-derivative of epibatidine in an analogous fashion, but only a partial separation was ever attained. However, it was found that the N-BOC-derivative could easily be separated on a Chiralcel OD stationary phase (3) leading to the discovery that the N-acetyl derivative behaves in an analogous fashion.

Resolution of epibatidine was attempted using a Chiral-AGP column as development work had demonstrated that the presence of lipophilic substituents and the availability of hydrogen bonding groups were important for chiral recognition (6). Figure 2a shows that baseline resolution of racemic epibatidine

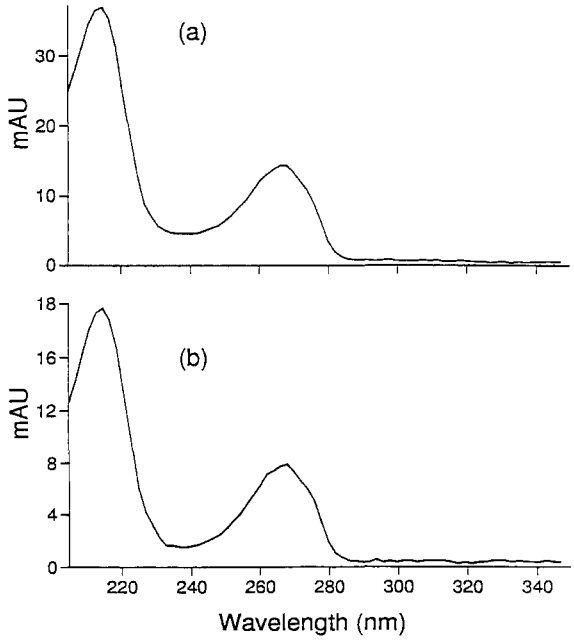

Figure 3. UV spectra obtained from on-line diode array detector of (a) enantiomer B from racemic mixture and (b) natural product.

could be achieved (t_1 = 6.68 mins, t_2= 8.34 mins; α = 1.28, R_s = 2.32). The use of the lipophilic ion-pair reagent pentane sulphonic acid in this system is worth noting. It was found that resolution could not be achieved using simple aqueous buffer systems as insufficient retention could be obtained but by addition of PSA, a significant increase in both retention and separation were gained. This allowed addition of an organic modifier which resulted in more efficient peaks thereby improving resolution. By injection of the racemate then injection of both the (+)- and (-)-enantiomers individually, the peaks are assigned as the first eluting enantiomer (-)-epibatidine.HCl and the second as (+)-epibatidine.HCl (4).

Figure 2b shows the injection of ca. 500ng of authentic epibatidine onto an identical HPLC system. The sample was known not to be pure as is evident from the chromatogram. Correlation of retention times from the racemate (t_2= 8.34 mins, epibatidine = 8.12 mins) strongly suggests that the natural product is the

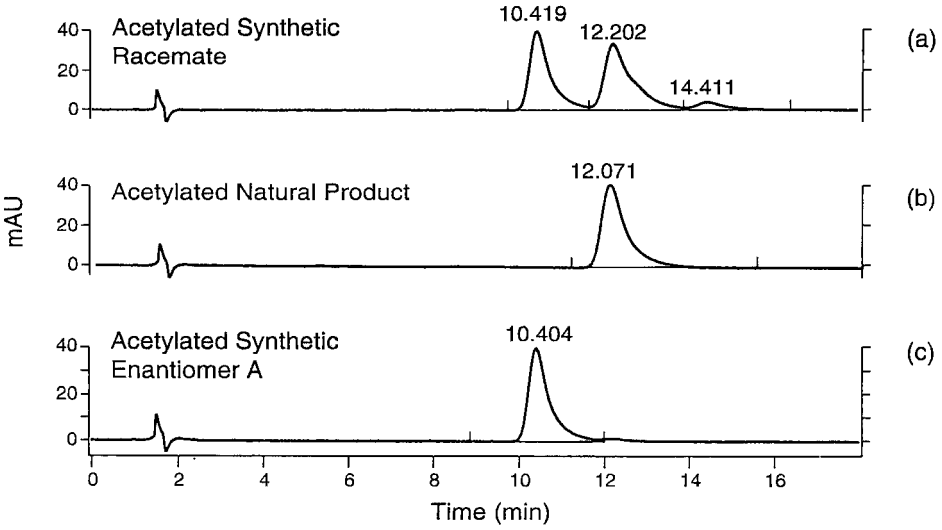

Figure 4. Chromatographic chiral separation using Chiralcel OD of N-acetyl epibatidine corresponding to (a) Synthetic racemic material (b) the natural product and (c) the opposite enantiomer to the natural product.

second eluting enantiomer which possesses the (+)- rotation as its HCl salt. Figure 2c shows that injection of the pure first eluting enantiomer indeed gives a peak at 6.78 minutes confirming that the natural product is the second eluting. Data obtained using the photodiode array detector showed that the UV spectra of the second eluting peak of the racemate (Figure 3a) exactly matched the UV spectrum of epibatidine (Figure 3b) under identical chromatographic conditions.

The resolution of N-acetylepibatidine is shown in figure 4a. The loss of the basic nitrogen through acetylation precludes separation on the Chiral-AGP, but methodology had already been devised for the resolution of N-BOC-epibatidine using a Chiralcel OD stationary phase (3). This was applied and optimised for the N-acetyl derivative (t_1 = 10.42 mins, t_2= 12.20 mins; α = 1.20, R_S = 1.59). Assignment of the peak elution order proved to be as for epibatidine i.e. the (-)-isomer elutes before the (+)-isomer. In an analogous experiment to that described above, natural N-acetylepibatidine is shown to correspond to the second eluting

peak (t_2 = 12.20 mins, N-acetylepibatidine = 12.07 mins; Figure 4b) demonstrating that this enantiomer corresponds to the natural product. Further confirmation is provided by injection of the pure (-)- enantiomer which is shown to elute at 10.40 minutes (Figure 4c) and by spiking approximately equal amounts of racemic N-acetylepibatidine with authentic material and chromatographing to give an increase in the peak area of the second eluting peak over the first.

CONCLUSIONS

Comparison of authentic epibatidine.HCl with synthetic (+)- and (-)- isomers shows the natural product to be the (+)- isomer. Efforts are continuing to elucidate the absolute configuration of this molecule by X-ray crystallography.

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REFERENCES

1. T.F. Spande, H.M. Garraffo, M.W. Edwards, H.J.C. Yeh, L. Pannell and J.W. Daly, *J. Amer. Chem Soc.*, 1992, **114**, 3475-78.

2. C.A. Broka, *Tetrahedron Lett.*, 1993, **34**, 3251-3254.

3. S.R. Fletcher, R. Baker, M.S. Chambers, S.C. Hobbs and P.J. Mitchell, *J. Chem. Soc., Chem. Comm.*, 1993, 1216-1218.

4. (+)-Epibatidine.HCl, $[\alpha]_D^{24}$ +34.7° (c=0.36, MeOH); t_r = 8.34 mins on Chiral- AGP system; t_r = 12.20 mins as N-acetyl derivative on Chiralcel OD system.

   (-)-Epibatidine.HCl, $[\alpha]_D^{24}$ -33.7° (c=0.16, MeOH); t_r = 6.68 mins on Chiral- AGP system; t_r = 10.42 mins as N-acetyl derivative on Chiralcel OD system.

5. Stalcup, A.M., Chang, S., Armstrong, D.W., Pitha, J. *J. Chromatogr.*, **513**, 181-194 (1990).

6. Hermansson, J., *Trends in Analytical Chemistry*, Vol. 8, No. 7, (1989).

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POLYMETHYLOCTILSILOXANE ADSORBED ON POROUS SILICA AS A PACKING MATERIAL FOR REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Packing material having 10, 20, 30, 40 and 50% loadings of polymethyloctilsiloxane on porous silica particles have been prepared and tested. Solvent extraction tests, measurements of physical properties and determinations of chromatographic parameters show that an initial loading of 40% gives a packing material with satisfactory chromatographic properties.

INTRODUCTION

Porous silica is the support material most widely used for reversed phase HPLC packings. Usually a

monomolecular layer of organosilane material is chemically bonded to the walls of the pores of this support so that this layer, the stationary phase, does not wash off into the mobile phase. Less widely used in reversed phase HPLC is an adsorbed (i.e. weakly bonded) stationary phase in which a layer of organic material, usually small (MW 50-100) molecules, is maintained on the surface by continuous replacement of desorbed molecules by similar molecules supplied from the mobile phase. The HPLC process in this case is called liquid-liquid chromatography. It is very versatile, with innumerable combinations of possible stationary phase molecular species, but tends to be inconvenient in practice because of the need to control the adsorption-desorption process [1].

In the present paper we show that large molecules (our example: polymethyloctilsiloxane, MW 6200), when adsorbed into the pores of chromatographic silica particles at sufficiently high loadings can have good chromatographics properties, even without the use of a special treatment to promote chemical bonding of the organic molecules to the silica surfaces.

EXPERIMENTAL

Reagents: methanol and dichloromethane (Lichrosolv grade, from Merck, Rio de Janeiro) were used without further purification. Carbon tetrachloride (Merck, Rio de Janeiro) was purified by distillation before use.

Davisil silica with a mean particle diameter of 10 μm was obtained from Alltech Associates, Inc.

Polymethyloctylsiloxane (PMOS) polymer was obtained from Petrarch Systems/Hüls America Inc. (product PS 140).

Method of Preparation of the Packing Materials: the silica was dried at 150°C for 24 hours prior to preparation of the packing material. Determined quantities of silica were added to solutions of polymethyloctylsiloxane dissolved in 60mL of dichloromethane to prepare packings with 10, 20, 30, 40 and 50% loadings. This mixture was slowly agitated at room temperature for three hours and then the solvent was evaporated, also at room temperature.

Columns: columns (125 x 3.4 mm id) were made from 316L grade stainless steel tubing. The internal surface was polished using a technique developed in our laboratories [2].

Columns were slurry packed using a 10% (w/v) slurry of the prepared packing material in CCl_4. A packing pressure of 38 MPa (Haskel High Pressure Packing Pump) was used, with methanol as propulsion solvent.

Columns were conditioned for five hours with mobile phase (methanol:water, 70:30, v/v) prior to testing.

Solvent extraction: the prepared packings were extracted at reflux temperature for six hours with each of three solvents (methanol, benzene and dichloromethane) using a modification of the method of Sanchez et al. [3]. Methanol was used because it is a polar solvent in wich PMOS is only slightly soluble; benzene because it is an apolar solvent in which PMOS is moderately soluble and dichloromethane because it is an even better solvent for PMOS.

Instrumentation: The chromatography was performed with a modular HPLC system equipped with a pneumatic injector (SSI model x3L), a Waters 510 pump, a UV-VIS absorbance detector (Waters Model 481) and a Waters 740

integrator. All measurements were carried out at ambient temperature. Two test mixtures were used during this study: (1) acetone, benzonitrile, benzene, toluene and naphthalene and (2) aniline and N,N-dimethylaniline both dissolved in mobile phase. Injections of 10 μL of these mixtures produced satisfactory chromatographic peaks with a x128 atenuation at 254 nm. The mobile phase was prepared volumetrically from individually measured aliquots of methanol and water, the mobile phase flow rate was set at 0.2 $mL.min^{-1}$.The column dead time, t_o, was determined using methanol as an unretained compound. Efficiency, resolution, capacity factor and peak asymmetry were determined for each chromatogram.

Elemental determinations on the packings were made using a Model 2400 Perkin Elmer CHN analyzer.

The surface areas of the silica support and of the prepared packings were determined by the conventional Brunauer-Emmett-Teller (BET) method [4] using a Model 2300 Micromeritics Flow Sorb II instrument.

Mean pore diameters (D_p) and specific pore volumes (V_p) of the silica and the packings were determined by mercury intrusion using a Model 9310 Micromeritics instrument.

The infrared spectrum of the liquid stationary phase was obtained from a film of the sample in a NaCl cell. For silica and the prepared packings, the spectra were obtained in KBr pellets compressed to 103 MPa. Transmission spectra were obtained with a Model 1600 Perkin Elmer FT-IR spectrophotometer.

RESULTS

Results from the solvent extractions are presented in Table 1. They indicate that, as the amount of PMOS

TABLE 1

Percentage of the Liquid Stationary Phase Extracted by Solvents.

% PMOS on Silica	% extracted with			% total extracted	% retained
	CH_3OH	C_6H_6	CH_2Cl_2		
10	0.2	3.3	2.0	5.5	4.5
20	0.2	12.0	2.0	14.2	5.8
30	0.2	14.5	2.0	16.7	13.3
40	0.3	21.4	1.5	23.2	16.8
50	0.3	25.4	4.4	30.1	19.9

deposited on the silica increases, the quantity of PMOS remaining after extraction also increases. These retained percentages are atributed to the PMOS that stays in the pores of the silica. Note that the methanol solvent extracts only a very small amount while benzene extracts a larger quantity according to the increase in the loading.

Figure 1 shows a linear increase in carbon load with the amount of PMOS used in the preparation of the packing between 10 and 40%. However, at 50%, the increase in carbon load is no longer linear, suggesting that further liquid phase may saturate the silica support.

The specific surface area (S_{BET}, Table 2) decreases as the amount of PMOS on the silica increases, up to 40%, after which the area stays practicality constant. This decrease presumably reflects the filling of the pores of the silica with the PMOS phase. Table 3 gives the values of the

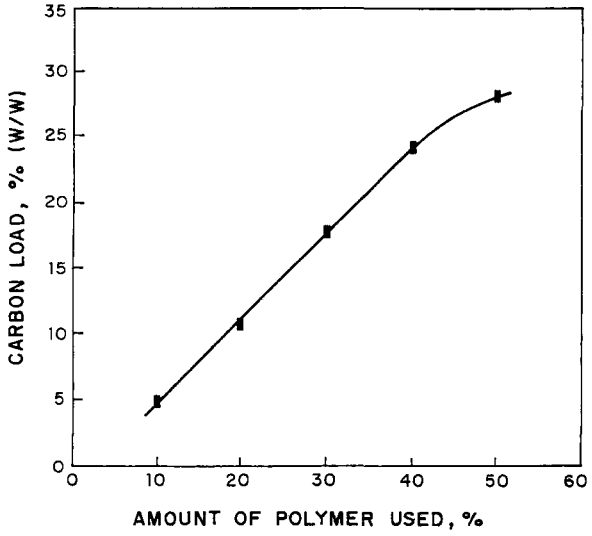

FIGURE 1 - Percentage of carbon in the prepared packings

TABLE 2

Specific Surface Area (S_{BET}) of the Silica and of the Packing Materials.

Sample	S_{BET} (m^2g^{-1})
silica	386.72
10% PMOS on silica	327.13
20% PMOS on silica	323.85
30% PMOS on silica	68.27
40% PMOS on silica	3.26
50% PMOS on silica	2.13

TABLE 3

Mean Pore Diameter (D_p) and Specific Pore Volume (V_p) Values of the Silica and Packing Materials

Sample	D_p (nm)	V_p (mL g^{-1})
Silica	9.9	1.24
10% PMOS on silica	10.2	1.08
30% PMOS on silica	10.5	0.84
40% PMOS on silica	10.8	0.56
50% PMOS on silica	12.5	0.43

mean pore diameter (D_p) and specific pore volume (V_p) of the packing materials. The increase in the percentage of the PMOS on silica results in a decrease of the specific pore volume to about 65% of its initial value as a consequence of the liquid phase loading.

The effect of carbon load on the specific surface area and specific pore volume of the prepared packing materials is illustrated in Figures 2 and 3, respectively. The pore volume decreases in a nearly linear manner with increase of the carbon load while the surface area decreases to a minimum at 40% PMOS on silica support, indicating that a layer of liquid phase has formed.

The infrared spectra of PMOS, of silica and of the prepared packing materials are shown in Figure 4. In these spectra the intensity of the signals characteristic of the silanol groups of the support (shoulder at 973.5 cm^{-1}) decreases as the percentages of PMOS on silica increase, up to 40%, confirming that

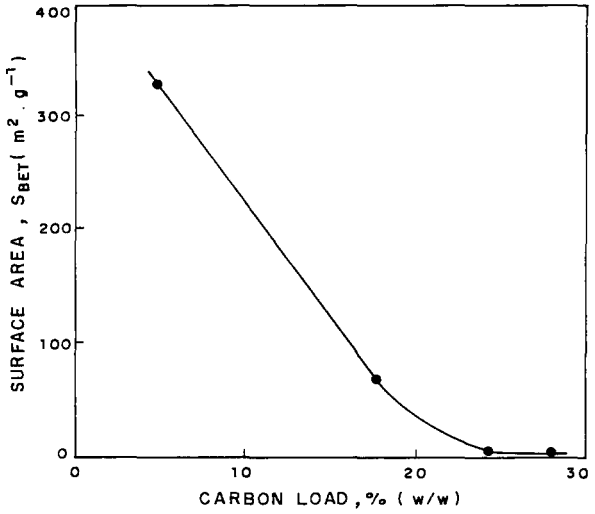

FIGURE 2 - Effect of carbon load on specific surface area of the packing materials

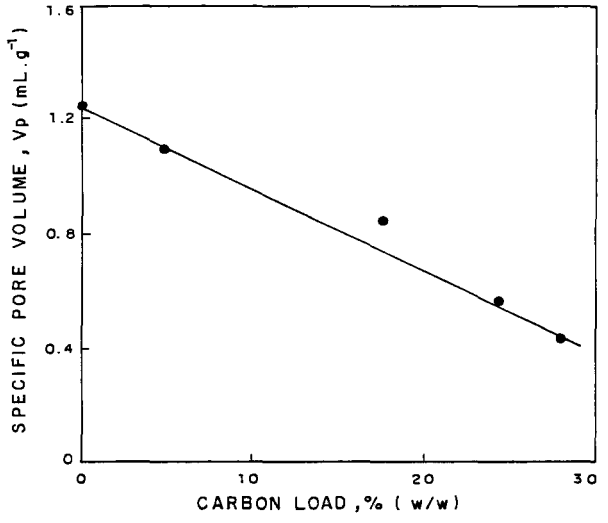

FIGURE 3 - Effect of carbon load on specific pore volume of the packing materials.

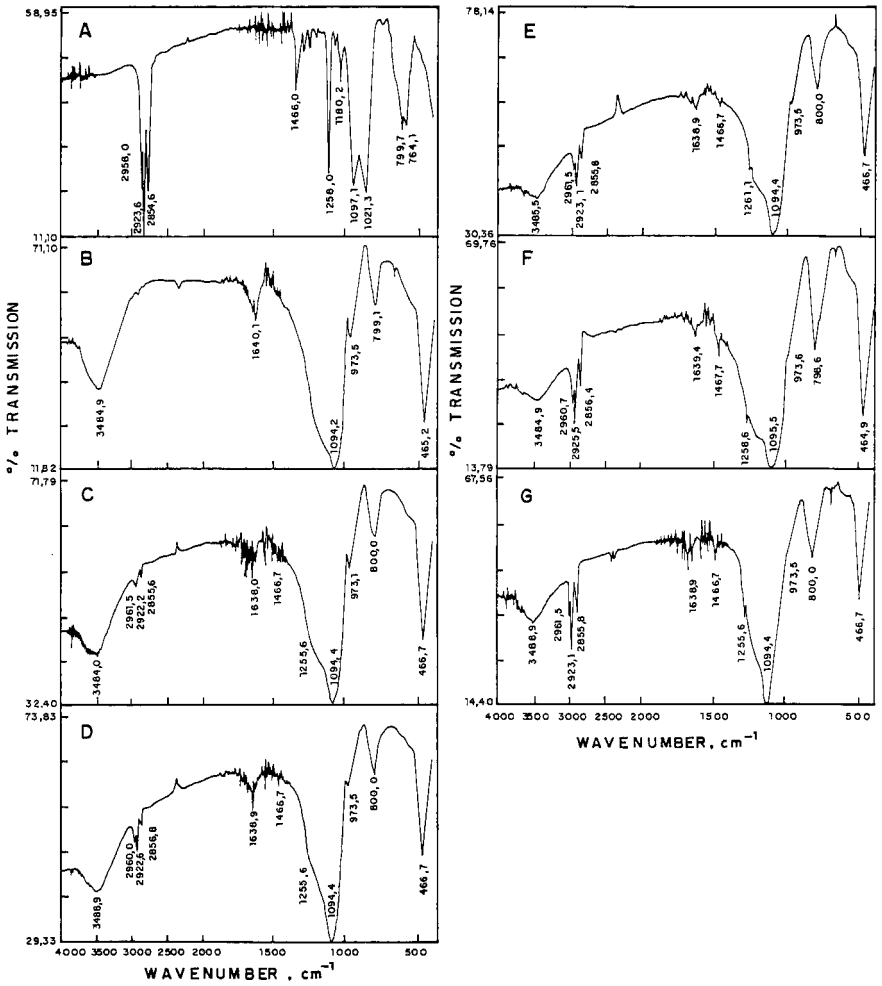

FIGURE 4 - Infrared spectra of: (A) PMOS, (B) silica, (C) 10% PMOS on silica, (D) 20% PMOS on silica, (E) 30% PMOS on silica, (F) 40% PMOS on silica and (G) 50% PMOS on silica.

TABLE 4

Chromatographic Parameters as a Function of the Amount of PMOS on Silica Using Test Mixture 1

% PMOS on silica	Chromatographic Parameters*				
	n** ($x10^3$)	n/meter** ($x10^3$, m^{-1})	k**	As**	Rs***
10	1.12	8.96	0.15	0.83	---
20	2.12	16.96	0.40	1.40	0.80
30	2.39	19.12	0.58	1.55	0.83
40	3.12	24.96	3.26	1.43	2.19
50	3.89	31.12	1.98	1.38	2.05

* Conditions of Determination: Column: 125 x 3.4mm id, mobile phase: methanol:water (70:30) at 0.2mL min^{-1}, detection: UV, 254nm

** Calculated for naphthalene

*** Calculated for toluene-naphthalene

a saturation of the porous silica has occurred, decreasing the number of exposed silanol groups.

The results obtained with test mixture 1 are given in Table 4. The amount of PMOS on silica affects all these parameters.

The retention factor (k) values show that the retention of naphthalene on columns packed with stationary phase having 10, 20 and 30% of PMOS on silica is low, limiting the resulting analysis. With 40 and 50% of PMOS the k values, for naphthalene, are

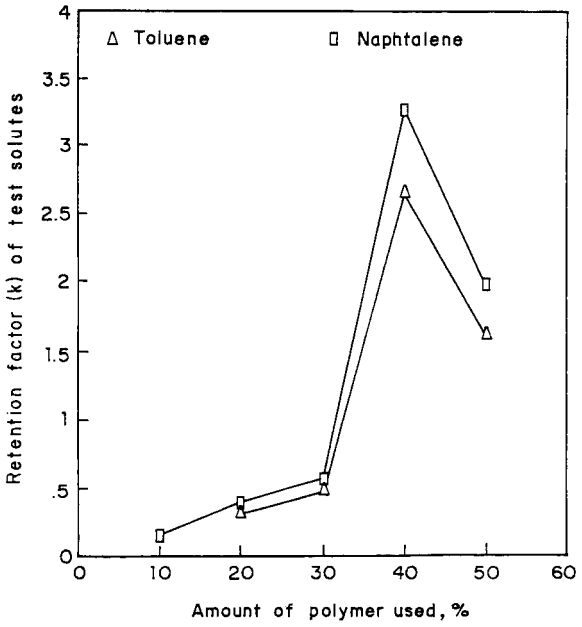

FIGURE 5 - The effect of the amount of polymer on the capacity factor of the test solutes toluene and naphthalene.

reasonable, due to the suitable interaction of the test compounds with both the mobile phase and the stationary phase ($1< k< 10$) [5].

Figure 5 shows the dependence of the retention factors of toluene and naphathalene on the loadings of the packing materials, indicating that the retention factor increases with increasing loading up to 40%, after which the solute retention decreases.

The observed resolutions show that columns packed with 10% PMOS on silica do not separate toluene and naphthalene. The same happens for acetone, benzonitrile and benzene. As the percentage of PMOS on silica

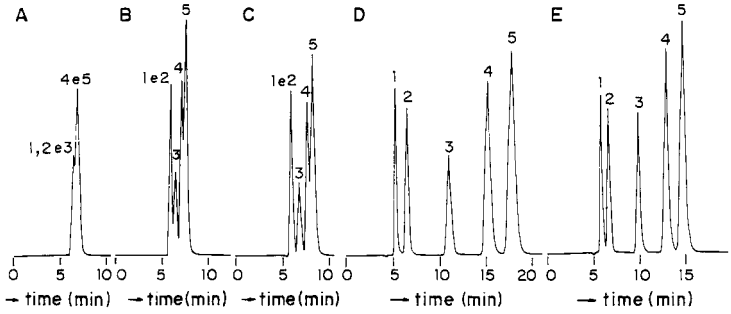

FIGURE 6 - Chromatograms obtained with the prepared packing materials: % PMOS on silica, (A) 10, (B) 20, (C) 30, (D) 40 and (E) 50. Test solutes : 1 = acetone , 2 = benzonitrile, 3 = benzene, 4 = toluene and 5 = naphthalene. Column: 125 x 3.4mm id, mobile phase: methanol: water (70:30, v/v), flow-rate: 0.2mL min^{-1}, pressure: 0 MPa, detection: UV, 254nm.

increases a better resolution of these compounds is observed, reaching acceptable separations at 40 and 50% PMOS. This can be visualized in the Figure 6 which shows the separation of the compounds of the test mixture 1.

The peak asymmetry, A_s, has been calculated for naphthalene. As can be seen in Figure 6, the toluene and naphthalene peaks in chromatograms A, B and C, corresponding to 10, 20 and 30% PMOS on silica, respectively, are not totally separated; therefore, the asymmetry values have been estimated. It should be noted that the asymmetry tends to decrease with increasing carbon load. At high loadings the asymmetry is acceptible for most purposes although it is still somewhat higher than the value (1.2) recommended by Snyder and kirkland [5] and by Poole and Poole [6].

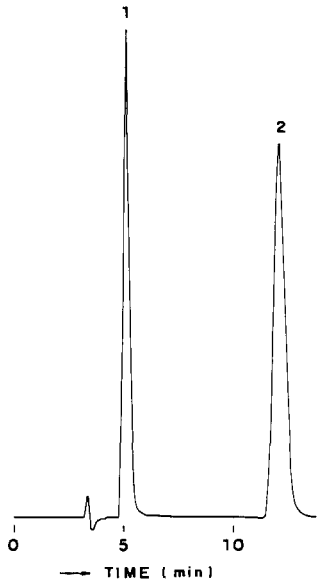

FIGURE 7 - Chromatogram obtained with the 40% PMOS on silica packing material.Test solutes: 1 = aniline and 2 = N,N-dimethylaniline. Column: 125 x 3.4mm id, mobile phase: methanol:water (70:30, v/v), flow-rate: 0.2mL.min^{-1}, pressure: 0MPa, detection: UV,254nm.

According to increase the loading, the peak became more symmetrical because of the increase of carbon load. Then, high loading helps to reduce peak asymmetry, but still present an asymmetry above that usually accepted ($A_s \leq 1.2$) [5].

Figure 7 shows the chromatogram obtained with basic solutes (test mixture 2) using a column packed with 40% PMOS packing material. The peaks for aniline and N,N-dimethylaniline are surprisingly symmetrical (with A_s values of 1.5 and 1.4, respectively) and

similar to that naphthalene (Table 4). This demonstrates that the silanol group activity in these packings is quite low, even though no immobilization or endcapping was included in their preparation.

DISCUSSION

Tests of porous silica particles having heavy loads of PMOS (up to 50%) show that, although the surface area of the particles decreases to near zero at 40% loading, the pore volume decreases linearly with the loading and is still at 65% its initial value with a 50% load. This suggests that the BET measurements of surface area are in some way obscured by the nature of the organic loading.

Below 40%, there is not an effective shielding of the silanol groups of the silica, thus prejudicing resolution. Above 40% a decrease of solute retention occurs, which can be interpreted in terms of the model presented by Lork and Unger [7], where the solute molecules are able to penetrate the stationary phase created by the n-alkyl groups only when the n-alkyl groups are sufficiently flexible and the possibility exists for lateral evasion. The n-alkyl groups that are very closely packed lose this degree of freedom; i.e., above a certain ligand loading the ability of a solute to penetrate into the stationary phase is strongly reduced.

The columns reported here give good chromatographic behavior for the 40 and 50% loaded packings which are economical and easy to prepare.

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REFERENCES

1 H. Engelhardt and M. Jungheim, Chromatographia, 29: 59-68 (1990)

2 T. A. Anazawa, F. Carraro, J. B. de Souza, K. E. Collins and I. C. S. Jardim, 14. National Meeting of the Brazilian Society of Chemistry, Caxambu, MG, (Brazil), 1991, QA-010.

3 E. F. Sanchez, J. A. Dominguez, J. G. Munoz and M. J. Molera, J. Chromatogr., 299: 151-158 (1984)

4 S. Brunauer, P. H. Emmett and E. Teller, J. Am. Chem. Soc., 60: 309-319 (1938)

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

6 C. F. Poole and S. K. Poole, Chromatography Today, Elsevier Science Publishing Company Inc., New York, 1991, p. 360

7 K. D. Lork and K. K. Unger, Chromatographia, 26: 115-119 (1988)

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IMPROVEMENT IN SELECTIVITY OF ANHYDROTRYPSIN-IMMOBILIZED DIOL SILICA COLUMN BY THE USE OF ELUENT CONTAINING CALCIUM ION

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ABSTRACT

The disadvantage of anhydrotrypsin-immobilized diol silica column, retention of basic peptides having no Arg or Lys at their C-termini and no retention of certain C-terminal Arg peptide, was overcome by using 10 mM acetate buffer (pH 6.0) containing 20 mM $CaCl_2$ as an eluent. The role of calcium ion was suggested to be attributable to masking of negatively charged sites which were present intrinsically on the diol silica and produced extrinsically on the support during activation and/or immobilization process.

INTRODUCTION

Since anhydrotrypsin (AHT), a catalytically inert derivative of trypsin in which the active site Ser residue is chemically converted to dehydroalanine residue, exhibits affinity toward peptides containing Arg or Lys at their C-termini, AHT-agarose is used for selective isolation of C-terminal peptide fragments from tryptic or chymotryptic digests of proteins

[1–5]. We recently prepared AHT–immobilized diol silica to improve the disadvantages of the AHT–agarose column such as slow separation speed and ligand leakage, and to use it as a precolumn of column–switching high–performance liquid chromatography (HPLC) system [6,7]. The AHT–diol silica column showed the expected excellent characteristics, but disadvantage in selectivity: a certain peptide was not retained on the column though having Arg at its C–termini, while half of peptides having no Arg or Lys at their C–termini were retained.

This paper describes the improvement in selectivity of AHT–diol silica column by the use of eluent containing calcium ion.

EXPERIMENTAL

Materials

Peptides used were purchased from the Peptide Institute (Osaka, Japan) and Sigma (St. Louis, MO, U.S.A.). Diol silica was prepared from Matrex silica beads (30–50 μm, 50 nm)(Amicon, Lexington, MA, U.S.A.) as described previously [8]. Other chemicals were of analytical–reagent grade. Deionized water (obtained with a Millipore RO–Q system) was used throughout this work.

Preparation of AHT column

The preparation method of AHT column was slightly modified as follows to immobilize larger quantities of AHT. Diol silica was activated with tresyl chloride (270 μl g^{-1}) for 20 min at room temperature. AHT was immobilized almost quantitatively by shaking the activated gel (300 mg) in 4 ml of 0.75 M phosphate buffer (pH 8.0) containing 15 mg AHT for 4 h at room temperature. The resulting gel was slurry–packed into a stainless–column (10 X 4.6 mm I.D.) after treatment with Tris–HCl buffer(pH 8.0) to remove excess active groups. The column was stored in 10 mM acetate buffer (pH 6.0) when not in use.

Evaluation of retention of peptides

The retention was evaluated as the percentage of peptide remaining on the AHT column after loading and washing, by using the column-switching system described previously [7]. The eluent for the loading and washing was 10 mM acetate buffer (pH 6.0) containing 20 mM $CaCl_2$ and delivered at a flow-rate of 0.5 ml/min. At 15 min after the injection of peptide solution (1 nmol/50 μl), a switching valve was changed for flushing the retained peptide from the AHT column onto an analytical column (Capcell Pak C_{18}; 150 X 4.6 mm I.D.). A straight-flushing mode was employed. The eluent for the flushing was CH_3CN-29 mM H_3PO_4 (3-25:97-75, v/v) containing 0.1 M $NaClO_4$, and delivered at a flow-rate of 1 ml/min. A typical chromatogram is shown in Fig. 1.

RESULTS

A preliminary study on the effect of calcium ion on selectivity of the AHT column prepared previously showed that it decreased non-selective adsorption of peptides having no Arg or Lys at their C-termini, but extinguished concurrently the retention of some of C-terminal Arg peptides and most of C-terminal Lys peptides [7]. We thought immobilization of larger quantities of AHT would be effective to overcome this disadvantage. A new column prepared in this study contained 50 mg AHT per gram, five times the amount immobilized on the previous column.

Table 1 shows the retentions of fifty peptides on the new AHT column. The peptides which had Arg or Lys at their C-termini and more than four or three amino acid residues, respectively, except Nos. 15 and 10, a C-terminal D-Arg peptide, were retained on the column when 10 mM acetate buffer (pH 6.0) was used as an eluent, and most of these retentions were nearly quantitative. However, five out of twenty-five peptides having no Arg or Lys at their C-termini were also retained on the AHT column. The retention of the five peptides to diol silica itself was fairly low except No. 37; 3% (No. 31), 62% (No. 37), 11% (No. 40), 7% (No. 41), 15% (No. 48).

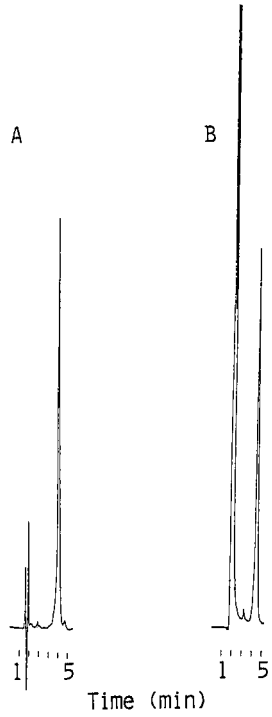

FIGURE 1. HPLC chromatograms of No. 7 obtained with (B) or without (A) the AHT precolumn.

These characteristics generally coincided with those of the previous AHT column [7].

The non-selective adsorption of peptides was suppressed completely by the addition of $CaCl_2$ (20 mM) to the eluent, while the retention of the C-terminal Arg peptides was hardly or only slightly affected. A C-terminal Arg peptide No. 15 became retained, though in small percentage, by the addition of $CaCl_2$. The retention of the C-terminal Lys peptides was generally reduced greatly by the addition of $CaCl_2$, but that of several peptides was hardly affected. Thus the selectivity of the AHT column was significantly improved.

TABLE 1
Retention of Fifty Peptides on the AHT Column

No.	Peptides[2]	Retention (%)[1] Carrier I[3]	Carrier II[3]
1	Tyr-Arg	0	0
2	Bz-Gly-Arg	0	0
3	Thr-Lys-Pro-Arg	96	100
4	Tyr-Ile-Gly-Ser-ArgNH_2	66	28
5	His-Leu-Gly-Leu-Ala-Arg	72	67
6	Tyr-Gly-Gly-Phe-Leu-Arg	89	86
7	Tyr-Gly-Gly-Phe-Leu-Arg-Arg	97	93
8	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Arg	96	92
9	Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg	87	68
10	Dnp-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg	0	0
11	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	95	100
12	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg	93	79
13	Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg	91	79
14	Tyr-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	105	95
15	Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg	0	9
16	Bz-Gly-Lys	0	0
17	Lys-Trp-Lys	99	91
18	Thr-Pro-Arg-Lys	81	0
19	Pro-Phe-Gly-Lys	57	11
20	Tyr-Gly-Gly-Phe-Met-Lys	82	51
21	Tyr-Gly-Gly-Phe-Leu-Lys	79	13
22	Ser-Ile-Gly-Ser-Leu-Ala-Lys	99	97
23	Val-His-Leu-Thr-Pro-Val-Glu-Lys	58	5
24	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys	82	69
25	Tyr-Pro-Phe	0	-[4]
26	Tyr-Tyr-Phe	0	-
27	Glu-Val-Phe	0	-
28	Met-Leu-Phe	0	-
29	Val-Ala-Ala-Phe	0	-
30	Gly-Arg-Gly-Asp	0	0
31	Arg-Pro-Lys-Pro	40	0
32	Phe-Gly-Gly-Phe	0	-
33	Phe-Leu-Glu-Glu-Val	0	0
34	Tyr-Pro-Phe-Pro-Gly	0	0
35	Tyr-Gly-Gly-Phe-Leu	0	0
36	Tyr-Gly-Gly-Phe-Met	0	-
37	Arg-Ser-Arg-His-Phe	72	0
38	Arg-Lys-Asp-Val-Tyr	0	0
39	Lys-Val-Ile-Leu-Phe	0	0

(*continued*)

TABLE 1 (*continued*)

No.	Peptides[2]	Retention (%)[1] Carrier I[3]	Carrier II[3]
40	Arg-Val-Tyr-Ile-His-Pro-Phe	84	0
41	Arg-Val-Tyr-Ile-His-Pro-Ile	73	0
42	Tyr-Pro-Phe-Pro-Gly-Pro-Ile	0	0
43	Tyr-Gly-Gly-Phe-Met-Arg-Phe	0	0
44	Ser-Met-Glu-Val-Arg-Gly-Trp	0	-
45	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	0	0
46	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	0	0
47	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu	0	0
48	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro	86	0
49	Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu	0	0
50	Arg-Arg-Leu-Ile-Glu-Asn-Ala-Glu-Tyr-Ala-Ala-Arg-Gly	0	0

1) Determined based on the peak area.
2) 1 nmole/50-μl injection.
3) I, 0.01 M acetate buffer (pH 6.0).
II, 0.02 M $CaCl_2$/0.01 M acetate buffer (pH 6.0).
4) Not determined.

To clarify how $CaCl_2$ could extinguish the non-selective adsorption, effect of its concentration on retention of selected peptides was investigated. As shown in Fig. 2, the retention of No. 40 was decreased rapidly with increasing concentration of $CaCl_2$, and extinguished at 1 mM. Nearly the same curve was also obtained by the addition of $MgCl_2$ (data not shown). The addition of NaCl was less effective for the suppression, the retention being extinguished at 30 mM. No. 15 became retained by the addition of $CaCl_2$, but no increase in retention depending on its concentration was observed. The addition of 20 mM $MgCl_2$ was also effective for the retention (data not shown). On the other hand, no retention was observed by the addition of NaCl at any concentrations. No substantial difference in effect between $CaCl_2$ and NaCl was observed in the case of No. 3, but its retention tended to decrease with increasing concentration of these salts. These results indicate that the suppression

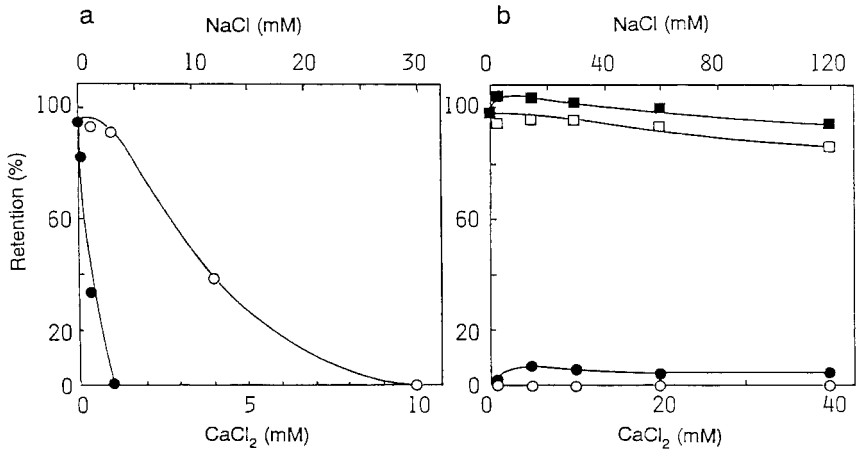

FIGURE 2. Effect of concentration of $CaCl_2$ (closed symbols) or NaCl (open symbols) on retention of peptides on the AHT precolumn. a, No. 40; b, No. 3 (□ ■), No. 15 (○ ●).

of the non-selective adsorption of peptides by calcium ion is not simply attributed to an increase in ionic strength.

Although the eluent containing 20 mM $CaCl_2$ was used in the above experiments, the disappearance of adsorption of No. 40 by using the eluent containing 1 mM $CaCl_2$, as shown in Fig. 1, reminded us that its concentration might be adequate to extinguish the non-selective adsorption of the other peptides and might result in an increase in the retention of the C-terminal Lys peptides. Actually, the retention of Nos. 31, 41 and 48 was 0–3% at 1 mM $CaCl_2$, and that of No. 18 was restored to 4%. The retention of No. 37, however, was 70%, indicating that the concentration of 20 mM was preferable for the complete suppression of the non-selective adsorption.

The new AHT column was satisfactorily stable throughout this study; no decrease in retention of No. 3 was observed after exposure for about 300 cycles to acidic eluents containing up to 25% acetonitrile during 6 months.

DISCUSSION

The data presented in this paper clearly indicated that the addition of $CaCl_2$ (20 mM) to eluent improved the selectivity of the AHT column, but decreased concurrently the retention of many C-terminal Lys peptides. Lower concentration of $CaCl_2$ increased the retention of the Lys peptides, but made impossible the complete suppression of the non-selective retention. The present AHT column, therefore, is not always usable for the selective isolation of C-terminal peptide fragments from tryptic or chymotryptic digests of proteins, though having effective selectivity as a precolumn of HPLC system. The use of arginylendopeptidase (mouse submaxillary protease) [9] instead of trypsin for digestion of proteins would compensate for the disadvantage of the AHT column. Kumazaki et al. reported that AHT prepared from *Streptomyces griseus* (S.G.) trypsin showed higher affinity than that from bovine trypsin for C-terminal Lys peptides [3]. Immobilization of AHT prepared from S.G. trypsin, therefore, would be another approach.

Among thirty peptides having no Arg or Lys at their C-termini, twelve were retained non-selectively in the previous study [7]. Five out of these twelve peptides were retained in this study. A careful investigation on amino acid composition of the twelve peptides showed that these were all basic peptides. We recently observed a similar phenomenon: two out of eighteen peptides having no aromatic amino acids at their C-termini were non-selectively adsorbed on an anhydrochymotrypsin (AHC)-immobilized column [10]. The two peptides are also basic. These facts suggest that some negatively charged site is present on the support or the ligands. As both AHT and AHC columns were prepared by using diol silica as a support and tresyl chloride as an activating reagent for immobilization of ligands, these common factors are more likely cause of the non-selective adsorption. The decrease in number of peptides adsorbed non-selectively by immobilizing larger quantities of AHT, as shown in this study, seems to support this assumption. However, the retention percentage of the five peptides to diol silica itself, except No. 37, was fairly low to explain the non-selective adsorption to the AHT column. Some change in property of the support, therefore, may occur during the

activation and/or immobilization process. In the case of No. 37, the adsorption is probably attributed to the diol silica itself. The presence of negative charge on diol silica is pointed out also by Schmidt et al. [11].

The difference in effect on suppression of the non-selective adsorption of peptides between calcium and sodium ion addition showed that the suppression by calcium ion was not simply attributed to an increase in ionic strength. The fact that magnesium ion shows nearly the same effect as calcium ion suggests that the non-selective adsorption is suppressed by divalent ions. These ions may mask specifically the negatively charged site as postulated above. The appearance of the retention of No. 15, an acidic C-terminal peptide, by the addition of calcium ion can be explained reasonably by the masking of the negatively charged site. At higher concentration, calcium ion may mask the negatively charged site which is present intrinsically on the diol silica, as shown by the example of No. 37. A diol silica showing less adsorption characteristics for basic peptides would be necessary for development of more excellent AHT column.

REFERENCES

1. S. Ishii, H. Yokosawa, T. Kumazaki, I. Nakamura, Methods in Enzymology, Vol. 91, Academic Press, New York, 1983, pp.378-383.

2. H. Yokosawa, S. Ishii, Biochem. Biophys. Res. Commun., 72: 1443-1449 (1976)

3. T. Kumazaki, T. Nakano, F. Arisaka, S. Ishii, Proteins: Struct. Funct. Genet., 1: 100-107 (1986)

4. T. Kumazaki, K. Terasawa,S. Ishii, J. Biochem., 102: 1539-1546 (1987)

5. S. Ishii, H. Yokosawa, S. Shiba, K. Kasai, Adv. Exp. Med. Biol., 120A: 15-27 (1989)

6. T. Ohta, T. Inoue, Y. Fukumoto, S. Takitani, Chromatographia, 30: 410-413 (1990)

7. T. Ohta, T. Inoue and S. Takitani, J. Chromatogr., 543: 59-67 (1991)

8. T. Ohta, K. Ishimura, S. Takitani, Chromatographia, 33: 113-116 (1992)

9. I. Schenkein, M. Levy, E. C. Franklin, B. Frangione, Arch. Biochem. Biophys., 182: 64–70 (1977)

10. T. Ohta, K. Ishimura, S. Takitani, J. Chromatogr., 637: 35–41 (1993)

11. D. E. Schmidt, Jr., R. W. Giese, D. Conron, B. L. Karger, Anal. Chem., 52: 177–182 (1980)

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APPLICATION OF SEPHADEX LH-20 CHROMATOGRAPHY FOR THE SEPARATION OF CYANOGENIC GLYCOSIDES AND HYDROPHYLIC PHENOLIC FRACTION FROM FLAXSEED

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ABSTRACT

A column chromatographic method, using Sephadex LH-20, for separation of cyanogenic glycosides and hydrophylic phenolic compounds from flaxseed is described. Results so obtained indicate that this method can be used as a first step in chromatographic separation and purification of linustatin, neolinustatin and phenolics compounds from flaxseed.

INTRODUCTION

Flaxseed (*Linum usitatissimum L.*) contains a number of anti-nutritional factors, amongst which cyanogenic glycosides are of most concern (1). Linustatin and neolinustatin have been identified as the major cyanogenic compounds in

flaxseed (2). Their content in flaxseed may be as high as 350 and 200 mg/100 g, respectively (3). Phenolic acids are the best-known group of phenolic compounds present in flaxseed (4,5). However, little is known about other classes of phenolic compounds present in the oilseed. As a first step in identification of such compounds, it was necessary to device a simple procedure for their isolation.

Application of Sephadex LH-20 gel filtration for separation of cyanogenic glycosides and hydrophylic phenolic compounds from flaxseed was intended. This method has been used previously for separation of phenolic compounds and glycosides from other plant materials (6-8).

MATERIALS AND METHODS

Defatted flaxseed was extracted with 80% (v/v) ethanol over a 1 h period at a seed to solvent ratio of 1:5 (w/v) at 65-70°C (9). The hydrophilic fraction of the extract was obtained by butanol/water (1:1, v/v) separation (6). The bottom aqueous layer in the separatory funnel was recovered and then lyophilized. Two grams of the residue so obtained was dissolved in methanol and applied to a C16/100 column (Pharmacia LKB, Uppsala, Sweden) packed with Sephadex LH-20 and eluated with methanol. Fractions (6 mL) were collected using a LKB Bromma 2112 redirac fraction collector (Pharmacia LKB, Uppsala, Sweden). The absorbance of methanolic solutions from each tube was measured at 280 nm. In addition, absorbance values at 490 nm and 726 nm were read after colour development reactions for sugars (10) and phenols (11), respectively. Eluates were then pooled into 5 fractions and weighed. UV spectra were obtained using

a Hewlett Packard 8452A diode array spectrophotometer. Fractions were also examined on TLC silica gel plates (Sigma, St. Louis, MD) using a chloroform/methanol/water (65:35:10, v/v/v) system for glycosides (12) and a butanol/acetic acid/water (3:1:1, v/v/v) system (13) for separation of phenolic compounds.

Sugar compounds were visualised after spraying the developed TLC plates with 0.1% orcinol in 75% sulphuric acid and heating for 5 min at 120°C (14). For phenolic compounds, plates were sprayed with a ferric chloride-potassium ferricyanide solution (15). In the case of sugars, raffinose and sucrose (Sigma, St. Louis, MO), as well as cyanogenic glycosides, linustatin and neolinustatin standards were also applied to TLC plates. The two cyanogenic glycosides were prepared as described elsewhere (16). For phenolic compounds, synapic acid, ferulic acid, nordihydroguaiaretic acid (NDGA), and glucopyranosyl sinapate were used as standards. Sinapic and ferulic acids are the main phenolic acids of flaxseed (4,5), and chemical structure of NDGA is very similar to that of flaxseed lignan (17). The standard glucopyranosyl sinapate was isolated from canola meal (18).

RESULTS AND DISCUSSION

The hydrophilic fraction of flaxseed extract was subjected to Sephadex LH-20 chromatography. The chromatogram (Figure 1) exhibited a large and two small peaks at 280 nm, the typical absorption wavelength for aromatic compounds, three maxima at 726 nm for phenolics, after color development (one large and two

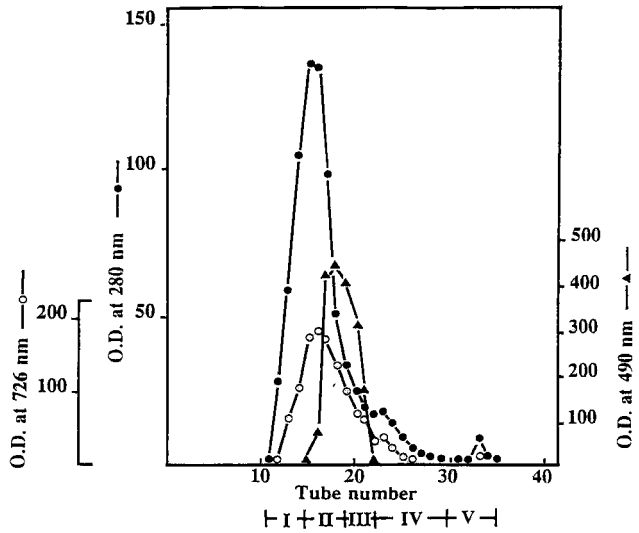

Figure 1. Sephadex LH-20 chromatography of hyhrophilic compounds from flaxseed separated on a 16/100 Pharmaica column using methanol as the eluting solvent.

small), and one maximum at 490 nm for sugars. The maxima for phenolics coincided with the UV peaks. The maximum observed for sugars occurred after the first phenolic peak.

Accordingly, five fractions (I-V) were separated after Sephadex LH-20 chromatography. These fractions contributed: I, 15.6%; II, 63.6%; III, 16.7%; IV, 3.6%; and V, 0.5% to the total amount of hydrophilic extracts.

Spraying of the TLC plates with the orcinol reagent indicated the presence of linustatin and neolinustatin in fractions II and III (Figure 2). In addition, raffinose was observed in fractions II, III, and IV and sucrose was present in

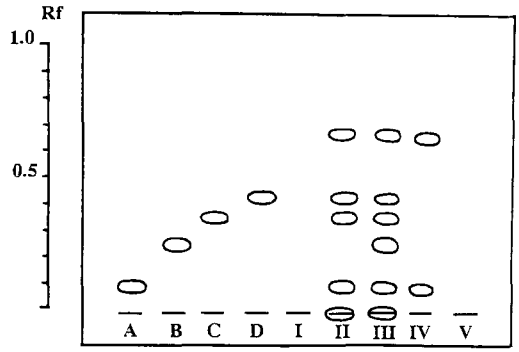

Figure 2. TLC separation of flaxseed sugars and glycosides. A silica gel phate and a solvent system consisting of chloroform/methanol/water (65:35:10, v/v/v) were used. Standards were: A, raffinose; B, sucrose; C, linustatin; D, neolinustatin. Fractions I to V were separated on a Sephadex LH-20 column.

fraction III. The most polar sugars (spot on the base line) were present in fractions II and III. Presence of less polar sugar (Rf=0.67) in flaxseed has already been reported in the literature (15).

TLC analysis showed that the isolated fractions contained numerous phenolic compounds (Figure 3). They were present in all five fractions but were dominated in fractions I to III. Polarity of the phenolic compounds was very different. Two polar compounds were present infraction I, one giving a long spot on the TLC plate. Fractions II, III, and V contained only simple phenolics. Three phenolics were visualized in fraction IV. Rf values of all hydrophylic phenolics were less than Rf of sinapic acid, ferulic acid and NDGA. However, Rf of one compound in fractions II-IV was very close to the Rf value of the glucopyranosyl sinapate. The hydrophylic phenolic fraction from flaxseed does

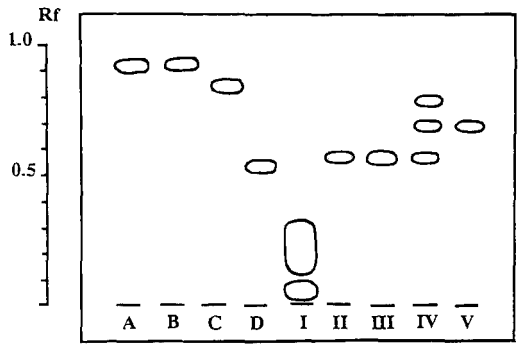

Figure 3. TLC separation of flaxseed phenolics. A silica gel plate and a solvent system consisting of n-butanol/acetic acid/water (3:1:1, v/v/v) were used. Standards were: A, sinapic acid; B, ferulic acid; C, nondihydroguaiaretic acid (NDGA); D, glucopyranosyl sinapate. Fractions I to V were separated on a Sephadex LH-20 column.

not show the presence of any free phenolic acids. Most probably glycosides of phenolics occur in this fraction.

UV spectra (Figure 4 and Table 1) of fractions I to III indicated similar absorption bands with maxima at 286, 284, and 282 nm and a shoulder at 308 nm. However, fraction III showed an additional shoulder at 290 nm. Absorption maxima for UV spectra of fractions IV and V were noted at shorter wavelengths of 270 and 274 nm. Fraction IV exhibited shoulders at 290 and 312 nm, fraction V at 282, 334 and 376 nm.

The chromatographic method described in this work may be employed as a preliminary step in separation of cyanogenic glycosides from flaxseed. Polar sugars obtained in fractions II and III can be easily removed by dissolving the

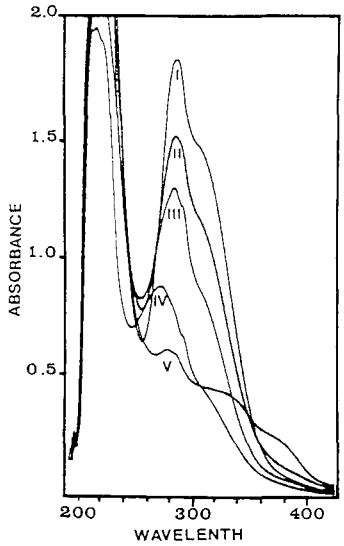

Figure 4. UV spectra of flaxseed extract fractions I to V separated on a Sephadex LH-20 column.

TABLE 1
UV spectral data of different fractions of flaxseed extract (λ_{max}, λ_{sh}, nm).

Fractions	λ_{max}	λ_{sh}
I	286	308
II	284	308
III	282	290, 308
IV	270	290, 312
V	274	282, 334, 376

extract in a chloroform/methanol mixture (15) and linustatin and neolinustatin may be further separated and recovered in pure forms using a reverse phase (RP) (2) or silica gel column chromatography (16). This would also allow the separation of compounds, especially individual pure compounds from fractions II and III and polar compounds from fraction I.

REFERENCES

1. W.M.N. Ratnayake, W.A. Behrens, M.R. Fisher, M.R. L'Abbe, R. Mongeau, J.L. Beare-Rogers, J. Nutr. Biochem., 3: 232-240 (1992).

2. C.R. Smith, D. Weisler, R.W. Miller, J. Org. Chem., 40: 507-510 (1980).

3. B.D. Oomah, G. Mazza, E.O. Kenaschuk, J. Agr. Food Chem., 40: 1346-1348 (1992).

4. H. Kozlowska, R. Zadernowski, F.W. Sosulski, Nahrung, 27: 449-453 (1983).

5. K.J. Dabrawski, F.W. Sosulski, J. Agric. Food Chem., 32: 128-130.

6. R. Amarowicz, H. Kozlowska, M. Shimoyamada, K. Okubo, Pol. J. Food Nutr. Sci., 28: 128-130 (1992).

7. R. Amarowicz, M. Shimoyamada, K. Okubo, H. Kozlowska, "Hemolytical activity of faba bean glucosides" in Proceedings of 1st European Conference on Legumin, Angers (France), 1992, pp. 433-434.

8. K.M. Johnston, D.J. Stern, A.C. Waiss, J. Chromatogr., 33: 539-541 (1968).

9. B. Nambisan, S. Sundersan, J. Assoc. Off. Anal. Chem., 67: 641-

10. M. Dubois, K.A. Gilles, J.K. Reber, P.A. Smith, Anal. Chem., 28: 350-356 (1956).

11. M. Naczk, F. Shahidi, Food Chem., 31: 159-164 (1989).

12. R. Amarowicz, J. Wanasundara, F. Shahidi, Nahrung, 37: 88-90 (1993).

13. R. Zadernowski, Acta Acad. Agricult. Techn. Olst., 21: 3-52 (1987).

14. K.G. Krebs, D. Heusser, H. Wimmer, "Spray Reagents" in Thin-Layer Chromatography, E. Stahl, ed., Springer Verlag, Berlin, Heidelberg and New York, 1990, pp. 854-911.

15. G.M. Barton, J.A.F. Gardner, Nature, 170: 249-251 (1952).

16. R. Amarowicz, X. Chong, F. Shahidi, Food Chem., 48: 99-101 (1993).

17. U. Wanasundara, R. Amarowicz, F. Shahidi, J. Agric. Food Chem., submitted.

18. J.E. Bakke, H.J. Klosterman, Proc. North Dacota Acad. Sci., 10: 18-22 (1956).

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STUDIES ON POLY (3-OCTADECYL PYRROLE) MODIFIED SILICA AS A REVERSED PHASE HPLC PACKING MATERIAL

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ABSTRACT

In this work poly(3-octadecyl pyrrole) (PODP) modified silica has been employed as a reversed phase material. The stationary phase has been characterized using a selection of test compounds and compared with polypyrrole and ODS phases. The stability of this stationary phase in acidic and basic media has been tested. Protein separations have been carried out on this phase. The results obtained indicate that this stationary phase is stable in acidic media and that the performance, with respect to stability, in basic media is similar to commercial ODS columns. Protein separations and basic compound separations have been achieved using this stationary phase.

INTRODUCTION

Separation of proteins and peptides using high-performance liquid chromatography (HPLC) has been extensively studied. A number of different modes of

chromatography have proven useful for such separations. These include size-exclusion, ion-exchange, affinity, hydrophobic interaction and reversed phase (1-3). Among them, reversed phase chromatography using alkyl (4-8) or phenyl (9) bonded silica has been widely used. Often aqueous-organic solvent systems with acids such as trifluoroacetic acid (TFA) (6-9), phosphoric acid (4-5) and perchloric acid (6) have been used. These eluents have a pH value of 2-3 and such conditions are known to promote the cleavage of the carbon chain from the silica in bonded phases (10-12). Separation of basic compounds has also proven difficult on silica-based reversed phase columns due to the solubility of silica under such conditions. Therefore, the development of a stationary phase which is more resistant to low and high pH eluents or alternatively more selective under moderate conditions is desirable.

The use of polymer materials may go some way towards providing such a stationary phase (12,13). In our laboratories conducting polymer based stationary phases have been developed (14-18). These phases possess several unique characteristics which make may make their use as chromatographic stationary phases attractive. Polymers, such as polypyrrole, are electrically conductive, chemically stable, electrochemically active and they can be easily coated on to silica to produce mechanically stable stationary phases. In addition they are capable of a diverse array of chromatographic interactions.

In other laboratories a derivative of pyrrole with a C-18 hydrophobic chain, 3-octadecyl pyrrole (3ODP), has been polymerised either electrochemically (19, 20) or chemically (21).

In this work, 3ODP has been absorbed onto silica and then polymerised. Synthesis of the polymer and characterisation of the stationary phase has been investigated. Stability in acidic and basic media has been examined and the suitability of the stationary phase for separation of proteins or basic compounds has been studied.

EXPERIMENTAL

Reagents and Materials

All reagents were Analytical Reagent (AR) grade unless otherwise stated. 3-octadecyl pyrrole monomer was synthesized using previously reported methods (19).

The chemical polymerization reagent employed was 0.2M $FeCl_3$. Chromatographic test compounds were benzene, toluene, dimethylphthalate (DMP), diethylphthalate (DEP), phenol, resorcin, aniline, N,N-dimethylaniline (DMA), caffeine, theophylline, nitrobenzene, acetophenone, benzoic acid, p-toluic acid, cytosine, uracil, procainamide and N-acetylprocainamide.

Protein samples included α-lactalbumin (LA), ovalbumin (OVA), bovine serum albumin (BSA), human serum albumin (HSA), myoglobin (MYO) and transferrin (TRAN). The proteins were dissolved in purified water and 0.05% (W/W) solutions were stored in a refrigerator.

Preparation of Packing Materials

1.0g of 3ODP was added to 20 ml CH_2Cl_2 in a 50ml beaker. Then, 10g of silica (Beckman, 10μm Ultrasphere, 80Å average pore size, 220 m^2/g specific surface area) were mixed with the solution in the beaker. A nitrogen stream was used to evaporate the CH_2Cl_2. 40 ml of 0.2 mol l^{-1} $FeCl_3$ solution was added to the slurry containing the 3ODP coated silica. Oxidation was carried out with vigorous stirring of the solution using nitrogen for 30 minutes. The particles were transferred to a Buchner filter and washed sequentially with purified water, acetone, dichloromethane, acetone and a mixture of water and acetone (50/50). Fine powder was removed by flotation in water. Finally, 5cm and 15cm x 4.6mm I.D. stainless steel columns were slurry-packed using acetonitrile as the solvent at 300 atm and 400atm respectively. A

Beckman ODS column (5μm, 80A pore size, 15cm x 4.6mm I.D) was used for comparative studies.

Chromatography

All isocratic chromatographic experiments on the PODP column were carried out using a Beckman 114M solvent delivery system, an Altex 210 valve (20μl sample loop), a Beckman 165 variable wavelength detector and an ICI DP-600 chart recorder. Gradient chromatographic experiments on the PODP column and stability tests in basic media were performed using a Dionex Series 4000i gradient pump and injector (50 μl), an ICI SD-2100 UV-VIS variable wavelength detector and a Kipp and Zonen BD41 chart recorder.

Eluents used for protein separations were,
(A) 0.1% TFA in water, and,
(B) 0.1% TFA in acetonitrile.
Stability tests in acid media were carried out by pumping 0.1% TFA in 50% water and 50% acetonitrile, flow rate = 1ml/min, through the column at 50°C. The temperature was controlled by a Waters column temperature controller.

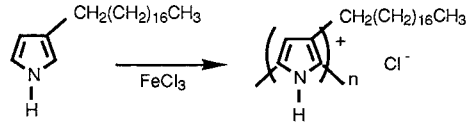
Stability tests in basic media were carried out by pumping 10mM phosphate buffer (pH=9) solution, containing 40% MeOH, at 1ml/min through the column at 80°C. Deterioration of column performance was tested using 20mM phosphate buffers (pH=3 and 7) containing 20% MeOH. All other chromatographic eluents were a mixture of methanol and water. The pH and ionic strength were adjusted using acetate buffers.

RESULTS AND DISCUSSION

Using the method described in the experimental section, poly(3-octadecyl pyrrole) (PODP) was deposited on the silica surface making the particles black in appearance.

Elemental analysis showed that the loadings of C, H, N and Cl were 3.54%, 0.56%, 0.31% and 1.13% by weight.

The presence of Cl indicated that chloride was incorporated as a counterion as follows:



The mole ratio of Cl to N (i.e. n) was approximately 1:4. This is lower than expected and may be due to the presence of $FeCl_4^-$ as a counterion in the polymer material as has been reported previously[18]. The surface area of the coated stationary phase was 172 m^2/g compared to 220m^2/g for the non-coated phase indicating that the polymer coating reduced the silica surface area by about 20%. Presumably some of the pores on the silica are blocked by the polymer coating.

A scanning electron micrograph (SEM) of the coated packing material was obtained (Figure 1). No significant changes were observed after coating even though a black deposit was visually obvious. This indicated that the coating was very thin.

Chromatographic Characterisation

Capacity factors as a function of methanol concentration were determined for 16 test compounds. Results are summarized in Figure 2. A distinct reversed phase interaction was observed with the retention of all species decreasing as the percentage of organic modifier was increased. Non-polar compounds, benzene and toluene have higher capacity factors than those obtained on PP/Cl previously (Table 1) which indicated that the PODP conducting polymer phase undergoes stronger hydrophobic interactions. Phenol and resorcin interacted weakly with the PODP

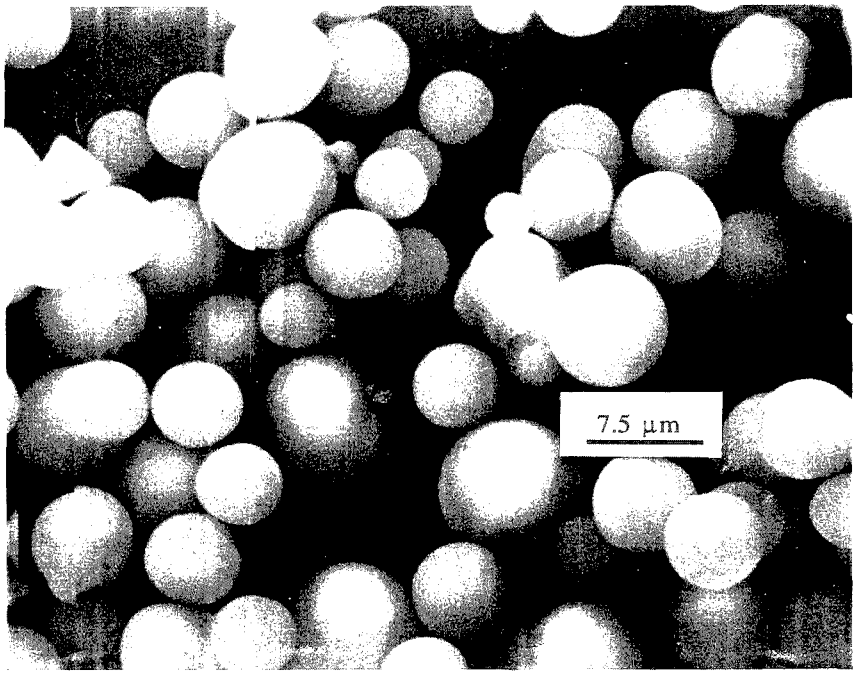


Figure 1 Electron micrograph of PODP coated silica

phase. This confirmed that polypyrrole based polymers do not strongly interact with proton donors as was observed for PP/Cl previously (18). The polar compounds DMP, DEP, nitrobenzene and acetophenone had fairly high retention values. Presumably, this is due to the contribution of the hydrophilic charged polypyrrole backbone. The large difference in capacity factors obtained for aniline and DMA further confirmed that hydrophobic interaction due to $-CH_3$ groups on the DMA molecule was significant. Retention of the basic compounds caffeine and theophylline was not high but benzoic and toluic acids were infinitely retained indicating that although there was an increase in hydrophobic character due to introduction of the alkyl chain to the pyrrole backbone, the anion exchange capability

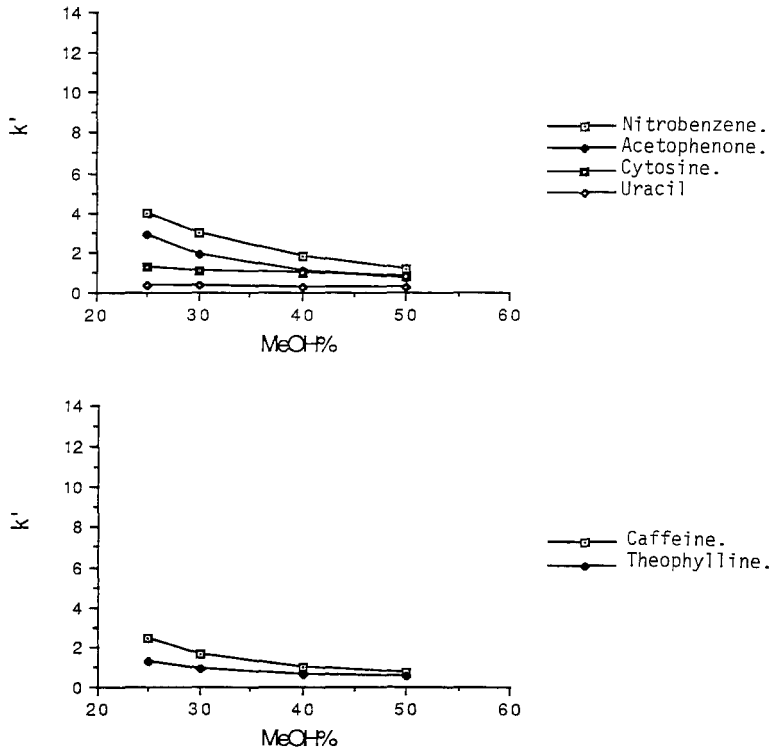


Figure 2 Plots of capacity factors vs methanol concentration in the eluents

Column: 4.6mm I.D.x50mm, 10μm PODP coated silica;

Eluent: Methanol and water (50/50) at 0.5ml/min.

Detector: UV-254 nm.

of the material is retained. Cytosine and uracil which are usually used to test cation exchange capability were weakly retained.

These results confirm that conducting polymer phases such as PODP possess multimodal chromatographic interaction capabilities with both hydrophobic and anion exchange character predominating but some cation exchange character also present.

TABLE 1 - Chromatographic retention characteristics
Comparison of PODP with Polypyrrole (PP)

Test Compound	Retention Index(1) On PP/Cl**	On PODP/Cl
Benzene	1.0	1.0
Toluene	1.6	1.8
DMP	2.0	0.6
DEP	2.1	1.4
Phenol	0.9	0.5
Aniline	8.0	0.5
DMA	22	2.1
Benzoic acid	∞	∞

* Eluent: 50% MeOH and 50% H_2O

* * Data from reference (18)

Injection volume = 20µl

(1) Retention Index = $\frac{\text{capacity factor test compound}}{\text{capacity factor benzene}}$

Buffers have often been used to effect the separation of basic compounds. A comparison of the PODP phase with the Beckman ODS phase was made using pH=7 and pH=3 buffers. The retention characteristics on PODP were markedly different from those observed on ODS (Table 2). ODS strongly interacted with non-polar toluene due to the expected reversed phase interaction while PODP did not. The much higher capacity factors observed for phenol, caffeine and N,N-dimethylaniline on ODS compared to PODP may also be due to the same interaction. Under neutral conditions retention of procainamide and N-acetylprocainamide were much higher on the PODP than on the ODS column. This may be due to cation exchange interaction with uncovered SiOH groups on the

TABLE 2 - Capacity factors on the PODP and ODS columns

	PODP		ODS	
Test compounds	pH = 7.0	pH = 3.0	pH = 7.0	pH = 3.0
toluene	7.88	7.12	>100	>100
phenol	1.44	1.17	8.59	7.91
caffeine	2.90	2.16	7.29	7.22
N,N-dimethylaniline	6.64	-*	93.4	-*
procainamide	24.94	1.38	1.78	0.80
N-acetylprocainamide	39.66	1.48	6.56	2.65

* No UV response obtained due to protonation

Column: ODS (5 µm, 80 Å pore size, 15 cm x 4.6 mm ID)

PODP (10 µm, 80 Å pore size, 15 cm x 4.6 mm ID)

Eluent = 20% MeOH, 80% H_2O, 20mM phosphate

Flow rate = 1.0 ml/min

Injection volume = 20µl ODS, 50µl PODP

Concentration of standards:

Phenol	8ppm
Caffeine	2ppm
DMP	2ppm
Toluene	80ppm
Procainamide	10ppm
N-acetylprocainamide	10ppm

PODP column. The greater retention of caffeine than that of phenol on the PODP indicated greater selectivity for basic compounds on the PODP.

Changes in the pH of the eluent had a slight influence on k' for toluene, phenol and caffeine on both phases. However, a significant decrease of k' for procainamide and N-acetylprocainamide was observed when the eluent was changed from 7.0 to 3.0. The capacity factors decreased by a factor of 2 on the ODS phase and a factor of 20 on the PODP phase. In the case of ODS this is probably due to protonation of residual silanol groups, preventing cation exchange interactions occurring. With the PODP phase this may also be the case. However as indicated above the conducting polymer phase is multimodal. This obviously results in more dramatic changes in retention as the result of pH is changed.

A further indication of the ion exchange capacity of the PODP phase were the observations recorded during changes in eluent pH. With the ODS column the effluent pH become constant after passing one column void volume while with the PODP column 10 times the void volume was required. The greater volumes required are a reflection of the ion exchange capabilities of the PODP column.

The column efficiency (N), in plates per metre, for selected test compounds (small molecules) are summarized in Table 3. The higher efficiency observed on the ODS column was at least in part due to the smaller particle size employed.

Following the above experiments with small molecules the chromatographic behaviour of selected proteins was investigated using this new polymer phase. The properties of the proteins used in this work and the corresponding chromatographic data obtained are summarised in Table 4. The retention sequence obtained on PODP was different from that observed on ODS based columns. For example, the retention sequence using PODP was MYO>BSA>OVA while it was found to be OVA>BSA>MYO using other ODS columns (9). The difference is probably due to the

TABLE 3 - Column efficiency(1)

	PODP	ODS
Test compounds		
phenol	9200	36200
caffeine	10000	37500

(1) Experimental conditions as in Table 2. (Eluent pH = 7.0).

N was calculated using = $5.54\ (\frac{t_r}{W_{1/2}})^2$

where - t_r = retention time.

$W\frac{1}{2}$ = peak width at half peak height.

See Experimental section for test conditions.

TABLE 4

Protein	Molecular Weight (X 1000)	pI (Isoelectric Point)	t_R (min)	Recovery (%)
α- Lactalbumin	14	5.1	1.07	92
Ovalbumin	44	4.6	0.84, 1.14, 3.35	23
BSA	66	5.3	4.8	47
HSA	66	4.7	4.5	69
Myoglobin	18	7.0	1.43, 17.3	88
Transferrin	77	5.0-6.0	5.2, 7.5	76

Gradient from 0.1% TFA in H_2O to 0.1% TFA in CH_3CN in 20 minutes at 1.0ml/min.

Sample loading = 50μl of 0.05% protein aqueous solutions

contribution of the hydrophilic pyrrole ring which enhances the protein retention. With most proteins the elution of two or more peaks was observed. The first peak may be the native protein and the second the denatured protein as has been reported by others (4). Recovery, calculated from peak areas with and without the column, was low for most proteins especially for ovalbumin.

Stability Test

Using a thermostatted column (50°C) and an acidic eluent containing 0.1% TFA the stability of the column under acidic conditions was tested using procedures described previously (10-12). The column was found to be stable. Even after passing 15000 column volumes of eluent, the retention of the small molecular probes listed in Table 2 had not changed.

The column resolution also did not change for these small molecules. Using the same conditions, the column was shown to be stable with respect to protein separations (Figure 3). The column performance was again unchanged after passing 15000 column volumes. The lack of stability of conventional carbon bonded silica phases when using strong acids such as TFA is well documented (22, 23). Obviously the pyrrole ring - C_{18} bond is not so susceptible to acid attack as the silane bond used to couple carbon chains to silica.

The stability of the PODP phase and ODS phase in basic media was then investigated using the procedure outlined in the experimental section. At certain intervals the flow of the high pH eluent was stopped and the above mentioned (Experimental) chromatographic eluents with pH = 3 or pH = 7 buffer were employed.

Retention of two test compounds, caffeine and phenol was used to test the column stability in basic media. On the PODP phase it was found that using a pH = 7 buffer

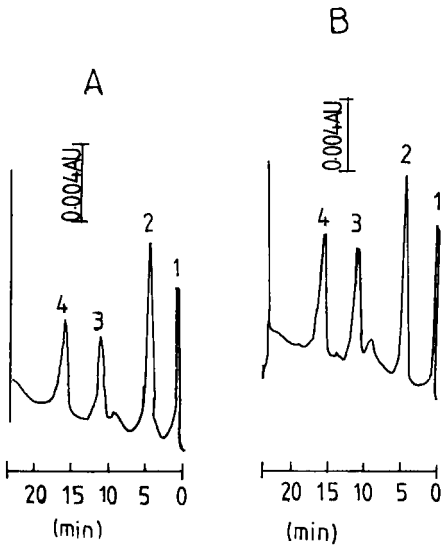


Figure 3 Separation of proteins after passing (A) 1170 and (B) 15300 column volumes stability test eluent.

(1) 25 μg oval bumin, (2) 50 μg BSA, (3 & 4) 25 μg myoglobin.

Column: 4.6 mm I.D x 50 mm, 10 μm PODP coated silica.

Stability test solvent: 50% H_2O and 50% CH_3CN with 0.1% TFA at 50°C at 1.0 ml/min;

Separation Eluent: Gradient from 0.1% TFA in H_2O to 0.1% TFA in CH_3CN in 20 minutes at 1.0 ml/min;

Detection: UV - 280 nm.

in the chromatographic eluent that the capacity factor for caffeine remained relatively constant while that for phenol decreased from 2.8 to approximately 2.2 after 500 hours exposure to the basic pH = 9 eluent. This resulted in a concomittant loss in selectivity over the period of the test. These changes were not apparent with the ODS phase over the same test period. It is possible that exposure to higher pH eluents causes some chemical transfomation in the conducting polymer stationary phase as has been discussed previously (24, 25).

The basic stability test was then repeated using a pH 9 buffer of higher buffering capacity (25 mM phosphate 50 mM borate) containing 40% MeOH. Retention of caffeine on PODP declined during the course of this test (as with the previous test) whereas it remained relatively stable on the ODS column. Column efficiency declined overall for both columns, but the effect was more significant on the ODS column, with approximately 55% loss in efficiency for phenol and caffeine compared with only a 20% loss of efficiency on the PODP. Silica based columns are know to be prone to dissolution at high pH. It appears that the conducting polymer protects against this. However, the exposure to high pH does cause some other chemical transformation to occur that influences chromatographic performance.

CONCLUSIONS

PODP coated silica has been synthesised and characterised using a group of test compounds. The novel properties of this new stationary phase have been revealed. The elution sequence of both small molecules and proteins revealed that the PODP phase has different selectivity to that of conventional ODS stationary phases. The stationary phase was stable under acidic reversed phase conditions but not basic. However the mechanism causing the changes observed at high pH appears to be different from that observed with silica. Separation of proteins was carried out using this stationary phase.

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REFERENCES

(1) F.E. Regnier, Chromatographia, 24 (1987) 241.

(2) F.E. Regnier, J. Chromatogr., 418 (1987) 115.

(3) M.T.W. Hearn, J. Chromatogr., 418 (1987) 3.

(4) S. Lin and B.L. Karger, J. Chromatogr., 499 (1990) 89.

(5) C.L. Flurer, C. Borra, F. Andreolini and M. Novotny, J. Chromatogr., 448 (1988) 73.

(6) K.K. Unger, G. Jilge, J.N. Kinkel and MT.W. Hearn, J. Chromatogr., 359 (1986) 61.

(7) M. Verzele, Y.-B. Yang, C. Dewaele and Berry, Anal. Chem., 60 (1988) 1329.

(8) N.E. Zhou, C.T. Mant and R.S. Hodges, Peptide Research, 3 (1990) 1.

(9) J.F. Fausnaugh, L.A. Kennedy and F.E. Regnier, J. Chromatogr., 317 (1984) 141.

(10) N. Sagliano, Jr., T.R. Floyd, R.A. Hartwick, J.M. Dibussolo and N.T. Miller, J. Chromatogr., 443 (1988) 155.

(11) J.L. Glajch, J.J. Kirkland and J. Kohler, J. Chromatogr. 384 (1987) 81.

(12) T. Uchida, T. Ohtani, M. Kasai, Y. Yanagihara, K. Noguchi, H. Izu and S. Hara, J. Chromatogr., 506 (1990) 327.

(13) V.A. Davankov, A.A. Kurganov, K.K. Unger, J. Chromatogr., 500 (1990) 519.

(14) H. Ge and G.G. Wallace, Anal. Chem., 61 (1989) 198.

(15) H. Ge and G.G. Wallace, Anal. Chem., 61 (1989) 2391.

(16) H. Ge and G.G. Wallace, J. Liq. Chromatogr., 13 (1990) 3245.

(17) H. Ge and G.G. Wallace, J. Liq. Chromatogr., 544 (1991) 305.

(18) H. Ge and G.G. Wallace, J. Chromatogr., 588, (1991) 25.

(19) J. Ruhe, T. Ezquerra and G. Wegner, Makromol. Chem., Rapid Commun., 10 (1989) 103.

(20) J. Ruhe, T.A. Ezquerra and G. Wegner, Synth. Met., 28 (1989) C177.

(21) K. Hong and M.F. Rubner, Thin Solid Films 179 (1989) 215.

(22) J.L. Glajch, J.J. Kirkland, J. Kohler. J. Chromatogr., 384, (1987), 81.

(23) N. Sagliano, T.R. Floyd, R.A. Hartwick, J.M. Disbusslo, N.T. Miller, J. Chromatogr. 443, (1988), 155.

(24) L. Yongfang, R. Quian, Synthetic Metals., 139-151, (1988), 2.

(25) G. Gustafsson, L. Lundstrom, B. Liedberg, C.R. Wu and O. Inganas., Synthetic Metals, 163-179, (1989), 31.

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COMPARISON OF AMINO ACID SEPARATIONS ON HIGH PERFORMANCE SILICA GEL, CELLULOSE, AND C-18 REVERSED PHASE LAYERS AND APPLICATION OF HPTLC TO THE DETERMINATION OF AMINO ACIDS IN *BIOMPHALARIA GLABRATA* SNAILS

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ABSTRACT

The separation of 18 amino acids on high performance silica gel, cellulose, and reversed phase bonded silica gel plates has been compared. Zones were detected with ninhydrin reagent. The ability to identify amino acids in Biomphalaria glabrata snail hemolymph and digestive gland-gonad complex (DGG) by HPTLC was studied, and alanine and aspartic acid were quantified in hemolymph and DGG by scanning densitometry. Analytical results were compared to those reported earlier using column ion exchange and reversed phase liquid chromatography and paper chromatography.

INTRODUCTION

In earlier studies in our laboratories, separations of 18 amino acids on silica gel, cellulose, ion exchange,

and reversed phase thin layer chromatography (TLC) plates and sheets were compared (1,2), and amino acids were determined by TLC in adult Echinostoma revolutum (Trematoda) (3). We have now extended these studies to amino acid separations on cellulose, silica gel, and chemically bonded C-18 modern high performance thin layer chromatography (HPTLC) plates, all of which contained a preadsorbent zone except the cellulose.

Most analyses of amino acids in biological samples have been performed using techniques other than TLC, i.e., gas-liquid chromatography and column ion exchange chromatography, particularly with automated amino acid analyzers. Recent reviews (4,5) have suggested the efficacy of TLC as an alternative method for the analysis of amino acids, and in this study we have used HPTLC to examine amino acids in the hemolymph and digestive gland-gonad complex (DGG) of the medically important snail, Biomphalaria glabrata. Results are compared with those reported earlier for amino acids in the hemolymph and digestive gland of Biomphalaria glabrata and other snail species based on ion exchange (6-8) and C-18 (9) column chromatography and paper chromatography (10,11) analyses.

EXPERIMENTAL

Sample Preparation

Snails with shell diameters of 10-20 mm were maintained in glass vessels with artificial spring water and fed a boiled lettuce diet for 7 days as previously described (12).

Amino acids were extracted from the DGG and hemolymph of pooled snails using the same procedures described earlier for carbohydrates (12). Typical sample sizes were 400 mg of DGG and 500 ul of hemolymph from a

pool of 5-10 snails. Extract solutions were stored in a refrigerator and used within two days of preparation. The effect of desalting on the chromatograms was tested by treating evaporated extract with 1 ml of 0.5% HCl in 95% ethanol for 24 hr (3) prior to TLC.

TLC Analysis

Standards of the amino acids listed in Table 1 were obtained from Sigma (St. Louis, MO). Stock standard solutions of each acid were prepared in 70% ethanol at concentrations of 1.00 mg/ml. TLC standards were prepared at concentrations of 100 and 500 ng/μl by dilution with 70% ethanol. For quantitative analysis, standards of alanine and aspartic acid were further diluted to 10.0 ng/μl with 70% ethanol.

Initial zones were applied using a 10 μl Drummond (Broomall, PA) digital microdispenser. For determination of R_f values and comparison of systems, 1.00 μl of the 500 ng/μl standards were spotted. For qualitative analysis of snail extracts, 5.00 μl of the 100 ng/μl standards and extracts reconstituted in 200 μl of solvent were applied. For quantitative analysis, 2.00, 4.00, 8.00 and 10.0 μl of the standards (20.0 to 500 ng) and extracts reconstituted in 100 μl of solvent were spotted.

The following 20 x 10 cm precoated thin layers were used: Whatman (Clifton, NJ) high performance silica gel LHPKDF with channels and preadsorbent area, catalog no. 4806-711 and C-18 chemically bonded silica gel with preadsorbent area, catalog no. 4800-820; E. Merck (Gibbstown, NJ) cellulose F, no. 15036-6; Merck RP-18F, CZ reversed phase chemically bonded C-18 silica gel with concentrating zone, no. 15498-6; and Merck RP-18WF, CZ water stable C-18 bonded silica gel layer with concentrating zone, no. 14235-6. All plates were 20 x 10

TABLE 1

hR_f Values of Amino Acid Standards on Reversed Phase Layers

	TLC System*					
	1	2	3	4	5	6
Aspartic acid	30	59	72	60	83	73
Arginine	28	4	35	28	86	82
Serine	55	36	69	50	82	73
Glycine	50	38	62	45	69	54
Tyrosine	91	77	88	68	77	67
Alanine	78	59	71	63	71	54
Glutamic acid	82	70	86	67	83	69
Proline	56	69	64	40	65	46
Cystine	11	12	39	33	85	84
Methionine	90	74	82	60	65	42
Lysine	31	84	27	24	74	79
Tryptophan	90	77	85	63	72	63
Valine	90	74	75	59	75	61
Threonine	78	52	68	50	72	57
Histidine	21	3	29	23	77	68
Phenylalanine	90	76	83	65	72	61
Leucine	90	77	81	62	75	63
Isoleucine	91	77	81	62	74	61

*Layers: 1,2 = Whatman C-18; 3,5 = Merck RP-18; 4,6 = Merck RP-18W; mobile phases: 1,3,4 = n-butanol-glacial acetic acid water (3:1:1); 2,5,6 = n-propanol-water (7:3)

cm except Whatman C-18, which was 20 x 20 cm. n-Butanol or 2-butanol-glacial acetic acid-water (3:1:1) and 70% aqueous n-propanol were employed as mobile phases.

Plates were developed in a Camag (Wilmington, NC) paper-lined, solvent vapor equilibrated HPTLC twin-trough chamber for a distance of 7 cm beyond the origin (11 cm for Whatman C-18), which required 45-150 min depending on the layer and mobile phase. The chromatogram was air dried in a fumehood, sprayed with ninhydrin detection solution (0.3 g ninhydrin in 100 ml of n-butanol plus 3 ml of glacial acetic acid), air dried for 5 sec, resprayed, and heated in an oven at 110°C for 10 min.

Quantification was carried out by scanning standard and sample zones at 610 nm with a Shimadzu (Columbia, MD) CS-930 scanner in the single beam, reflectance mode. The absorption maximum for scanning was determined by measuring the in situ spectrum of a standard zone using the visible (400-700 nm) spectral mode of the densitometer. The concentration of amino acid was calculated as μmol/g for DGG and μmol/ml for hemolymph from the scan areas of the standard zones and a bracketed sample zone, the amount of sample taken, the reconstitution volume of the sample extract, and the volumes of standards and sample spotted.

RESULTS AND DISCUSSION

A large number of silica gel, cellulose, and reversed phase systems suggested by a search of the literature and our previous experience (1,2) were tested for resolution of the standards of 18 important essential and nonessential amino acids. Most of these compounds were likely to be found in B. glabrata snails based on the results of previously published studies. hR_f values (R_f x 100) for the amino acids in the best systems, as evidenced by their efficiency (compactness of zones) and selectivity (separation of zone centers), are shown in Tables 1 and 2. Different recipes of ninhydrin and fluorescamine (13) spray reagents found in the paper chromatography and TLC literature were also compared, and the butanol-acetic acid formulation of ninhydrin gave the best sensitivity, stability, and color differentiation. Depending on the layer, amino acid zones were various shades of purple, blue, orange, yellow, or red. In general, amino acids formed compact bands on silica gel, cellulose, and Merck C-18 plates and were somewhat more diffuse on Whatman C-18 plates.

TABLE 2

hR_f Values of Amino Acid Standards on Normal Phase Layers

	TLC System*			
	1	2	3	4
Aspartic acid	28	27	26	58
Arginine	18	18	17	2
Serine	26	30	27	40
Glycine	26	32	28	43
Tyrosine	46	58	53	78
Alanine	38	32	32	55
Glutamic acid	69	56	50	64
Proline	45	32	28	50
Cystine	10	11	9	30
Methionine	60	59	51	72
Lysine	15	13	10	4
Tryptophan	55	63	57	82
Valine	60	56	49	68
Threonine	34	32	32	53
Histidine	14	14	11	17
Phenylalanine	68	61	55	80
Leucine	79	61	55	78
Isoleucine	78	59	54	77

*Layers: 1 = cellulose; 2, 4 = Whatman silica gel; 3 = Merck silica gel; mobile phases: 1 = 2-butanol-glacial acetic acid water (3:1:1); 2, 3 = n-butanol-glacial acetic acid-water (3:1:1); 4 = n-propanol-water (7:3)

The migration sequences indicated by the data in Tables 1 and 2 do not clearly demonstrate that a different mechanism was operative for the normal phase (silica gel, cellulose) and reversed phase (C-18) plates, but suggest that the separations were most likely governed by a combination of adsorption, partition, and/or solubility (2). The possible variations among different brands of the same type of stationary phase is demonstrated in Table 1 for arginine and histidine, which have very low R_f values on Whatman C-18 plates but high

values on Merck RP-18 and RP-18W. The unique selectivities of the systems caused the amino acids to be resolved into several diverse groups with differing mobility on each plate, which would allow a large number of binary and larger amino acid mixtures to be separated based on the data in Tables 1 and 2. However, only the following compounds were completely resolved from all others on any single plate: histidine and cystine on Whatman C-18 developed with n-butanol-acetic acid-water (3:1:1); threonine, alanine, and cystine on cellulose developed with 2-butanol-acetic acid-water (3:1:1); arginine and cystine on Merck silica gel developed with n-butanol-acetic acid-water (3:1:1); methionine, aspartic acid, and histidine on Whatman silica gel developed with n-propanol-water (7:3); and arginine and cystine on Merck RP-18 developed with n-butanol-acetic acid-water (3:1:1).

Extracts of B. glabrata DGG and hemolymph were developed in all of the systems involving butanol-acetic acid water mobile phases and with n-propanol-water (7:3) on Whatman silica gel, and the resulting chromatograms were very complex. A desalting procedure (3) was applied to the extracts to determine if a small degree of trailing in some of the sample chromatograms could be decreased or eliminated. It was found that extract chromatograms were essentially identical with or without desalting.

Comparison of R_f values and zone colors and intensities between samples and standards in the various systems indicated that DGG and hemolymph extract chromatograms were similar and that most, if not all, of the 18 amino acids were probably present in both extracts. However, only the seven completely resolved compounds mentioned above, i.e., histidine, cystine, threonine, alanine, arginine, methionine, and aspartic acid, could be definitely identified. Based on zone

intensities, methionine and cystine were present in very low amounts, while the other five amino acids were major components. It was not possible to definitely confirm the presence or absence of the other acids, despite the use of a number of different types of high performance plates. These findings cast doubt on qualitative and quantitative results reported earlier (10,11) for amino acids in B. glabrata based on paper chromatography, which is considerably less efficient than HPTLC.

Earlier publications on the qualitative and quantitative analysis of amino acids in B. glabrata hemolymph also reported the presence of many amino acids, but results were not consistent. Stanislawski et al. (8) found 17 of the 18 amino acids we investigated present by ion exchange chromatography at levels ranging from 8.1 to 90.6 μmol/L; tryptophan was not reported. Schnell et al. (9) determined 15 of the 18 acids at concentrations greater than 13 μmol/L in hemolymph and 0.2 μmol/g in digestive gland using C-18 HPLC, along with traces of methionine and tryptophan; cystine, proline, and histidine were not found in either sample. Gilbertson et al. (7) found 15 of the 18 acids in hemolymph at levels between 1-57 μmol/L, plus a trace of tyrosine; neither tryptophan nor cystine were reported. Using paper chromatography, Dusanic and Lewert (11) identified lysine, glycine, proline, threonine, and leucine, and Targett (10) identified all of the 18 acids except tryptophan. In a study of the hemolymph of five species of freshwater snails not including B. glabrata, Gilbertson and Schmid (6) detected 18 different amino acids by ion exchange chromatography, among which serine, glutamic acid, aspartic acid, glycine, threonine, and alanine were the major compounds in all species.

To demonstrate the applicability of quantitative HPTLC to amino acid analysis in snails, DGG and hemolymph

extracts were analyzed for two of the completely resolved acids, i.e., alanine on cellulose developed with 2-butanol-acetic acid-water (3:1:1), and aspartic acid on Whatman silica gel developed with n-propanol-water (7:3). Calibration curves for the two acids had linearity coefficients of 0.99 over the range of standards spotted. The results for alanine were 106 μmol/L in hemolymph and 0.326 μmol/g in the DGG, while the corresponding values for aspartic acid were 30.3 μmol/L and 0.508 μmol/g. These values are in the range of those found earlier for B. glabrata by column LC: 27 (7), 98 (8), and 107 (9) μmol/L for alanine in hemolymph and 1.1 μmol/g (9) in the digestive gland, and 16 (9), 21 (7), and 58 (8) μmol/L for aspartic acid in hemolymph and 0.45 μmol/g (9) in the digestive gland.

The variations described above in reported qualitative and quantitative results for amino acids in B. glabrata snails are due not only to the different analytical methods employed, but also to differences in strain, development, nutrition, and maintenance of the snails.

Our present and earlier (1,2) studies demonstrate that HPTLC can be used for the separation, detection, identification, and quantification of amino acids in selected mixtures. However, the method has limited applicability to the analysis of amino acids in freshwater snails because of the complexity of the mixture present in aqueous extracts. Of the procedures proposed in the literature, column ion exchange chromatography (amino acid analyzer) appears to be the method of choice for optimal qualitative and quantitative analysis of snail amino acids.

REFERENCES

1. Sleckman, B.P. and Sherma, J., J. Liq. Chromatogr. 5, 1051 (1982)

2. Sherma, J., Sleckman, B.P., and Armstrong, D.W., J. Liq. Chromatogr. 6, 95 (1983)

3. Bailey, R.S., Jr. and Fried, B., Int. J. Parasitol. 7, 497 (1977)

4. Bhushan, R., in Handbook of Thin Layer Chromatography (Sherma, J. and Fried, B., editors), Marcel Dekker, Inc., New York, 1991, pp. 353-387

5. Blackburn, S. (editor), CRC Handbook of Chromatography: Amino Acids and Amines, vol. II (Zweig, G. and Sherma, J.,editors-in-chief), CRC Press, Boca Raton, FL, 1989

6. Gilbertson, D.E. and Schmid, L.S., Comp. Biochem. Physiol. 51B, 201 (1975)

7. Gilbertson, D.E., Etges, F.J., and Ogle, J.D., J. Parasitol. 53, 565 (1967)

8. Stanislawski, E., Becker, W., and Mueller, G., Comp. Biochem. Physiol. 63B, 477 (1979)

9. Schnell, S., Becker, W., and Winkler, A., Comp. Biochem. Physiol. 81B, 1001 (1985)

10. Targett, G.A.T., Ann. Trop. Med. Parasitol. 56, 61 (1962)

11. Dusanic, D.G. and Lewert, R.M., J. Inf. Dis. 112, 243 (1963)

12. Anderton, C.A., Fried, B., and Sherma, J., J. Planar Chromatogr.-Mod. TLC 6, 51 (1993)

13. Sherma, J. and Touchstone, J.C. Anal. Lett. 7, 279 (1974)

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DETERMINATION OF A CEFUROXIME AND AMINOPHYLLINE/THEOPHYLLINE MIXTURE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high-performance liquid chromatographic method for the simultaneous determination of cefuroxime and aminophylline/theophylline has been developed. Cefuroxime was separated from aminophylline/theophylline within 8 min using a octadecylsilane column and a mobile phase consisting of a 10:1 mixture of 0.1 M acetate buffer pH 3.4 - acetonitrile at ambient temperature. Orcinol was used as internal standard and the analytes were monitored at 254 nm. The method was free of interference from degradation products related to any of the analytes. Drug to internal standard peak area ratios were linear ($r^2 \geq 0.9998$) for each analyte with good precision (RSD $\leq 2.1\%$) and accuracy ($\leq 2.3\%$).

INTRODUCTION

Cefuroxime is a cephalosporin antibiotic intended for parenteral administration. Cefuroxime for injection is presented as the sterile

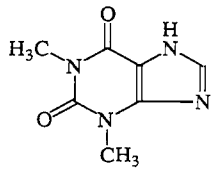
sodium salt in a sealed container and is sometimes admixed by the addition of an appropriate amount of aminophylline or theophylline injection.

Methods for the simultaneous determination of cefuroxime and aminophylline/theophylline in a mixture have not been reported, although there are HPLC methods used separately for the analysis of cefuroxime (1-3) and aminophylline/theophylline (4-6). The HPLC assay method reported herein for cefuroxime and aminophylline/theophylline was modified from a stability-indicating assay for cefuroxime reported in the Code of Federal Regulations (CFR) (1). The HPLC method was shown to be stability-indicating with respect to both cefuroxime and aminophylline/theophylline in a single injection.

EXPERIMENTAL

Reagents and Chemicals

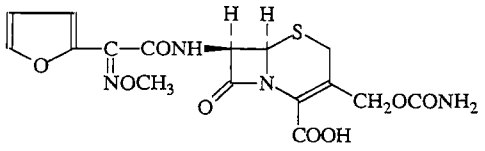
The chemical structures of the analytes are shown in Figure 1. Cefuoxime sodium was a gift from Glaxo, Inc. (Research Triangle Park, NC 27709). Aminophylline and theophylline were obtained from Sigma Chemical Co. (St. Louis, MO 63178). Orcinol monohydrate (5-methylresorcinol, J.T. Baker, Phillipsburg, NJ 08865) was obtained for use as the internal standard. All chemicals and solvents used were the highest grade of commercially available materials. A pH 3.4 acetate buffer was prepared by the addition of 50 mL of 0.1M sodium acetate into a 1000 mL volumetric flask followed by 0.1M acetic acid to volume.



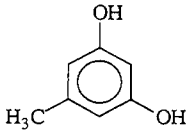
THEOPHYLLINE

$(C_7H_8N_4O_2)_2.H_2NCH_2CH_2NH_2.2H_2O$

AMINOPHYLLINE



CEFUROXIME



ORCINOL

Figure 1. Chemical structures of the analytes.

Apparatus

The liquid chromatography system consisted of a Beckman Model 110B Pump (San Ramon, CA 94583), an Alcott Model 738 Autosampler (Norcross, GA 30093) and an ABI Model 759A UV/VIS Absorbance Detector (Foster City, CA 94404). Separation was accomplished on an Beckman Ultrasphere Octadecylsilane (C18) column (150 mm x 4.6 mm i.d., 5 μm) equipped with a 50 mm C18 guard column. The mobile phase consisted of a 10:1 v/v mixture of 0.1M acetate buffer, pH 3.4 and acetonitrile. The mixture was filtered through a 0.45 μm membrane filter and degassed by sonication prior to use. The operating conditions for this system were as follows: flow rate, 2 mL/min; detector

wavelength, 254 nm; injection volume, 50 μL; and column temperature, 23 ± 1°C. Data acquisition and reduction were performed on an HP Model 3394A Integrator (Hewlett-Packard Company, Avondale, PA 19311).

Preparation of Standard and Sample Solutions

An internal standard solution was prepared by dissolving a weighed amount (45 mg) of orcinol monohydrate in 50 mL of water to give a 900 μg/mL solution. The solution was filtered, protected from light, and prepared fresh every week. A combined cefuroxime and aminophylline/theophylline standard solution was prepared by accurately weighing 18 mg of cefuroxime sodium and 2.4 mg of aminophylline or 1.9 mg of theophylline, transferring the powders to a 100-mL low actinic volumetric flask, water added to volume, and manual shaking for 30 sec. The solutions were stored in a refrigerator (4°C) and used within one week (7). Accurately pipetted volumes of 62.5, 125, 250, 500, and 1000 μL of the combined standard solution were each mixed with 500 μL of the internal standard solution and diluted to 1.5 mL with water to give concentrations in the range of 7.5 -120 μg/mL for cefuroxime sodium, 1 - 16 μg/mL for aminophylline and 0.8 - 13 μg/mL for theophylline. Five point calibration curves were constructed for each analyte. Additional dilutions of the combined standard solution were prepared in water to serve as spiked samples for each analyte to determine accuracy and precision of the method. Samples to be assayed

were mixed with 500 μL of the internal standard solution and diluted to 1.5 mL with water. Standards and samples were placed in autosampler vials and 50 μL aliquots were injected into the HPLC system. Quantitation was based on linear regression analysis of peak area ratios of each drug to internal standard versus their concentration in μg/mL.

RESULTS AND DISCUSSION

The aim of this study was to modify the CFR isocratic HPLC assay for cefuroxime to determine both cefuroxime and aminophylline/theophylline in a single injection. The method would need to be free of interference from degradation products related to either analyte. The HPLC procedure for cefuroxime reported in the CFR was initially investigated for use in this study (1). The cefuroxime mobile phase consisted of a 10:1 v/v mixture of 0.1M acetate buffer pH 3.4-acetonitrile and a column packed with 5 μm hexylsilane (C6). It was found that the cefuroxime and aminophylline peaks overlapped (2.35 vs 2.26 min, respectively) on the C6 column. When the column was changed to a 5 μM octysilane (C8), both drugs were still not well separated (5.82 vs 5.51 min, respectively). A 10 μm octadecylsilane (C18) column was then placed in the HPLC system and showed suitable resolution of the cefuroxime and aminophylline peaks (4.01 vs 5.50 min, respectively). Further investigations of various octadecylsilane columns from different manufacturers indicated that the best peak resolution and peak shapes were obtained using a Beckman ultrasphere octadecylsilane

column. A typical chromatogram from the ultrasphere C18 column is shown in Figure 2-I.

Determination of both cefuroxime and aminophylline/theophylline was performed using internal calibration. Calibration curves were generated by least-square regression of the drug/internal standard peak area ratios versus the concentration of each drug. The regression analysis showed linearity for cefuroxime over the 7.5 - 120 μg/mL range and for aminophylline/theophylline over the 1-16/0.8-13 μg/mL range with correlation coefficients $\geq$ 0.9998 (n = 5).

Percent error (accuracy) and precision of the method were evaluated using spiked samples. The results shown in Tables 1 and 2 indicate that the procedure gives acceptable accuracy and precision.

A study of any interferences from degradants of each drug was conducted. Solutions containing 90 μg/mL cefuroxime and 12 μg/mL aminophylline or 10 μg/mL theophylline in water were allowed to degrade by standing at ambient temperature (23 $\pm$ 1°C) or under room lighting for 9 days, or by heating for 6 hours in an 80°C oven. The chromatogram from the degraded sample shown in Fig. 2-II demonstrates that there were no interferences noted at the retention times of cefuroxime, aminophylline/theophylline, or orcinol (internal standard).

The chromatographic assay thus provides a rapid, precise and selective method for the simultaneous determination of both cefuroxime

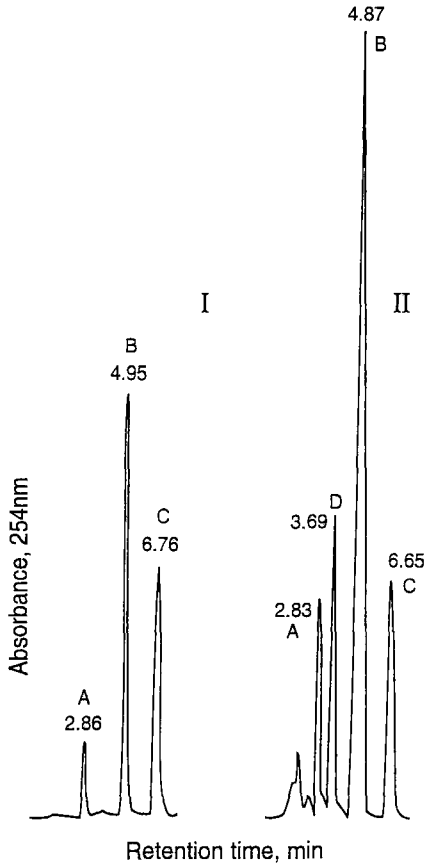


Figure 2. Typical chromatograms of a typical mixture (I) and a degraded mixture (II) containing aminophylline/theophylline (A), cefuroxime (B), orcinol (C) and an unknown degradation product. (D).

Table 1 - Inter-day Precision and Accuracy of Cefuroxime and Aminophylline /Theophylline

Concentration added, (μg/mL)	Concentration found, (μg/mL)	n	Relative Error (%)	RSD (%)
Cefuroxime Sodium				
15.00	14.84 ± 0.18	6	1.1	1.2
45.00	44.16 ± 0.47	5	1.9	1.1
90.00	89.28 ± 0.50	6	0.8	0.6
Aminophylline				
2.028	1.981 ± 0.033	6	2.3	1.6
6.085	6.034 ± 0.032	5	0.8	0.5
12.17	12.02 ± 0.102	6	1.3	0.8
Theophylline				
1.616	1.593 ± 0.025	6	1.4	1.6
4.848	4.830 ± 0.044	6	0.4	0.9
9.695	9.705 ± 0.091	6	0.1	0.9

Table 2 - Intra-day Precision and Accuracy of Cefuroxime and Aminophylline/Theophylline

Concentration added, (μg/mL)	Concentration found, (μg/mL)	n	Relative Error (%)	RSD (%)
Cefuroxime Sodium				
15.00	14.73 ± 0.11	5	1.8	0.7
45.00	43.97 ± 0.13	5	2.3	0.3
90.00	89.00 ± 0.08	5	1.1	0.1
Aminophylline				
2.028	1.985 ± 0.042	5	2.1	2.1
6.085	6.027 ± 0.047	5	0.9	0.8
12.17	11.94 ± 0.131	5	1.9	1.1
Theophylline				
1.616	1.609 ± 0.032	5	0.4	2.0
4.848	4.899 ± 0.012	5	1.1	0.3
9.695	9.746 ± 0.080	5	0.5	0.8

and aminophylline/theophylline in a single injection. The procedure is suitable for the analysis of freshly manufactured materials and it also provides a reliable estimate of the potency of stored samples.

REFERENCES

1. 21 Code of Federal Regulations Ch. 1 (4-1-87 Edition), p. 340.

2. P.A. Coomber, J.P. Jefferies and J.D. Woodford. High-performance liquid chromatographic determination of cefuroxime. *Analyst.* 107 (1982) 1451.

3. V. Springolo and G. Coppi. Comparison between HPLC and microbiological assay of some cephalosporins. *Boll. Chim. Farm.*, 122 (1983) 104.

4. C.A. Lau-Cam and R. W. Roos. Simultaneous high-performance liquid-chromatographic determination of theophylline and ethylenediamine dosage forms as their dansyl derivatives. *J. Liq. Chromatogr.*, 14 (1991) 1939.

5. H. Zhang and Y. Chen. HPLC determination of theophylline and phenobarbital in compound aminophylline tablets. *Yiyao-Gongye*, 18 (1987) 221.

6. R.H. Gannon and R.M. Levy. Interference of third-generation cephalosporins with theophylline assay by high performance liquid chromatography. *Am. J. Hosp. Pharm.*, 41 (1984) 1185.

7. The Merck Index, 11th Ed., Merck and Co., Rahway, NJ, (1989) p. 300.

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DETERMINATION OF [^{14}C] GLUTAMINE SPECIFIC ACTIVITY IN PLASMA

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ABSTRACT

Glutamine is a key amino acid participating in various metabolic pathways. Study of the physiology of its carbon skeleton has been hampered by lack of a simple and precise method to measure its [^{14}C] specific activity in plasma. The present report describes an automated, sensitive and specific method to determine plasma [^{14}C] glutamine specific activity which can be used to measure glutamine carbon turnover and substrate-product interactions in vivo. An orto-phtalaldehyde derivative is analysed on a reverse phase column by UV detection. With this procedure μMol amounts of glutamine and other plasma compounds can be assayed for [^{14}C] specific activity. The method is sufficiently fast (25 samples in 24 hrs) and reproducible (CV$<$5.5%) for accurate measurements in a large volume of samples.

INTRODUCTION

Glutamine is the most abundant amino acid in humans and plays an important role in many physiologic processes (1). Constituting more than 60% of the free intracellular amino acid pool and about 25% of amino acids in plasma (2), it is the major interorgan carrier of ammonium ions (3) and a key nutrient for cells of the gastrointestinal tract (4) and immune system (5,6). Moreover, glutamine has been shown to participate in the regulation of protein metabolism (7) and gluconeogenesis (8).

Despite its unique characteristics and importance in various physiologic processes, relatively little is known of glutamine metabolism in humans. Major limitations have been the expense involved in determination of plasma [^{13}C] glutamine enrichments and the lack of a precise and convenient method to measure plasma [^{14}C] glutamine specific activity. Our knowledge is thus limited to its net balance across tissue beds (8) and turnover of its nitrogen (9-15). Glutamine fractional extraction, uptake and release across tissue beds, turnover of its carbon skeleton, and its incorporation into other metabolites (e.g. glucose and alanine) in vivo remains unknown.

Herein we describe a high performance liquid chromatography method to measure the specific activity of [^{14}C] glutamine (and potentially other amino acids) in plasma which should prove useful for further characterization of glutamine physiology in humans.

MATERIALS AND METHODS

Reagents

Amino acids standards, tetrahydrofurane, 2-mercaptoethanol and orto-ophtalaldehyd (OPA) were purchased from Sigma (St. Louis, MO). [U-^{14}C] glutamine was supplied by Amersham (Buckinghamhire, UK).

Apparatus

Chromatography analysis was performed with a Pharmacia High Performance Liquid Chromatography (HPLC) system using HPLC Manager System (Pharmacia, Uppsala, Sweden) as software for controlling the HPLC modules and P.E. Nelson 2600 Chromatography Data System (Perking Elmer Nelson, Cupertino, CA) as software for integrations and calculations. The hardware consisted of a 2157 Autoinjector with a 500 μl injection loop, two 2248 High Pressure Pumps, a 2155 Column Oven, a VWM 2141 UV Detector and a HeliFrac Fraction Collector. The software was installed on a Compaq computer Pro Linea 4/25s (Compaq, Houston, Tx). The samples were run on a reverse phase C18 column (IB-Sil, 5μ, 4.6 x 250 mm, Phenomenex, Torrance, CA) at 30°C. Eluting fractions were counted on a Wallac 1411 Liquid Scintillation Counter (Wallac, Turku, Finland).

Plasma preparation

Blood samples were collected in vials containing sodium fluoride and immediately placed on ice. Plasma was prepared by centrifuging within 30 min,

and an internal standard (25 nmole P-Fluoro-DL-Phenylalanine, F-Phe) was added to each 3 ml of plasma. This plasma volume was thereafter extracted with 3 ml of 10% perchloric acid. The sample was vortexed, centrifuged, and the supernatant was diluted according to the method of Smith and Panico (16) by adding 0.5 ml 4 mM Na-acetate buffer pH 4.8 and after that adjusted to pH 4.8 by adding 5N potassium hydroxide. The sample was stored at -25°C before analysis.

Chromatography

Before analysis, the sample was thawed and subsequently dried by vacuum evaporation (Speed Vac, Savant, Farmingdale, N.Y.). Subsequently, the sample was reconstituted in 750 μl distilled water, centrifuged and filtered (Millipore, 0.45 μ). Equal amounts of sample and derivatization mixture were loaded into the sample loop, reacted for 3 min, and thereafter 500 μl was injected on the column. The procedure was automated by a vial containing the derivatizing agent in a defined position on the autosampler.

The derivatization agent was prepared by dissolving 100 mg ortho-ophtalaldehyd in 2 ml methanol, adding 80 μl 2-mercaptoethanol and finally 0.2 M Na-borate to pH 9.5 (16). The mobile phase was created by a discontinuous gradient of buffer A (80% 0.05 M Na-acetate pH 5.9, 19% methanol, 1% tetrahydrofurane) and buffer B (80% methanol, 20% 0.05 Na-acetate pH 5.9) with a run time of 30 min at a flow rate of 1 ml/min. Each run started with 10% buffer B in the gradient, 30% at 2 min, 40% at 13 min, 60% at 15 min, and

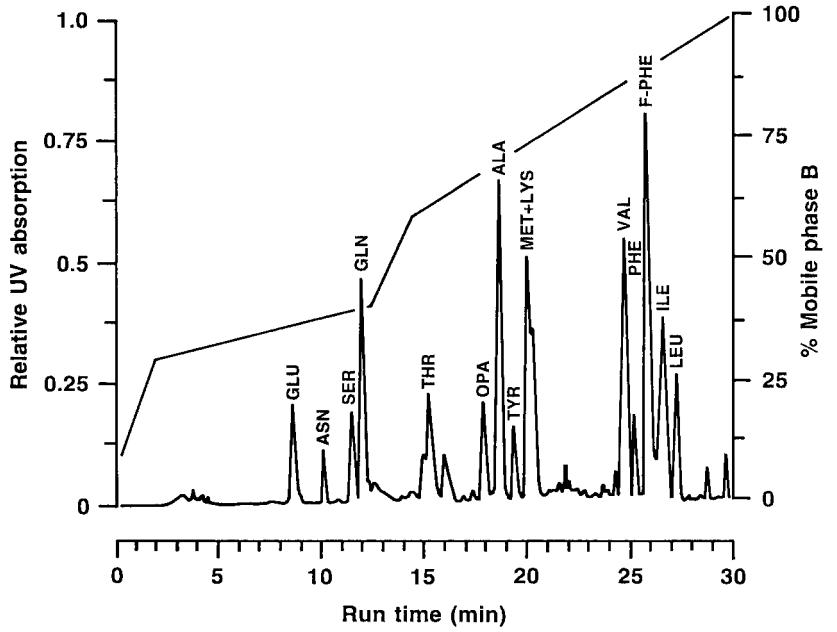

Figure 1. Chromatogram of human plasma processed for determination of ^{14}C specific activity in glutamate, glutamine and alanine (concentrated from 3000 μl to 750 μl, injection volume 250 μl). Positions of other identified amino acids and the derivatizing agent (OPA) are also indicated.

100% buffer B at 30 min (fig. 1). The peaks of the amino acid derivatives were detected at wavelength 360 nm. The system was then recalibrated with 10% buffer B for 10 min, total run time was therefore 40 min. Eluates corresponding to the peaks of glutamate, glutamine and alanine were collected in scintillation vials before counting.

Calculations

Standard curves of glutamate, glutamine and alanine were prepared in duplicate with serial dilutions of the amino acids in concentrations of 5, 2.5, 1.3,

0.6, 0.3 and 0.15 mM. Since glutamate had a lower plasma concentration and a higher absorbance, 5 mM was omitted and 0.08-0.02 mM included in the glutamate standard curve. In one set of standard samples 25 nmoles of F-Phe was added to each tube as an internal standard. These standards went through the extraction procedure as described for the plasma samples (internal standard curve). Another set of standards were analyzed directly on the HPLC system without the addition of F-Phe (external standard curve).

The internal standards were used to calculate plasma concentrations of glutamate, glutamine and alanine. This was expressed as the ratio between peak area of the amino acid in question and that of the F-Phe peak. This assumes that any degradation in our preparation affects each amino acid to the same extent. This proved to be the case as long as storage of samples was kept shorter than 3 months.

The external standards were used to calculate the exact amount of substrate occurring in each peak eluting off the column. Consequently, the specific activity of the amino acid in question could be calculated by dividing the scintillation counts (dpm, disintegrations per minute) in the peak by the amount of substrate expressed in μmoles in the peak eluate:

Specific activity (SA, dpm/μMol) =

Substrate counts (dpm)/Substrate amount (μMol)

Experiments

Four healthy volunteers (2 males, 2 females, aged 47 to 54 years, body weight 59-86 kg, body mass index 24.7 ± 1.9 kg/m^2) received a primed,

continuous intravenous infusion of [U-^{14}C] glutamine (25 μCi, 0.25 μCi/min) over five hours. An arterial line was placed in the radial artery of the non-dominant arm and kept open with a continuous infusion of saline (25ml/h). Four blood samples were drawn every 20 min over the last hour and processed as described above. Glutamine turnovers were calculated according to the steady state equation of Steele (17).

This protocol was approved by the local Ethical Committee.

RESULTS

A typical chromatogram of human plasma is depicted in figure 1. Amino acid and OPA peaks are identified by the eluting profile of the corresponding standards.

When either standards or unlabelled plasma were spiked with [U-^{14}C] glutamine prior to deproteinization and derivatization, [^{14}C] counts could be detected only in the glutamine peak, indicating no artifactual generation of glutamate from glutamine during sample preparation. Glutamine recovery corrected for volume loss during deproteinization ranged 89-99% (n=16 coefficient of variation 3.3%). Similar recoveries were found for glutamate (88-99%) and alanine (91-98%) with coefficient of variation of 3.9 and 4.5, respectively).

To assess the effect of storage on interassay variability, plasma from a subject was processed as described above, stored in aliquots at -25°C and run on 6 different occasions over 3 months. As shown in Table 1, there was no

TABLE 1

Stability of Glutamate, Glutamine and Alanine in Extracted Plasma Stored for 0-12 Weeks before Analysis.

Weeks		0	2	4	6	8	12	C.V.
Glutamate	(μMol/L)	38	39	32	34	36	38	5.3
	(dpm/μMol)	62	61	71	58	57	63	4.8
Glutamine	(μMol/L)	448	468	435	451	435	446	3.7
	(dpm/μMol)	723	712	708	718	721	724	2.3
Alanine	(μMol/L)	396	382	392	378	385	372	2.1
	(dpm/μMol)	68	62	66	64	68	63	4.3

C.V. = Coefficient of Variation.

significant loss of either glutamate, glutamine or alanine and the coefficients of variation for each amino acid were less than 5.5%.

Plasma concentrations, specific activities and rates of glutamine carbon turnover for 4 volunteers infused with [U-^{14}C] glutamine to steady state are given in Table 2. Infused radioactivity did not appear in amino acids other than glutamate, glutamine and alanine. Plasma glutamine carbon turnover averaged 334 umol/kg/hr using [U-^{14}C] glutamine comparable to rates reported using [2-^{15}N] glutamine as a tracer (9-15).

DISCUSSION

The precolumn derivatization with OPA is convenient for rapid analysis on a reverse phase column. Plasma glutamine has also been determined by ion exchange amino acid analyzers (18). This, however, is a time consuming

TABLE 2

Plasma Glutamine Concentration, Specific Activity and mean Turnover in Four Healthy Volunteers

	Concentration (μMol/L)				Specific Activity (dpm/μMol)				Turnover (μMol/kg/Hr)
Time (min)	0	20	40	60	0	20	40	60	
Subject 1	480	570	480	400	894	820	937	958	386
Subject 2	690	630	760	640	1595	1289	1342	1689	206
Subject 3	750	780	760	780	844	866	926	949	391
Subject 4	540	650	590	640	1069	1075	1285	1190	334
Mean	615	658	620	615	1101	1013	1123	1197	334
SEM	55	38	68	77	149	93	96	150	38

procedure that involves expensive equipment. The present method permits determination of plasma concentrations and specific activities of glutamine in 25 samples in 24 hours including time for daily sample preparation.

The use of OPA as the derivatizing agent for the measurement of glutamine has previously been described by Lindroth and Mopper (19). Smith and Panico reported an automated (OPA) method for determination of amino acids in the picomole range on a reverse phase column (16). However, application of picomole amounts is insufficient for detection of [^{14}C] counts in human plasma amino acids because of the limits on the amounts of radioactivity that can be administered to humans.

The present method permits determination of specific activity in micromole amounts of glutamine. Milliliter amounts of sodium fluoride plasma is extracted,

evaporated for plasma water and reconstituted to give a 4-fold concentration of sample. A discontinuous gradient is used to give the optimal separation of glutamate, glutamine and alanine. Our results indicate that precise and reproducible values can be obtained from plasma sample volumes as low as 2 ml when tracer infusion doses in humans are below 250 mrem. As evaluated by plasma samples spiked with [^{14}C] glutamine, less than 10% of the activity is lost during analysis with no spill-over to other compounds. Reproducibility is acceptable with an interassay variation of less than 5%.

Although glutamine turnover rates found in the present study using [U-^{14}C] glutamine as a tracer are comparable to those reported using [2-^{15}N] glutamine as a tracer (9-15), the advantage of using [^{14}C] glutamine as tracer is that one can measure the fractional extraction, uptake and release of glutamine carbon skeleton across tissue beds and conversion of glutamine carbon into other substrates.

In conclusion, the present study describes a rapid and precise measurement of plasma glutamine specific activity in human plasma which should prove useful for characterizing glutamine physiology.

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REFERENCES

1. N. Bulus, E. Cersosimo, F. Gishan and N.N. Abumrad, Metabolism, 38 (Suppl 1): 1-5 (1989)

2. J. Bergstrom, P. Fiirst, L.-O. Noree, E. Vinnars, J.Appl.Physiol., 36: 693-697 (1974)

3. T.C. Welbourne, Am.J.Physiol., 253: F1069-F1076 (1987)

4. E.M. Neptune, Am.J.Physiol., 209: 329-333 (1965)

5. M.S.M Ardawi, E.A. Newsholme, Biochem.J., 212: 835-842 (1983)

6. J. Crawford, H.J. Cohen, J.Cell.Physiol., 124: 275-282 (1985)

7. F. Hammarqvist, J. Wernerman, A.M. Rustom, A. von der Decken, E. Vinnars, Ann.Surg., 209: 455-461 (1989)

8. E. Bergman, R.N. Heitmann, Fed.Proc., 37: 1228-1232 (1978)

9. D. Darmaun, D. Matthews, D. Bier, Am.J.Physiol., 251: E117-E126 (1986)

10. D. Darmaun, M. Rongier, J. Koziet, J.-J. Robert, Am.J.Physiol., 261: E713-E718 (1991)

11. D. Darmaun, P. Dechelotte, Am.J.Physiol., 260: E326-E329 (1991)

12. D. Matthews, M. Marano, R. Campbell R, Am.J.Physiol., 264: E848-E854 (1993)

13. D. Darmaun, D.E. Matthews, D.M. Bier, Am.J.Physiol., 255: E366-E373 (1988)

14. D. Matthews, G. Pesola G, R. Campbell, Am.J.Physiol., 258: E948-E956 (1990)

15. D. Matthews, G. Campbell, Am.J.Clin.Nutr., 55: 963-970 (1992)

16. R.J. Smith, K.A. Panico, J.Liq.Chromat., 8: 1783-1795 (1985)

17. R. Steele, Ann.N.Y.Acad.Sci., 82: 420-430 (1959)

18. T.L. Perry, S. Hansen, J. Kennedy, J.Neurochem., 24: 587-589 (1975)

19. P. Lindroth, K. Mopper, Anal.Chem., 51: 1667-1674 (1979)

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A METHOD FOR QUICK DETERMINATION OF BILE ACIDS IN BILE OF PATIENTS WITH BILIARY LITHIASIS

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ABSTRACT

A single high-performance liquid chromatographic method is described for determining and measuring the major conjugated bile acids in human bile. Four different chromatographic conditions were applied using C-18 columns and an isocratic solvent system. An effective one-step purification with Sep-pak C-18 was adopted. The advantages of this method are an improved separation of the conjugated bile acids within a short period of time. The detection limit was 0.0125 µg/µl and linearity was up to 6

µg/µl. Recovery was always up to 96.6 %, this being sufficient for routine clinical application during bile acids evaluation in biliary lithiasis.

INTRODUCTION

Nowadays, biliary lithiasis presents an important health problem due to its high subject rate and economic repercussions.

Recent reports (1) have shown that bile acid secretion is altered in biliary lithiasis patients. Therefore it is necessary to develop an easy and rapid method to determine conjugated bile acids in the human bile and to evaluate its feasible alterations in hepatic and biliary diseases.

The classic methods that have been used to detect bile acids have many disadvantages: the gaseous-liquid chromatography requires a lot of time and the enzymatic kits only determine the total but not individual bile acids. The high-performance liquid chromatographic method (HPLC) is the suitable one for rapid and simultaneous bile acids determination (2,3,5,6). Previous to the bile acid separation by HPLC, purification of samples must be done in order to remove proteins, biliary pigments and other compounds that can interfere with it (8). Many methods have been used, the liquid-liquid extraction, Amberlite resins, etc., but the most recent method is that based on the use of discarded cartridges like octadecylsilane that show an improved recovery and a shorter analysis time.

The purpose of the present study is to evaluate a method to determine bile acids in human bile and to apply it to routine clinical evaluation in biliary lithiasis (2).

MATERIAL AND METHODS

Reagents: The sodium salts of taurocholic acid (TC), glycocholic acid (GC), taurochenodeoxycholic acid (TCDC), taurodeoxycholic (TDC), glycochenodeoxycholic acid (GCDC), glycodeoxicholic acid (GDC), taurolithocolic acid (TLC), glycolithocolic acid (GLC), and dexamethasone were supplied by SIGMA (St. Louis MO, USA). The water was purified with a Mili-Q water purification system purchased from MILLIPORE (Bedford MA, USA). Methanol and acetonitrile were HPLC-gradient from MERCK (Darmstard, FRG). All other chemicals were of analytical grade. The C-18 Sep-Pak cartridges were also purchased from MILLIPORE.

Apparatus: The HPLC system was made up of a L-6200 solvent delivery pump, a L-4250 detector and a D-2500 integrator by MERCK-HITACHI. The injector was a 7125 Reodyne with a 20 µl loop.

Operating conditions: The HPLC method was performed under isocratic conditions at room temperature. Analyses were performed on different reversed-phase C-18 columns: µBondapack C18 (10 µm particle size, 300 x 3.9 mm ID, WATERS); Spherisorb ODS 2 (5 µm, 150 x 3.9 mm, SUGELABOR); Lichrospher RP-18 (5 µm, 250 x 4 mm, MERCK); Lichrospher RP-18 (5 µm, 125 x 4 mm, MERCK).

The different mobile phases used were prepared daily with known quantities of phosphate or acetate buffer and with different percentages of methanol or acetonitrile. The pH was adjusted with phosphoric acid. All solvents were filtered through a 0.45 µm filter (type HA, MILLIPORE). Details of the chromatographic conditions applied in the HPLC systems are shown in Table 1. The flow in most of them was 1 ml/min. and the detection was performed at 205 nm and 0.04 aufs.

The chromatographic parameters: capacity factor (K') and selectivity (alpha) were determined as defined by Snyder and Kirkland (4). Peak areas were quantified by the integrator and it was calibrated by conjugated bile acids standards. To prepare a mixture of the eight conjugated bile acids, we dissolved each bile acid individually in methanol at a concentration of 1 mg/ml; 100 µl of each solution was then combined, evaporated under nitrogen stream and redissolved in 1 ml of the adequate mobile phase.

Sample preparation: Human bile was obtained by gallbladder punction during surgery in patients with symptomatic biliary lithiasis (n=10). This bile was immediately frozen. 500 µl of the human bile sample was centrifuged for 5 min. at 10.000 g without loss of bile acids in the precipitate. After that, an aliquot of 100 µl of the supernatant was diluted with 5 ml of 70 mM phosphate buffer at pH 7.0 and with 100 µg of dexamethasone added as an internal standard. The solution was mixed with a vortex mixer for 1 minute, after which the mixture was passed through a C-18 Sep-pak cartridge

Table 1-. Chromatographic systems.

SYSTEMS	COLUMN	MOBILE PHASE	%	FLOW
SYSTEM 1	COLUMN uBONDAPAK	acetonitrile/phospate buffer 10 mM pH 4.5	(30 / 70)	1
SYSTEM 2	COLUMN MERCK 125	methanol/acetate buffer 50 mM pH 4.3	(70 / 30)	0.8
SYSTEM 3	COLUMN MERCK 250	methanol/acetate buffer 50 mM pH 4.3	(70 / 30)	1
SYSTEM 4	COLUMN SPHERISORB	methanol/ acetate buffer 50 mM pH 4.3	(70 / 30)	1

conditioned previously with 5 ml of methanol, 10 ml of water and 5 ml of phosphate buffer 70 mM pH 7. The mixture was eluted successively with 10 ml of water, 3 ml of acetone 10%, 10 ml of water and 5 ml of methanol. The last fraction which contained the conjugated bile acids was evaporated to dryness under a nitrogen stream at 40ºC, and the residue was redissolved in 500 µl of the mobile phase and filtered through a 0.45 µm filter (MILLIPORE, type HV) before being injected into the HPLC (20 µl).

RESULTS

We applied different chromatographic columns in order to determine conjugated bile acids in human bile and obtained the best results with the Lichrospher RP-18 (5 µ m, 250 x 4 mm, MERCK) column. Chromatographic parameters are shown in Table 2 and chromatograms in Figure 2and in Figure 3. The elution order of the bile acids depends on the number of OH groups: trihydroxylated bile acids are eluted first, secondly dihydroxylated and finally monohydroxylated.

Table 2-. Chromatographic systems parameters. A=alpha; ND = non detected.

	TR	TR'	K	A
SYSTEM 1				
TC	8.56	6.12	2.50	
GC	13.16	10.72	4.39	1.75
TCDC	16.75	14.31	5.86	1.33
TDC	19.80	17.36	7.11	1.21
GCDC	35.84	33.40	13.68	1.92
GDC	41.71	39.27	16.09	1.17
TLC	N.D			
GLC	N.D			
SYSTEM 2				
	TR	**TR'**	**K**	**A**
TC	3.00	2.36	1.90	
GC	4.63	3.39	2.73	1.43
TCDC	5.90	4.66	3.75	1.37
TDC	6.80	5.56	4.48	1.19
GCDC	7.93	6.72	5.41	1.20
GDC	9.28	8.04	6.48	1.19
TLC	11.80	10.56	8.51	1.31
GLC	16.44	15.20	12.25	1.43
SYSTEM 3				
	TR	**TR'**	**K**	**A**
TC	5.71	3.78	1.95	
GC	6.98	5.05	2.61	1.33
TCDC	9.10	7.17	3.71	1.42
TDC	10.48	8.55	4.43	1.19
GCDC	11.66	9.73	5.04	1.13
GDC	13.34	11.41	5.91	1.17
TLC	17.58	15.65	8.10	1.37
GLC	23.36	21.43	11.10	1.37
SYSTEM 4				
	TR	**TR'**	**K**	**A**
TC	4.99	2.85	1.33	
GC	6.79	4.65	2.17	1.63
TCDC	7.62	5.48	2.56	1.17
TDC	8.58	6.44	3.00	1.17
GCDC	11.11	8.97	4.19	1.39
GDC	12.70	10.56	4.93	1.17
TLC	17.12	14.98	7.00	1.41
GLC	22.20	20.06	9.37	1.33

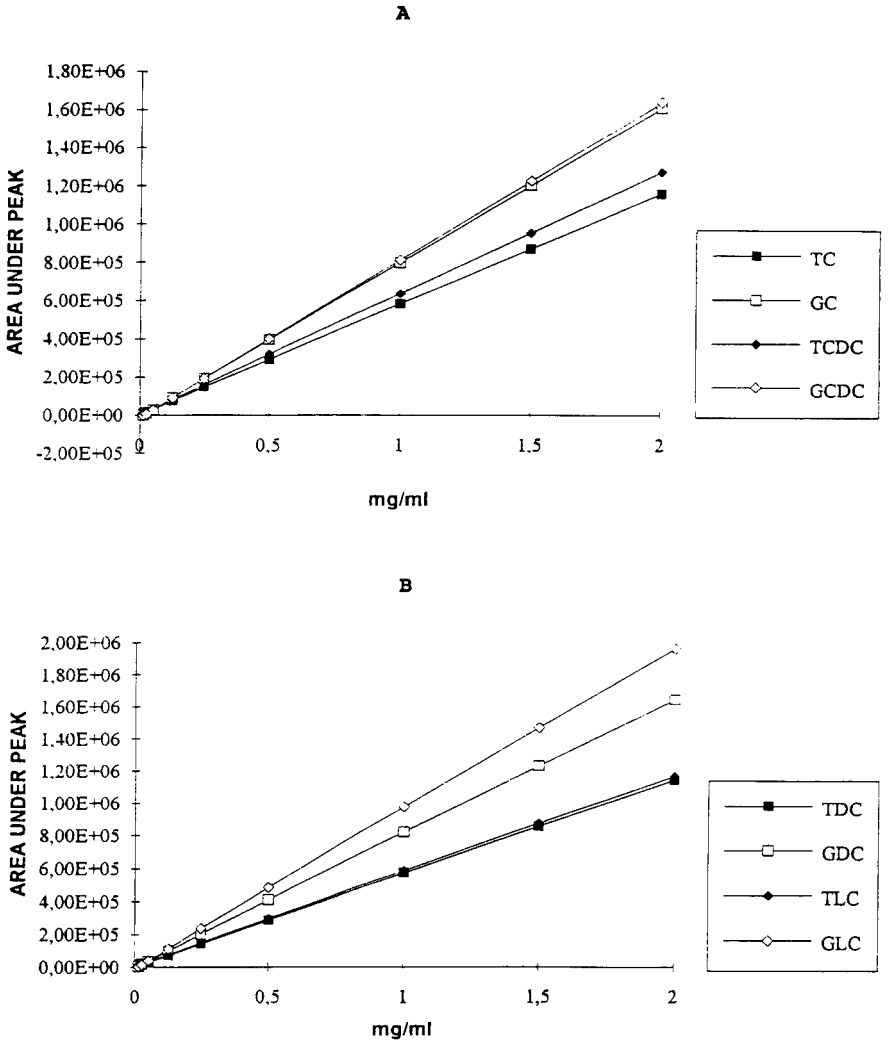

Fig 1-. Calibration curves. Correlation factors are: rTC: 0.996; rGC: 0.996; rTCDC: 0.998; rTDC: 0.997; rGCDC: 0.998; rGDC: 0.998; rTLC: 0.998; rGLC: 0.997.

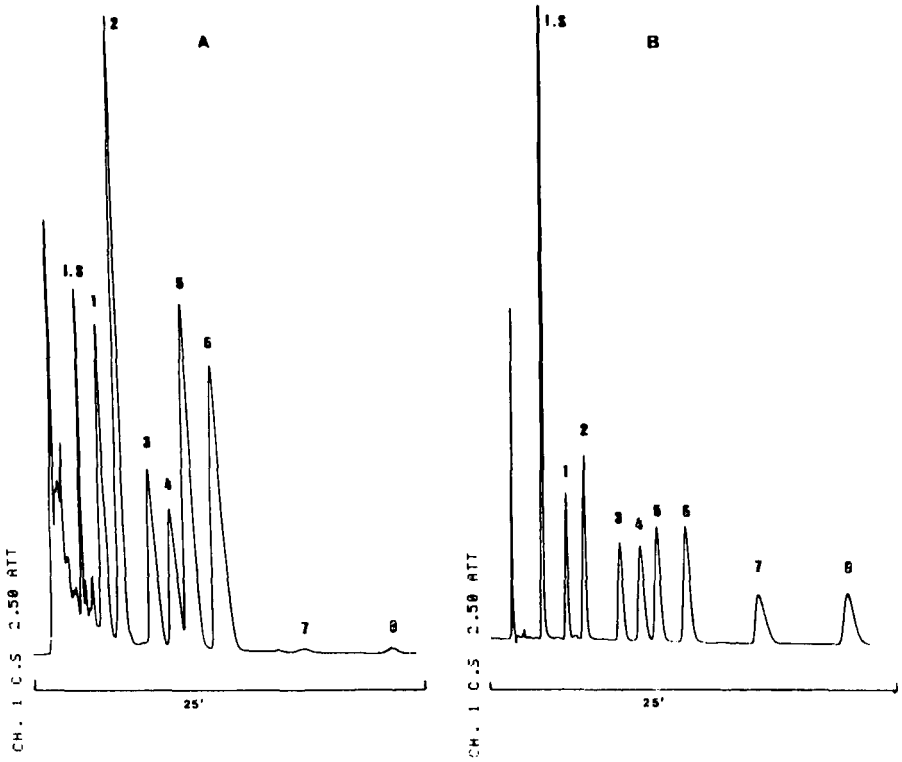

Fig 2- .A) Pathological Human bile chromatogram.

.B) Chromatogram of eight conjugated bile acids standards: 1. TC; 2. GC; 3. TCDC; 4. TDC; 5. GCDC; 6. GDC; 7. TLC; 8. GLC. I.S.= internal standard: dexamethasone.

Calibration: Known amounts of standards solutions of each bile acid were analysed in order to graph the calibration curves, which are shown in Figure 1. Correlation factors ranged from 0.996 to 0.999. The detection limits found were in the range of 2.5 ng/l, and the linearity was observed up to 6 µg/l.

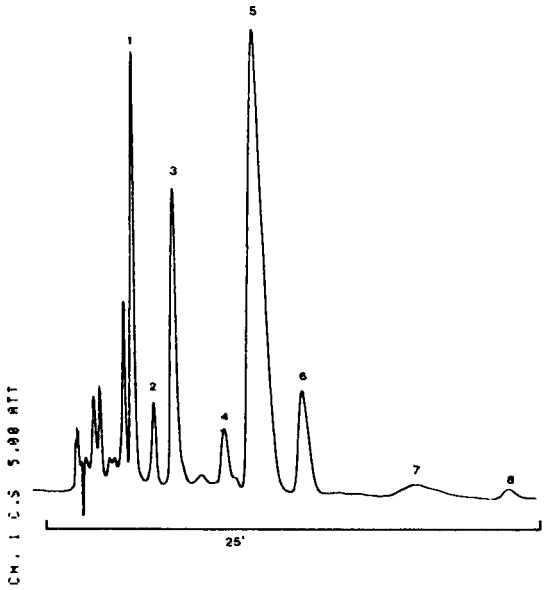

Fig 3-"Normal" Human Bile chromatogram.

Table 3-. Reproduction of human bile acids, C.V.= coefficient of variation

	TR	AREAS
n=5	*CV %*	*CV %*
TC	0.13	0.51
GC	0.10	0.79
TCDC	0.15	1.86
TDC	0.17	1.67
GCDC	0.16	0.95
GDC	0.20	1.99
TLC	0.25	5.94
GLC	0.26	1.71

Reproducibility: Reproducibility was tested by analysing 20 µl taken from the same stock bile sample on 5 different days. Results are shown in Table 3.

In order to determine the recovery, the bile samples were prepared by adding known amounts of each bile acid. The samples were subjected successively to purification by Sep-pak C 18 and determined by HPLC as described above. The recovery was always over 96.6 %, this being sufficient quantity for a routine clinical analysis. The results are shown in Table 4.

DISCUSSION

At present, high-performance liquid chromatographic methods are widely used for the assay of the conjugated bile acids by other authors (2, 5-14), but we have studied several chromatographic parameters in an effort to optimise the separation of the bile acids in human bile.

The reversed-phase is the appropriate system to separate non-polar and non-ionized substances; however, ionized substances like bile acids are separated on reversed-phase columns by different modifications: suppression ionic or the addition of a paired ionic chromatography (PIC) reagent (Waters). We do not consider the PIC to be adequate due to the low pH it needs. The acidification of the mobile phase entails a lot of undesirable problems: the functional groups of the column gel may be partially covered by these acid groups, reducing the separation efficiency and also means a shorter column time life. We used the ion suppression mode

Table 4-. Recovery (%) of bile acids from bile samples treated with Sep-pak, C.V.= coefficient of variation.

n=4	BILE mg/ml	ADDED mg/ml	EXPECTED mg/ml	FOUND mg/ml	RECOVERY %	CV %	R MEAN %	CV %
TC	0.8265	0.4	1.226	1.232	100.5	4.919	100.795	3.434
	0.9016	0.2	1.101	1.114	101.09	1.95		
GC	2.289	0.4	2.689	2.718	101.07	4.208	100.195	3.250
	2.437	0.2	2.637	2.619	99.32	2.293		
TCDC	0.9154	0.4	1.416	1.163	107.66	5.461	103.925	3.559
	1.020	0.2	1.222	1.222	100.9	1.658		
TDC	0.616	0.4	1.078	1.078	106.1	5.385	107.22	3.909
	0.646	0.2	0.9167	0.9167	108.34	2.433		
GCDC	1.892	0.4	2.336	2.336	101.9	3.906	101.075	3.296
	2.082	0.2	2.288	2.288	100.25	2.687		
GDC	1.450	0.4	1.933	1.933	103.78	3.54	102.45	3.076
	1.557	0.2	1.775	1.775	101.12	2.612		
TLC	0.041	0.4	0.5024	0.5024	113.78	4.331	116.14	5.065
	0.038	0.2	0.2827	0.2827	118.49	5.799		
GLC	0.072	0.4	0.4651	0.4651	98.42	4.512	97.62	4.570
	0.0795	0.2	0.2706	0.2706	96.82	4.629		

in which the pH of the mobile phase is adjusted according to the pK of the substances to be analysed. The conjugated bile acids show low pK values (pK taurine=2 and pK glycine=4.5) with the purpose of increasing the solubility at the intestinal pH. We adjusted the pH of the mobile phase at 4.3 because that pH confers improved separation and selectivity to the chromatographic system and an adequate control of the retention time.

The effect of the particle size was also studied. By using Lichrospher RP 18 (250 x 4 mm, MERCK), we could see that the 5 μm particle size fit better to the bile acid size than when another particle size (10 μm) is used, with a consequent loss of peak symmetry (system 1).

The effect of different buffers (phosphate and acetate) was also evaluated; although most of the authors think phosphate is better than acetate (6, 8, 11, 14), we have obtained an improved baseline and separation with acetate buffer (5).

Loss of resolution was observed with the use of acetonitrile instead of methanol. We think methanol is the most adequate solvent, although it has an inherently ultraviolet (UV) cut-off at 205 nm where conjugated bile acids are optimally detected; However acetonitrile as a solvent produces longer retention times for TLC and GLC, and also problems with the solubility of these previous bile acids (system 1).

The use of a shorter column (system 2) makes it possible to shorten the analysis time but with a loss of selectivity. We think the suitable column length is 250 mm (systems 3 and 4) and that system 1 shows an excessive length with excessive retention times.

The purification of the bile acids extract before HPLC analysis is achieved by Sep-pak C 18 cartridges (14). We consider that the precondition of the cartridge with the system methanol, water and phosphate buffer is a crucial step for successful purification. Also the use of phosphate buffer as a solvent of bile sample improves purification and removes interfering peaks, although the pH should never be over 7, because the solubility of cartridge silica gel increases dramatically over pH 7.

In conclusion, an isocratic HPLC method allows for the rapid and simultaneous determination of eight conjugated bile acids in pathological biles and makes it possible to evaluate the feasible involvement in biliary lithiasis. We think the method is not sensitive enough to determine bile acids in serum, because they are present in quantity hundreds of time less. The only disadvantage of this technique is that it does not determine unconjugated bile acids, although they are present in a very low percent in human bile.

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BIBLIOGRAPHY

1. M.C. Batesoon, Lancet, 2, 8518 (1986) 1265-7.

2. W. Swobodnik, U. Klüppelberg, J.G. Wechsler, M. Volz, G. Normadin and H. Ditschunerl, J. Chromatogr., 339 (1985) 263-271.

3. I. Iwata and E. Yamasaki, J. Biochem., 56 (1964) 424-431.

4. L.R. Snyder and J.J. Kirkland, Introduction to modern liquid chromatography. J.Wiley & Sons. Inc, New York 1979.

5. S. Scalia, J. Liquid Chromatogr., 10 (1987) 2055-80

6. P.S. Tietz, J.L. Thistle, L.J. Miller and N.F. LaRusso, J. Chromatogr., 336 (1984) 249-257.

7. F. Nakayama and M. Nakagaki, J. Chromatogr., 183 (1980) 287-293.

8. S. Scalia and M . Guarneri, Il Farmaco, 45 (1990) 797-805.

9. A. Hernanz and R. Codoceo, Clin. Chim. Acta, 145 (1985) 197-203.

10. K.D.R. Setchell and J. Worthington, Clin. Chim. Acta, 125 (1982) 135-140.

11. S.S. Rossi, J.L. Converse and A.F. Hofmann, J. Lipid Res., 28 (1987) 589-595.

12. C.N. Carducci, M.Matejka, H. Erquien, N. Labonia and A. Mamianettia, J. Chromatogr., 337 (1985) 91-97.

13. H.J. Wildgrube, U. Füssel, H. Lauer and H. Stockhausen, J. Chromatogr., 282 (1983) 603-608.

14. C.P. Pang, S.D. Mok, P.K. Lam, J. Varma and A.K.C. Li, Clin. Biochem., 23 (1990) 85-90.

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HPLC/EC ASSAY OF THE DISTRIBUTION OF THE ANTIMALARIAL ARTEETHER INTO FAT AND MUSCLE TISSUE FOLLOWING INTRAVENOUS ADMINISTRATION

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ABSTRACT

An HPLC/electro-chemical method with an internal standard was developed for the analysis of arteether in fat and muscle tissue giving good recovery and accuracy with moderate precision. Following an intravenous administration of arteether (11.6 mg/Kg in a micro-emulsion), it was found that after 15 minutes the arteether concentration was 18.4±13.1 μg/g (15.8% of administered dose) in fat and 1.35±0.25 μg/g (5.8% of dose) was found in muscle tissue. The concentration of arteether in fat tissue initially dropped very rapidly ($t_{1/2}$ = 14 min), followed by a much slower rate of elimination ($t_{1/2}$ = 34 hr). Using the same dose, vehicle, and route of administration, arteether had been reported to be cleared from plasma at a higher rate ($t_{1/2}$ = 10 min).

INTRODUCTION

Arteether is a new semi-synthetic antimalarial drug derived from the natural product artemisinin (also known as Qinghaosu) that has been shown to be particularly effective against chloroquine-resistant parasites.[1,2] Artemether (the ethoxy group of arteether is replaced by a methoxy group) is a closely related antimalarial that has recently been introduced into commercial use in China in a dosage form

consisting of a simple solution of artemether in a vegetable oil vehicle intended for intramuscular injection. The plasma pharmacokinetics of artemisinin and artemether have been reported[3,4] and it has been generally found that the disappearance of artemether from plasma following I.M. administration is very slow. Following the intramuscular administration of arteether (vegetable oil vehicle) to dogs, the plasma concentration initially was found to fall fairly rapidly ($t_{1/2\alpha}$ = 0.84 hr), then much more slowly ($t_{1/2\beta}$ = 27.9 hr) as the drug was slowly released from the depot injection.[5,6] In our laboratories, it was found that when arteether was intravenously administered (in a fat micro-emulsion vehicle) to rats, that the concentration of the unchanged drug dropped very rapidly ($t_{1/2}$ = 10.0 ±0.6 min) during the 1.5 hour observation period, and that within this time period the concentration of the several metabolites exceeded the concentration of the parent compound.[7]

Since the tissue distribution of arteether had not previously been reported, one of the primary objectives of the present study was to develop an analytical method that would be suitable for fat and muscle tissue analysis. A second objective of the project was to gain some insight as to whether the very rapid fall ($t_{1/2}$ = 10.0 ±0.6 min) of arteether following the intravenous administration was the primarily the result of the rapid metabolism or whether the rapid fall might have been the result of the partitioning of unchanged arteether (which has an extremely high log P value) into fat depots.

EXPERIMENTAL

Arteether was synthesized from artemisinin using a previously reported procedure.[8] The arteether was given by intravenous administration of an oil/water micro-emulsion that was prepared within 24 hr of the animal dosing. The procedure used for the preparation was similar to a general procedure used for the extemporaneous preparation of oil soluble cancer-chemotherapeutic agents that are given by intravenous administration.[9] Under aseptic conditions, a 100.0 mg/mL solution of arteether in ethanol was slowly added dropwise (10 µL/min) to a vigorously stirred commercial fat emulsion (Liposyn II®, 20%, Abbott Laboratories) to give a final arteether concentration of 6.0 mg/mL. Male Wistar rats were anesthetized with sodium pentobarbital (60 mg/kg), then each animal was administered arteether (11.6 mg/kg in the micro-emulsion) by intravenous bolus injection into the femoral vein.

After sacrifice of the rat at the appropriate time interval, approximately 200 mg fat sample (or muscle tissue) was transferred to a teflon/glass homogenizer along with 20 µl of the internal standard (a 250 µg/ml solution of the propyl analog of arteether). Then, 1.2 ml of ethyl acetate:acetone (1:1) was added to the sample, the sample was homogenized, and then the sample was centrifuged. The supernatant was transferred to a conical vial and evaporated to a small volume under a stream of nitrogen in a water bath (< 50°), then adjusted to a final volume of approximately 250 µl (occasionally 200-500 µl). The extract was then transferred to the deoxygenation chamber of the HPLC injector.

The sample was degassed with argon for 1 min, then the sample was transferred directly into the 20 µl capacity loop of the HPLC injector by means of pressurization with argon gas using a closed system to avoid the introduction of atmospheric oxygen into the HPLC loop injector. The HPLC column consisted of a 4.7 mm x 11 cm a C-18 cartridge type of column (Whatman Particil ODS-3, 5 µ particle size) using a mobile phase of 70% (v/v) methanol in 0.1 M aqueous ammonium acetate at 1.0 ml/min. The HPLC mobile phase was deoxygenated by flowing argon gas (mobile phases continually refluxed in a distillation-condenser at 40° with 30 ml/min argon flow). The detector (LC-3A, Bioanalytical Systems, Inc.) was used in the reductive mode, with the potential of the mercury/gold electrode set to -0.9 volts. The analytical method was calibrated using an internal standard peak ratio method using simple solutions of arteether and the internal standard. The method was then validated using fat and muscle that had been spiked with known quantities of arteether.

RESULTS AND DISCUSSION

During the preliminary studies on the development of an analytical method that would be suitable for the assay of arteether (which is extremely lipophilic) in fat samples, a number of approaches to selectively remove arteether from the endogenous lipids were investigated. One approach that was successful was to use a small normal-phase silica gel column to selectively remove the arteether from the fat sample prior to analysis using the reversed-phase HPLC analytical column. However, it was later found that the concentration of arteether in the fat samples of dosed rats was actually high enough that such an elaborate sample work-up was not needed but might prove to be useful in the future if very low concentrations were

encountered. All of the results reported here utilized the simple homogenization, one-step extraction procedure described in the experimental section.

To validate the assay method, known quantities of arteether were added to fat samples then the standard assay was used (each sample was prepared and extracted in triplicate). To verify the long-term reproducibility of the method, additional samples were prepared, extracted, and assayed (in triplicate) on subsequent days. The results of these validation experiments (Table 1) showed that the extraction gave essentially quantitative recovery (107.6% ±7.4%) for the 15 μg/g samples. The amount found to be present (15.9 ±1.3 μg/g) using the internal standard calibration method was within the experimental error of the amount of arteether that had been added (15.0 μg/g) to the fat sample. When the fat samples were spiked at 60 μg/g the recovery and accuracy was also to be satisfactory. However with regard to the precision of the method at either 15 or 60 μg/g, small standard deviations (±2.6% for 15 μg/g and ±10.7% for 60 μg/g) were found for results obtained on any single day, but somewhat larger standard deviations (±8.7% for 15 μg/g and ±24.1% for 60 μg/g) were obtained for the variation of the results over several days.

The assay method was also evaluated by spiking muscle tissue with varying quantities of arteether, and then applying the standard assay method. The results of these experiments (Table 2) showed that the amount found to be present in the samples was equal to the amount that had been added to the sample within the standard deviation of the assay.

Previous studies for arteether using this route of administration and vehicle had revealed plasma kinetics that indicated that arteether might be extensively taken-up by tissue compartments within the first 15 minutes.[7] For this reason, our initial efforts at examining tissue levels of arteether focused on the 15 minute sampling time (Table 3). The HPLC/EC chromatograms of the 15 min fat samples (Fig.1, panel B) of the animals dosed with arteether were found to contained large amounts of arteether and the HPLC/EC chromatograms of the fat of negative control animals (Fig. 1, panel C) were found to be free of any significant interference. The concentration of arteether in the 15 min fat sample was found to be 18.4 μg/g (Table 3) and only 1.35 μg/g of arteether was found in muscle tissue. If one assumed that fat[10] comprised 10% of the total body weight and that muscle[11] comprised 50% of total body weight, those tissue concentrations corresponded to 15.8% of the initial dose as unchanged arteether in fat tissue and 5.8% of the dose as unchanged

TABLE 1. Determination of the Recovery, Accuracy, and Precision of the Assay for Fat Samples Spiked with Arteether.

Day	Amount added	Amount found[a]	% of added[b]	% Recovery[c]
1	15 μg/g	17.4 ±0.9 μg/g	116.0 ±6.0	106.4 ±25.6
2	15	14.9 ±0.3	99.3 ±2.0	115.5 ±18.3
3	15	15.4 ±0.1	102.7 ±0.6	100.9 ±4.4
average		15.9 ±1.3[d]	106.0 ±8.7[d]	107.6 ±7.4[d]
4	60	42.7 ±4.3	71.2 ±7.2	79.2 ±8.6
5	60	61.4 ±7.6	102.3 ±12.7	77.4 ±13.9
6	60	70.1 ±6.7	116.8 ±11.1	115.0 ±27.1
average		58.1 ±14.0[d]	96.8 ±23.3[d]	90.5 ±21.2[d]

a Three fat samples were separately extracted and analyzed each day. Each sample was spiked with the internal standard and the assay was calibrated using a simple solution of arteether and the internal standard.
b The amount of arteether found as a percentage of the amount that had been added to each of the three samples.
c The absolute recovery of arteether based on the size of the arteether peak alone without reference to the internal standard.
d Standard deviation of the variation between days.

TABLE 2. Determination of the Accuracy and Precision of the Assay for Muscle Tissue Samples Spiked with Arteether.

Sample	Amount added	Amount found	Percent found
1	1.84 μg	1.63 μg	88.6%
2	1.87	1.68	89.8
3	9.81	9.45	96.3
4	10.48	12.17	116.1
			97.8 ±12.7

arteether in muscle tissue. Using the same dose, route of administration, and vehicle; it had been previously reported[7] that at 15 min the plasma concentration of arteether was only 3.03 μg/ml (approx. 2% of dose) of unchanged arteether along with significant concentrations of 12 metabolites. Thus it would appear that even as early as 15 min following intravenous administration, a very large portion of the dose had undergone metabolic transformation, but of the unchanged drug that remains in the body at that time, the vast majority of the unchanged drug resided in fat tissue.

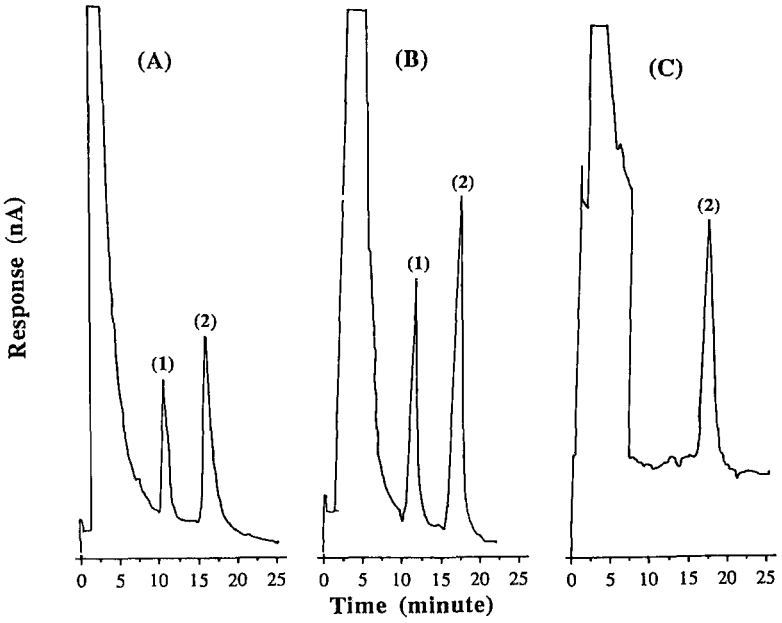

FIGURE 1: HPLC analysis of fat samples- A: fat sample spiked with arteether (1) and the internal standard (2), B: fat sample obtained 15 minutes after dosing found to contain 13 µg arteether per gram of fat, C: negative control fat sample containing only the internal standard.

TABLE 3. The distribution of Parent Compound into Fat and Muscle Tissue 15 Minutes after an Intravenous Injection of Arteether (11.6 mg/Kg).

		Arteether in tissue		Percent of Dose [a]	
Wt. of rat	dose	Fat	Muscle	Fat	Muscle
0.53 Kg	5.83 mg	6.8 µg/g	1.34 µg/g	5.8%	5.8%
0.49	5.39	32.7	1.60	28.2	6.9
0.41	4.51	15.9	1.12	13.7	4.8
0.48 Kg	5.24 mg	18.4±13.1	1.35±0.24 µg/g	15.8%	5.8 % avg.

a Conversions of µg/g in tissue to percent of dose values were based on the assumption that fat[10] comprised 10% of total body weight and muscle tissue[11] comprised 50% of total body weight.

TABLE 4. Kinetics of the Distribution of Arteether to Fat Tissue Following Intravenous Administration (11.6 mg/Kg).

0.25 hr	0.50 hr	1.0 hr	3.0 hr	5.0 hr	20.0 hr
13.63	4.76	2.56	2.50	1.98	
12.75	1.78	0.40	0.69	0.42	
1.70	2.82	3.06	1.83	1.83	1.03
2.46	5.48	3.41	3.99	4.00	2.14
avg. 7.63 µg/g	3.70 µg/g	2.36 µg/g	2.26 µg/g	2.06 µg/g	1.58 µg/g

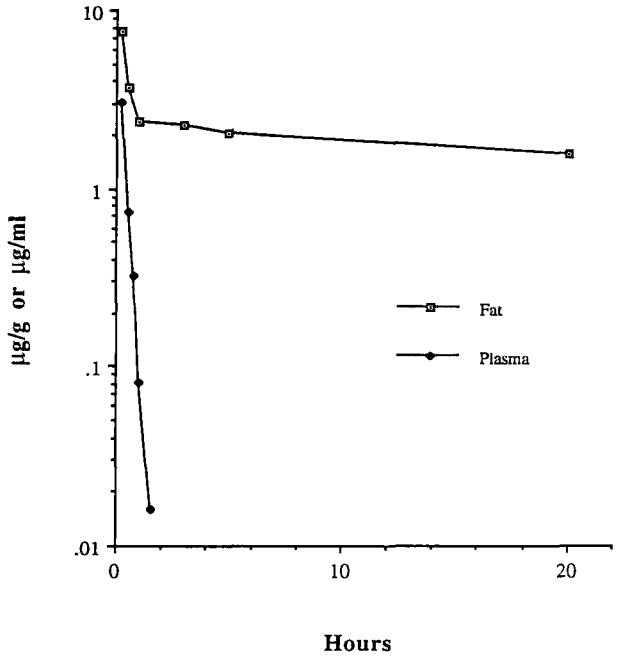

FIGURE 2: Concentration of unchanged arteether in plasma and fat tissue. The plasma analysis method and data had been previously published[7]

In an examination of the kinetics of the distribution of unchanged arteether in fat tissue (Table 4, Fig. 2), It was found that the concentration of arteether in fat dropped very quickly during the first half-hour ($t_{1/2}$ = 14 min, C_0 = 15.6 μg/g) followed by a much slower elimination over 20 hours ($t_{1/2}$ = 34 hr, C_0 = 2.4 μg/g). During the first 1.5 hr, the plasma concentration of unchanged arteether dropped very rapidly ($t_{1/2}$ = 10.0 ±0.6 min, Fig. 2). During the first 1.5 hr, it had been previously reported from these laboratories, that there were 12 different metabolites of arteether detected in plasma, several of which attained concentrations higher than arteether during that same time period.[7]

In summary, it would appear that when arteether was intravenously administered, that there was a very rapid uptake of the drug into fat (15.8% of dose at 15 min.), but that there was also a extremely rapid metabolic transformation during the first 60 minutes. By 60 minutes, the fat concentration had dropped to 2.36 μg/g (2.1% of dose) and to 1.58 μg/g (1.3% of dose) at 20 hr. In other words, a large portion of the unchanged arteether that remained at any one time period was found in fat, but because of rapid metabolic transformation, the concentration of arteether in both plasma and fat falls very rapidly during the first 60 minutes. After 60 minutes, only a very small fraction of the dose remained which was very slowly eliminated from fat over the 1 to 20 hr period.

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REFERENCES

1. X. D. Luo, C. C. Shen, *Medicinal Research Reviews*, **7**: 29-52 (1987).

2. D. L. Klayman, *Science*, **228**: 1049-1055 (1985).

3. Z. Zhou, Y. Huang, G. Xie, X. Sun, Y. Wang, L. Fu, H. Jian, X. Guo, G. Li, *Journal of Liquid Chromatography*, **11**: 1117-1137 (1988).

4. H. A. C. Titulaer, J. Zuidem, C. B. Lugt, *International Journal of Pharmaceutics*, **69**: 83-92 (1991).

5. A. Benakis, C. Schopher, M. Paris, C. T. Plessas, P. E. Karayannokos, I. Dondas, S. T. Plessas, G. Skalkeas, *European Journal of Drug Metabolism and Pharmacokinetics*, **16**: 325-328 (1991).

6. V. Melandez, J. O. Peggins, T. G. Brewer, A. D. Theoharides, *Journal of Pharmaceutical Sciences*, **80**: 132-138 (1991).

7. H. T. Chi, K. Ramu, J. K. Baker, C. D. Hufford, I. S. Lee, Y. L Zeng, J. D. McChesney, *Biological Mass Spectrometry*, **20**: 609-628 (1991).

8. A. Brossi, B. Venugopalan, L. D. Gerpe, H. J. C. Yeh, J. L. Flippen-Anderson, P. Buchs, X. D. Luo, W. Milhous, W. Peters, *Journal of Medicinal Chemistry*, **31**: 645-650 (1988).

9. C. L. Fortner, W. R. Grove, D. Bowie, M. D. Walker, *American Journal of Hospital Pharmacy*, **32**: 582-584 (1975).

10. L. L. Reed, F. Yamaguchi, W. E. Anderson, L. B. Mendel, *Journal of Biological Chemistry*, **86**: 147-174 (1930).

11. L. E. Gerlowski, R. K. Jain, *Journal of Pharmaceutical Sciences*, **72**: 1103-1127 (1983).

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ON-LINE TRACE ENRICHMENT OF MIFENTIDINE IN PLASMA USING COLUMN-SWITCHING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The purpose of this study was to develop for the determination of mifentidine, a new long-acting histamine H_2 receptor antagonist, in plasma using a column-switching HPLC with a ultraviolet (UV) detection. Changing the pH influenced the species equilibria of mifentidine and these were well monitored and quantitated. This method showed a excellent precision with good sensitivity and speed, and a detection limit of 10 ng/mL. The total analysis time per sample was less than 20 min and mean coefficients of variation for intra- and inter- assay were both less than 3 %. The method has been successfully applied to plasma samples from rats receiving oral administration of mifentidine.

INTRODUCTION

Mifentidine, a new (imidazolylphenyl) formamidine derivative and a novel class of H_2-receptor antagonists that exhibit strong gastric antisecretory activity in different animal models (1), has been recently investigated. Few high performance liquid chromatographic (HPLC) methods have been reported for the determination of mifentidine in biological samples (2,3). In analyzing pharmaceutical formulations and biological samples, typical sample preparations such as liquid-liquid, liquid-solid extractions, solvent evaporations, and sample reconstitutions with suitable solvents are quite time-consuming and often give rise to loss of sample from incomplete extraction. In order to avoid these problems and accommodate the large number of samples commonly encountered in many pharmaceutical laboratories, a column-switching technique (4 -10) which allows on-line trace enrichment using precolumn without an extraction procedure is the best candidate for the determination of general drugs. This study describes an automated HPLC with the direct injection of plasma for the determination of mifentidine in plasma using the column-switching technique.

MATERIALS AND METHODS

Reagents

Pure sample of mifentidine (imidazole phenyl formamidine) was provided by Il-yang Pharm. Co., Ltd. (Suwon, Korea). Acetonitrile and methanol were HPLC grade (E. Merck, Darmstardt, Germany) and the water was deionized with NANOpure II (Barnstead, Iowa, USA). All other reagents were of analytical grade including phosphoric acid and potassium phosphate.

Standard Solutions and Plasma Samples

A stock solution was prepared by dissolving mifentidine in methanol and diluted to the appropriate concentrations with a phosphate buffer (pH 2.5). Spiked plasma samples containing mifentidine in the range of 10 - 500 ng/mL were prepared by adding aliquots of mifentidine stock solution to plasma.

Animal Treatment

Male Fisher F344 rats (KRICT animal laboratory, Taejon, Korea) weighing 140 - 160 g were used. After overnight fasting, the left femoral artery was cannulated and 10 mg/kg of mifentidine was orally administered to the conscious rats. The blood was collected at intervals of 0.25,0.5,1,2,4,8,12,18 and 24 h, heparinized, and centrifuged for 10 min at 3,000 x g to obtain plasma.

Chromatographic System

The HPLC system consisted of Waters 501 pump (Milford, MA, USA), Spectra-Physics Model SP 8800 pump (Santaclala, CA, USA), a Rheodyne 7125 injector (Cotati, CA, USA), a ten-port multifunction valve (Valco, Houston, TX, USA) and Spectra-Physics 8450 uv/vis detector. Data handling was performed by a Spectra-Physics 4270 computing integrator. The instrument arrangement for ten-port column-switching system is shown in Figure 1.

The precolumn (20 x 3.9 mm i.d.) was tap-filled with LiChrosorb RP-8 (25 - 40 μm, Merck) and changed after injection of 100 samples. A guard column was GuarPak Nova-Pak C8 (Waters Assoc.) and the analytical column was Ultracarb 5 ODS 20 (250 x 4.6 mm i.d., Phenomenex, Torrance, CA, USA).

The washing solvent was 0.05 M phosphate buffer (pH 2.5) at a flow rate of 0.5 mL/min. The mobile phase was

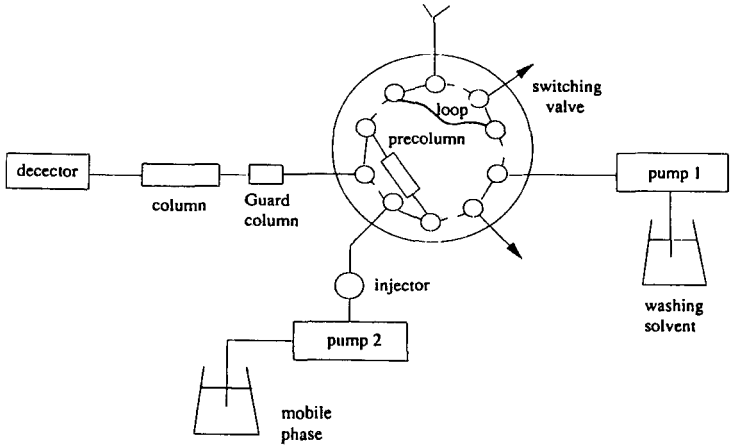


FIGURE 1: Schematic diagram of ten-port switching system (———— inject - - - - load).

methanol - 0.05 M phosphate buffer (pH 2.5) (1:9, v/v). The column temperature was ambient and the wavelength of detection was at 266 nm.

Analytical Procedure

A 100 μL of the spiked plasma or plasma samples was injected. The sequence of the sample analysis included the following three steps and required about 20 min.
Step I (0 - 5 min) : The plasma sample was injected onto the precolumn. Possible polar interfering plasma components were washed out to waste. Guard column and analytical column were equilibrated with the mobile phase.
Step II (6 - 15 min) : Washing solvent passed through to waste. The retained components were eluted from the precolumn to guard column/analytical column in back-flush mode by the mobile phase.

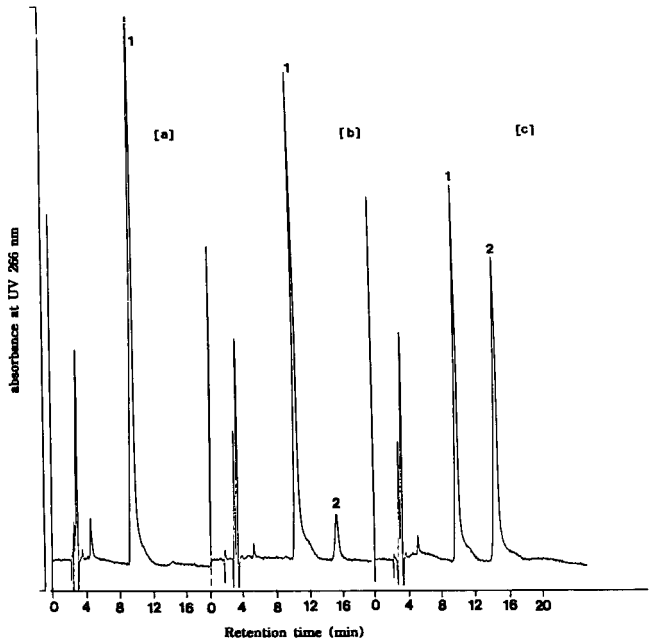


FIGURE 2: Chromatograms of 500 ng/mL mifentidine in the various pH buffers (a) pH 2.5, (b) pH 7.5 and (c) pH 9.0. Peaks: 1 = neutral form; 2 = protonated form.

Step III (16 - 20 min) : The eluted drugs were separated in the analytical column. Meanwhile precolumn was re-equilibrated with the washing solvent for the next injection.

RESULTS AND DISCUSSION

Chromatography

Chromatograms of mifentidine standard solution in the various pH values of phosphate buffer are shown in Figure 2. Figure 2 shows the effect of pH on the species

equilibria of mifentidine. According to Haaksma et al.(11) it was of interest to consider that protonation on the imino nitrogen of amidine group results in the resonance-stabilized amidinium cation, where the positive charge is delocalized over a plane of sp^2-hybridized atoms. In this study, these species of equilibria are shown. Chromatogram (a) shows a dominant neutral species of mifentidine at pH 2.5. Chromatogram (b) and (c) indicate protonated mifentidine at pH 7.5 which has pKa value of 8.82, due to the loss of proton from the amidine, and diprotonated mifentidine at pH 9.0 which has pKa value of 5.64, due to the loss of a proton from the protonated imidazole ring.

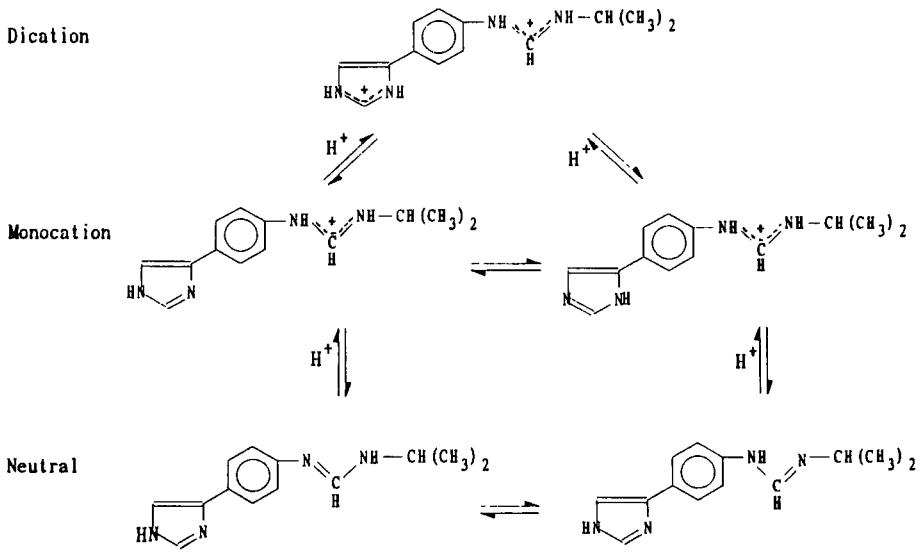
It indicated that mifentidine may exist above physiological pH 7.4 as a mixture of protonated and neutral species in their possible tautomeric forms (Scheme 1).

Therefore, to obtain good resolution of the neutral species of mifentidine in plasma, pH 2.5 phosphate buffer was chosen for mobile phase.

Chromatogram of blank plasma, spiked plasma, and plasma from a rat after oral administration of 10 mg/kg mifentidine are shown in Figure 3. As shown in Figure 3a, there was no interfering peaks at the retention times of mifentidine and its protonated form. Figure 3b and c show distinct, well-resolved peaks.

Column-Switching Procedure

In column-switching technique, it was necessary to choose the precolumn packing material, washing solvent and washing time in such a way that mifentidine and its protonated form would be completely adsorbed while the interfering endogenous components in plasma would be washed out from the precolumn to the waste port.



SCHEME 1: Ionic and tautomeric species of mifentidine.

LiChrosorb RP-8 (25 - 40 μm), a nonpolar octylsilane bonded phase adsorbent, was chosen for precolumn packing because of its strong adsorptivity for mifentidine and its protonated form at acidic pH, stability at pH 1 - 7 and easy availability.

To obtain high percent recovery of mifentidine as the neutral species and to clean the plasma components from the precolumn, 0.05 M phosphate buffer (pH 2.5) was chosen for washing solvent.

Washing the precolumn with the buffer at a flow rate of 0.5 mL/min for 5 min was good enough to get good peaks and clean chromatograms.

Recovery and Reproducibility

A series of five plasma samples spiked with mifentidine in the range of 10 - 500 ng/mL was assayed.

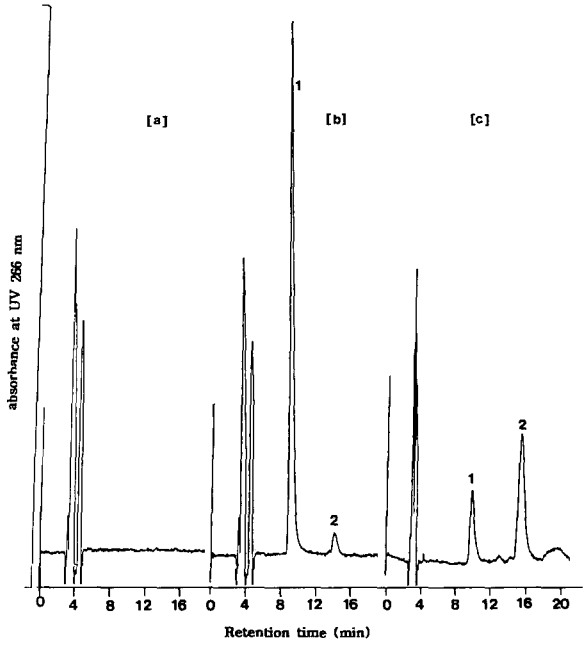


FIGURE 3: Chromatograms of (a) blank plasma, (b) blank plasma spiked with mifentidine (400 ng/mL) and (c) plasma of a rat 30 min after 10 mg/kg oral administration of mifentidine. Peaks: 1 = neutral form ; 2 = protonated form.

TABLE 1

Intra- and Inter-Assay Reproducibility of Mifentidine in Plasma Samples (n = 5)

Amount Added (ng/mL)	Amount Found (ng/mL)		Coefficient of Variation (%)	
	Intra	Inter	Intra	Inter
10	9.50	9.54	2.7	2.8
50	45.80	46.40	2.0	3.7
100	96.30	95.60	3.0	3.2
400	373.50	373.80	0.8	1.3
500	457.00	462.50	1.5	2.7

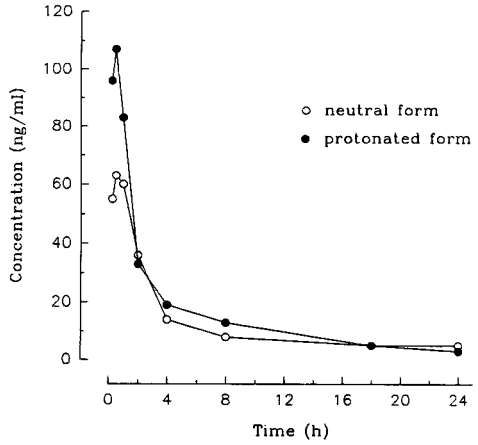


FIGURE 4: Plasma concentrations versus time curve of a rat after a 10 mg/kg oral administration of mifentidine.

Recovery of mifentidine in plasma was quantitative with a mean value of 93.8 ± 2.7 %.

Precision (defined as the coefficient of variation of replicate analysis) of the assay for mifentidine was evaluated over the concentration range studied (Table 1). The coefficient of variation for intra- and inter- assay were less than 3 %.

Application of the Method to Biological Samples

The present method was ideally suitable for mifentidine quantitation in plasma samples. Figure 4 shows the plasma concentration versus time plot of mifentidine and its protonated form after single oral administration of 10 mg/kg mifentidine to rats. Plasma concentrations of mifentidine and its protonated form are maximum 0.5 h and almost below the detection limit 18 h after oral administration.

In conclusion, the present work provided that column-switching technique was an ideal method for mifentidine quantitation from microvolumes of plasma samples without laborious sample manipulations because of its excellent precision, sensitivity and speed.

REFERENCES

1. A. Giachetti, F. Pagani, R. Micheletti, A. brambilla, E. Cereda, and A. Donetti, Agents and Actions, 16: 173-175 (1985)

2. B. P. Imbimbo, O. R. Urso, G. Thieme, B. Sturn, B. Ueckert, A. Vidi, H. Ladinsky, and S. Daniotti. Br. J. clin. Pharmac., 26: 407-413 (1988)

3. M. Jahanshahi, K. Pattichis, and J. W. Gorrod, J. Chromatogr., 575: 75-85 (1992)

4. W. Voelter, T. Kronbach, K. Zech, and R. Huber, J. Chromatogr., 239: 475-482 (1982)

5. R. Wyss and F. Bucheli, J. Chromatogr., 424: 303-314 (1988)

6. H. S. Lee, E. J. Kim, O. P. Zee, and Y. J. Lee, Arch. Pharm. (weinheim), 322: 801-806 (1989)

7. H. S. Lee, O. P. Zee, and K. I. Kwon, J. Chromatogr., 528: 425-433 (1990)

8. Y. J. Lee and H. S. Lee, Chromatographia, 30: 80-84 (1990)

9. H. S. Lee, O. P. Zee, B. H. Woo, and Y. J. Lee, J. Environ. Sci. Health, A26: 1253-1266 (1991)

10. H. S. Lee, H. C. Shin, S. S. Han, and J. K. Rho, J. Chromatogr., 574: 175-178 (1992)

11. E. E. J. Haaksma, B. Rademaker, K. Kramer, J. Ch. Eriks, A. Bast, and H. Timmerman, J. Med.Chem., 30: 208-211 (1987)

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SEPARATION AND DETERMINATION OF CARBADOX, NITROFURAZONE, NITROFURANTOIN, FURAZOLIDONE, AND FURALTADONE IN THEIR MIXTURES BY THIN LAYER AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The separation and quantitative determination of five drugs, namely carbadox, nitrofurazone, nitrofurantoin, furazolidone and furaltadone, in their various mixtures of 3, 4 or 5 components was investigated. Two types of mobile phases were examined for the TLC separation of the drugs, chloroform/acetonitrile/formic acid and chloroform/acetone. Two other mobile phases were also examined for the high performance liquid chromatographic determination of them, acetonitrile/sodium acetate and acetonitrile/sodium dihydrogen phosphate. The resolution of the chromatograms was studied in both cases and

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also the regression lines of the quantitative determination were described. The absolute detection limits of the determination were in the range of 0.2-1.6 ng for the five compounds.

INTRODUCTION

Among the drugs that are used extensively in veterinary medicine there are some groups of artificial substances with antimicrobial action either on gram positive or gram negative microorganisms. Nitrofurazone (2-[(5-nitro-2-furanyl) methylene]-hydrazinecarboxamide), nitrofurantoin (1-(5-nitro-2-furfurylidene-1-amino) hydantoin), furazolidone (3-(5-nitrofurfurylideneamino)oxazolidinone) and furaltadone (5-morpholinomethyl-3-(5-nitrofurfurylideneamino)-2-oxazolidinone) belong to the group of nitrofuranes and they are chemically characterized by the presence of 5- nitro groups in their molecules as a requisite for antimicrobial activity [1]. In figure 1 the chemical formulas of these substances are given, emphasizing the similarity of their chemical structure with the exception of carbadox. In the following text the substances will be referred by numbers as in fig. 1.

Carbadox ((2-quinoxalinylmethylene) hydrazine- carboxylic acid methyl ester N,N' -dioxide) is used to increase the rate of weight gain, improve feed efficiency and prevent swine dysentery and bacterial enteritis (treponema hyodysenteria) [2], but many times it is used during unnecessary prolonged periods. carbadox and its metabolites (desoxycarbadox) are suspected carcinogens and their use should be restricted to a bare minimum [3]. Within EEC the upper level of carbadox concentration permitted by the Feed Additives Committee is 0.0050 % (50 $g.ton^{-1}$) [4].

Furazolidone and nitrofurazone are fed to poultry at low levels (5-50 $g.ton^{-1}$) as growth promoters. At intermediate levels (50-200 $g.ton^{-1}$) they are used in both poultry and swine husbandry for the prevention of diseases such as fowl cholera, coccidiosis blackhead and swine enteritis. Nitrofurazone, furazolidone and furaltadone are also used in the control and treatment of mastitis in dairy cattle [5-8].

Several methods have been reported in the literature for the separation and determination of carbadox or nitrofurans in animal feed or biological tissue

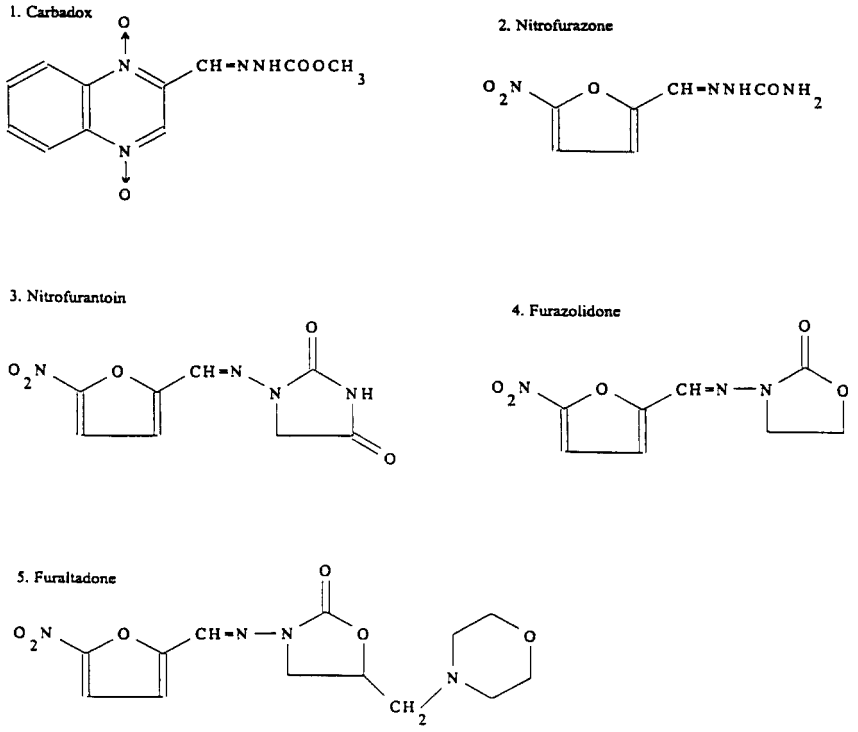

Fig. 1. Formulas of the compounds examined with HPTLC and HPLC.

matrices. They can be classified to spectroscopic methods (as the colorimeric methods for carbadox and furazolidone-nitrofurazone in feeds determination approved by AOAC) [9] and liquid chromatographic methods. The former have some drawbacks like time consuming procedures and poor specificity. In addition the later (LC methods) have better reproducibility and lead to higher recoveries.

Another approach to the analysis of nitrofurans is the application of gas chromatography [10]. Practically, a method of analysis must be capable of reliably determining some hundredths of ppm to be suitable for routine analysis of such compounds [2,11-13].

In this work a comparative study of two chromatographic techniques is described for the determination of the above set of drugs. Thin layer chromatography with two different mobile phases has been applied for the separation of the five compounds in their mixtures and high pressure liquid chromatography with two other mobile phases for the determination of these five compounds in their mixtures.

MATERIALS AND METHODS

Reagents and solutions

The stock solutions of 1, 2, 3, and 5 were prepared by dissolving these compounds (Sigma) in ethanol and the stock solution of 4 was prepared in chloroform (HPLC grade). The concentration of the compounds in all the above solutions was 500 $mg.l^{-1}$ and they were stored in the dark at -18°C. It must be noted that the solutions must be protected from UV and fluorescent light during storage and handling.

The standard working solutions were prepared in a concentration range between 0.1 to 5.0 $mg.l^{-1}$ by dilution of the stock solutions with chloroform. Various mixtures of the compounds were prepared in the same way, with three (1,2,4), (3,4,5), (1,4,5), four (1,2,4,5) or five compounds (1,2,3,4,5) for the TLC separation experiments.

Instrumental Conditions for TLC

The mobile phases examined were the folowing:
i) chloroform / acetonitrile / formic acid, 87/10/3 (v/v) and
ii) chloroform / acetone, 70/30 (v/v).
The plates were Merck No 5631 type, 10X10cm covered with silica gel.
The volume of the solutions was 1 µl or 2 µl and was injected at 2 cm from the lower edge of the plate.
The chromatograms were developed by spraying the spots with pyridine vapours and the spots were detected at 366 nm by means of a Gamag TLC Scanner II cabinet.

Instrumental Conditions for HPLC

The mobile phases examined were the folowing:

i) acetonitrile / sodium acetate 0.01 M, 20/80 (v/v), pH = 5 and

ii) acetonitrile / sodium dihydrogen phosphate 0.05 M, 20/80 (v/v), pH = 4.

A Jasco 880-PU High Pressure Liquid Chromatograph was used for all the determinations. The flow rate was 1.6 $ml.min^{-1}$ for mobile phase (i) and 0.75 $ml.min^{-1}$ for mobile phase (ii).

The chromatographic column was a Lichrospher RP-18, 5 μm particle size, stainless steel, with dimensions 250X4 mm, kept at 30°C. The detector was a Jasco 870-UV spectrophotometer operated at 365 nm.

RESULTS AND DISCUSSION

Thin Layer Chromatographic Separation

Two different experiments were carried out to check the TLC behavior of the five drugs. In the first experiment mixtures of standard solutions prepared freshly were examined (concentration of each compound 1 or 5 $mg.l^{-1}$) and in the second experiment simple standard solutions prepared one day before were examined to detect the appearance of dissociation products (concentration of each compound 5 $mg.l^{-1}$).

In the first experiment the chloroform / acetone (mobile phase ii) was proven more efficient than the chloroform / acetonitrile / formic acid (mobile phase i) for the separation of the drugs in their various mixtures. In figs. 2 and 3 the resulting plates are shown schematically. Ternal and quaternaly mixtures are well separated with both mobile phases but the quintiple mixture of the drugs was sufficiently separated only with mobile phase (ii). With the mobile phase (i) the spots of carbadox and nitrofurantoin were partially overlapped. It must be noted here that carbadox is a self fluorescent compound that can be seen directly by eye on the plate when present in a significant concentration (more than 1 $mg.l^{-1}$). No second spots were developed when the solutions were freshly prepared and rapidly injected on the plates. In the second experiment two spots from each substance were developed, the second being probably dissociation products. With the first mobile phase (fig. 4) the two spots of furaltadone were not sufficiently distinguished while with the second

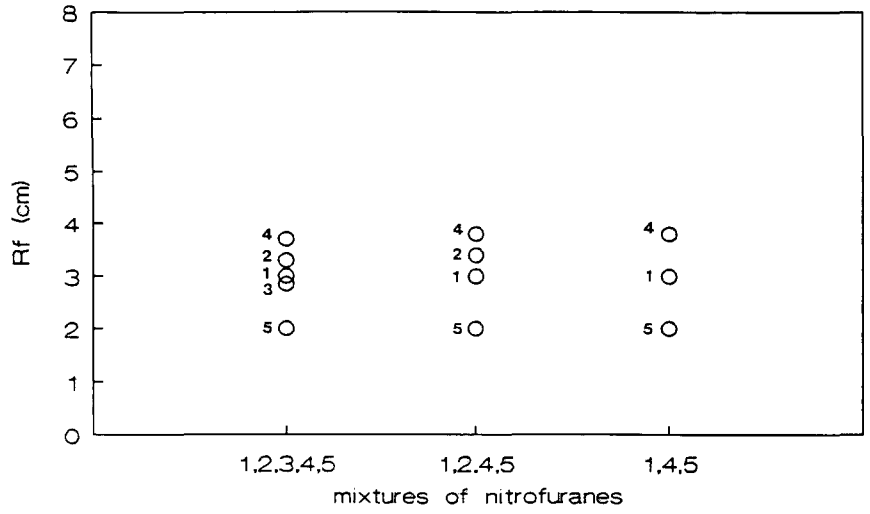

Fig. 2. TLC plates from various mixtures of the drugs. The mobile phase (i) consisted from chloroform, acetonitrile and formic acid. The numbers of drugs as in fig. 1.

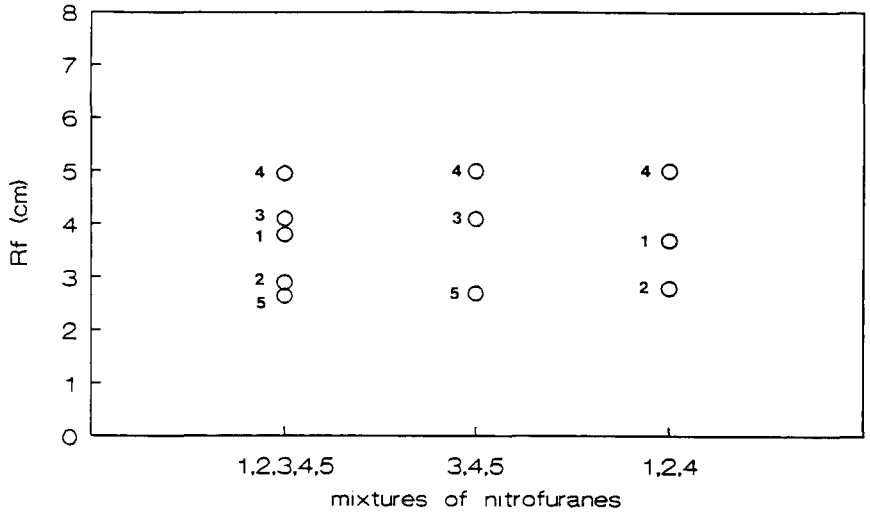

Fig. 3. TLC plates from various mixtures of the drugs. The mobile phase (ii) consisted from chloroform and acetone. The numbers of drugs as in fig. 1.

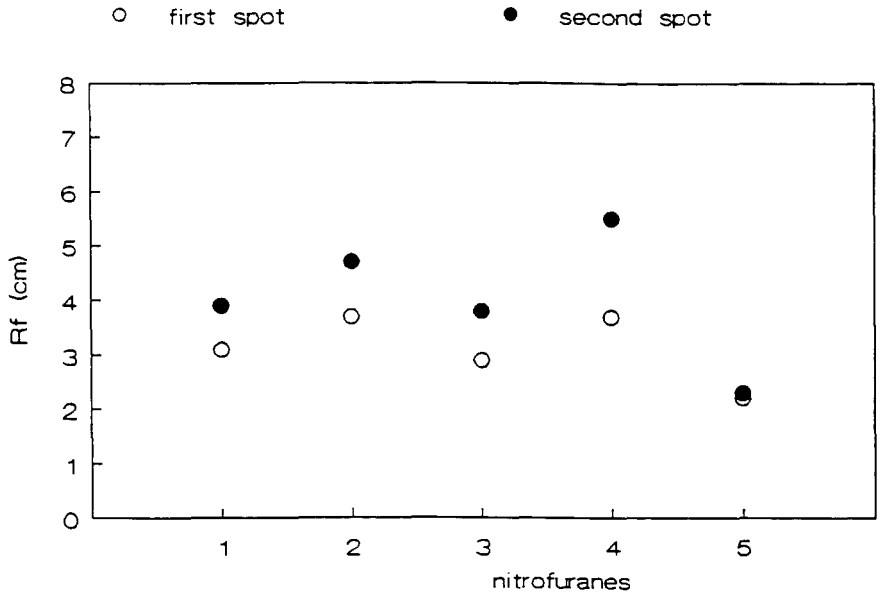

Fig. 4. TLC plates from standard solutions of the drugs. The mobile phase (i) consisted from chloroform, acetonitrile and formic acid. The numbers of drugs as in fig. 1.

mobile phase (fig. 5) carbadox showed no second spot and all the others were clearly separated.

Characteristics of the HPLC Chromatograms

In this mode two different mobile phases were examined also. In fig 6 two different chromatograms obtained from a mixture of the five substances (1 mg. l^{-1} each) and a mixture of four substances (drugs 1 and 2, 0.5 mg.l^{-1} each, furazolidone and furaltadone 1 mg.l^{-1} each, no nitrofurantoin) are given. The mobile phase used was acetonitrile / sodium dihydrogen phosphate (mobile phase ii) and it was proved that the peaks of nitrofurantoin and furazolidone were not resolved at all although when present one of them the resolution was good. On the other hand no secondary peaks from dissociation products were appeared during the time needed for the elution of the drugs.

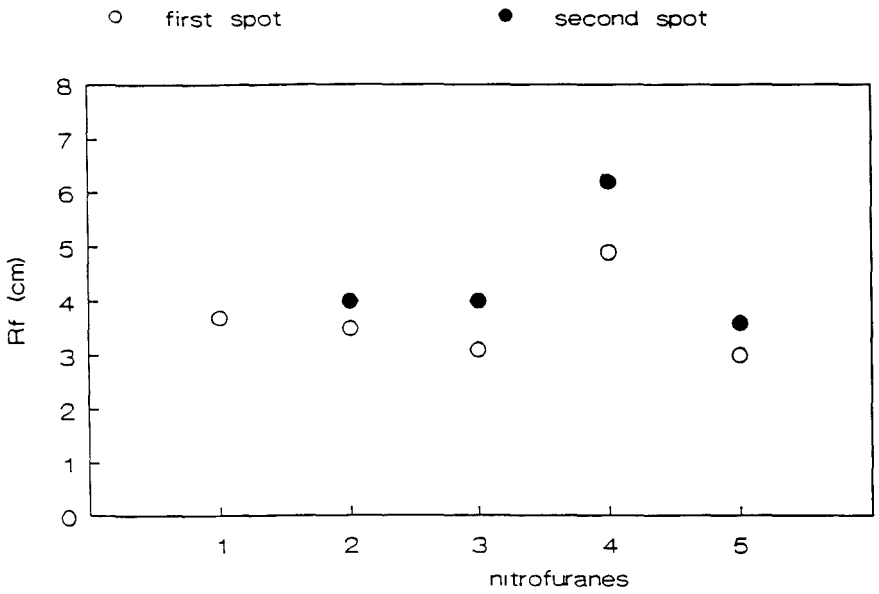

Fig. 5. TLC plates from standard solutions of the drugs. The mobile phase (ii) consisted from chloroform and acetone. The numbers of drugs as in fig. 1.

In fig. 7 two typical chromatograms of a mixture of the five substances (1 mg. l^{-1} each) developed with acetonitrile / sodium acetate (mobile phase i) are given. The two runs were taken from the same mixture with an hour delay between each other in order to examine the effect of light on the degradation of the drugs. From the second run it was obvious that the four last peaks (i.e. nitrofurazone, nitrofurantoin, furazolidone and furaltadone) had lost about 20 % of their heights whereas the carbadox peak seemed to be stable. In addition, two secondary peaks appeared just before the second and third peak, indicating probably the presence of at least two different products. This was explained because nitrofurans undergo photochemical reactions which result to the production of other chemical compounds. These products from nitrofurazone and nitrofurantoin in many cases develope a second peak during the elution while furazolidone remains with a single peak. However even furazolidone gives a dissociation product but its retention time is the same as the parent drug

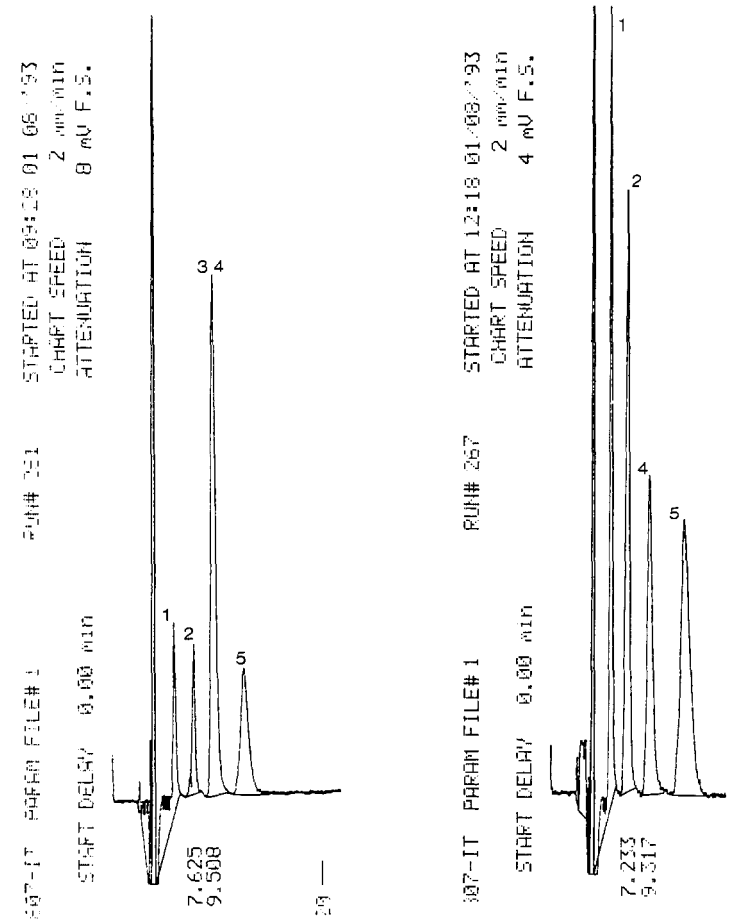

Fig. 6. HPLC chromatograms from quintiple mixture of the drugs. The mobile phase (i) consisted from acetonitrile and CH_3COONa. The numbers of drugs as in fig. 1. The second elution was done 1 hour later than the first.

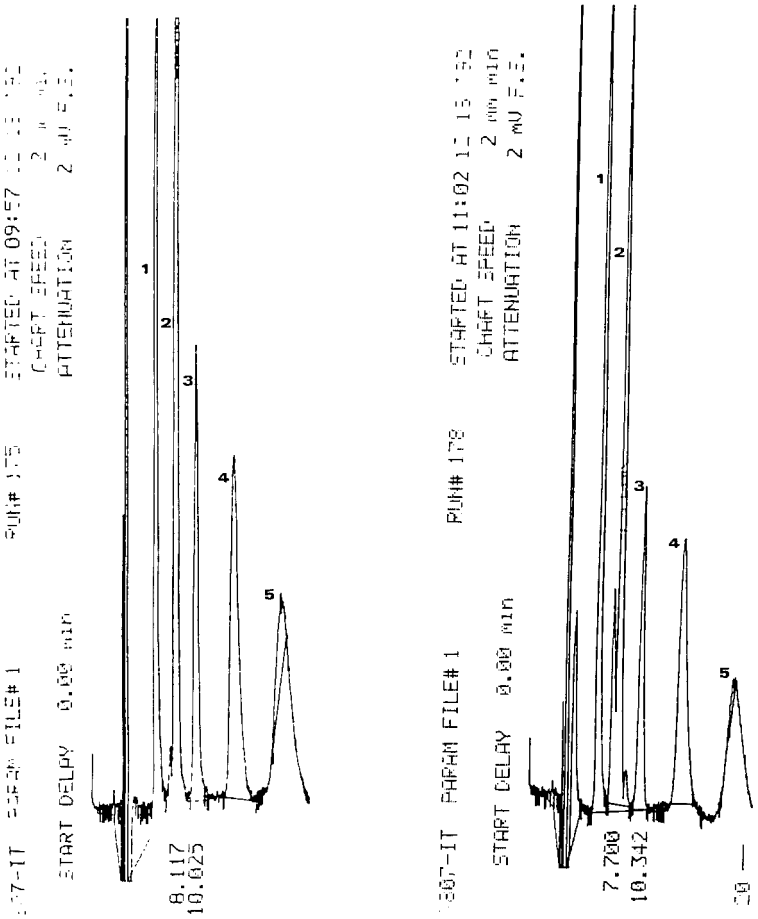

Fig. 7. HPLC chromatograms from quintiple and quaternaly mixtures of the drugs. The mobile phase (ii) consisted from acetonitrile and NaH_2PO_4. The numbers of drugs as in fig. 1. The first elution shows the ovelapped peaks of nitrofurantoin and furazolidone. The second elution was the quaternaly mixture without nitrofurantoin.

TABLE 1

Retention times and Resolution factors of the HPL Chromatographic separation of mixtures of five and four drugs with equal concentrations (1 mg. l^{-1}) for the two mobile phases respectively.

Substance	Retention time (min)		Resolution factor	
Mobile phase	(i)	(ii)	(i)	(ii)
1. carbadox	5.9	5.8	-	-
2. nitrofurazone	7.5	7.5	1.6	1.5
3. nitrofurantoin	9.8	9.4	2.0	1.4
4. furazolidone	13.0	9.4	2.1	0
5. furaltadone	17.2	13.3	1.6	1.4

with the mobile phase used. This behavior has been proposed [14] even as a distinguishing technique between nitrofurazone and furazolidone.

The resolution factors calculated for the two mobile phases (for 4.4 % of the peak height) are given in table 1. For mobile phase (i), it was proved that the resolution between the five substances of the mixture was very good and practically for all of them baseline separations were achieved. For mobile phase (ii) it was proved that no resolution can be achieved between nitrofurantoin and furazolidone while for the rest of the substances baseline resolution is achieved.

Regression Analysis of HPLC Determination of the Drugs

Various standard solutions of the drugs in the concentration range 0.1-5.0 mg. l^{-1} were injected in the chromatographic column to prepare the calibration curves of the separate determinations while passing the mobile phase (i). The regression data calculated are given in details in table 2, and the respective regression lines are plotted in the diagram of fig. 8.
The sensitivity of the determinations decreases in the order carbadox, nitrofurazone, nitrofurantoine, furazolidone and furaltadone, which is similar to the order of their elution. Also the linearity is better for carbadox and

TABLE 2

Regression data of the HPL chromatographic determination of the five drugs for mobile phase (i).

Substance	Regression equation	Correlation coefficient	Confid. limits of slope (95%)
1. carbadox	Y = 10.5 + 221X	0.998	210-233
2. nitrofurazone	Y = 14.4 + 138X	0.989	123-154
3. nitrofurantoin	Y = 14.6 + 66X	0.996	55-76
4. furazolidone	Y = 15.1 + 34X	0.977	27-40
5. furaltadone	Y = 10.4 + 21X	0.963	18-25

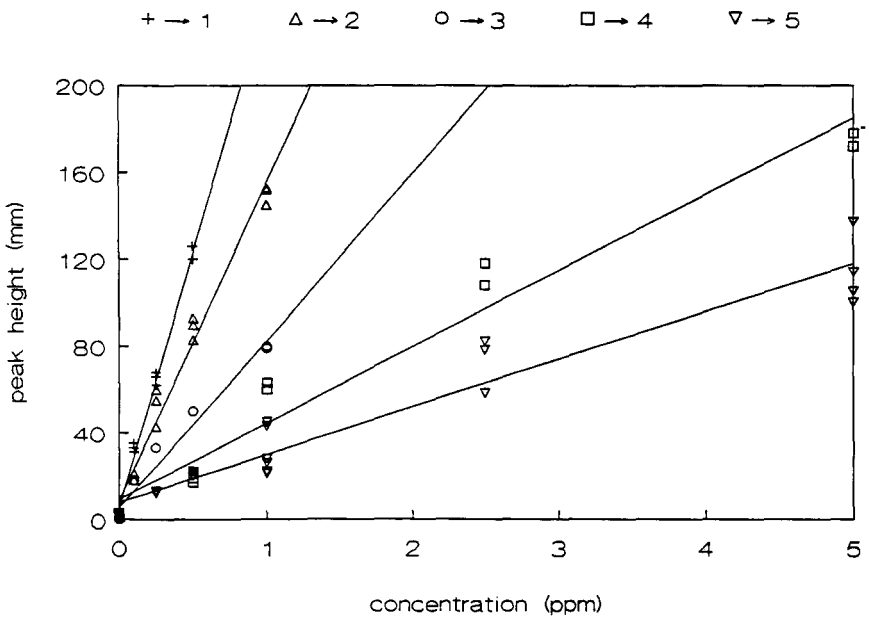

Fig. 8. Regression lines and experimental points of the HPLC determination.

nitrofurantoin than for the other drugs. The detection limits expressed by means of concentration in the injected solution were calculated as follows: carbadox 0.011 mg. l^{-1}, nitrofurazone 0.015 mg. l^{-1}, nitrofurantoine 0.022 mg. l^{-1}, furazolidone 0.060 mg. l^{-1} furaltadone 0.084 mg. l^{-1}. The corresponding absolute detectable quantities for 20 μl injection volume were as follows: carbadox 0.2 ng, nitrofurazone 0.3 mg, nitrofurantoine 0.4 ng, furazolidone 1.2 ng, furaltadone 1.6 ng.

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REFERENCES

1. Nitrofuran derivatives: In Veterinary Pharmacology and Therapeutics. Ed. N.H. Booth and L. McDonald, The Iowa State University Press / 1982, pp.767-769.

2. Roubal J.E., Munnsand R.K. and Shimoda W., J. Assoc. Off. Anal. Chem., 68, 653 (1985).

3. Jager L.P. and Vroomen L.H., Tijdschr - Diergeneeskd, 115, 727 (1990).

4. Code of Federal Regulations, Title 21, sec. 556100 (1984).

5. Ryan J.J., Lee Y.C., Dupont J.O. and Charbonneau C.F., J. Assoc. Off. Anal. Chem., 58, 1227 (1975).

6. Botsoglou N.A., J. Agric. Food Chem., 36, 1224 (1988).

7. Heotis J.P., Mertz J.L., Herrett R.J., Diaz J.R., Van Hart D.C. and Olivard J., J. Assoc. Off. Anal. Chem., 63, 720 (1980).

8. Kaniou I.P., Tsoukali E., Epivatianos P. and Zachariadis G., Journal of Environm. Science and Health, A28, 645 (1993).

9. Official Methods of Analysis of the Association of Official Analytical Chemists, 14th ed., AOAC Inc., USA (1984).

10. Thorpe V.A., J. Assoc. Off. Anal. Chem., 61, 88, (1978).

11. Cieri U.R., J. Assoc. Off. Anal. Chem., 62, 168 (1979).

12. De Graaf G.J. and Spierenburg T.J., J. Assoc. Off. Anal. Chem., 68, 658 (1985).

13. Schmid P., Mooser A.E. und Koch H., Mitt. Gebiete Lebensm. Hyg., 81, 461 (1990).

14. Parks O.W., J. Assoc. Off. Anal. Chem., 72, 567 (1989).

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HPLC DETERMINATION OF A VINCRISTINE, DOXORUBICIN, AND ONDANSETRON MIXTURE IN 0.9% SODIUM CHLORIDE INJECTION

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ABSTRACT

A high performance liquid chromatography procedure has been developed for the assay of a vincristine, doxorubicin, and ondansetron mixture in 0.9% sodium chloride injection. The separation and quantitation are achieved on a phenyl column at ambient temperature using a mobile phase of 50:50 v/v 0.02 M phosphate buffer, pH 5.4-acetonitrile at a flow rate of 1.0 mL/min with detection of all three analytes at 233 nm. The separation is achieved within 15 min with sensitivity in the ng/mL range for each analyte. The method showed linearity for vincristine, doxorubicin, and ondansetron in the 0.36 - 3.6, 10.0 - 100, and 11.95 - 119.7 μg/mL ranges, respectively. Accuracy and precision were in the 1 - 3% and 0.2 - 3.3% ranges, respectively, for all three compounds. The limits of detection for vincristine, doxorubicin, and ondansetron were 90, 200 and 200 ng/mL, respectively, based on a signal to noise ratio of 3 and a 20 μL injection.

INTRODUCTION

A mixture of vincristine, doxorubicin, and ondansetron is highly effective in the treatment of certain types of cancer. Interest in our laboratories in the stability and compatibility of the drug mixture over time in 0.9% sodium chloride injection required the development of an HPLC method. A search of the literature indicated that an HPLC method was not available to assay for all three compounds concurrently in a single injection.

Vincristine has been previously analyzed by radioimmunoassay (1), TLC (2) and HPLC (3). The radioimmunoassay method also measured vinblastine and the sensitivity was in the low ng/mL range for both compounds. The TLC separation was achieved on alumina using a dual development technique, first with ethyl acetate, and then a 3:1 mixture of ethyl acetate - ethanol. The HPLC separation is used as the official USP XXII assay for the drug substance. It involves chromatography of the drug on an octylsilane analytical column equipped with an octadecylsilane guard column. The mobile phase is 70:29.5:0.5 methanol-water-diethylamine (pH adjusted to 7.5) and the analyte was detected at 297 nm using a 2 mL/min flow rate.

Assay methods for doxorubicin have included spectrophotometry (4), electrochemistry (5,6), microbiological agar diffusion (4), HPLC (7-10) and TLC (11). The HPLC methods are the most common of the procedures reported and have involved the separation of the drug on

silica, cyanopropyl, octyl, or octadecylsilane columns. The official USP XXII assays for doxorubicin drug substance and injection utilize reverse-phase chromatography on an octadecylsilane column (10).

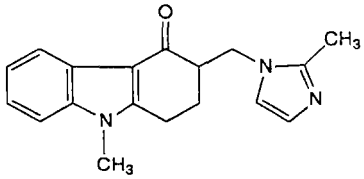
Ondansetron has been assayed by high performance thin-layer chromatography (HPTLC) and HPLC methods (12-14). The HPTLC method was developed especially for plasma samples, but the sample throughput was low and the equipment is not generally available in most laboratories. The HPLC assays used either a silica column with an aqueous-organic mobile phase or a cyanopropyl column operated in the reverse-phase mode.

In this paper, an isocratic HPLC assay is presented that will simultaneously analyze for vincristine, doxorubicin, and ondansetron in 0.9% sodium chloride injection using a single injection. The compounds are separated on a phenyl column using a buffered aqueous -acetonitrile eluent. The separation is achieved within 15 min at ambient temperature with sensitivity in the ng/mL range.

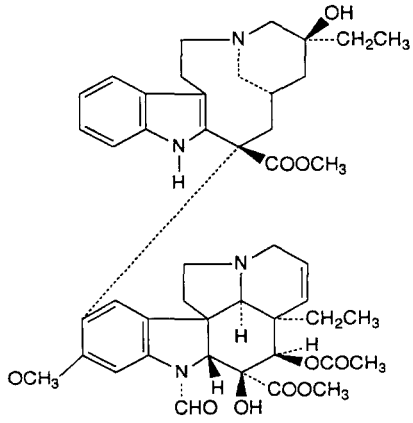
EXPERIMENTAL

Reagents and Chemicals

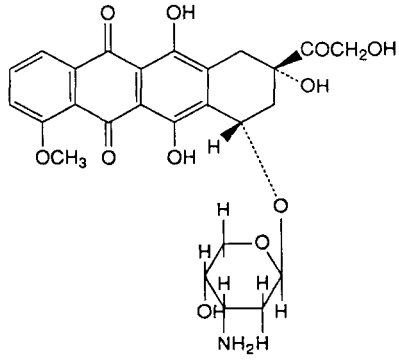
The structure formulae of the compounds studied are shown in Figure 1. Vincristine and doxorubicin were purchased from the United States Pharmacopeial Convention, Inc. (Rockville, MD 20852). Ondansetron (Batch C662/116/1) was a gift from Glaxo, Inc. (Research



ONDANSETRON



VINCRISTINE



DOXORUBICIN

Figure 1 - Chemical structures of compounds studied.

Triangle Park, NC 27709). Acetonitrile (J.T. Baker, Phillipsburg, NJ 08865) was HPLC grade and water was purified by a cartridge system (Continental Water Systems, Roswell, GA 30076). Monobasic sodium phosphate and sodium hydroxide were Baker analyzed reagents.

Instrumentation

The chromatographic separation was performed on an HPLC system consisting of a Waters Model 501 pump (Milford, MA 01757), an Alcott Model 728 auto-sampler (Norcross, GA 30093) equipped with a 20 μL loop, a Beckman Model 163 variable wavelength UV-VIS detector (Fullerton, CA 92634) and a Shimadzu Model CR-3A integrator(Columbia, MD 21046). Separation was accomplished on a 30 cm phenyl column (3.9 mm i.d., 10 μm particle size, Waters μ-Bondapak, Milford, MA 01757). The mobile phase consisted of 50:50 v/v 0.02M aqueous monobasic potassium phosphate, pH 5.4 (adjusted with 1 N sodium hydroxide)-acetonitrile. The mobile phase was filtered through a 0.45 μm Nylon-66 filter (MSI, Westborough, MA 01581) and degassed by sonication prior to use. The flow rate was set at 1 mL/min. The detector was set at 233 nm.

Preparation of Standard Solutions

A combined standard solution containing vincristine, doxorubicin, and ondansetron was prepared by accurately weighing 0.195 mg of vincristine sulfate, 5.45 mg of doxorubicin hydrochloride, and 7.50 mg of ondansetron hydrochloride, transferring to a 50-mL volumetric flask,

manually shaking for 10 min and 0.9% sodium chloride injection added to volume. This combined standard solution along with 4:10 and 1:10 dilutions made from the combined standard solution gave solutions containing 0.36, 1.40 and 3.60 μg/mL of vincristine, 10.0, 40.2, and 100.0 μg/mL of doxorubicin, and 11.95, 47.9 and 119.7 μg/mL of ondansetron expressed as the free base concentrations. Three point calibration curves were constructed for each analyte. Additional dilutions (2:10 and 8:10) of the combined standard solution were prepared in 0.9% sodium chloride injection to serve as spiked samples for each analyte to determine accuracy and precision of the method. Quantitation was based on linear regression analysis of analyte peak height versus analyte concentration in μg/mL.

RESULTS AND DISCUSSION

The goal of this study was to develop an isocratic HPLC assay for the analysis of a vincristine, doxorubicin and ondansetron mixture in 0.9% sodium chloride injection. The mixture is typical of a chemotherapy regimen that would be administered to a cancer patient. Stability studies of the mixture would require an assay procedure that would detect and quantitate each analyte with reasonable accuracy and precision.

There are no reports in the scientific literature describing a separation of these three analytes in a single mixture. Initial studies to

develop a single isocratic HPLC method for the three compounds involved the use of underivatized silica and cyanopropyl columns with various mobile phases containing acetonitrile-aqueous phosphate buffers. The vincristine peak eluted > 14 min and showed tailing on both columns with varied mobile phases. Doxorubicin and ondansetron eluted in that order and generally gave sharp peaks with some overlap on the silica column to less overlap of the peaks on the cyanopropyl column. Changes in mobile phase composition did not significantly improve the resolution of the three compounds on either column. Using an octylsilane column and a 60:40 aqueous pH 5.4 phosphate buffer-acetonitrile, the doxorubicin and ondansetron peaks were not well resolved, but the vincristine peak was well-separated from the other analytes with an 8 min retention time. Also, a deactivated octylsilane column was investigated and, while it generally provided excellent separation of the three compounds, a split peak was observed at higher concentration levels of ondansetron. Next, two commercial brands of octadecylsilane columns were studied. Even though the better separation was achieved on one of the brands using a mobile phase of 50:50 aqueous pH 6.5 phosphate buffer-acetonitrile at 1 mL/min. the peaks were still too close to one another and greater resolution was needed.

Our attention turned to the use of a phenyl column for the separation of the three analytes. Using various proportions of 0.02M pH

5.4 phosphate buffer - acetonitrile as mobile phases, the best separation and excellent resolution of the three analytes were obtained using a 50:50 v/v phosphate buffer-acetonitrile mobile phase with a total run time of 15 min. It was discovered later in these studies that the phenyl column also allowed the separation of methylparaben from the analytes, an important consideration since both ondansetron and vincristine commercial injections contained significant amounts of methylparaben as a preservative. Thus, the phenyl column with a mobile phase consisting of 50:50 v/v 0.02 M phosphate buffer pH 5.4 - acetonitrile was selected for the assay. A typical chromatogram showing the separation of the three analytes is shown in Figure 2.

In the acetonitrile-phosphate buffer mobile phase, the absorption maxima for vincristine, doxorubicin, and ondansetron were 254, 233, and 216 nm, respectively. It was decided to use 233 nm as the detection wavelength for the mixture since this was the wavelength that provided the best accuracy and precision data for all three components.

The HPLC method showed concentration versus absorbance linearity for vincristine, doxorubicin, and ondansetron in the 0.36 - 3.6, 10.0 - 100.0 and 11.95 - 119.7 μg/mL ranges, respectively at 233 nm. Table 1 gives other analytical figures of merit for each analyte. A photodiode array detector (Model 990, Waters Associates, Milford, MA 01757) was used to verify that none of the degradation products of any of the three analytes would interfere with the quantitation of each drug

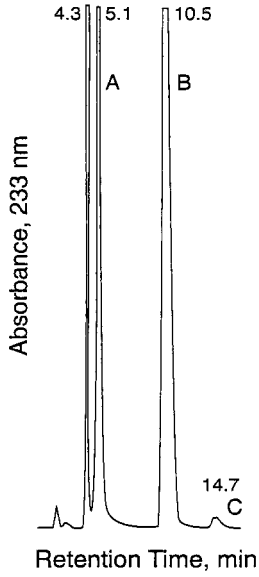


Figure 2- Typical HPLC chromatogram of doxorubicin (A), ondansetron (B) and vincristine (C) on phenyl column with acetonitrile - aqueous phosphate buffer pH 5.4 mobile phase. The peak at 4.3 min retention time is methylparaben, a component in ondansetron and vincristine injections. See Experimental Section for assay conditions.

at 233 nm. These experiments were performed on solutions of the three drugs in 0.9% sodium chloride injection after they has been degraded for 6 hr at 80°C in both 1.0N acid and 1.0N base.

Percent error and precision of the method were evaluated using spiked samples containing each analyte. The results shown in Table 2 indicate that the procedure gives acceptable accuracy and precision for all three analytes.

TABLE 1

Analytical Figures of Merit For Doxorubicin, Ondansetron, and Vincristine.

Analyte	r^{2a}	System Suitability[b]	LOD[c] ng/mL	k′	Theoretical Plates[d]	Tailing Factor[e]	Rs
Doxorubicin	0.9993	1.52	200.0	1.19	419	2.0	
							5.08
Ondansetron	0.9998	0.49	200.0	3.40	1591	1.5	
							2.94
Vincristine	0.9999	0.74	90.0	5.20	1127	1.5	

[a] Range examined from 0.36 - 3.60 μg/mL vincristine(n = 9) 10.0 - 100 μg/mL doxorubicin (n = 9), and 11.95 - 119.7 μg/mL ondansetron (n = 9). Mobile phase consisted of 50:50 v/v 0.02 M phosphate buffer, pH 5.4-acetonitrile at 1.0 mL/min with detection at 233 nm.

[b] RSD % of 6 replicate injections at 2.9 μg/mL vincristine, 80.0 μg/mL doxorubicin, and 95.7 μg/mL ondansetron at 233 nm.

[c] Limit of detection, S/N = 3.

[d] Calculated as $n = 16\ (t/w)^2$

[e] Calculated at 10% peak height.

TABLE 2

Accuracy and Precision Using Spiked Drug Samples

	Concn Added (μg/mL)	Concn Found[a] (μg/mL)	Percent Error	RSD (%)
Vincristine	0.72	0.70 ± 0.004	2.40	0.58
	2.90	2.87 ± 0.009	1.14	0.30
Doxorubicin	20.1	19.92 ± 0.38	0.90	1.91
	80.4	79.50 ± 1.47	1.12	1.85
Ondansetron	23.9	24.63 ± 0.82	3.05	3.33
	95.8	96.70 ± 0.15	0.94	0.16

[a] Based on n = 3.

Intra-day variabilities of the assay for vincristine, doxorubicin, and ondansetron expressed as % RSD were 1.58, 1.39 and 0.87% (n = 4), respectively. Inter-day variabilities of the assay for these drugs were 0.74, 1.52 and 0.49% (n = 30 over 6 days), respectively.

In summary, a phenyl column with an aqueous 0.02 M pH 5.4 buffer-acetonitrile mobile phase has been shown to be amenable for the separation and quantitation of a vincristine-doxorubicin-ondansetron mixture in 0.9% sodium chloride injection. This study suggests that the HPLC method can be used to investigate the chemical stability of all three drugs in sodium chloride injection.

ACKNOWLEDGMENTS

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REFERENCES

1. Teale, J.D., Clough, J.M. and Marks, V., Radioimmunoassy of Vinblastine and Vincristine Br. J. Clin. Pharmacol., 4, 169 (1977).

2. Cone, N.J. Miller, R. and Neuss, N., Alkaloids of Vinca rosea Linn. XV Analysis of Vinca Alkaloids by Thin-Layer Chromatography, J. Pharm. Sci., 52, 688 (1963).

3. The United States Pharmacopeia XXII - National Formulary XVII. The United States Pharmacopeial Convention, Rockville, MD (1990). pp 1449.

4. Federal Register 41, 14184 Section 450.24, April 2, 1976.

5. Rao, G. M., Lown, J.W. and Plambeck, J.A, Electrochemical Studies of Antitumor Antibiotics III Daunorubicin and Adriamycin, J. Electrochem. Soc., 125, 534 (1978).

6. Sternson, L. A. and Thomas, G., Differential Pulse Polarographic Analysis of Adriamycin in Plasma, Anal. Lettr., 10, 99 (1977).

7. Federal Register 43, 44836 Section 436.322 September 29, 1978.

8. Hulhoven, R. and Desager, J.P., Quantitative Determination of Low Levels of Daunomycin and Daunomycinol in Plasma By High performance Liquid Chromatography, J. Chromatogr., 125, 369 (1976).

9. Eksborg, S., Reversed-Phase Liquid Chromatography of Adriamycin and Daunorubicin and Their Hydroxyl Metabolites Adriamycinol and Daunorubicinol, J. Chromatogr., 149, 225, (1978).

10. The United States Pharmacopeia XXII - National Formulary XVII, The United States Pharmacopeial Convention, Rockville, MD (1990) p 478; First Supplement USP-NF, (1990) pp 2122-2123.

11. Arcamone, F., Cassinelli, G., Franceschi, G., Penco, S., Pol, C. Redaelli, S. and Selva, A., "International Symposium on Adriamycin", S.K. Carter, A. DiMarco, M. Ghime, I.H. Krakoff and G. Mathe, Eds., Springer-Verlag, Berlin (1972) pp 1-22.

12. Colthup, P.V., in Recent Advances in Thin-Layer Chromatography, Dallas,F.A., Read, H. Ruane, R.J. and Wilson, I.D., Eds., Plenum, New York (1988) pp 179-185.

13. Personal Communication, Fox, J., Glaxo, Inc., Research Triangle Park, NC 27709 (1991).

14. Colthup, P.V., Felgate, C.C.. Palmer, J.L and Scully, N.L. Determination of Ondansetron in Plasma and its Pharmacokinetics in the Young and Elderly, J. Pharm. Sci., 80, 868 (1991).

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QUANTITATIVE ANALYSIS OF POLYAMINES AT TRACE LEVELS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN HIGH SALT SOLUTIONS. APPLICATION TO SEAWATER

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ABSTRACT

This paper presents an original method to determine polyamines (putrescine, spermidine and spermine) by HPLC in high salt solutions. Sample preparation consists in many steps such as lyophilisation and dansylation before analysis. This method offers the advantages to be reproducible, linear and sensitive, as example we applied this technique to seawater and determined polyamines at trace level.

INTRODUCTION

Ubiquity and physiological importance of the polyamines in living systems (animal and plant) explain the numerous methods described in the last few years for their analysis and quantification.

High performance liquid chromatography with fluorimetric detection is mainly used with O-phthaldehyde (1) or dansylchloride (2, 3) as fluorescent agents.

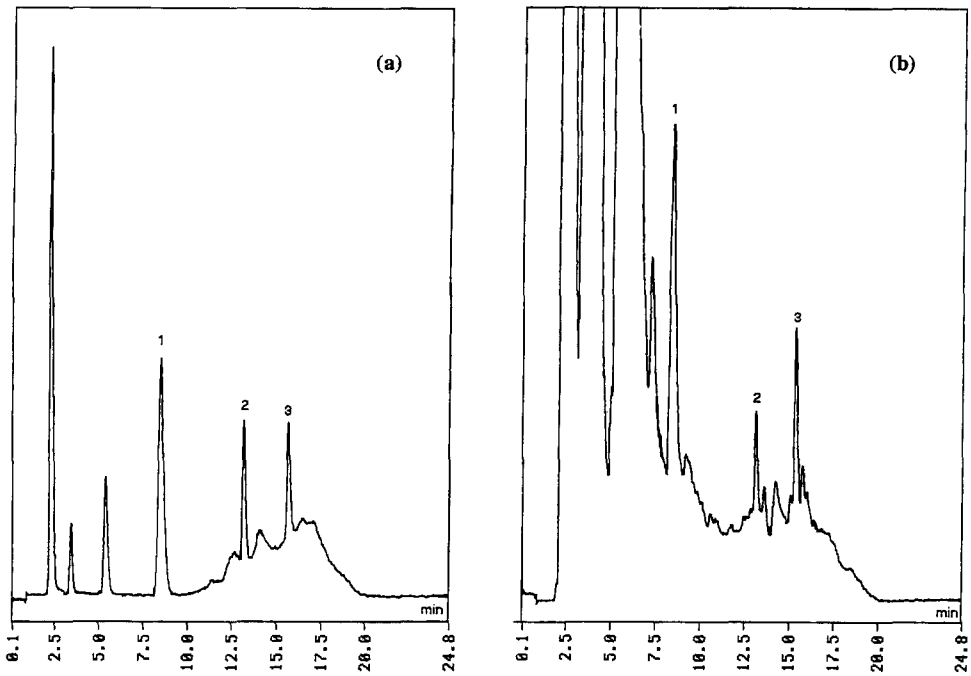

FIGURE 1 (a and b)

Chromatograms Of A Standard (Pu (1), Spd (2), Spm(3)) (Figure 1a) And One Of Seawater Sample (Figure 1b).
Chromatographic Conditions, See Material And Methods.

In this paper, we describe an original method to determine polyamines (Putrescine (Pu), Spermidine (Spd) and Spermine (Spm)) in high salt synthetic solutions (NaCl 35 o/oo). Many steps are necessary to prepare the extract before dansylation and separation on reversed phase column. In particular, the first and most important problem consists in removing salt without loss of polyamines.

In the present study we have applied this method to sea water and have detected free polyamines at trace levels.

REAGENTS AND SOLVENTS

Sodium hydrogenphosphate, sodium chloride, sodium carbonate, perchloric acid were of analytical reagent grade and benzene, acetone, acetonitrile, methanol were HPLC grade solvent (Merck, Darmstadt, Germany).

Dansyl chloride, putrescine, spermidine and spermine were purchased from Sigma (St Louis, MO, USA).

APPARATUS

The HPLC system consisted of two model 420 pump (Kontron) coupled with a high pressure mixer, an autosampler MSI660 (Kontron) with a 7110 Rheodyne injection valve fitted with a 20 ul loop.

Separation was achieved on Ultrasphere ODS column (250 x 4.6 mm ID, 5 um) protected by a Brownlee RP 18 guard column (30 x 4.6 mm ID 5 um). A gradient was realized in 25 min with two mobile phases A : (methanol-acetonitril 50 : 50 V/V) and B (NaH_2PO_4 0.01 M, pH 4.4) at a flow rate of 1.0 $ml.min^{-1}$: 5 min 20% B, 2 min 20% B to 11% B, 5 min 11% B to 0% B, 3 min 0% B to 20% B, 10 min 20% B. Solvents and mobile phases were filtered on 0.45 u (Millipore) before used.

Detection was accomplished using a spectrofluorimeter (SFM 25, Kontron) at an excitation wave length of 360 nm, and emission wave length of 510 nm. This HPLC system was controlled by a microcomputer Data system 450 (Kontron).

EXTRACTION PROCEDURE AND DANSYLATION

Synthetic mixtures with added amounts of Pu, Spd, Spm and seawater were treated in parallel.

5 ml NaCl 35 o/oo with Pu, Spd, Spm
or 5 ml seawater

Lyophilisation

Polyamine extraction + 3 ml methanol

Centrifugation 4000 tr/min 10 min
Evaporation of the surpernatant under vacuum

+ 200 ul $HClO_4$ 5%

Dansylation + 200 ul Na_2CO_3 saturated

overnight in dark + 200 u l dansyl chloride (5 mg/ml in acetone)

Extraction with 2 ml C_6H_6 (twice one ml)

Evaporation under vacuum

+ 200 ul methanol
Filtration (0.45 um) and injection into the HPLC system

TABLE I

Pu	Spd	Spm
894 ± 237.2	203 ± 65.4	1050 ± 169.7

Polyamine Contents in Sea water Expressed in pg/ml (X ± SE), N = 5.

RESULTS AND DISCUSSION

The first step in the extraction procedure of polyamines consists of removing as much salt as possible from the high salt solution before dansylation. Indeed, this derivatization procedure requires favorable conditions of pH, reaction time and ionic strength. Lyophilisation is a good technique to remove water and replace it with methanol without any loss of polyamines because sodium chloride is less soluble in this solvent . The linearity of the procedure was tested from 0.015 ng to 0.5 ng injected, for Pu, Spd, and Spm in NaCl 35 o/oo solution. The linear regression equation are the following : Pu : y = 59.49 x - 0.364 (r = 0.995), Spd : y = 84.95 x -0.076 (r = 0.999), Spm : y = 66.74 x - 0.138 (r = 0.999). Polyamines were identified with the method of constant additions. No internal standard was used in reference to our previous paper which discussed the choice of diaminohexane (DAH) as internal standard (4). Figure 1 shows chromatograms of a standard mixture (Pu, Spd, Spm) (Figure 1a) and one of seawater (Figure 1b).

In the seawater tested in our study, polyamines are at trace level (Table I).

Intra assay coefficients of variation for sea water are the following (N = 5) Pu : CV = 0.26, Spd : CV = 0.51, CV = 0.36 ; these results are good when analysis is performed at such a sensitivity. Indeed, the limits of the detection were 5 pg, 5 pg, 1.5 pg injected for Pu, Spd and Spm respectively, with a signal to noise ratio of 2.

This method is reproducible and sensitive ; it enables to determine polyamines at trace levels in seawater.

It will be interesting to study polyamine concentrations in sea water samples drawn from different environments. Applications are numerous if there exists a relationship between the level of free polyamines in sea water and living activity.

REFERENCES

1. J.L. Corbin, B.M. Marsh, G.A. Peters. Plant Physiol. 90 : 434-439 (1989).

2. H.E. Flores, A.W. Galston. Plant Physiol. 69 : 701-706 (1982).

3. S. C. Minocha, R. Minocha C. A. Robie. J. Chromatogr, 511 : 177-183 (1990).

4. C. Cann-Moisan, J. Caroff, E. Girin. J. Chromatogr. 574 : 134-136 (1992).

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DETERMINATION OF IVERMECTIN RESIDUES IN MEAT AND LIVER BY HPLC AND FLUOROMETRIC DETECTION

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ABSTRACT

The method describes the analysis of ivermectin in meat and liver at a quantification limit of 5 µg/kg. This level is lower than the maximum residue limit (MRL) imposed for EEC countries. The absolute detection limit was 250 pg. The recoveries ranged from 70 % to 88 %. Ivermectin was extracted by a mixture of acetonitrile and water, purified on Bond Elut C_{18} columns and converted to a fluorescent derivative using trifluoroacetic anhydride and N-methylimidazol. The analysis was performed on a liquid chromatograph fitted with a µ- Bondapak C_{18} column and ivermectin was detected by fluorescence spectroscopy. The method has already been used for routine analysis.

INTRODUCTION

Ivermectin is a broad-spectrum antiparasitic agent and extensively used for food-producing animals. The formula of ivermectin is given in figure 1.
Ivermectin is a mixture of two homologous compounds (H2B1a and H2B1b) differing from each other by one methylene group. H2B1a is the major compound

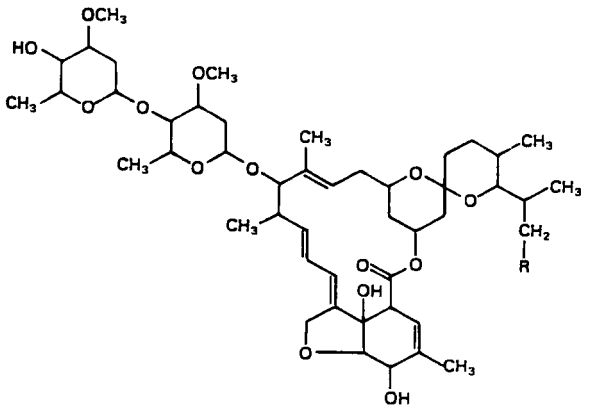

FIGURE 1. Structural Formula of Ivermectin
H2B1a: R = CH_3; H2B1b: R = H

and therefore marker substance for residue analysis. Tissue residue distribution and metabolism of tritium-labeled ivermectin have been studied in animal tissues (1) and examined thoroughly in swine (2) and have shown residues in numerous tissues and fluids, highest levels were found in bile, fatty tissues and liver.

In European countries the reglementation 675/92 EEC (3) allows a maximum residue limit (MRL) of 15 µg H2B1a/kg liver and of 20 µg H2B1a/kg fatty tissue. Recent analytical methods used high-performance liquid chromatography (HPLC) to analyze and to quantify ivermectin in feeds, serum, blood, plasma, milk and animal tissues, for the detection ultraviolet spectrophotometry (4-10) and fluorometry (11-14) were used.

Our aim was to develop a method allowing us to execute the surveillance program imposed to each member state of the European Community by the Council Directive 86/469/EEC (15). It was important to quantify ivermectin with accuracy in positive samples down to 15 µg/kg tissue and to have a method suitable for routine-analysis. Sample preparations were adapted following the method described by Th. Reuvers and al. (4) combined with the fast derivatization procedure of ivermectin to a fluorescent derivative from P. De Montigny and al. (14).

EXPERIMENTAL

Apparatus

A Vortex super mixer from Lab-Line Instruments (Illinois, USA) was used for the extraction procedure. Centrifugations were achieved with a centrifuge GLC-2B from Sorvall (Dupont, USA). The solvents were evaporated using a Rotavapor-R from Büchi (Switzerland) and a Reacti-Therm heating module from Pierce (Illinois, USA). Purification of the extracts on Bond Elut C_{18} columns were achieved using a Baker-10 extraction system (J.T. Baker, NJ., USA). HPLC analyses were performed with a 5000 liquid chromatograph from Varian (USA). Ivermectin was detected with an LS-4 fluorescence spectrometer from Perkin-Elmer (USA). The chromatograms were registered with a recorder A-41 from Ankersmit (The Netherlands) (paper speed: 2 mm/min).

Solvents and reagents

Acetonitrile and methanol were delivered by Labscan (Dublin, Ireland), N-methylimidazol GC 99% by Aldrich (Steinheim, Germany) and trifluoroacetic anhydride 99 % by Sigma (St. Louis, USA).

Standard and standard solutions

Ivermectin standard was supplied by Merck, Sharp and Dohme B.V. (The Netherlands) (purity: 90.07%). The working standard solution contained 1.8 µg ivermectin/ ml methanol and was used for the standard curves and to spike the samples.

Columns

For the purification procedure Analytichem Bond Elut C_{18} columns (6 ml) from Varian (CA, USA) were used. The HPLC column was a µ- Bondapak C_{18}, 10 µm column (3.9 mm X 300 mm) from Millipore-Waters (USA).

Sample preparation

Extraction procedure of ivermectin from meat and liver

4 g minced meat or liver were mixed with 40 ml acetonitrile and 3.5 ml water in a closed 100 ml glass centrifuge tube. The mixture was shaken on a Vortex during 2 minutes and then centrifuged at 2000 rpm during 10 minutes. The solvent was transferred into a 100 ml brown round bottom flask. The extraction procedure was repeated once more with 20 ml acetonitrile and 3.5 ml water. Both extracts were joined together and evaporated until 6 ml using the Rotavapor-R. No acetonitrile may be left in the flask. Finally 6 ml water were added to the extract.

Purification procedure of ivermectin from meat and liver

The Analytichem Bond Elut C_{18} columns were conditioned successively with 4 ml acetonitrile and 4 ml acetonitrile/water (1:9). The extracts were passed through the columns and the columns were dried over a period of 10 minutes by air aspiration. Ivermectin was then eluted with 5 ml acetonitrile which were evaporated afterwards under a stream of nitrogen.

Derivatization of ivermectin

To the dried residue were added successively:

1. 150 µl trifluoroacetic anhydride / acetonitrile (1:2; v/v)
2. 100 µl N- methylimidazol / acetonitrile (1:1; v/v)

The tubes were shaken, closed and stored in the dark, since the fluorescent derivative of ivermectin is very sensitive to light.

Chromatography

A 10 µl or 50 µl portion of the extract was injected into the liquid chromatograph at room temperature using a C_{18} µ-Bondapak reversed-phase

column and a flow rate of 1.5 ml/min. The eluent was methanol/water (95:5; v/v). Ivermectin was detected with a fluorescence detector (excitation wavelength: 364 nm; emission wavelength: 470 nm; slits: 15 and 20; fixed scale: 2).

RESULTS AND DISCUSSION

Meat and liver samples were collected in slaughterhouses. Meat from cattle and pigs was taken randomly, not only fatty tissues, since all kinds of meat will be eaten by the consumer.

The HPLC analyses of the ivermectin standard resulted in a linear response within the range of 2 to 30 ng. The correlation coefficient was 0.9980. Table 1 summarizes the recoveries, standard deviations and coefficients of variation of ivermectin in meat and liver. Each result is the mean of five different extractions. The recovery studies were equally good for both matrixes, 70% to 88% could be reached. The quantification limit was 5 µg ivermectin/kg meat or liver.

The absolute detection limit was 250 pg without being disturbed by a higher background (fixed scale : 20). This is shown in figure 2 A. The same figure shows a typical chromatogram of a standard solution (2 B), a blank meat sample (2 C) and a spiked meat sample (2 D). The retention time for ivermectin was 11 minutes under the described LC conditions.

The sample preparation procedure provided clean extracts. For the purification we chose Bond Elut C_{18} columns, with which a very good reproducibility could be reached. The conversion of ivermectin to a fluorescent derivative was very fast and resulted in a high sensitivity. The derivative has to be protected from light. The stability was checked and showed a degradation of 50% in three hours when tubes containing the fluorescent derivative of ivermectin were exposed to daylight. Quantifications were done using a standard curve; samples with high concentrations were diluted or less extraction material was used. Daily analysis of 12 samples could be done without difficulties. To keep the HPLC column in good conditions, this one was rinsed afterwards with 30 ml methanol and reconditioned before reuse. 100 meat samples were analyzed until now, 3 samples were positive for ivermectin ranging from 15 to 30 µg/kg. 30 liver

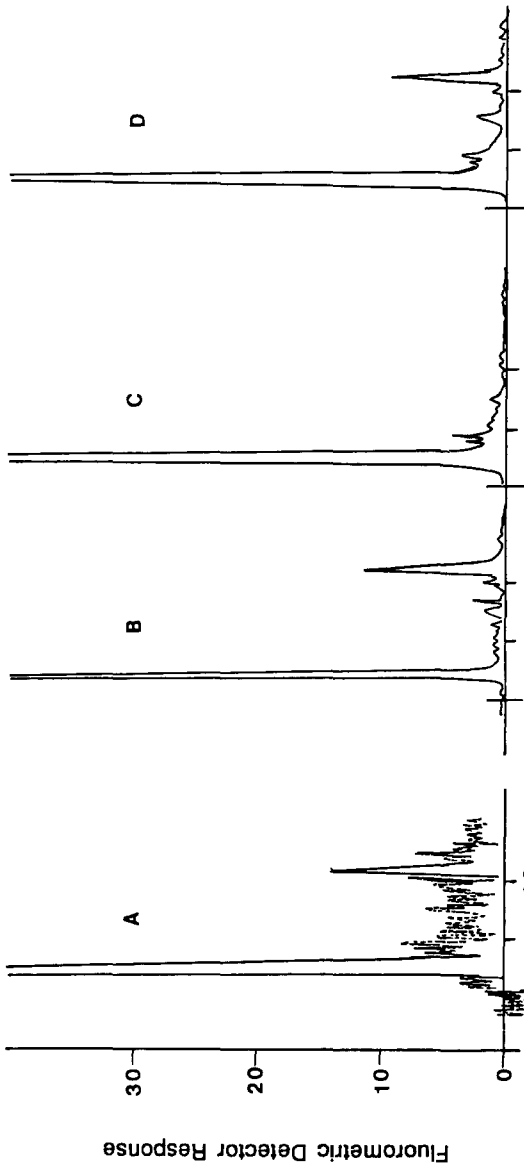

FIGURE 2. Chromatograms of Ivermectin. -A. Absolute Detection Limit (250 pg Ivermectin); -B. Standard Solution (3.6 ng Ivermectin); -C. Blank Meat Sample; -D. Spiked Meat Sample (22.5 µg Ivermectin/kg Meat); Injection volume : 10 µ l; Fixed Scale of the Fluorescence Spectrometer : 20 (A), 2 (B, C, D).

TABLE 1

Recovery Data of Ivermectin in Meat and Liver by HPLC and Fluorometric Detection

Added quantity (µg/kg)	Mean recovery (n = 5) (µg/kg)		Standard deviation		Coefficient of variation (%)		Recovery (%)	
	Meat	Liver	Meat	Liver	Meat	Liver	Meat	Liver
11.25	-	07.95	-	1.15	-	14.43	-	70.68
22.50	17.91	17.67	0.67	1.03	3.74	05.85	79.59	78.53
45.00	36.10	39.79	2.64	4.47	7.31	11.23	80.22	88.42
90.00	77.32	-	4.14	-	5.36	-	85.91	-

samples were analyzed, 1 sample contained 5.6 µg ivermectin/kg liver.
The method has proved to be suitable as a routine analysis method for ivermectin residues at a level below the maximum residue limit imposed by the EEC reglementation.

ACKNOWLEDGEMENT

We wish to thank Mrs. L. Vifquin for technical assistance and Mr. J. De Groof for statistical analyses.

REFERENCES

1. S.H.L. Chiu, R.P. Buhs, E. Sestokas, R. Taub, T.A. Jacob, J. Agric. Food Chem., 33: 99-102 (1985)

2. S.H.L. Chiu, E. Sestokas, R. Taub, M.L. Green, F.P. Baylis, T.A. Jacob, A.Y.H. Lu, J. Agric. Food Chem. 38: 2079-2085 (1990)

3.Reglementation (EEC) N° 675/92 of 18 march 1992, Off. J. of the Eur. Comm. N° L 73/8 of 19.03.1992

4. Th. Reuvers, R. Diaz, M.M. Depozuelo, M. Ramos, Anal. Chem. Acta 275: 353-358 (1993)

5. A. Fox, D.W. Fink, Analyst, 110: 259-261 (1985)

6. D.D. Oehler, J.A. Miller, J. Assoc. Off. Anal. Chem., 72: 59-61 (1989)

7. S.J. Doherty, A. Fox, D.W. Fink, J. Assoc. Off. Anal. Chem., 73: 931-934 (1990)

8. C.M. Dickinson, J. Chromatogr., 528: 250-257 (1990)

9. S.H.L. Chiu, E. Sestokas, R. Taub, J. Chromatogr. 433: 217-224 (1988)

10. J.V. Pivnichny, A.A. Lawrence, J.D. Stong, J. Chromatogr. Sci. 25: 181-186 (1987)

11. P.C. Tway, J.S. Wood, G.V. Downing, J. Agric. Food Chem., 29: 1059-1063 (1981)

12. R. Chiou, R.J. Stubbs, W.F. Bayne, J. Chromatogr., 416: 196-202 (1987)

13. S.V. Prabhu, T.A. Wehner, P.C. Tway, J. Agric. Food Chem., 39: 1468-1471 (1991)

14. P. De Montigny, J.S.K. Shim, J.V. Pivnichny, J. Pharm. Biomed. Analysis, 8: 507-511 (1990)

15. Council Directive (86/469/EEC) of 16 september 1986 on the determination of residues in animals and in fresh meat. Off. J. of the Eur. Comm. N° L 275/36 of 26.09.1986

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LIQUID CHROMATOGRAPHY CALENDAR

1994

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

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

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

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

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

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

JUNE 1 - 3: International Symposium on Hormone & Veterinary Drug Residue Analysis, Congress Centre Oud Sint-Jan, Brugge, Belgium. Contact:

Prof. C. Van Peteghem, University of Ghent, L:aboratory of Food Analysis, Harelbekestraat 72, B-9000 Ghent, Belgium.

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

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

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

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

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

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

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

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

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

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

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

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

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

1995

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

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

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

JUNE 15 - 17: 50th Northwest/12th Rocky Mountain Regional Meeting, ACS, Park City, Utah. Contact: J. Boerio-Goates, Chem Dept, 139C-ESC, Brigham Young Univ, Provo, UT 84602, USA.

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

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

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

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

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

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

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

1996

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

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

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

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

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

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

1997

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

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

1998

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

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

1999

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

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

2000

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

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

2001

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

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

2002

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

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

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Following are acceptable reference formats:

Journal:

1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18: 1979-1998 (1985)

Book:

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

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

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

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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.