JOURNAL OF LIQUID CHROMATOGRAPHY

VOLUME 18 NUMBER 13 1995

JOURNAL OF LIQUID CHROMATOGRAPHY

July 1995

Aims and Scope. The journal publishes papers involving the applications of liquid chromatography to the solution of problems in all areas of science and technology, both analytical and preparative, as well as papers that deal specifically with liquid chromatography as a science within itself. Included will be thin-layer chromatography and all models of liquid chromatography.

Identification Statement. *Journal of Liquid Chromatography* (ISSN: 0148-3919) is published semimonthly except monthly in May, August, October, and December for the institutional rate of $1,450.00 and the individual rate of $725.00 by Marcel Dekker, Inc., P.O. Box 5005, Monticello, NY 12701-5185. Second Class postage paid at Monticello, NY. POSTMASTER: Send address changes to *Journal of Liquid Chromatography*, P.O. Box 5005, Monticello, NY 12701-5185.

Volume	Issues	Institutional Rate	Individual Professionals' and Student Rate	Foreign Postage: Surface	Foreign Postage: Airmail to Europe	Foreign Postage: Airmail to Asia
18	20	$1,450.00	$725.00	$70.00	$110.00	$130.00

Individual professionals' and student orders must be prepaid by personal check or may be charged to MasterCard, VISA, or American Express. Please mail payment with your order to: Marcel Dekker Journals, P.O. Box 5017, Monticello, New York 12701-5176.

CODEN: JLCHD8 18(13) i-viii, 2507-2722 (1995)
ISSN: 0148-3919

Printed in the U.S.A.

JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

Indexing and Abstracting Services. Articles published in *Journal of Liquid Chromatography* are selectively indexed or abstracted in:

■ Abstracts Journal of the Institute for Scientific Information of the Russian Academy of Sciences ■ Analytical Abstracts ■ ASCA ■ Berichte Pathologie ■ BioSciences Information Service of Biological Abstracts (BIOSIS) ■ CAB International ■ Cambridge Scientific Abstracts ■ Chemical Abstracts ■ Chemical Reactions Documentation Service ■ Current Awareness in Biological Sciences ■ Current Contents/Life Sciences ■ Current Contents/Physical and Chemical Sciences ■ Engineering Index ■ Excerpta Medica ■ Physikalische Berichte ■ Reference Update ■ Saltykov-Shchedrin State Public Library ■ Science Citation Index

Manuscript Preparation and Submission. See end of issue.

This journal is also available on CD-ROM through ADONIS™ beginning with the 1991 volume year. For information contact: ADONIS, Marketing Services, P.O. Box 839, Molenwerf 1, 1000 AV Amsterdam, The Netherlands, Tel: +31-20-6842206, Fax: +31-20-6880241.

The journals of Marcel Dekker, Inc. are available in microform from: University Microfilms, Inc., 300 North Zeeb Road, Ann Arbor, Michigan 48106-1346, Telephone: 800-521-0600; Fax: (313) 761-1203.

Contributions to this journal are published free of charge.

Effective with Volume 6, Number 11, this journal is printed on acid-free paper.

JOURNAL OF LIQUID CHROMATOGRAPHY

Volume 18, Number 13, 1995

CONTENTS

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2507-2526 (1995)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC CHARACTERIZATION OF DIOL BONDED PHASES SYNTHESIZED VIA A HYDRIDE INTERMEDIATE

JOSEPH J. PESEK[1], MARIA T. MATYSKA[1], HELENA HEMPHÄLÄ[2], AND PAUL CHRISTENSEN[3]

[1]*Department of Chemistry*
San José State University
One Washington Square
San José, California 95192-0101
[2]*The Royal Institute of Technology*
Stockholm, Sweden
[3]*Lockheed*
1111-Lockheed Way
B/195B, 0148-50
Sunnyvale, California 94089

ABSTRACT

The chromatographic characterization of two diol stationary phases for HPLC prepared via a hydride intermediate followed by hydrosilation is described. The bonded materials are the result of the reaction of 7-octene-1,2-diol or allyl glycidyl ether with silica hydride. Characterization in the reverse phase mode was done via a standard mixture of three polycyclic aromatic hydrocarbons (PAHs). In the normal phase mode, retention data of model compounds in several different eluents were compared to each other and with data available from the literature. ESCA analysis is used to monitor for the presence of reduced Pt catalyst and when it is detected its effects can be seen in the chromatographic data.

INTRODUCTION

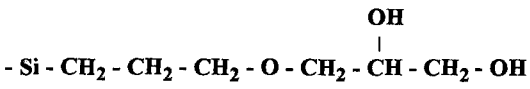
In recent years diol columns have found growing acceptance and application among modern stationary phases. The most common use of diol materials is in normal phase chromatography where extensive characterization has already been reported (1). Applications include: sugars (2), xanathates (3), chalcones (4), aromatic, polyaromatic and phenol compounds (5). In the case of phenols, it appears that retention is the result of donor-acceptor interactions. Another study measured retention on the diol stationary phase of more than 300 aromatic compounds extracted from plants and foodstuffs (6). Despite the obvious advantages of the diol columns in normal phase chromatography, a number of interesting reverse phase applications have been reported as well. A comparison of phospholipid separations has been made between diol, amino and silica columns (7). Sugars can also be separated in the reverse phase mode but a post column reaction with copper-amine is necessary for detection (8). An interesting reverse phase application involves the separation and quantitation of the drug verapamil, used for the treatment of heart disease, and its major metabolite norverapamil (9).

Among the other applications for diol bonded materials, size exclusion chromatography has been among the most important. It has been used to separate water-soluble polymers (10), proteins (11) with molecular weights ranging from 17,000-440,000, hemoglobin subunits (12) and carrageenans (13). Another approach involves immobilizing an active species through the diol moiety which can then be used in high-performance affinity chromatography (14). Cyclodextran has also been immobilized on a diol column for direct serum assays of drug enantiomers (15). The diol bonded phase has also found application in thin-layer chromatography where its performance is often compared to amino and cyano plates (16). A variety of mixtures containing vitamins, pesticides, steroids as well as other natural substances have been compared on these three types of TLC plates(17). Other comparisons among the cyano, amino and diol plates include nucleosides (18), benzodiazepine derivatives (19) and a two-dimensional separation of polyaromatic hydrocarbons involving both a reverse phase and a normal phase development has been reported (20).

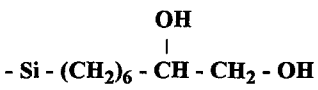
Until recently, the only synthetic route to producing a diol phase involved the bonding of 3-glycidyloxypropyltrimethoxysilane to silica followed by acid hydrolysis of the epoxide group to the diol. However, this method can result in a variety of products instead of the epoxide intermediate and diol product (21). Therefore it is not unusual that similar separations reported in the literature are not identical due to the variability of the bonded material. We have reported another approach to the synthesis of a new phase using a hydride intermediate followed by hydrosilation with the olefin 7-octene-1,2-diol

which results in a "true" diol bonded material (22). The goal of this study is to chromatographically characterize this new diol bonded material and compare it to phases which are synthesized from allyl glycidyl ether as well as typical results from the literature. The structures of the two types of diol phases are shown below:

a) Diol via allyl glycidyl ether :



b) Diol via 7-octene-1,2-diol :



The effects of hydride on the surface in comparison to hydroxide as well as evaluations of similar phases made by conventional organolsilanization have been addressed in another study (23).

EXPERIMENTAL

Materials

The silica gel used was Vydac 101 TP lot # 900201 - The Separations Group (Hesperia, CA, US) - (BET surface area, S_{BET}= 106 m^2/g ; particle diameter, dp = 6.6 μm - all data supplied by manufacturer). The solvents were HPLC grade from Fisher Scientific (Pittsburgh, PA, US) and J.T.Baker (Phillipsburg, NJ, US). Solutes were from Aldrich (Milwaukee, WI, US), Sigma (St. Louis, MO, US), Eastman Organic Chemicals (Rochester, NY, US) and Matheson Coleman & Bell (Norwood, OH, US).
The column Selectivity Test Mixture for Liquid Chromatography (Polycyclic Aromatic Hydrocarbons, SRM 869) was from the National Institute of Standards & Technology (Gaithersburg, MD, US). Samples were diluted in the mobile phases (weights were always less than 10 μg per injection volume - 5 μL - to avoid overloading of the column). Solvent flow was 2 mL/min. The determination of t_0 was carried out using n-pentane as an inert solute by measuring the maximum negative deflection when it was injected into a heptane mobile phase.

Columns

Three types of columns were used: diol via allyl glycidyl ether (AGE - white), diol via allyl glycidyl ether with reduction of the catalyst (AGE - gray) and diol via 7-octene-1,2 - diol (7-OD). The carbon content and surface coverages of the three columns are shown in Table I. The synthesis and spectroscopic characterization of diol-bonded silicas were described earlier (22).

Instrumentation

All chromatographic measurements were made with a Hewlett-Packard (Avondale, PA, US), model 1050 liquid chromatographic system equipped with quaternary gradient pump, automatic injector, variable wavelength UV detector and computer data station. Columns were slurry-packed (10% w/v bonded silica in 9:1 v/v CCl_4/methanol) into 15-cm x 0.46-cm I.D. stainless steel tubes (Altech Co., Deerfield, Il, US) using a Haskel (Burbank, CA, US) pneumatic pump at 40 MP. Methanol was the driving solvent. The ESCA data were obtained with a Perkin-Elmer Model 5600 Multitechnique Spectrometer (Lockheed, CA, US).

Procedures

For ESCA analysis, the silica-powder samples were coated onto double-side-sticky tape, mounted on a sample stage and evacuated to a base pressure of about $1x10^{-9}$ Torr. The H_2PtCl_6 reference catalyst was tested similarly on an inert gold-plated substrate. To enhance the magnitude of the spectral peaks, the sample stage was tilted to a take-off angle of 70^0 with respect to the normal surface plane. A monochromatic Al Kα X-ray source (1486.6 eV) with a 7-mm filament operating at 400 W irradiated the samples. A hemispherical-electrostatic-field analyzer detected the kinetic energies of the emitted photoelectrons. The test-spot sizes for the silica-powder samples and the catalyst were 800x2000 μm and 400 μm, respectively. ESCA-survey spectra between 0 and 1100 eV were first collected with a 187.85 eV pass energy and a 0.40 eV resolution to identify the primary elements present. To enhance spectral resolution for various elements, ESCA-multiplex spectra were then obtained with a 58.70 eV pass energy and a 0.25 eV resolution. The binding energies were normalized to the C 1s line at 284.6 eV. All ESCA data were collected at ambient temperature.

TABLE 1.
Carbon content and surface coverages of diol silica samples

No	Sample	Corrected % C *	α_R μmol/m²
1.	Vydac-diol (AGE)-gray	4.58	4.25
2.	Vydac-diol (AGE)-white	4.15	3.63
3.	Vydac-diol (7-OD)	5.05	3.66

* - % C after subtracting the amount before hydrosilation reaction.

RESULTS AND DISCUSSION

Reverse Phase

SRM 869 has proven to be a useful tool for the classification of bonded phase type. The relative retention of two PAHs, tetrabenzonaphthalene (TBN) to benzo[a]pyrene (BaP), is used to determine if a phase is polymeric, monomeric or intermediate (24). According to the classification scheme developed, α TBN/BaP is <1.0 if the phase is polymeric and >1.7 if it is monomeric. Between 1.0 to 1.7 the phase is classified as intermediate. The method specifies a mobile phase composition of 85% acetonitrile (MeCN)/water. However, under these conditions incomplete separation of the mixture was obtained on the AGE (white) and 7-OD columns. It was found that an eluent composition of 60% acetonitrile/water gave adequate separations in order for the relative retentions to be determined on the two phases. Using this mobile phase and according to classification for SRM 869, the 7-OD column behaves like a monomeric phase ($\alpha_{TBN/BaP}$ = 1.74), while AGE behaves like an intermediate material ($\alpha_{TBN/BaP}$ = 1.34).

Figure 1 shows the retention of the three components of SRM 869 on the two diol columns at 50% acetonitrile/water. For the component which shows highest retention on two columns, TBN, retention is in the order 7-OD>AGE(white). The result for 7-OD is expected since it should be more hydrophobic than AGE with the bonded moiety consisting of two hydroxyl groups and an alkyl chain of six methylene groups. AGE has an additional oxygen (ether linkage) and a shorter alkyl chain (four methylene groups). Figure 1 also shows that on the two columns for PAHs with the same F number (25)

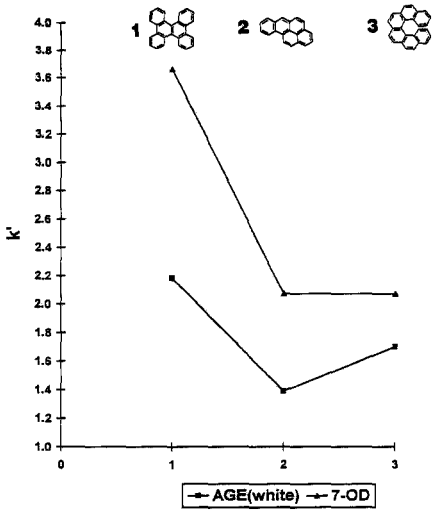


FIGURE 1. k' values of test mixture for liquid chromatography (SRM 869) on diol bonded phases: **AGE (white)** - diol via allyl glycidyl ether and **7-OD** - diol via 7-octene-1,2-diol . Mobile phase: 50 % acetonitrile in water (v/v), solutes: 1 - TBN (Tetrabenzonaphthalene), 2 - BaP (Benzo[a]pyrene), 3 - PhPh (Phenanthro[3,4-c]phenanthrene). Flow rate 2 mL/min.

(TBN and PhPh, F =13, F = number of double bonds + number of of primary and secondary carbons - 0.5 x number of non aromatic rings) longer retention is observed for the more planar of the two compounds (TBN). Recently we have tested the behavior of various tetracyclines in the reverse phase mode using high percentages of water and low pH (26). Excellent separations were obtained for many tetracycline mixtures and their degradation products with no evidence of column deterioration under aggressive mobile phase conditions.

Normal Phase

Aromatic Compounds-

In order to characterize the new materials and compare them to other diol phases aromatic compounds were chosen as test solutes despite their low k' values, because abundant data exists in the literature. Table II gives log k' values for a series of aromatic

TABLE 2
Retention data of aromatic compounds on diol bonded phases

No	SOLUTES	k'		
		1	2	3
1	Biphenyl	0.24	0.11	0.24
2	Naphtalene	0.23	0.11	0.24
3	Fluorene	0.32	0.12	0.33
4	p-Xylene	0.12	0.12	
5	Toluene	0.13	0.12	
6	m-Xylene	0.12	0.12	
7	Pyrene	0.49	0.12	0.64
8	p-Dibromobenzene	0.15	0.12	
9	Styrene	0.15	0.11	
10	1-Methylnaphthalene	0.22	0.12	
11	2-Methylnaphthalene	0.22	0.12	
12	Chlorobenzene	0.15	0.12	
13	Benzene	0.14	0.13	0.09
14	Phenanthrene	0.39	0.15	0.47
15	Anthracene	0.40	0.15	
16	Fluoranthene	0.34	0.20	0.67
17	Durene	0.13	0.26	
18	Chrysene	0.76	0.27	
19	Nitrobenzene	1.34	0.30	
20	Benzo(k)fluoranthene	0.59	0.32	
21	Acetophenone	0.91	0.36	
22	Perylene	0.66	0.37	

(1); Age(white) column, mobile phase-heptane
(2); 7-OD column, mobile phase-heptane
(3); LiChrosorb 100 from Merck (data from literature[5]), mobile phase-heptane

compounds on the two materials synthesized for this study as well as data obtained from the literature (5) for a commercial diol column (Merck LiChrosorb). These results indicate that the 7-OD column is less hydrophilic than either the AGE (white) or the Merck diol based on data from identical solutes. For virtually all of the aromatic solutes studied, 7-OD had the lowest k' with heptane as the mobile phase. A much different order of retention is observed for the same compounds on the AGE (white) and the commercial diol . In almost all cases

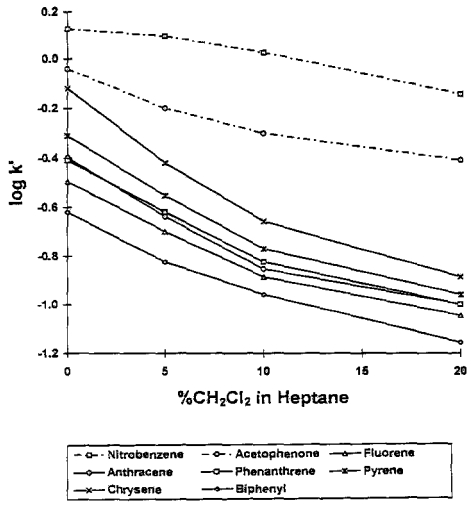


FIGURE 2. Retention data of some aromatic compounds on AGE (white) column with methylene chloride-heptane as the mobile phase. For notation of column see FIGURE 1. Flow rate 2 mL/min.

where literature data is available, the Merck material has a higher k' value. As indicated above, incomplete hydrolysis or the presence of other species which can form during the conventional bonding process may account for this difference. In direct comparisons using heptane as the mobile phase, only benzene has lower retention on the commercial diol. However, the k' is small and no information is given in the literature on the determination of t_0 so that the difference can be easily due to this discrepancy.

Further documentation of the strong polar characteristics of the AGE (white) phase is given in Figure 2. Retention for the unsubstituted aromatic solutes generally increases as the number of aromatic rings increases: biphenyl (2) < fluorene, phenanthrene, anthracene (3) < pyrene, chrysene (4). The shape of the curve is consistent with the donor-acceptor retention mechanism. As the amount of modifier in the mobile phase is decreased, retention (log k') increases faster at lower modifier concentrations. Lower amounts of modifier enhance the donor-acceptor interaction taking place between the aromatic rings of the solutes and the diol moiety of the bonded phase. In contrast the two solutes with a functional group (nitrobenzene and acetophenone) that can hydrogen bond with the diol species display higher k' values and are less affected as the amount of modifier is increased.

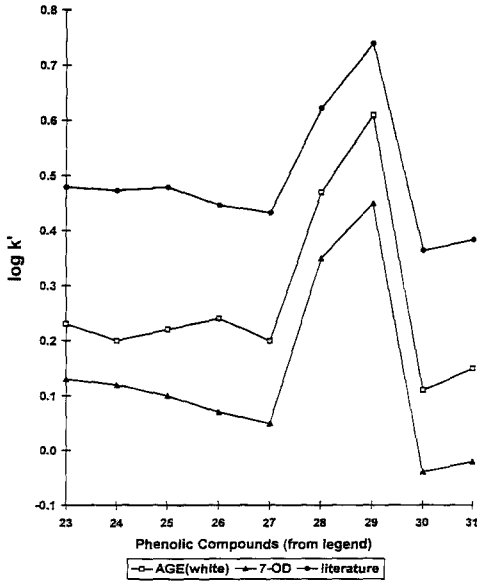


FIGURE 3. Comparison of the retention data of phenols on diol columns with data from the literature (5). Mobile phase heptane-chloroform (50:50 v/v) for AGE (white) and 7-OD columns (For notation of columns see FIGURE 1). Data from literature (5)(for Merck diol column) are with the mobile phase isooctane-chloroform (50:50 v/v). The solutes: **23** ; 4-Bromophenol, **24** ; 3-Chlorophenol, **25** ; 4-Chlorophenol, **26** ; 4-Fluorophenol, **27** ; Naphtol-2, **28** ; 3-Nitrophenol, **29** ; 4-Nitrophenol, **30** ; Phenol, **31** ; 4-Phenylphenol. Flow rate 2 mL/min.

The behavior of another class of aromatic compounds, phenols, is shown in Figure 3 for the two diol columns synthesized for this study as well as for the Merck diol. For the nine compounds studied, the same relative order for each compound on the three columns is observed: 7-OD < AGE (white) < Merck diol (literature), consistent with the hydrophilic/hydrophobic nature of the materials as explained above. At the lowest amounts of modifier in the mobile phase, the AGE (white) column displays lower retention than the commercial diol for all of the phenol solutes. While some differences in retention may be due to the effects of heptane instead of isooctane as the primary mobile phase constituent, the major trends observed are probably too great to be accounted for by this change alone.

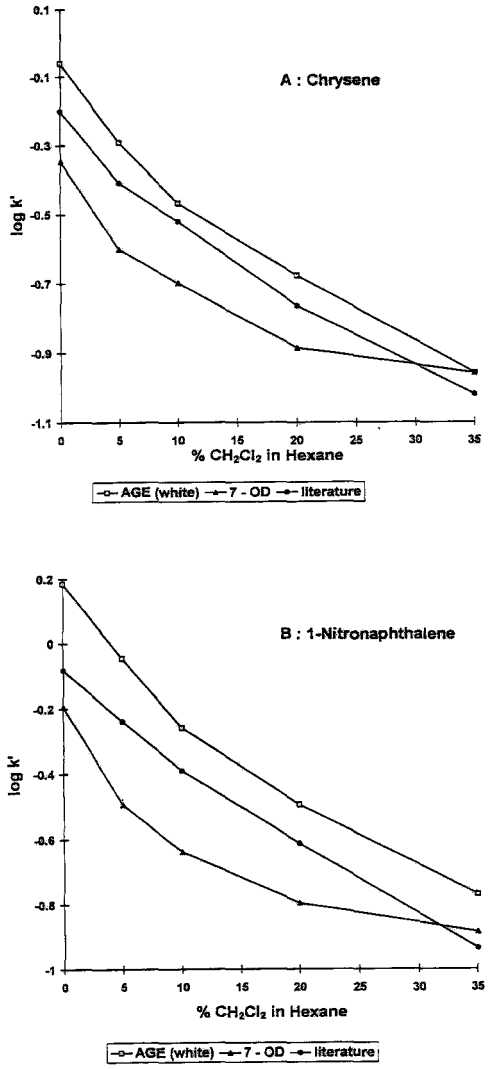
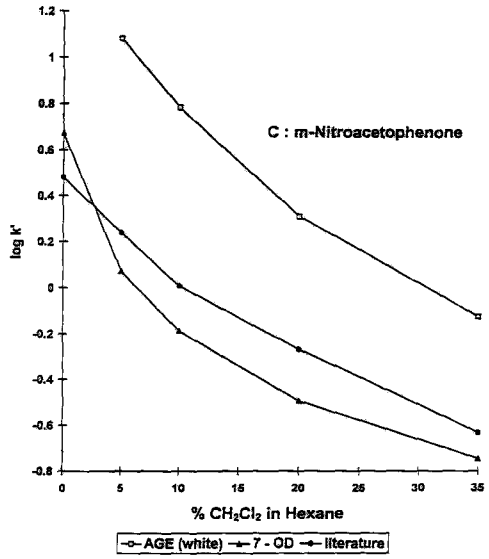


FIGURE 4. Retention data of model compounds on diol columns with methylene chloride-hexane as eluent (for notation of columns see FIGURE 1). Flow rate 2 mL/min. The literature data are from reference number 1 (for DuPont diol column). A ; data for chrysene, B ; data for 1-Nitronaphthalene, C ; data for m-Nitroacetophenone, D ; data for Benzyl alcohol.



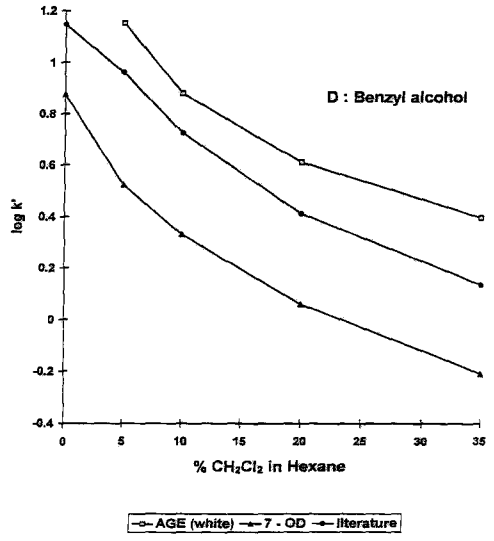


FIGURE 4 (continued)

An illustration of the variability between diol columns is shown in Figure 4 where the two materials in this study are compared to another commercial source (DuPont) using the same solutes in the same mobile phase as in a previous report (1). In the case of AGE (white), retention is consistently longer for all compounds at all modifier concentrations than for the DuPont - diol column. In the case of the least hydrophilic material, 7-OD , only nitroacetophenone in the absence of modifier has greater retention than the commercial column.

Steroids-

A clearer example of the trend observed above can be seen for very highly retained solutes such as steroids. Data from the previous study (1) on the commercial diol column cited above were based on varying amounts of methylene chloride in hexane as the mobile phase to elute five test steroids. Three of the same steroids and a fourth similar in structure to the other two test compounds were used in evaluating the two new diol materials. For AGE (white) only the two least retained steroids could be easily eluted (Figure 5A) in 100% methylene chloride. In order to obtain k' values < 10 for corticosterone and prednisone on the AGE (white) material, it was necessary to modify methylene chloride with 2-propanol. For highly retained solutes the AGE (white) column synthesized for this study is more polar than the DuPont diol column (1). On the 7-OD column the steroids could be eluted in hexane modified with methylene chloride (Figure 5B). In this case, retention for all the test compounds was less than that observed on the DuPont diol column. This result confirms all of the previous observations that 7-OD material is less hydrophilic than any of the diol columns based on hydrolyzed propyl glycidyl ether moieties.

In terms of a practical separation involving the four steroids tested above, the k' values are sufficiently different that it is necessary to use a gradient in order to do the analysis in a reasonable time frame. The AGE (white) column has the strongest retention for the steroids and the greatest range of k' values under a given set of isocratic conditions. Therefore, the separation of the four solutes was optimized on the AGE (white) column and the results are shown in Figure 6A. Good separation in just over three minutes is achieved using 2-propanol as the modifier in methylene chloride with a gradient from 2.5% to 15% between 1 and 2.5 min in the chromatographic run. The same gradient was applied to the 7-OD column (Figures 6B). The very short retention and incomplete separation of the four steroids on the 7-OD column for this same gradient is consistent with its properties as less hydrophilic than the AGE (white) phase.

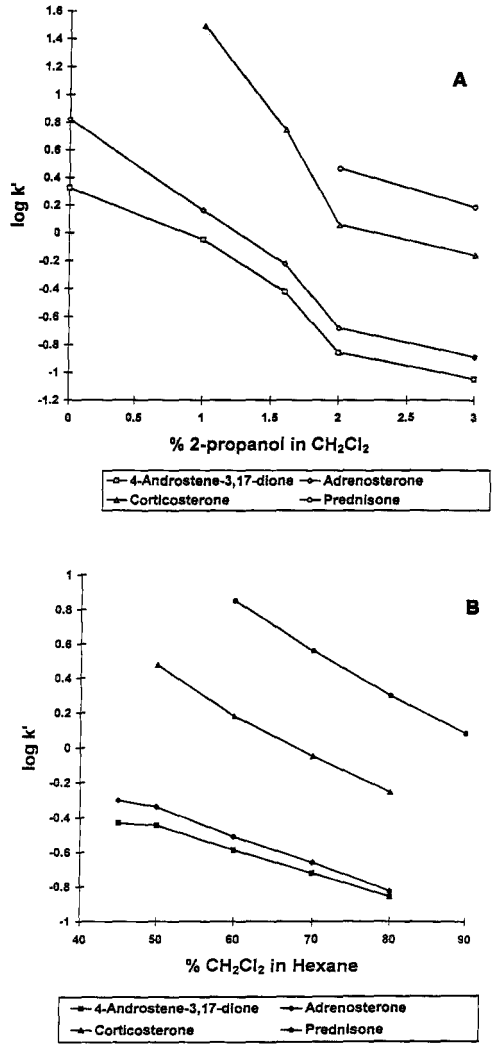


FIGURE 5. Retention data of steroids on: A ; AGE (white) column with 2-propanol-methylene chloride as eluent and B ; on 7-OD column with methylene chloride-hexane as the mobile phase. Flow rate 2 mL/min. For notation of columns see FIGURE. 1.

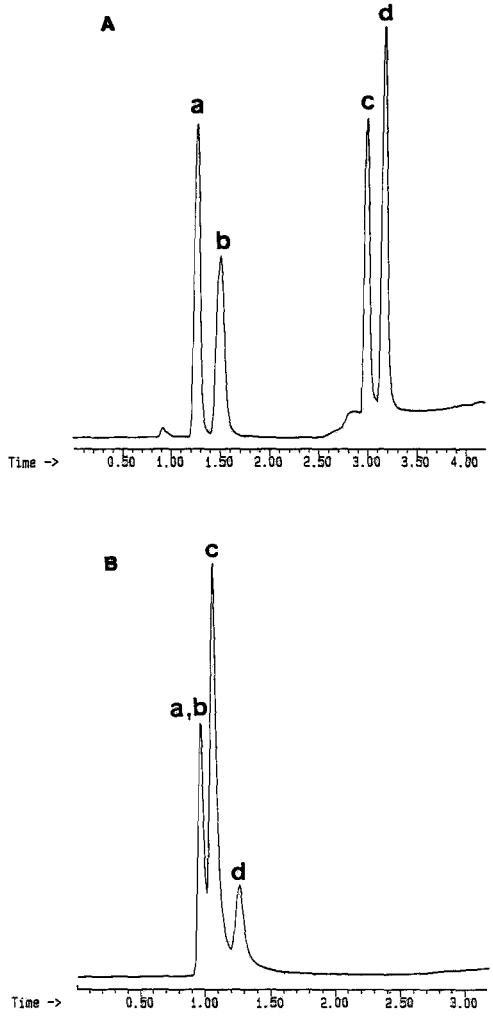


FIGURE 6. Gradient separation of steroids (a ; 4-Androstene-3,17-dione, b ; Adrenosterone, c ; Corticosterone, d ; Prednisone). Gradient conditions: 2-propanol as the modifier in methylene chloride from 2.5% to 15% between 1 and 2.5 min. in the chromatographic run.
A ; AGE (white), B ; 7 - OD and C ; AGE (gray) columns (for notation of columns see Experimental).

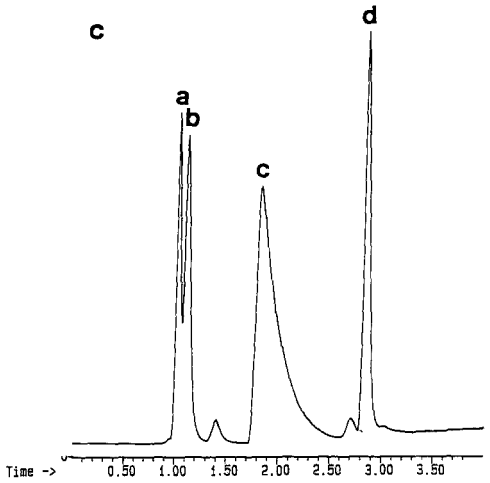


FIGURE 6 (continued)

Pt Reduction

When a large amount of catalyst was used (10 fold increse) it was noted that the final product was gray. This material was packed into a column for testing. The result of the steroids separation is shown in Figure 6C. The interesting feature of this chromatogram is the broad unsymmetrical peak (c) observed for the third component (corticosterone). By making the gradient shallower, the fourth peak becomes broader as well. With a very shallow gradient the first two peaks (a,b) have significant retention and become broader and unsymmetrical. This effect is most likely due to the presence of Pt on the surface (see discussion below) which apparently has a strong interaction with the steroid solutes. The chromatographic behavior can be explained by both the presence of Pt on the surface and the use of 2-propanol as the modifier. At low k' values, interaction between the solute and the stationary phase is relatively small so that there is no significant effect of the Pt. When retention is longer but there is still no significant amount of modifier in the mobile phase, the solute can display strong adsorption at the Pt sites on the stationary phase. Such an effect is observed for the third peak (c) in Figure 6C. Once there is an appreciable quantity of 2-propanol in the mobile phase it will mask the Pt so that the solute does not interact with these adsorption sites but just the organic moiety. The effect of 2-propanol is not suprising since it is the solvent used to dissolve the

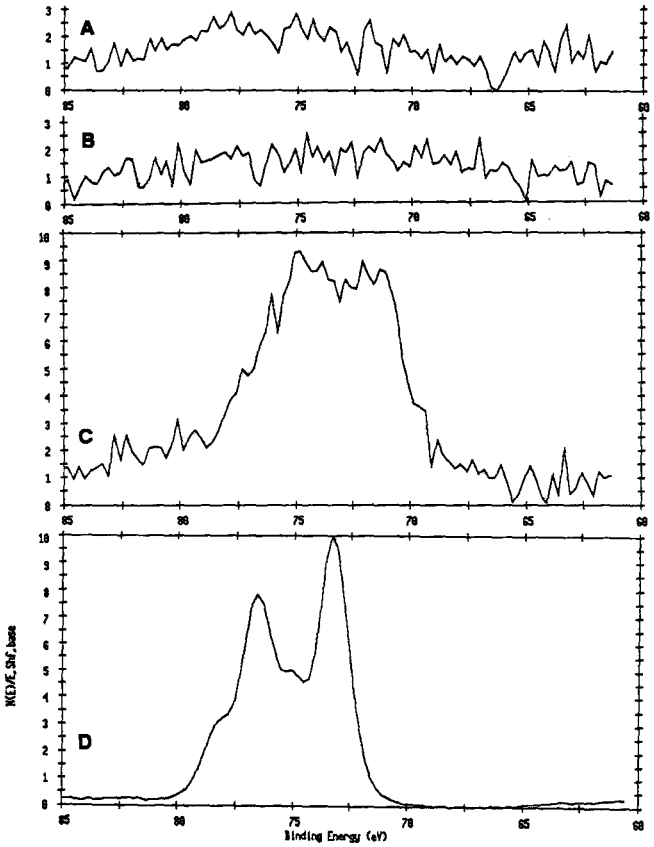


FIGURE 7. Pt 4f region of the ESCA spectra of: A ; bare Vydac TP silica, B ; Vydac-H TP (hydride intermediate) silica, C ; AGE (gray) column material (diol via allyl glycidyl ether with reduction of the catalyst) and D ; catalyst - hexachloroplatinic acid.

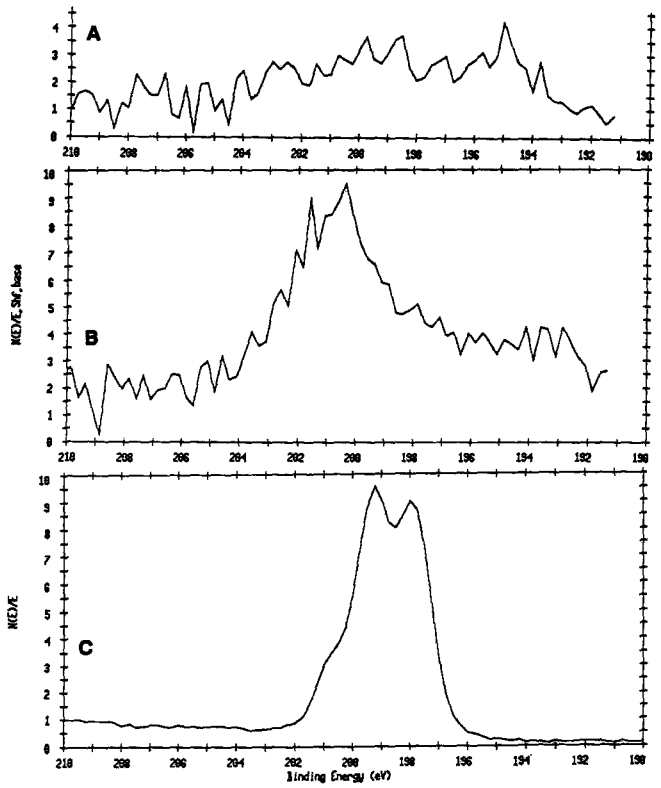


FIGURE 8. Cl 2p region of the ESCA spectra of: A ; Vydac-H TP (hydride intermediate) silica, B ; AGE (gray) column material (for notation of column see FIGURE 7) and C ; catalyst - hexachloroplatinic acid.

hexachloroplatinic acid (Spier's catalyst) for the bonding reaction (hydrosilation). It is interesting to note that such a dramatic effect on chromatographic behavior is caused by a very small amount (.03 atom %) of Pt on the surface. This results illustrates the importance of preventing catalyst deposition during the bonding reaction.

Both the chromatographic data reported above and the color of the AGE (gray) material suggest that some reduction and deposition of the catalyst has occurred. This can be conclusively proven through ESCA which determines the elemental composition of only the surface of a material (27). Figures 7A and 7B show the high resolution ESCA spectra of the platinum 4f region for bare Vydac TP silica and the same hydride intermediate used to make the AGE columns. No Pt is detectable (<0.01 atom %) on the surface of either material. In comparison, Figure 7C shows the same region for the AGE (gray) column material. The Pt 4f doublet is visible above the noise at a level estimated to be around 0.03 atom %. Additionally, the same high resolution scan was obtained for the catalyst, hexachloroplatinic acid, used to bond the AGE via the hydride intermediate (Figure 7D). From the position of the peaks in the two spectra, it can be concluded that the majority of the platinum which is on the AGE (gray) material is in the elemental state. Since the catalyst is soluble under the reaction conditions, the Pt(0) detected must be the result of reduction through interaction with the hydride surface. This observation is also supported by DRIFT spectra which show a measurable decrease in the Si-H stretching band for the gray material in comparison to the white product (22). Further confirmation of the change in oxidation state of the Pt containing material is shown in Figure 8, the high resolution ESCA spectra of the Cl 2p region. The bare silica (Figure 8A) shows no evidence of Cl on the surface. The 2p doublet is visible in the spectrum of the AGE (gray) material near 200 eV. However, for the catalyst the lower energy peak appears near 197 eV. Therefore, it can be concluded that the Cl on the bonded material is not coordinated to Pt(IV) as is the case for the catalyst but is probably free chloride ion which is adsorbed on the surface after the platinum is reduced. It is clear that a relatively small amount of Pt on the surface can lead to a darkening of the product and have a considerable effect on the chromatographic properties of some solutes.

CONCLUSIONS

This study presents chromatographic data on two types of diol phases synthesized via a hydride intermediate. One phase, similar to commercially available products, is made by bonding allyl glycidyl ether while the other contains only a hydrocarbon chain and the diol functional group. In both the reverse phase and normal phase experiments, the 7-OD (diol via 7-octene-1,2 - diol) material is clearly more hydrophobic than the AGE (diol via allyl

glycidyl ether) column. The AGE column behaves similarly to those obtained via conventional organosilanization. The potential advantages of the new materials include their pH stability proven in previous studies (23,28) for the same bonding scheme, variable hydrophillicity for normal phase applications depending on whether the AGE or 7-OD phase is selected and the existence of a single product diol phase with no chance of by-products for the 7-OD material.

ACKNOWLEDGMENTS

Partial support of this research was provided by the National Science Foundation (CHE 9119933) and by the Camille and Henry Dreyfus Foundation through a Scholar (JJP) award. The authors wish to thank the Separations Group for donation of the Vydac silica.

REFERENCES

1. A. W. Salotto, E. L.Weiser, K. P.Caffey, R. L. Carty, S. C. Racine and L.R. Snyder, J. Chromatogr., 498 (1990) 55.

2. B. Herbreteau, M. Lafosse, L. Morin-Allory and M. Dreux, Chromatographia, 33 (1992) 325.

3. Ch. Zhou, A. Bahr and G. Schwedt, Lihua Jianyan, Huaxue Fence, 27 (1991) 203.

4. B. Walczak, L. Morin-Allory, J. R. Chretien, M. Lafosse and M. Dreux, Chemom. Intell. Lab. Syst., 1 (1986) 79.

5. A. M. Siouffi, M. Righezza and G. Guiochon, J. Chromatogr., 368 (1986) 189.

6. M. Luebke, J. L. Le Quere and D. Barron, J. Chromatogr., 646 (1993) 307.

7. R. M. Sheeley, W. J. Hurst, D. M. Sheeley and R. A. Martin Jr.,J. Liq. Chromatogr., 10 (1987) 3173.

8. M. Abbou and A. M. Siouffi, J. Liq. Chromatogr., 10 (1987) 95.

9. A. T. Kacprowicz, R. O. Fullinfaw and R.W. Bury, J. Chromatogr., 337 (1985) 412.

10. D. P. Herman and L. R. Field, J. Chromatogr. Sci. 19 (1981) 470.

11. P. Roumeliotis and K. K. Unger, J. Chromatogr., 218 (1981) 535.

12. V. Baudin-Chich, M. Marden and H. Wajcman, J. Chromatogr., 437 (1988) 193.

13. G. Sworn, W. M. Marrs and R. J. Hart, J. Chromatogr., 403 (1987) 307.

14. T. Ohta, T. Inoue, Y. Fukumoto and S. Takitani, Chromatographia, 30 (1990) 410.

15. J. Haginaka and J. Wakai, Anal. Sci., 8 (1992) 137.

16. W. Jost and H. E. Hauck, GIT-Suppl. 3 (1988) 74.

17. H. E. Hauck and W. Jost, Am. Lab. 22 (1990) 44.

18. T. Cserháti, J. Chromatogr. Sci., 29 (1991) 210.

19. T. Cserháti and H. E. Hauck, J. Chromatogr., 514 (1990) 45.

20. A. M. Siouffi, J. Kantasubrata, M. Righezza, E. Mincsovics and E. Tyihak, Proc. Int. Symp. Instrum. High Perform. Thin-Layer Chromatogr., 3rd Ed. R. E. Kaiser Inst. Chromatogr., Bad Duerkheim, Germ. !985. p. 201.

21. B. Porsch, J. Chromatogr. 653 (1993) 1.

22. J. J. Pesek and M. T. Matyska, J. Chromatogr., in press.

23. M. C. Montes, C.van Amen, J. J. Pesek and J. E. Sandoval, J. Chromatogr., in press.

24. L. C. Snyder and S.A. Wise, Anal. Chem., 56 (1984) 504.

25. J. F. Schbron, R. J. Hurtubise and H. F. Silver, Anal. Chem., 49 (1977) 2253.

26. M.T. Matyska, J.J. Pesek and A.M. Siouffi - submitted for publication - Chem.Anal.

27. J.F. Watts, "An Introduction to Surface Analysis by Electron Spectroscopy", Oxford Univ. Press 1990.

28. J. E. Sandoval and J. J. Pesek, Anal. Chem., 63 (1991) 2634.

Received: January 28, 1995
Accepted: February 16, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2527-2541 (1995)

AN IMPROVED CHIRAL RECOGNITION MODEL FOR RESOLVING N-ACYL-α-ARYL-ALKYLAMINES ON PIRKLE-TYPE π-ACIDIC CHIRAL STATIONARY PHASES

MYUNG HO HYUN*, CHUNG SIK MIN, YOON JAE CHO, AND MYOUNG SEON NA

Department of Chemistry
Pusan National University
Keumjeong-Ku, Pusan 609-735
South Korea

ABSTRACT

An improved chiral recognition model for resolving N-acyl-α-arylalkylamines on Pirkle-type π-acidic chiral stationary phases (CSPs) has been proposed based on the chiral resolution trends of a homologous series of N-acyl-α-(1-naphthyl)ethylamines on CSPs derived from N-(3,5-dinitrobenzoyl)-(R)-phenylglycine (CSP **1**) and N-(3,5-dinitrobenzoyl)-(S)-leucine (CSP **2**). The chiral recognition model proposed has been evidenced by its successful application to the explanation of the chromatographic resolution trends of a homologous series of N-acetyl-α-(1-naphthyl)alkylamines on CSP **1** and **2** and a homologous series of N-acyl-α-(1-naphthyl)ethylamines and N-acetyl-α-(1-naphthyl)alkylamines on CSP **5**, which has the same structure as CSP **1** except the tether direction.

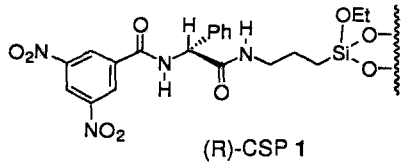
INTRODUCTION

Chiral recognition models proposed for the discrimination of the two enantiomers of racemates by certain liquid chromatographic chiral stationary phases (CSPs) have been often successfully utilized in developing improved CSPs and in predicting the possibility of resolving certain recemates on a given CSP.[1] For example, a CSP derived from α-(6,7-dimethyl-1-naphthyl)-isobutylamine has been designed based on the chiral recognition model proposed for resolving N-acyl-α-arylalkylamines on a CSP derived from the 3,5-dinitrobenzamide of (R)-phenylglycine (CSP **1**) and has been proved to be excellent in resolving various racemates including N-(3,5-dinitrobenzoyl)-α-amino esters.[2,3,4] However, it should be noted that chiral recognition models might be improved or modified as more chromatographic and/or spectroscopic data are accumulated.[5]

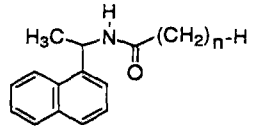
Recently, we had the chance of resolving N-acyl-α-(1-naphthyl)ethylamines **3** on a CSP derived from the 3,5-dinitrobenzamide of (S)-leucine (CSP **2**) and we expected that the chromatographic resolution behaviors of **3** on CSP **2** should be identical with those reported on CSP **1** based on the chiral recognition model proposed previously.[2] However, surprisingly, the elution orders and the resolution trends of **3** on CSP **2** were not consistent with those expected from the chiral recognition model proposed. Therefore, herein, we systematically reinvestigate the chromatographic resolution behaviors of a homologous series of **3** on CSP **1** and **2** and propose an improved chiral recognition model which can rationalize the discrepancies between the chromatographic resolution behaviors on CSP **1** and **2**. The chiral recognition model proposed is also tested by additional chromatographic resolution experiments.

EXPERIMENTAL

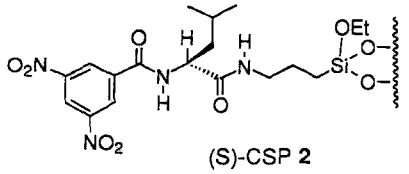
Chromatographic resolution data were collected with an HPLC system consisting of a Waters model 510 pump, a Rheodyne model 7125 injector with a



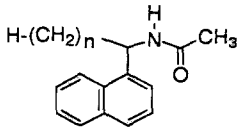
(R)-CSP **1**



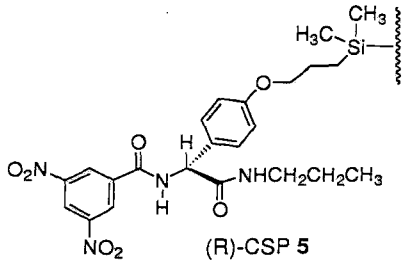
3



(S)-CSP **2**



4



(R)-CSP **5**

20 μl sample loop, a Youngin model 710 absorbance detector with a 254 nm UV filter and a Youngin D520B computing integrator. The chiral columns packed with CSP **1** and **2** were available from the Regis Chemical Company, Morton Grove, Illinois, U.S.A. All chromatographic resolution experiments were performed using 2-propanol-hexane (10:90 or 20:80, v/v) as a mobile phase with a flow rate of 2 ml/min at 20 °C. Column void volume was measured by injecting 1,3,5-tri-*t*-butylbenzene.[6] All analytes used in this study were prepared by the procedures described previously.[2]

RESULTS AND DISCUSSION

The dipole-stacking chiral recognition model proposed previously for resolving N-acylated α-arylalkylamines on CSP **1** implies the π-π interaction

between the 3,5-dinitrobenzoyl group of the CSP and the α-aryl group of the analyte and the simultaneous dipole-stacking between the amide dipoles of the CSP and the analyte.[2,3] Another possible hydrogen-bonding chiral recognition model proposed includes the π-π interaction between the 3,5-dinitrobenzoyl group of the CSP and the α-aryl group of the analyte and the simultaneous hydrogen bonding interaction between the amide N-H hydrogen of the CSP and the carbonyl oxygen of the analyte.[2] In each model, the phenyl group of CSP **1** plays a role as a steric barrier which interferes with the analyte in its approaching to the CSP from the side of the phenyl group of the CSP. In this instance, the isobutyl group at the chiral center of CSP **2** can play a role similar to that of the phenyl group of CSP **1** and in consequence, the chromatographic resolution behaviors of N-acylated α-arylalkylamines on CSP **2** are expected to be identical with those on CSP **1** in the sense of chiral recognition. However, the chromatographic resolution behaviors of N-acyl-α-(1-naphthyl)-ethylamines **3** on CSP **2** are exactly opposite to those on CSP **1** as shown in Table 1 and Figures 1 and 2.

Table 1 summarizes the data concerning the resolution of N-acyl-α-(1-naphthyl)-ethylamines **3** on CSP **1** and **2**. The chromatographic resolution results shown in Table 1 are graphically illustrated in Figures 1 and 2. As shown in Figure 1, the capacity factor of the more retained (S)-enantiomer on CSP **1** decreases more rapidly than that of the (R)-enantiomer and, in consequence, the separation factor, which is defined as the ratio of the capacity factor of the second eluted enantiomer to that of the first eluted enantiomer, decreases continuously as the N-acyl chain of **3** increases in length. In contrast, the capacity factor of the less retained (R)-enantiomer on CSP **2** decreases more rapidly than that of the (S)-enantiomer and, in consequence, the separation factor on CSP **2** increases continuously as the N-acyl chain of **3** increases in length as shown in Figure 2. Based on the chiral recognition model proposed previosly,[2] CSP **1** and **2** are also expected to show opposite elution orders in resolving N-acyl-α-(1-naphthyl)-ethylamines **3** because their absolute configurations are opposite to each other. However, identical elution orders were observed in the resolution of N-acyl-α-(1-naphthyl)-ethylamines **3** on CSP **1** and **2** as shown in Table 1 and Figures 1 and 2.

TABLE 1
Resolution of N-Acyl-α-(1-naphthyl)-ethylamines **3** on CSP **1** and CSP **2**.[a]

		CSP **1**				CSP **2**			
3	n[b]	k_1'[c]	k_2'[d]	α[e]	Conf.[f]	k_1'[c]	k_2'[d]	α[e]	Conf.[f]
	1	8.44	17.00	2.01	S	5.51	5.51	1.00	
	2	6.51	12.38	1.90	S	3.67	4.65	1.27	S
	3	5.03	9.27	1.84	S	2.81	3.59	1.28	S
	4	4.53	7.97	1.76	S	2.29	3.16	1.38	S
	6	3.51	5.64	1.61	S	1.72	2.51	1.46	S
	7	3.35	5.28	1.58	S	1.58	2.39	1.51	S
	9	3.00	4.54	1.51	S	1.36	2.13	1.57	S
	11	2.69	3.94	1.46	S	1.22	1.96	1.61	S
	13	2.54	3.73	1.47	S	1.13	1.86	1.65	S
	15	2.35	3.42	1.46	S	1.05	1.74	1.66	S
	17	2.20	3.18	1.45	S	0.98	1.63	1.66	S

a: See the experimental section for the chromatographic conditions. Mobile phase was 20 % 2-propanol in hexane. b: Length of the N-Acyl alkyl chain [$(CH_2)_n$-H] of analyte **3**. c: Capacity factor of the first eluted enantiomer. d: Capacity factor of the second eluted enantiomer. e: Separation factor. f: Absolute configuration of the second eluted enantiomer.

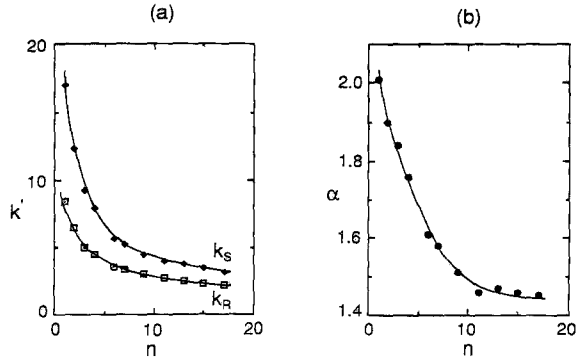


FIGURE 1. The trends of (a) capacity factors and (b) separation factors for resolving N-acyl-α-(1-naphthyl)-ethylamines **3** on CSP **1**. Chromatographic conditions are given in the experimental part.

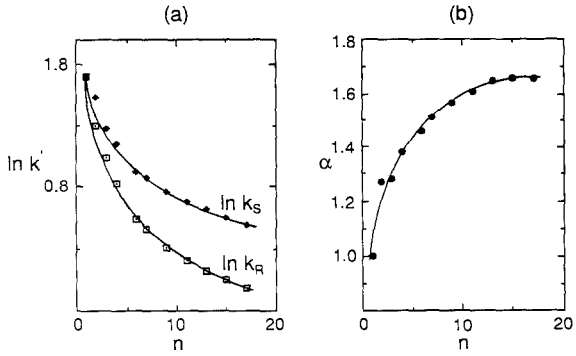


FIGURE 2. The trends of (a) capacity factors and (b) separation factors for resolving N-acyl-α-(1-naphthyl)-ethylamines **3** on CSP **2**. Chromatographic conditions are given in the experimental part.

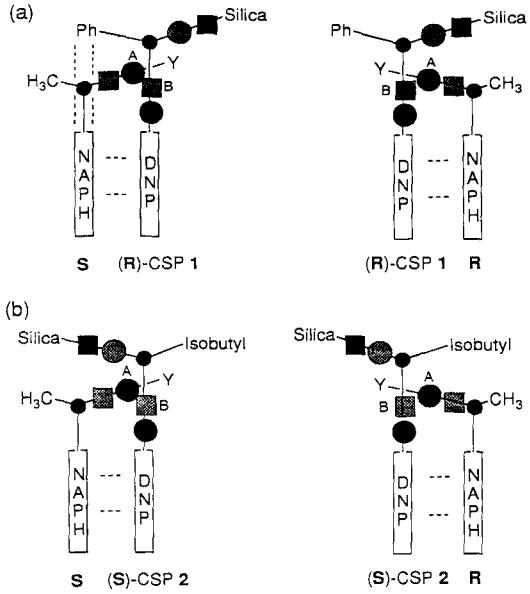


FIGURE 3. The proposed chiral recognition model for resolving N-acyl-α-(1-naphthyl)-ethylamine on (R)-CSP **1** or (S)-CSP **2**. Small solid circle : Methine hydrogen toward the viewer. Large solid circle : Carbonyl oxygen toward the viewer. Large gray circle : Carbonyl oxygen away from the viewer. Solid square : Amide hydrogen toward the viewer. Gray square : Amide hydrogen away from the viewer.

To rationalize those discrepancies between the chromatographic resolution behaviors on CSP **1** and **2**, we propose from study of the CPK molecular models a new chiral recognition model as is shown in Figure 3. In the model shown in Figure 3, (R)-CSP **1**, (S)-CSP **2** and N-acyl-α-(1-naphthyl)ethylamine are schematically represented in conformations which are presumed to be in their lowest energy and hence preferentially populated as described previously.[2] In Figure 3a, (R)-CSP **1** and the analyte are proposed to interact through the face-to-face π-π complexation between the 3,5-dinitrophenyl group (DNP) of the CSP and the 1-naphthyl group (NAPH) of the analyte and through the hydrogen bonding between the carbonyl oxygen (A) of the analyte and the amide N-H hydrogen (B) of the CSP. In this instance, the edge of the 1-naphthyl group (NAPH) of the (R)-analyte confronts the face of the phenyl group of the CSP, invoking the face-to-edge π-π interaction which has been considered as an associative force between aromatic rings.[7] Therefore, the transient diastereomeric (R,S)-complex formed between (R)-CSP **1** and the (S)-analyte might be more stable than the (R,R)-complex and consequently the (S)-enantiomer is retained longer than the (R)-enantiomer on CSP **1**. In this event, the N-acyl alkyl chain (denoted by Y in the model) of the (S)-analyte is oriented alongside the direction of the connecting tether of the CSP and eventually intercalates between the adjacent strands of the CSP. The intercalation of the N-acyl alkyl chain of the (S)-analyte might be evidenced by the trends in the capacity factors of the two enantiomers shown in Figure 1a. The more rapid decrease in the capacity factor of the (S)-analyte than that of the (R)-analyte, which is shown in Figure 1a, suggests in the normal phase chromatography that unfavorable steric interactions are occurring for the (S)-analyte. In consequence, the stability of the originally more stable (R,S)-complex decreases as the N-acyl alkyl chain increases in length and the separation factor decreases continuously as shown in Figure 1b.

The model of Figure 3b for the interaction between (S)-CSP **2** and the analyte shows the same face-to-face π-π complexation between the 3,5-dinitrophenyl group (DNP) of the CSP and the 1-naphthyl group (NAPH) of the analyte and the same hydrogen bonding between the carbonyl oxygen (A) of the analyte and the amide N-H hydrogen (B) of the CSP as that of Figure 3a. In

TABLE 2

Resolution of N-Acetyl-α-(1-naphthyl)-alkylamines **4** on CSP **1** and CSP **2**.[a]

		CSP **1**				CSP **2**			
4	n[b]	k_1'[c]	k_2'[d]	α[e]	Conf.[f]	k_1'[c]	k_2'[d]	α[e]	Conf.[f]
	2	5.76	10.73	1.86	S	7.36	8.29	1.13	R
	3	4.50	8.75	1.94		5.00	6.18	1.24	
	4	3.81	7.89	2.07		4.25	5.74	1.35	
	5	3.34	7.22	2.16		3.68	5.17	1.40	
	7	2.72	6.14	2.26		2.93	4.29	1.46	
	9	2.39	5.60	2.34		2.51	3.77	1.50	
	13	2.01	4.94	2.46		2.16	3.41	1.58	
	15	1.81	4.41	2.43	S	1.95	3.06	1.57	R

a: See the experimental section for the chromatographic conditions. Mobile phase was 20 % 2-propanol in hexane. b: Length of the alkyl chain [-$(CH_2)_n$-H] at the chiral center of analyte **4**. c: Capacity factor of the first eluted enantiomer. d: Capacity factor of the second eluted enantiomer. e: Separation factor. f: Absolute configuration of the second eluted enantiomer. For blanks, the elution orders have been determined to be the same as others in the series based on the TRAC technique.[3]

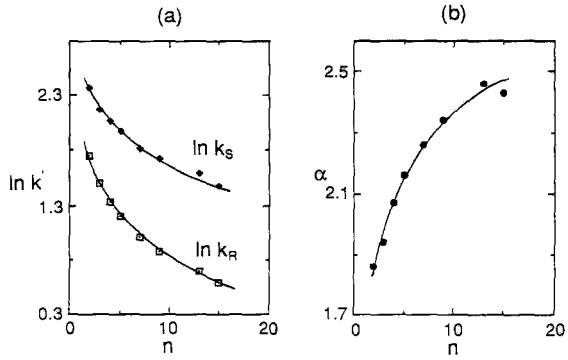


FIGURE 4. The trends of (a) capacity factors and (b) separation factors for resolving N-acetyl-α-(1-naphthyl)-alkylamines **4** on CSP **1**. Chromatographic conditions are given in the experimental part.

this instance, the N-acyl alkyl chain (denoted by Y in the model) of the (R)-analyte is directed between the adjacent strands of bonded phase and toward the silica support. When the N-acyl alkyl chain of the analyte is methyl, the stability of the (S,S)- and the (S,R)-complex is presumed to be identical from the separation factor (α = 1.00) for resolving N-acetyl-α-(1-naphthyl)-ethylamine (n = 1 in Table 1) on CSP **2**. As the N-acyl alkyl chain increases in length, however, the intercalation of the N-acyl alkyl chain of the (R)-enantiomer between adjacent strands of bonded phase experiences more difficulty and the retention of the (R)-analyte decreases more rapidly than that of the (S)-analyte (Figure 2a). In consequence, the stability of the (S,R)-complex decreases and the separation factor increases continuously with the (S)-enantiomer being retained longer on the column (Figure 2b).

The chiral recognition model proposed might be tested by the resolution trends of N-acetyl-α-(1-naphthyl)alkylamines **4** on CSP **1** and **2**. The chromatographic resolution results of N-acetyl-α-(1-naphthyl)alkylamines **4** on CSP **1** and **2** are summarized in Table 2 and the resolution trends are drawn in Figures 4 and 5. As shown in Figure 4, the resolution trends of N-acetyl-α-(1-naphthyl)alkylamines **4** on CSP **1** are exactly opposite to those (shown in Figure 1) of N-acyl-α-(1-naphthyl)ethylamines **3** on CSP **1**. The resolution trends of N-acetyl-α-(1-naphthyl)alkylamines **4** on CSP **2**, which is shown in Figure 5, are quite similar to those shown in Figure 2 for N-acyl-α-(1-naphthyl)ethylamines **3** on CSP **2** but the elution orders are opposite.

All of those resolution trends shown in Figure 4 and 5 can be rationalized based on the chiral recognition model proposed in Figure 3. In resolving N-acetyl-α-(1-naphthyl)alkylamines **4** on CSP **1** and **2**, the methyl group at the chiral center of the analyte in the chiral recognition model shown in Figure 3 is changed to varying alkyl group whereas the N-acyl group is fixed as acetyl. In this instance, the intercalation of the N-acyl alkyl chain of the analyte does not influence the enantioselectivity any more. Instead the alkyl group at the chiral center of the analyte is considered to influence the enantioselectivity based on the chiral recognition model shown in Figure 3. Namely, the alkyl group at the chiral center of the less retained (R)-analyte is directed alongside the connecting

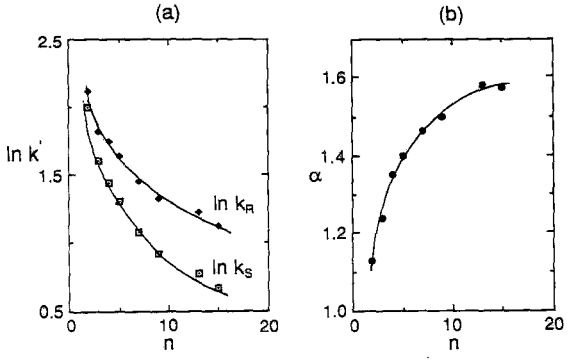


FIGURE 5. The trends of (a) capacity factors and (b) separation factors for resolving N-acetyl-α-(1-naphthyl)-alkylamines **4** on CSP **2**. Chromatographic conditions are given in the experimental part.

tether of CSP **1** and presumably intercalates between adjacent strands of bonded phase. In this instance, the capacity factor of the less retained (R)-analyte on CSP **1** should decrease more rapidly than that of the more retained (S)-analyte and the separation factor should increase continuously as the alkyl group at the chiral center of analyte **4** increases in length. These expectations are consistent with the trends shown in Figure 4.

Similarly, the alkyl group at the chiral center of the (S)-enantiomer of analyte **4** is oriented to the direction of the connecting tether of CSP **2** and probably intercalates between the connecting tethers. In this event, lengthening the alkyl chain at the chiral center of the analyte diminishes the retention of the (S)-enantiomer on CSP **2** more significantly than that of the (R)-enantiomer and consequently increases the separation factor continuously with the (R)-enantiomer being retained longer as is shown in Figure 5. Note again that the retention of the two enantiomers on CSP **2** is initially (when the alkyl group at the chiral center of the analyte is methyl *i.e.* n = 1) the same (see Table 1).

Resolution of N-acyl-α-(1-naphthyl)ethylamines **3** and N-acetyl-α-(1-naphthyl)alkylamines **4** on CSP **5**, the structure of which is identical with that of CSP **1** except the tether direction,[8] might be expected to show the reversed

TABLE 3
Resolution of N-Acyl-α-(1-naphthyl)-ethylamines **3** and N-Acetyl-α-(1-naphthyl)-alkylamines **4** on CSP **5**.[a]

analyte	n[b]	k_1'[c]	k_2'[d]	α[e]	Conf.[f]
3	1	6.55	8.11	1.24	S
	2	6.41	6.91	1.07	S
	3	5.23	5.82	1.11	
	4	4.89	5.58	1.14	S
	6	4.00	4.80	1.20	
	7	3.71	4.57	1.23	
	9	3.20	4.09	1.28	
	11	2.83	3.74	1.32	
	13	2.56	3.48	1.36	
	15	2.34	3.22	1.38	S
	17	2.17	3.02	1.39	S
4	2	2.13	2.51	1.18	S
	3	1.84	2.21	1.20	
	4	1.73	2.03	1.17	
	5	1.64	1.84	1.12	
	7	1.51	1.51	1.00	
	9	1.34	1.34	1.00	
	13	1.02	1.14	1.12	R
	15	0.90	1.05	1.17	R

a: See the experimental section for the chromatographic conditions. Mobile phase was 10 % 2-propanol in hexane for resolving **3** and 20 % 2-propanol in hexane for resolving **4**. b: Length of the N-Acyl alkyl chain [-$(CH_2)_n$-H] of analyte **3** or the alkyl chain [-$(CH_2)_n$-H] at the chiral center of analyte **4**. c, d, e, f: See the foot notes c, d, e and f of **TABLE 2**.

resolution trends compared to those on CSP **1** based on the chiral recognition model proposed in Figure 3. The tether direction of CSP **5** is exactly opposite to that of CSP **1**. Therefore, it is not difficult to imagine that the intercalation effects should be reversed.

Table 3 summarizes the resolution results of N-acyl-α-(1-naphthyl)-ethylamines **3** and N-acetyl-α-(1-naphthyl)alkylamines **4** on CSP **5**. The resolution trends are graphically shown in Figure 6 and 7. In resolving N-acyl-α-(1-naphthyl)ethylamines **3** on CSP **5**, the capacity factor of the less retained (R)-enantiomer decreases more rapidly than that of the more retained (S)-enantiomer and consequently, the separation factor increases continuously as is shown in Figure 6. These trends are exactly opposite to those on CSP **1** and are consistent with those expected from the chiral recognition model proposed in Figure 3. Note that the direction of the tether of the CSP in the chiral recognition model shown in Figure 3a is now reversed and the N-acyl alkyl chain (denoted by Y in the model) of the less retained (R)-enantiomer is oriented to the direction of the tether. In this instance, the N-acyl alkyl chain of the (R)-enantiomer intercalates between adjacent strands of bonded phase, the retention of the (R)-enantiomer decreases more rapidly than that of the (S)-enantiomer and the separation factor increases continuously as the N-acyl alkyl chain of the analyte increases in length. The minimum in the separation factor at n = 2 noted in Figure 6 might be a consequence of conformational factors as described previously.[9,10]

In resolving N-acetyl-α-(1-naphthyl)alkylamines **4** on CSP **5**, the alkyl group at the chiral center of the (S)-enantiomer is expected to be directed alongside the tether of the CSP and intercalate between adjacent strands of bonded phase based on the chiral recognition model proposed. In this instance, the retention of the more retained (S)-enantiomer should decrease more readily than that of the (R)-enantiomer and the separation factor should decrease continuously as the alkyl group at the chiral center of the analyte increases in length. As expected, the capacity factor of the (S)-enantiomer decreases more readily than that of the (R)-enantiomer as shown in Figure 7. In this case, however, the capacity factor of the initially more retained (S)-enantiomer becomes equal to that of the (R)-enantiomer when the alkyl chain at the chiral

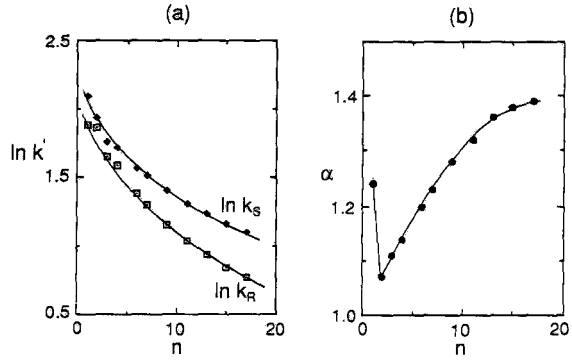


FIGURE 6. The trends of (a) capacity factors and (b) separation factors for resolving N-acyl-α-(1-naphthyl)-ethylamines **3** on CSP **5**. Chromatographic conditions are given in the experimental part.

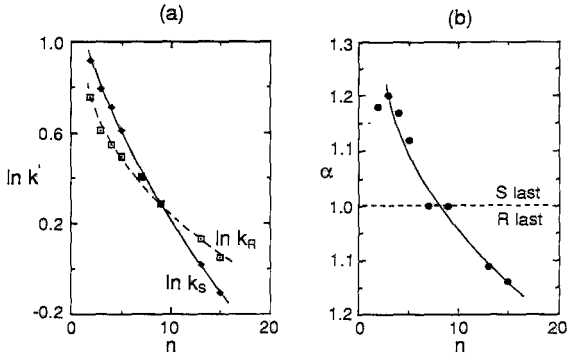


FIGURE 7. The trends of (a) capacity factors and (b) separation factors for resolving N-acetyl-α-(1-naphthyl)-alkylamines **4** on CSP **5**. Chromatographic conditions are given in the experimental part.

center of the analyte reaches at a certain length and after that (R)-enantiomer is retained longer than the (S)-enantiomer as the alkyl chain at the chiral center of the analyte increases in length further, resulting in the inversion of elution order. The overall resolution trends shown in Figure 7 are exactly consistent with those expected from the chiral recognition model proposed.

At this stage, it should be noted that the chromatographic trends described herein for resolving a series of N-acyl-α-(1-naphthyl)alkylamines on CSP **1** and **2** are closely related to earlier observations made for a series of N-acyl-α-(2-fluorenyl)alkylamines as a reviewer pointed out.[11] In the earlier paper, the chromatographic trends for a series of N-acyl-α-(2-fluorenyl)alkylamines have been explained by two competing, opposite sense chiral recognition mechanisms termed the "dipole-stacking" and "hydrogen-bonding" process. However, the two competing chiral recognition mechanisms can not explain the chromatographic resolution trends of N-acyl-α-(1-naphthyl)alkylamines on CSP **5**. In this instance, the chiral recognition model proposed in this study is thought to be more general.

In summary, in this study, we proposed a chiral recognition model which can rationalize the chromatographic trends of resolving N-acyl-α-(1-naphthyl)-ethylamines **3** on Pirkle-type π-acidic CSPs such as CSP **1** or **2**. Based on the chiral recognition model proposed, the chromatographic resolution behaviors of N-acetyl-α-(1-naphthyl)alkylamines **4** on CSP **1** and **2** and the chromatographic resolution behaviors of N-acyl-α-(1-naphthyl)ethylamines **3** and N-acetyl-α-(1-naphthyl)alkylamines **4** on CSP **5**, which has the same structure as CSP **1** except the tether direction, have been successfully rationalized. From these results, we conclude that the chiral recognition model proposed in Figure 3 is quite convincing. However, it should be reminded that mechanistic hypotheses can be disproven but not proven[1,5] and the chiral recognition model proposed in this study might be disproved or solidified as more chromatographic and/or spectroscopic data are accumulated. The efforts to acquire more solid evidences to support the chiral recognition model proposed are currently underway in our laboratory.

ACKNOWLEDGMENT

This work was supported by grants from the Organic Chemistry Research Center sponsored by the Korea Science and Engineering Foundation and from the Basic Science Research Program, Ministry of Education, Korea (BSRI-95-3410).

REFERENCES

1. Pirkle, W. H. and Pochapsky, T. C., *Chem. Rev.*, 89, 347(1989)
2. Pirkle, W. H., Welch, C. J and Hyun, M. H., *J. Org. Chem.*, 48, 5022(1983)
3. Pirkle, W. H., Hyun, M. H. and Bank, B., *J. Chromatogr.*, 316, 585(1984)
4. Pirkle, W. H. and Hyun, M. H., *J. Org. Chem.*, 49, 3043(1984)
5. Pirkle, W. H., Welch, C. J. and Hyun, M. H., *J. Chromatogr.*, 607, 126(1992)
6. Pirkle, W. H. and Welch, C. J., *J. Liq. Chromatogr.*, 14, 1(1991)
7. Grossel, M. C., Cheetham, A. K., Hope, D. A. O. and Weston, S. C., *J. Org. Chem.*, 58, 6654(1993) and references cited therein.
8. The detailed synthetic procedure of CSP **5** will be reported elsewhere.
9. Hyun, M. H., Cho, S. M., Ryoo, J.-J. and Kim, M. S., *J. Liq. Chromatogr.*, 17, 317(1994)
10. Hyun, M. H., Jin, J. S., Ryoo, J.-J. and Jyung, K. K., *Bull. Kor. Chem. Soc.*, 15, 497(1994)
11. Pirkle, W. H. and Däppen, R., *J. Chromatogr.*, 404, 107(1987)

Received: January 27, 1995
Accepted: April 12, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2543-2558 (1995)

NORMAL-PHASE LIQUID CHROMATOGRAPHY ON AMINO-BONDED-PHASE COLUMN OF FLUORESCENCE DETECTED PESTICIDES

F. GARCÍA SÁNCHEZ, A. NAVAS DÍAZ, AND A. GARCÍA PAREJA

Departamento de Química Analítica
Facultad de Ciencias
Universidad de Málaga
29071 Málaga, Spain

ABSTRACT

A liquid chromatographic method with fluorimetric detection is developed for the simultaneous determination of aminocarb, carbaryl, carbendazim, fuberidazole and 1-naphthylacetamide. An amino-bonded column was used and the optimun mobile phase was selected through fitting a mathematical model to experimental data. Separations were accomplished in less than 20 min. Recoveries from spiked grapes ranged from 90 % to 103 % and detection limits between 5.5 x 10^{-3} mg kg^{-1} for fuberidazol and 0.08 mg kg^{-1} for 1-naphthylacetamide and relative standard deviation lower than 6 %.

INTRODUCTION

In normal-phase liquid chromatography only a few quantitative rules exist for predicting how change in mobile phase composition will affect sample

retention [1-3] and locating the overall optimal set of chromatographic conditions is often an unsuccesful trial and error procedure. However, as multifactor methods have been developed for optimization of reverse-phase liquid chromatographic separations [4] they would provide a means to optimization of normal-phase liquid chromatographic separation of pesticides.

Aminocarb, carbaryl, carbendazim, fuberidazol and 1-naphthylacetamide (1-NA) are two insecticides, two fungicides and a plant growth regulator, respectively. They can be found in fruits, vegetables, cereals and other types of crops as a consequence of the pre- and post-harvest treatment. Methods for carbaryl determination include liquid chromatography (LC) [5-8] and gas chromatography (GC) [9,10], also aminocarb is determined by LC [5,11] and GC [12,13]. Determination of carbendazim is mainly by LC [14-16] and also by GC with nitrogen-phosphorous detection after derivatization [17]. Fuberidazol is determined by GC with flame ionization detector after derivatization [18] and no LC methods are available. Determination of 1-NA is by LC [19]. So, the simultaneous determination of the five pesticides is unavailable at present by GC or LC, mainly as a result of their thermal lability or their polar character.

In residue analysis, GC gives usually lower detection limits than LC. However, LC facilitates the direct determination of acidic and thermally labile pesticides without the need for derivatization. For the more polar samples an amino-bonded column is preferable to separations on silica or alumina and because of the nature of the five pesticides a normal-phase LC on amino-bonded column determination is developed. In this work, a multifactor chromatographic optimization is used to find the optimum chromatographic system. The deduced mobile phase is used to develop a rugged residue method of the five fluorescent pesticides in grapes.

EXPERIMENTAL

Instrumentation

The measurements were performed with a Merck-Hitachi liquid chromatograph (Darmstadt, Germany) which incorporates a L-6200 pump, AS-4000 autosampler, L-4250 UV-visible detector and D-6000 interface. Integration was made with a PC/AT computer and the instrumental parameters were controlled by Hitachi-Merck HM software. A Perkin-Elmer LS-50 fluorescence detector (Beaconsfield, UK) placed in series with and after the UV-visible spectrophotometer, is equipped with a xenon discharge lamp and two monochromators. Software Fluorescence Data Manager (FLDM) (LC program) and RS232C interface was used to send information to an external computer. For graphical recording, a NEC Silenwriter2 S60P laser printer was connected to the spectrofluorimeter.

The calculations were made with PC-MATLAB software (MatWorks Inc., Sherborn, MA). To resolve the linear system with more equations than unknowns was used orthogonal factorization. Of the many solution vectors, orthogonal factorization found the best solution in a least squares sense. Surface graphs were obtained by using Surfer software (Golden Software, Golden, CO).

Reagents

The solvents used were tetrahydrofuran (THF), methanol (MeOH), dichloromethane (CH_2Cl_2) and water gradient grade Lichrosolv (Merck); acetic acid, propan-2-ol, and acetonitrile pro analysis (Merck). Water was distilled and deionized or LC grade. The pesticide aminocarb (99%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany); carbaryl (Pestanal, 99%), carbendazim

(Pestanal, 99%) and fuberidazol (Pestanal, 99%) were supplied by Riedel-deHäen (Hannover, Germany) and 1-naphthylacetamide. The solvents were previously sonicated for 30 min and filtered through 0.2 μm Nylon filters. Other chemicals used were celite (particle size 0.01-0.04 mm), sodium acetate and tetramethylammonium hydrogen sulphate (TMA) (Merck).

The stock standard solutions of aminocarb, carbaryl, 1-NA and fuberidazole of 1 g l^{-1} and carbendazim at 0.2 g l^{-1} were prepared by dissolving the pesticide in methanol and stored at 4 °C. Working solutions were prepared by dilution with tetrahydrofuran.

Buffer solution of pH 5.6 was prepared from 0.2 M acetic acid/sodium acetate, and that of pH 6.7 from 0.05 M acetic acid/sodium acetate. This solutions were filtered through 0.2 μm Nylon filters.

Extraction

50 g of grapes were chopped in a food chopper and transfered to a blender cup to be blended with 100 ml of acetonitrile, 25 ml water and 10 g of celite, for 15 min. The homogenate is filtered through fritted glass Buchner funnel (coarse porosity) under reduced pressure, the filtrate transfered to a 500 ml volumetric flask and diluted with acetonitrile: water (50 + 50).

An aliquot (10 ml) of the extract in acetonitrile:water was evaporated to dryness by a rotary evaporator. The residue was taken up in 3 ml of methanol, this solution filtered through 0.2 μm Nylon membranes filters and the filter cake washed with 2 ml of methanol. Finally, the residue was dissolved in 5 ml of THF. This solution was used for the analytical determination.

Chromatographic conditions

The extracts redissolved in THF are analyzed using an analytical column Spherisorb S5 amino, 250 x 4.6 mm id (Phase Separations, U. K.). Flow rate is

TABLE 1
Mobile Phase Composition and Wavelength Program

Time min	THF %	10 mM TMA %	Buffer pH 6.7 %	λ_{exc} nm	λ_{em} nm
0	99.8	0.2	0	255	367
3.6				280	333
7.0				282	340
10.0	99.8	0.2	0		
11.0	98.9	0.1	1	310	345
13.0				285	318
20.0	98.9	0.1	1	285	318

1 ml min^{-1} and injection volume 20 µl. The mobile phase composition and wavelength program for fluorimetric detection are detailed in Table 1. Measure peak area response at retention times of aminocarb (3.42 min), carbaryl (3.80 min), 1-NA (10.23 min), fuberidazol (11.90 min) and carbendazim (15.18 min). A linear regression curve of concentration versus peak area is calculated.

Recovery test

Grapes were spiked with a solution of pesticides in methanol at the levels indicated in Table 2 after checking for the absence of aminocarb, carbaryl, carbendazim, fuberidazole and 1-NA. They were subjected to the whole procedure and determined by the LC method.

RESULTS AND DISCUSSION

The structures of aminocarb, carbaryl, carbendazim, fuberidazole and 1-NA, are presented in Figure 1 in which is indicated the chemical name (IUPAC)

TABLE 2

Estimated Parameters

Compound	a	b	c	d	e
Aminocarb	-1.4580	324.7109	0.0145	-2.6779	-3.2481
Carbaryl	-1.3087	203.1251	0.0131	-1.5930	-2.0303
1-NA	-1.7409	205.0411	0.0175	-1.6388	-2.0452
Fuberidazole	-1.4823	232.4747	0.0149	1.6965	2.3269
Carbendazim	-1.1644	22.1096	0.0117	0.2391	-0.2229

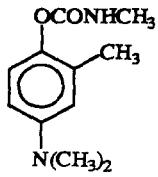
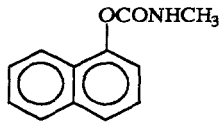
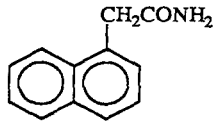
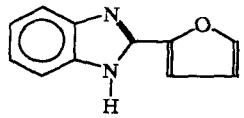
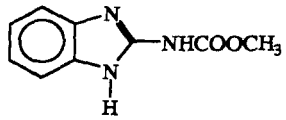
STRUCTURE	RETENTION TIME (min.)	IUPAC NAME
	3.42	4-Dimethylamino-m-tolyl methylcarbamate (Aminocarb)
	3.80	1-Naphthyl methylcarbamate (Carbaryl)
	10.23	1-naphthylacetamide
	11.90	2-(2-furyl) Benzimidazole (Fuberidazole)
	15.18	Methyl benzimidazol-2-yl carbamate (Carbendazim)

FIGURE 1. Structure of the pesticides

and common name [20]. These compounds posses enough natural fluorescence to enable their detection without pre- or post-column derivatization steps. The use of selective fluorescence detector minimizes interferences of coextractives and permits very sensitive determinations, so fluorimetric detection was selected. To maximize sensitivity and selectivity the maximum emission wavelength of each pesticide must be used. From this a wavelengths program for the best mixture resolution is deduced. In Table 1 are the wavelengths selected.

Generally is observed that more polar samples present additional problems in separations on silica or alumina and because of the polar character and hydrogen bonding properties of the studied pesticides an amino-bonded column was selected for the separation. Optimization of mobile phase composition was initially performed by trial and error procedures. Thus, retention data were obtained for a single solvent, methanol (MeOH), dichloromethane (CH_2Cl_2) and tetrahydrofuran (THF) (Solvent strength parameter on aminopropyl column: 0.24, 0.13 and 0.11, respectively); with MeOH the sample is not retained, with CH_2Cl_2 carbendazim, fuberidazole and 1-naphthalenacetamide are strongly retained and by using THF as mobile phase only carbendazim is retained. Binary mixtures of CH_2Cl_2-MeOH, CH_2Cl_2-propan-2-ol, THF-propan-2-ol, THF-water, -acetic acid, -acetate buffer pH 5.6 and acetate buffer of pH 6.7 and ternary mixtures of THF-water-acetate buffer pH 5.6, or THF-propan-2-ol-acetic acid, at different proportions were tested but no adequate separation selectivity was achieved.

Thus, the trial and error procedures were not satisfactory for optimization of the chromatographic system and a multifactor optimization strategy based in the "windows diagrams" [21] was used to predict the mobile phase giving optimal separation. From the previously tested systems, a combination of THF with controlled amounts of aqueous solutions gave the best results. An increase in the water concentration of the mobile phase decreases retention of the

pesticides, so the amino-bonded phase functioned in the normal mode. Because of the basic nature of solutes the addition of surfactants such as TMA (tetramethylammonium hydrogen sulphate) is successful in supressing anomalous retention behaviour and peak shape degradation [22]. So, a binary system THF-TMA aqueous was tuned in composition following a factorial design [16] to select the overall optimum. Experiments were carried out to determine the retention times of each pesticide. The results for each pesticide were fitted to a full second-order polynomial model, including a mathematical interaction effect:

$$\log K = aX_1 + bX_2 + cX_{12} + dX_{22} + eX_1X_2 \qquad (1)$$

where K is the capacity factor for each pesticide *a*, *b*, *c*, *d* and *e* are parameters of the model and X_1 and X_2 represent the percentage in mobile phase of THF and TMA, respectively. Table 2 contains the best least-squares estimates for the parameters of eq.1 for each of the five pesticides. Table 3 gives predicted and observed values of K for the separation under optimal conditions; the close agreement between predicted and observed values demonstrated the ability of the fitted equation to accurately predict capacity factors. Fig. 2 is a representation of resolution in vertical axis against a variable chromatographic factor for the worst separated pair of compounds (1-NA and fuberidazole). The value of the chromatographic factor that corresponds to the top of the tallest peak gives the best possible separation of the worst separated pairs of compounds; the conditions located by the tallest point in the Figure 1 corresponds to a mobile phase composed of 99.8 % THF and 0.2 % TMA 10 mM. This mobile phase separates the five pesticides but the peak of carbendazim has a low efficiency (effective plate number N = 37), to increase efficiency for carbendazim a mobile phase of composition 98.9 % THF, 0.1 % TMA 10 mM and 1 % buffer acetate pH 6.7 is needed. So, to achieve the best separation a gradient elution as the indicated in Table I was used. Chromatographic peaks were examined by asymmetry factor measurements and an asymmetry factor of 1.25 and effective

TABLE 3
Predicted and Observed Capacity Factors at the Optimum Conditions

	Aminocarb	Carbaryl	1-NA	Fuberidazole	Carbendazim
Predicted K	0.15	0.23	1.95	2.51	3.80
Observed K	0.14	0.26	2.39	2.95	3.98
Δk	0.01	0.03	0.44	0.44	0.18

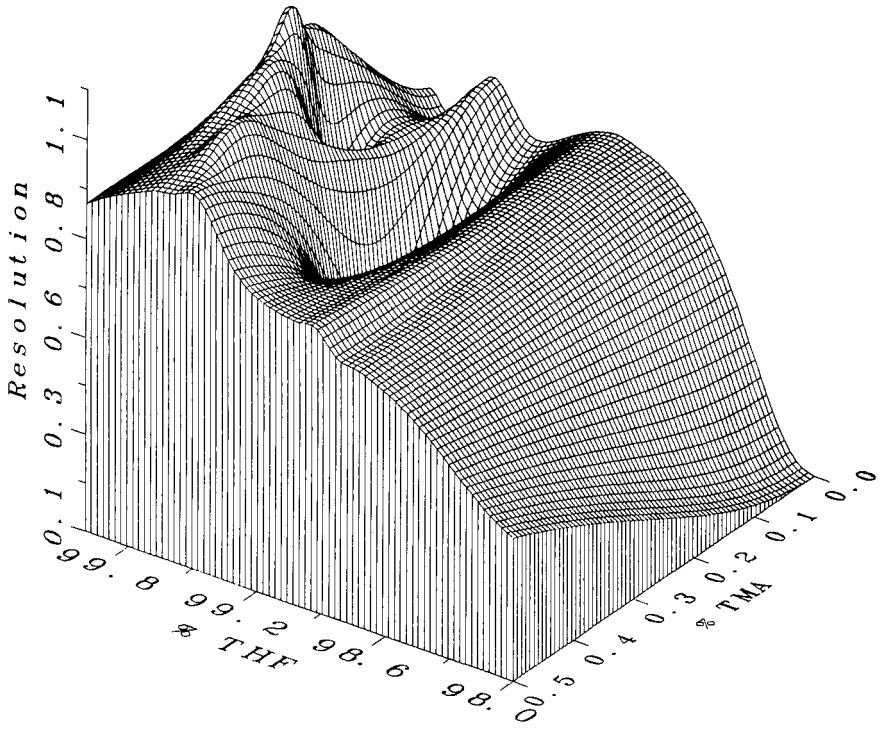


FIGURE 2. Predicted resolution of 1-naphthylacetamide and fuberidazole as a function of percent THF and TMA.

plate number N = 142 for carbendazim were obtained.

Calibration graphs were obtained by plotting the peak areas against the pesticides concentration. The correlation coefficients of the linear regression curves were 0.999, 0.996 and 0.999, over the concentration range 0.1-2 μg ml^{-1} for aminocarb, carbendazim and 1-NA respectively, 0.995 over the concentration range between 0.1-1.5 μg ml^{-1} for carbaryl and 0.998 over the concentration range between 5-100 ng ml^{-1} for fuberidazol.

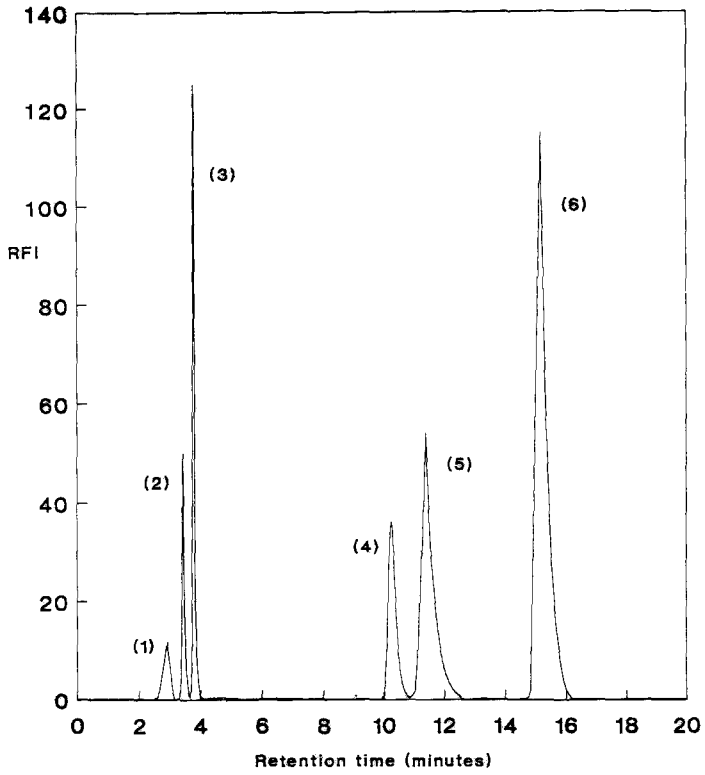


FIGURE 3. Chromatogram of grapes spiked. (1) peak from the matrix, (2) 0.8 μg ml^{-1} aminocarb, (3) 0.8 μg ml^{-1} carbaryl, (4) 0.8 μg ml^{-1} 1-NA, (5) 40 ng ml^{-1} fuberidazole, (6) 0.8 μg ml^{-1} carbendazim,

Application

Aminocarb, carbaryl, carbendazim, fuberidazol and 1-NA can be found in fruits, vegetables, cereals and other types of crops. Grapes were chosen for evaluation of the developed method.

TABLE 4
Recovery of Pesticides from Grapes[a]

Compound	Taken mg kg^{-1}	D_L[b] mg kg^{-1}	C_Q[c] mg kg^{-1}	Recovery %	RSD %
Aminocarb		0.07	0.25		
	1.00			95.0	5.3
	4.00			98.7	4.4
	8.00			100.0	3.1
Carbaryl		0.02	0.07		
	1.00			90.0	4.1
	4.00			94.0	3.1
	8.00			98.7	2.6
1-NA		0.08	0.26		
	1.00			90.0	3.5
	4.00			102.5	2.6
	8.00			99.4	0.8
Fuberidazole		5.5×10^{-3}	18.5×10^{-3}		
	0.05			95.4	2.6
	0.20			99.5	3.1
	0.40			100.8	0.8
Carbendazim		0.02	0.07		
	1.00			95.0	5.7
	4.00			96.2	4.3
	8.00			98.7	3.2

[a] n = 3, [b] detection limit for a signal-to-noise ratio = 3, [c] quantification limit for signal-to-noise ratio = 10.

Three different grape samples were spiked prior the extraction at three levels with a mixture of the pesticides in methanol. Extraction was performed with acetonitrile-water which is widely used for residue screening in non-fatty foods [23-27]. The extract in acetonitrile is evaporated to dryness, redissolved in methanol, concentrated to a 1 ml and diluted with THF. A previous redissolution in methanol is needed to impede residue precipitation. The chromatogram of an extract is shown in Fig. 3. In Table 4 the recoveries obtained are show. It can be observed that the recoveries are excellent giving values between 90 % and 102.5 %. The detection limit defined as the amount that gave a signal to noise ratio of 3 is between 5.5 x 10^{-3} mg kg^{-1} for fuberidazol and 0.08 mg kg^{-1} for 1-naphthylacetamide. The precision given by the relative standard deviation (RSD) is good and below of 6 %.

CONCLUSIONS

This study demonstrated that aminocarb, carbaryl, carbendazim, fuberidazole and 1-NA may be recovered simultaneously from food samples and then quantitatively determined by normal-phase LC on amino-bonded column. Because the unsuccessful separation based on systematic change in the mobile phase a graphical method is developed for optimization of normal-phase liquid chromatographic separation of pesticides.

ACKNOWLEDGEMENT

This work was supported by DGICYT (Proyecto PB93-1006).

REFERENCES

1. L. R. Snyder, T. C. Schunk, Anal. Chem., 54, 1764-1772 (1982).

2. M. C. Hennion, C. Picard, C. Combellas, M. Caude, R. Rosset, J. Chromatogr., 210, 211-228 (1981).

3. R. J. Hurtubise, A. Hussain, H. F. Silver, Anal. Chem., 53, 1993-1997 (1981).

4. D. L. Massart, B. G. M. Vandeginste, S. N. Deming, Y. Michotte, L. Kaufman, Chemometrics: a Textbook, Elsevier, Amsterdam, 1988, p. 305.

5. R. T. Krause, J. Assoc. Off. Anal. Chem., 68, 726-733 (1985).

6. J. G. Brayan, P. R. Haddad, G. J. Sharp, S. Dilli, J. M. Desmarchelier, J. Chromatogr., 447, 249-255 (1988).

7. C. H. Marvin, I. D. Brindle, C. D. Hall, M. Chiba, J. Chromatogr., 503, 167-176 (1990).

8. S. Kawai, K. Goto, K. Kano, T. Kubota, J. Chromatogr., 442, 451-454 (1988).

9. P. N. Kendrick, A. J. Trim, J. K. Atwal, P. M. Brown, Bull. Environ. Contam. Toxicol., 46, 654-661 (1991).

10. H. Bagheri, C. S. Creaser, J. Chromatogr., 547, 345-353 (1991).

11. M. B. Thomas, P. E. Sturrock, J. Chromatogr., 357, 318-324 (1986).

12. A. Cassista, V. N. Mallet, Chromatographia, 18, 305-308 (1984).

13. G. M. Richardson, S. V. Quadri, J. Agric. Food Chem., 35, 877-878 (1987).

14. L. F. López, A. G. López, M. V. Riba, J. Agric. Food Chem., 37, 684-687 (1989).

15. F. Sánchez-Rasero, T. E. Romero, C. G. Dios, J. Chromatogr., 538, 480-483 (1991).

16. D.M: Gilvydis, S.M. Walters, J. Assoc. Off Anal. Chem., 73, 753-761 (1990).

17. H. Steinwandter, Frezenius'Z Anal. Chem., 321, 599-600 (1985).

18. L. Ogierman, J. Chromatogr., 210, 83-92 (1981).

19. W. P. Cochrane, M. Lanouette, R. Grant, J. Assoc. Off Anal. Chem., 63, 145 (1980).

20. The British Crop. Protection Council (Ed.) The Pesticide Manual. A World Compendium. 1983.

21. B. Sachok, J. J. Stranahan, S. N. Deming, Anal. Chem., 53, 70-74 (1981).

22. C. F. Poole, S. K. Poole, Chromatography Today, Elsevier, Amsterdam, 1991, pp. 415.

23. Analytical Methods Committee, Analyst, 102, 869-872 (1977).

24. L. J. Carson, J. Assoc. Off. Anal. Chem., 64, 714-719 (1981).

25. M. B. Taccheo, C. Spessotto, B. Bresin, L. Bagarolo, Pestic. Sci., 15, 612-615 (1984).

26. S. R. Priebe, J. A. Howell, J. Chromatogr., 324, 53-63 (1985).

27. Pesticide and Industrial Chemical Residues, Official Methods of Analysis, AOAC, Arlington, 1990, Chap. 10.

Received: January 27, 1995
Accepted: February 16, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2559-2568 (1995)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ISOLATION AND SPECTROSCOPIC CHARACTERIZATION OF THREE MAJOR METABOLITES FROM THE PLASMA OF RATS RECEIVING RAPAMYCIN (SIROLIMUS) ORALLY*

C. PAUL WANG**, HENG-KEANG LIM, KELVIN W. CHAN, JOANN SCATINA, AND SAMUEL F. SISENWINE
Division of Drug Metabolism
Wyeth-Ayerst Research
CN 8000
Princeton, New Jersey 08543

ABSTRACT

Three major metabolites of rapamycin (M2, M3, and M5) were isolated from pooled plasma of orally dosed rats. Metabolites were extracted from the plasma with ethyl acetate/methanol prior to isolation by HPLC using a Supelcosil SPLC-18, 5μm, 10 x 250 mm column. The mobile phase was a methanol/ammonium acetate linear gradient system. The isolated metabolites were characterized by negative ion FAB MS, ion-spray MS and ion-spray MS/MS. Metabolite M2 is oxygenated in the southern portion of rapamycin and the macrolide ring is opened. M3 is a structural isomer of rapamycin where the lactone ring is opened. M5 is O-demethylated on the C41 methoxy moiety and the macrolide ring is intact.

*This work was presented at the sixth North American ISSX Meeting, Raleigh, NC, October, 1994.

**To whom all correspondence should be addressed.

INTRODUCTION

Rapamycin, [3S-[3R*[S*(1R*,3S*,4S*)],6S*,7E,9S*,10S*,12S*,14R*,15E,17E,19E, 21R*,23R*,26S*,27S*,34aR*]]-9,10,12,13,14,21,22,23,24,25,26,27,32,33,34a-Hexadecahydro-9,27-dihydroxy-3-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c] [1,4]oxaazacyclohentriacontine-1,5,11,28.29(4H,6H,31H)-pentone, (Figure 1), an antitumor and antifungal agent isolated from the fungus *Streptomyces hygroscopicus* (1,2,3), has been found to have potent immunosuppressive activity while exhibiting little toxicity in primates (4,5). Rapamycin is currently under clinical trials as an immunosuppressive agent. The drug demonstrates a synergistic effect when co-administered with cyclosporine (6,7).

We have reported the isolation and characterization of two major *in vitro* degradation products following incubation of rapamycin at 37°C in rat bile or ammonium acetate (pH 8.0) (8). Degradation product A was a macrolide ring-opened hydrolysis product of rapamycin where the C25 ester bond has been hydrolyzed. Degradation product B was a ring-opened isomer of rapamycin. Christians et. al. have isolated two metabolites after *in vitro* metabolism of rapamycin in human liver microsomes and rat small intestinal microsomes (9). One of the metabolites was 41-O-demethyl-rapamycin. The second was a hydroxylated metabolite. However, the identification of *in vivo* plasma metabolites of rapamycin has not yet been reported. The present study was therefore conducted for the isolation and structural characterization of rapamycin metabolites in the plasma of rats after receiving rapamycin orally. A semi-preparative HPLC system was developed for the separation of metabolites.

EXPERIMENTAL

Materials

Rapamycin was obtained from Wyeth-Ayerst Research, Rouses Point, NY. It was formulated in a vehicle consisting of 2% (v/v) ethanol, 0.03% (w/v) Polysorbate 80, 0.37% (v/v) Phosal 50 PG and 96.7% (v/v) water at a concentration of 1.05 mg/ml for the doses used in the study. Ammonium acetate (HPLC grade) was obtained from J.T. Baker (Phillipsburg, NJ). All solvents used in the study were HPLC grade.

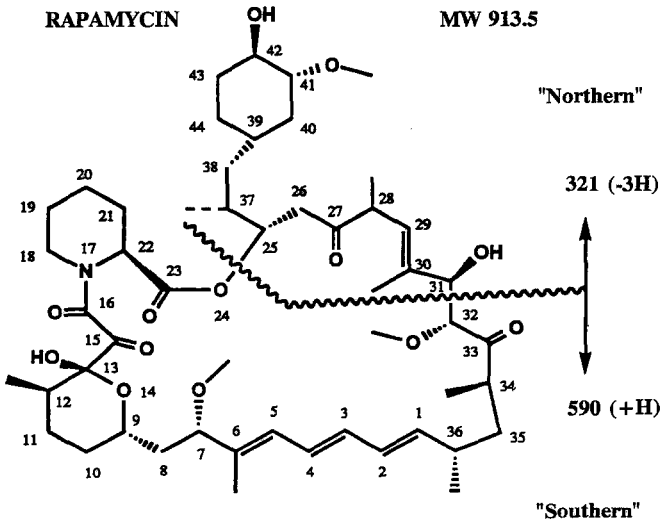


FIGURE 1 Chemical Structure of Rapamycin and its Major Fragmentation Pathway

Animals and Treatment

The study consisted of sixty-four Sprague-Dawley rats (Charles River Laboratory), weighing between 257 and 324 g. Two animals received only vehicle and were used as control. The remaining animals were dosed by gastric intubation with rapamycin at 10.5 mg/kg in a volume of 10 ml/kg. Blood was collected via cardiac puncture into tubes containing heparin at 1 hr post-dosing and immediately placed on ice. Plasma was separated by centrifugation at 2300 rpm for 10 min prior to freezing at -80°C until analysis.

Instrumentation

The HPLC system consists of a Waters 600E system controller and pump (Waters Associates, Milford, MA), a Waters 490E programmable multi-wavelength detector, a Waters U-6K manual injector and a Hewlett-Packard 3390A integrator. Isolation of metabolites was

TABLE 1

Stepwise gradient system used for the isolation of rapamycin metabolites[a]

Time (min)	Flow rate (ml/min)	Percent methanol	Percent 0.05M ammonium acetate
0.0	2.0	62	38
80	2.0	84	16
95	2.0	84	16
100	2.0	62	38

[a]Linear gradient was used between each time point; column was Supelco SPLC-18, 250 x 10.0 mm, 5 μm.

achieved using Supelcosil SPLC-18, 5 μm, 10 x 250 mm column (Supelco, Bellefonte, PA). A Sciex TAGA 6000A upgraded to API III ion spray-mass spectrometer operated under negative ion mode was used for the structural elucidation of metabolites.

Isolation of Rapamycin Metabolites in Rat Plasma

A total of 242 ml of plasma was used for metabolite isolation by HPLC. Five ml aliquots of plasma were mixed with 300 μl of methanol to which 10 ml of ethyl acetate was added and the samples were shaken for 20 min. Following centrifugation at 2500 rpm for 20 min, the ethyl acetate extract was removed and dried under nitrogen. The remaining aqueous layer was re-extracted as above and the combined organic layers were dried under nitrogen prior to reconstitution in 1 ml of methanol/water (65/35). Isolation of metabolites was by HPLC using a Supelco semipreparative SPLC-18 column and a mobile phase gradient listed in Table 1. Detection was by UV absorbance at 276 nm. Eluates containing each of the three individual peaks (M2, M3, and M5) were collected and pooled following repeated injections of the sample. After removal of methanol using a Savant speed-vac concentrator, the remaining aqueous layer was lyophilized to dryness. The dried samples were stored at -80°C prior to being characterized by ion spray mass-spectrometry.

Structural Elucidation of Metabolites by Mass Spectrometry

Metabolites were analyzed using ion spray-mass spectrometry (MS and MS/MS modes). The sample was initially dissolved in either dichloromethane or absolute ethanol and an aliquot was removed for analysis. Samples were then evaporated to dryness under nitrogen at room temperature. Prior to analysis, the residue was dissolved in 80 µl of 20% 4 mM ammonium acetate in methanol. The solution was infused at 4 µL/min into the mass spectrometer using a Harvard Syringe Pump. Mass spectra were acquired at Cornell University using a Sciex TAGA 6000E upgraded to an API III and a home made ion sprayer (10). The mass spectrometer was calibrated in the negative ion mode up to 2000 daltons with polypropylene glycol prior to analysis; final optimization of sprayer position and mass spectrometer conditions for maximum sensitivity was carried out with rapamycin standard (1 mg/mL). The reconstituted residue was analyzed by infusion into the mass spectrometer at
-3.2 Kv and a declustering potential of -68 V. The product ion mass spectra were acquired at collision energies of 50-53 eV and at collision gas pressure of approximately 5 x 10^{12} atoms/cm^2 of Argon.

RESULTS

Identification of Rapamycin and its Metabolites in Rat Plasma

Metabolites M2, M3, and M5 were isolated from a semipreparative column using a gradient mobile phase system. A typical HPLC chromatogram showing separation of rapamycin and its metabolites is shown in Figure 2. The molecular weights (MW) and possible metabolic transformation of these metabolites are summarized in Table 2. Further insight into the possible sites of metabolic transformation was provided by tandem mass spectrometry (MS/MS) as described below. The product ion mass spectrum of rapamycin shows two complimentary diagnostic product ions at m/z 321 and 590 which correspond to the "northern" and "southern" portions of the molecule (Figure 1), respectively. The latter two product ions are useful for monitoring biotransformation of the rapamycin molecule by the mass shift technique (11).

M2: The full scan mass spectrum of M2 shows the presence of a low abundance deprotonated molecular anion at m/z 928.6 which is 16 daltons more than rapamycin. This suggests a

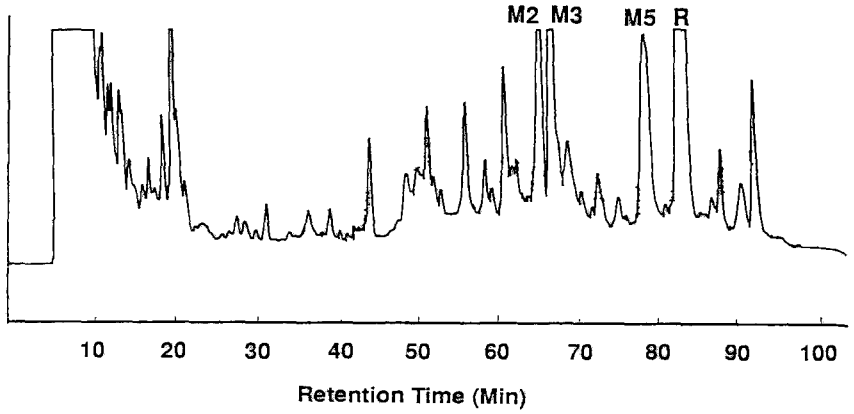


FIGURE 2 HPLC (Supelco LC-18, 25 cm x 10 mm, 5 μm) Chromatogram of Pooled 1 Hour Post-dosing Plasma from Rats Dosed Orally with Rapamycin Showing M2, M3, M5, and Rapamycin (R). The major metabolite peaks were over scale because data was acquired with low attenuation for the collection of metabolite eluates.

TABLE 2

Summary of MS analysis of major plasma metabolites of rapamycin in rats

Metabolite Number	MW[a]	Possible Metabolic Transformation
M2	929	monohydroxylation or epoxidation
M3	913	ring opened isomer
M5	899	monodemethylation

[a]MW based on nominal monoisotopic mass.

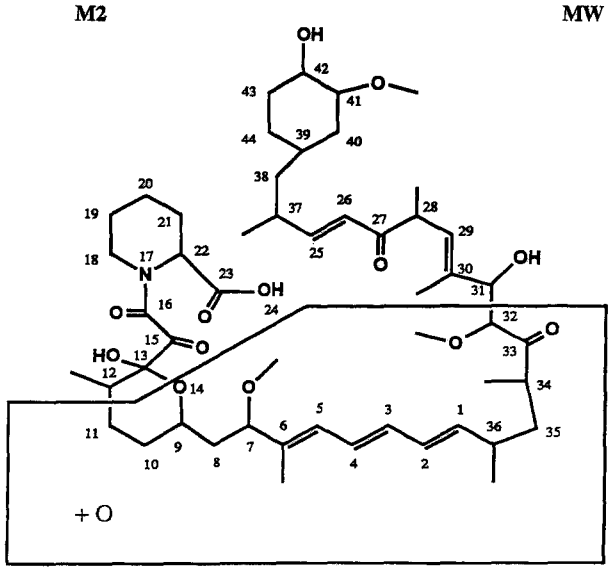


FIGURE 3 Proposed Site of Metabolic Transformation of Rapamycin to Metabolite M2

biotransformation by either aliphatic hydroxylation or epoxidation. The site of biotransformation can be located from the product ion mass spectrum which shows the complimentary fragment ions at m/z 321 and 606. The fragment ion at m/z 321 suggests that the "northern" portion of rapamycin is unchanged. That biotransformation occurred at the "southern" portion of rapamycin is inferred from the ion at m/z 606 which is 16 daltons higher than the corresponding fragment ion from the parent compound. Furthermore, the base peak at m/z 240 indicates that the change is unlikely on carbons 12 to 23. The proposed site of metabolic transformation of M2 is depicted in Figure 3.

M3: This metabolite has the same deprotonated molecular anion (m/z 912.6) as rapamycin in the full scan ion spray mass spectrum. That this metabolite is isomeric with rapamycin is indicated by the similarity of its product ion mass spectrum to that of rapamycin. M3 has been identified and reported as a degradation product of rapamycin (8). Figure 4 shows the structure of M3.

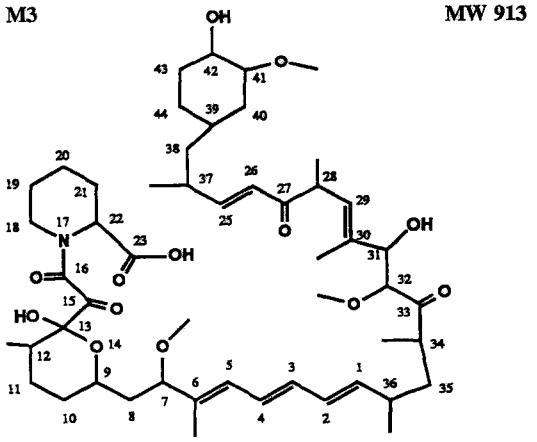


FIGURE 4 Proposed Structure of M3

M5: The full scan ion spray mass spectrum of M5 shows the presence of a low abundance deprotonated molecular anion at m/z 898.6 which is 14 daltons less than rapamycin. This would correspond to the loss of a methyl group. The product ion mass spectrum shows the presence of two complimentary fragment ions at m/z 307 and 590. The weak fragment ion at m/z 590 suggests that the "southern" portion of the rapamycin molecule is intact. Therefore, biotransformation must have occurred on the "northern" portion of rapamycin and is corroborated by the fragment ion at m/z 307 which is 14 daltons less than the corresponding "northern" fragment of rapamycin. Examination of the "northern" portion of rapamycin indicates a metabolic soft spot at carbon 41 which would likely be O-demethylated. The fragment ion at m/z 321 in the product ion mass spectrum may suggest the presence of another minor isomeric demethylated metabolite in this sample, that is, either one of the metabolic soft spots on carbon 7 or 32 in the "southern" portion of rapamycin is likely to be O-demethylated. The structure of M5 is proposed in Figure 5.

DISCUSSION

A semipreparative HPLC procedure using a C-18 column for the isolation of three major plasma metabolites of rapamycin (M2, M3, and M5) has been developed successfully. The

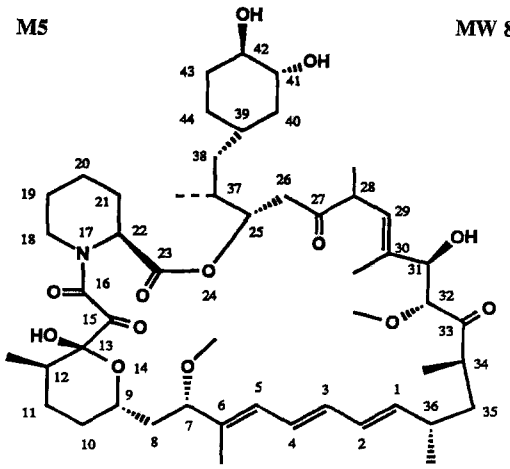


FIGURE 5 Proposed Structure of M5

baseline separation of these metabolites was achieved using a reverse phase gradient system. Metabolites M2, M3, and M5 were isolated and identified. All other minor metabolites were not investigated due to their lower quantities. In our experience, these metabolites are not stable; thus, sample handling is critical for the successful isolation of the metabolites. Rats were given a high dose to produce large quantities of metabolites and therefore to facilitate metabolite isolation and identification. Structural elucidation by mass spectrometry indicated that metabolites M2, M3, and M5 are the oxygenated, ring opened and O-demethylated metabolites of rapamycin, respectively. M3, a degradation product of rapamycin found *in vitro* (8), was also present *in vivo*. The M5 metabolite identified in this study may be the same as the previously reported *in vitro* O-demethylated metabolite of rapamycin at carbon 41 (9). The pattern of metabolism of rapamycin is similar to that of FK506, another immunosuppressive agent, in which major metabolites also produced via demethylation and oxygenation (12).

ACKNOWLEDGEMENT

We would like to acknowledge professor J. Henion at Cornell University for use of the Sciex TAGA 6000E for acquisition of the data.

REFERENCES

1. S.N. Sehgal, H. Baker, C. Vezina. Rapamycin (AY-22989), a new antifungal antibiotic. Fermentation, isolation and characterization. J. Antibiot., 28: 729-32 (1975).

2. C. Vezina, A. Kudelski, and S.N. Sehgal. Rapamycin (AY-22989), a new antifungal antibiotic.I. Taxonomy of the producing streptomycete and isolation of the active principle. J. Antibiot., 28: 721-26 (1975).

3. C.P. Eng, S.N. Sehgal, C. Vezina. Activity of rapamycin (Ay-22989) against transplanted tumors. J. Antibiot., 37: 1231-37 (1984).

4. R.Y. Calne, S. Lim, A. Somaan, D. St. J. Collier, S.G. Pollard, D.J.G. White. Rapamycin for immunosuppression in organ allografting. The Lancet, 227 (1989).

5. S.N. Sehgal and J.Y. Chang: Rapamycin: a new immunosuppressive macrolide. Transplant. Immunol. Lett. 7: 12-14 (1990).

6. P.M. Kimball, R.H. Kerman, D.B. Kahan. Production of synergistic but non-identical mechanisms of immunosuppression by rapamycin and cyclosporine. Transplantation, 51: 486-90 (1991).

7. B.D. Kahan, S. Gibbons, N. Tejpal, S.M. Stepkowski, and T.C. Chou. Synergistic interactions of cyclosporine and rapamycin to inhibit immune performances of normal human peripheral blood lymphocytes in vitro. Transplantation 51: 232-39 (1991).

8. C.P. Wang, K. Chan, R.A. Schiksnis, J. Scatina, S.F. Sisenwine. High performance liquid chromatographic isolation, spectroscopic characterization and immunosuppressive activities of two rapamycin degradation products. J. Liq. Chrom., 17(16): 3383-92 (1994).

9. U. Christians, M. Sattler, H.M. Schiebel, C. Kruse, H.H. Radeke, A. Linck, K.-FR. Sewing. Isolation of two immunosuppressive metabolites after in vitro metabolism of rapamycin. Drug Metab. Dispos., 20: 186-91 (1992).

10. A.P. Bruins, T.R. Covey, and J.D. Henion. Ion spray interface for combined liquid chromatography/atmospheric pressure ionization mass spectrometry. Anal Chem., 59: 2642-46 (1987).

11. F.P. Abramson. Mass spectrometry in pharmacology. In: Suelter CH, Watson JD, editors. Biomedical applications of mass spectrometry. New York: John Wiley & Sons, 289-347 (1990).

12. K. Iwasaki, T. Shiraga, K. Nagase, Z. Tozuka, K. Noda, S. Sakuma, T. Fujitsu, K. Shimatani, A. Sato, M. Fujioka. Isolation, identification, and biological activities of oxidative metabolites of FK506, a potent immunosuppressive macrolide lactone. Drug Metab. Dispos., 21 (6): 971-7 (1993).

Received: February 17, 1995
Accepted: March 6, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2569-2578 (1995)

SEPARATION AND IMPROVEMENT IN DETECTION OF POLYCYCLIC AROMATIC HYDROCARBONS BY REVERSE-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING MICELLAR MOBILE PHASE AND FLUORESCENCE DETECTOR

M. R. HADJMOHAMMADI AND M. H. FATEMI
Institute of Chemistry
Mazandaran University
P.O. Box 311
Babolsar, Iran

ABSTRACT

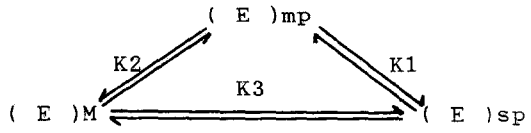
Separation and investigation of Detection Limit (LOD) of Polycyclic Aromatic Hydrocarbons ,PAH,compounds with RP-HPLC using fluorescence detection were studied . Lower LOD for these compounds were observed because of higher fluorescence intensity in the prescence of micellar mobile phase as compared to those of hydroorganics. The codition for optimum separation and enhancement of fluorescence intensity by variation of concentration of coorganic solvent (2-Propanol) and SDS were obtained. Selectivity factor and fluorescence intensity were the basic factors for the optimization of separation and improvement in detection respectively . The condition obtined were 3% of 2-Propanol in 0.035 M SDS.

INTRODUCTION

The PAH compounds are carcinogenic so that the identification and determination of these compounds in environment are very important .Micellar Liquid Chromatography ,MLC, with the use of fluorescence detector has a very good selectivity and sensitivity(1).The use of surfactant solution at concentration below critical micelle concentration ,CMC, as the mobile phase began in the beginning of 1970 and was named Ion-Pair Chromatoghraphy (2).Armstrong and Henry for the first time used the aqueous surfactant solution with concentration above CMC as the mobile phase in RP-HPLC which was named MLC (3,4) .

The advantages of micellar mobile phase over hydroorganic one are nontoxcicity , nonflammability ,its lower price and selectivity change because of increasing in the number of equilibrium.The higher fluorescence intensity of some compounds in micellar media as compared to hydroorganic mobile phases causes lower LOD with the use of fluorescence detector (5) .

The distribution of sample in hydroorganic mobile phase occurs between stationary and mobile phase while in MLC the sample molecule could be distributes between micelle-stationary phase,micelle-mobile phase and mobile -stationary phase, which could improve separation . The distribution of sample in micellar media are shown as the following :



K1=distribution coefficient of sample between mobile and stationary phase
K2=distribution coefficient of sample between mobile phase and micelle
K3=distribution coefficient of sample between micelle and stationary phase
(E)mp=Representation of sample in aqueous mobile phase
(E)M= Representation of sample in micelle
(E)sp=Representation of sample in stationary phase

The capacity factor of the sample is related to the above coefficients and is shown by the following equation (6,7) :

$$1/k' = ([M]mp . K2)/R.K1 + 1/R.K1 \quad (1)$$

Where R is a constant its value depending on the amount of phase ratio.

Equation (1) shows that there is a linear relationship between 1/K' vs micelle concentration [M]mp . The value of K2 can be obtained by dividing intercept to slope of this line. The value of K2 represents the tendency of the sample for interaction with micelles . Since the value of K1 for nonpolar compounds is very high, equation (1) can be written as the following(8) :

$$1/K' = [M]mp/R.K3 \quad (2)$$

$$K3 = K1 / K2$$

Micellar media can increase the fluorescence intensity because the quenching of the excited molecule by collision is decreased (9) . The reports show that efficiency of micellar mobile phase is lower than hydroorganic mobile phase (10) .To overcome this problem a coorganic solvent such as 2-PrOH was added to micellar mobile phase and column held at the temperature of 40'c.

In this project separation and improvement of detection of some PAH compounds by MLC were done with optimization of coorganic solvent and surfactant concentration.

EXPERIMENTAL

The PAH compounds used were from Fluka Company.In some case these compounds were purified by sublimation and recrystallization. The stock solutions were prepared in methanol with concentration range of 100-400 ppm . De-ionized double distilled water that was filtered by 0.45μm filter for the preparation of mobile phase .The needed mobile phase was made by dilution of 0.400M SDS solution with water and coorganic solvent . The HPLC pump were model 6000A with UV detector model 440A and fluorescence detector model 420-Ac all from Waters Company (Waters Assoc. Inc., Milford , MA).

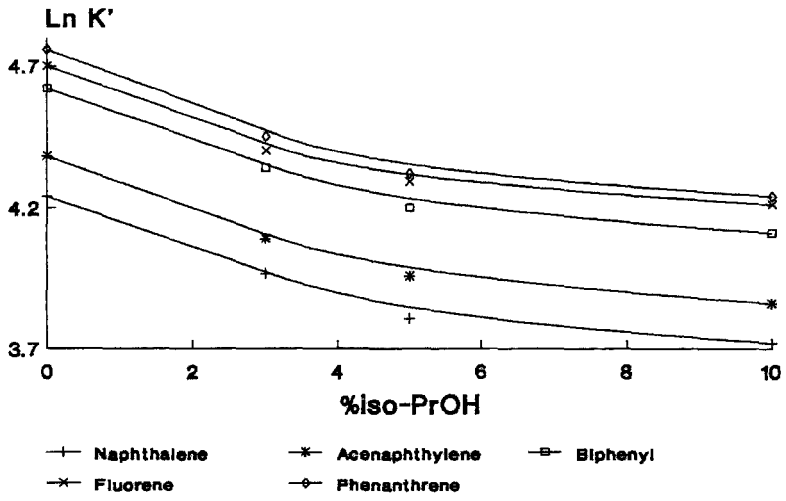


FIGURE 1. Effect of iso-PrOH percentage on K'

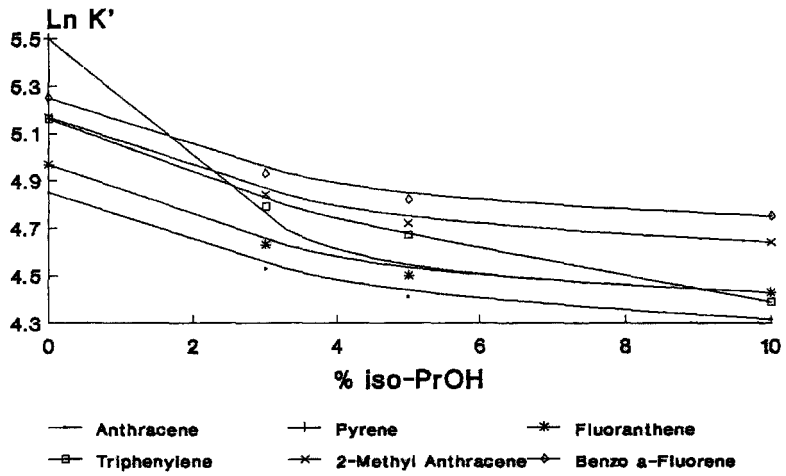


FIGURE 2. Effect of iso-PrOH percentage on K'

RESULTS and DISCUSSIONS

A)Effect of coorganic solvent on resolution

Effect of coorganic solvent in mobile phase on K' for PAH compounds were shown in fig. 1 & 2 . The results show a decrease in K'values with increase in concentration of coorganic solvent in mobile phase .The value of selectivity factor decreases as the percentage of coorganic solvent increases in mobile phase for all PAH compounds except for Triphenylene and 2-Methyl-Anthracene as shown in fig.3 . The viscosity and assymetric factor increases at higher concentration of 2-Propanol in mobile phase . The minimum amount of coorganic solvent needed for improvment in efficiency was about 3% and obtined experimentally.

B) Effect of SDS concentration on K' and selectivity factor

I) Relationship between K'and SDS concentration

Results shown in table 1 and 2 represent a linear relation between 1/K' vs SDS concentration .The distribution coefficient of sample between mobile phase and micelle (K2) derived from equation (1) by dividing slope to intercept of this line . The value of K2 can be only measured for Acenaphthene because of its lower hydrophobicity as compared with the other PAH compounds ,which they have negative intercepts . The negative intercept is related to the tendency of direct transfer of sample molecule from micelle to stationary phase.In these cases equation (2) gives better result and 1/K'vs surfactant concentration is quite linear.

II) Effect of SDS concentration on selectivity factor

The selectivity factor between adjucent peaks decreases for all PAH compounds (except for compounds 4,5

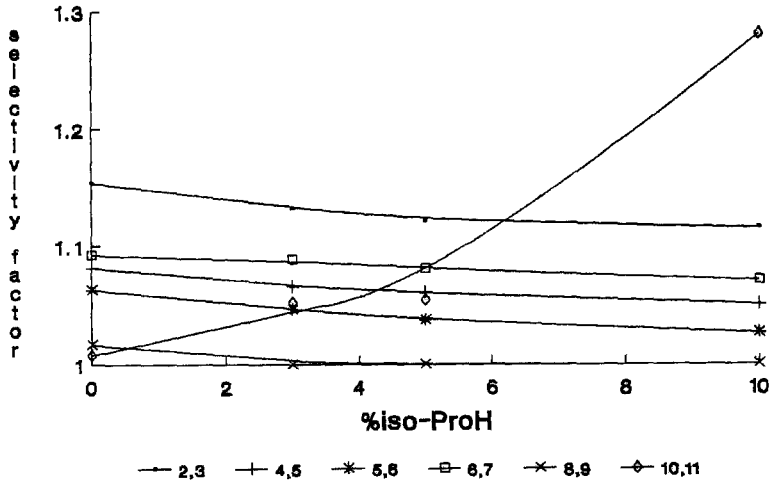


FIGURE 3. Effect of iso-PrOH percentage on selectivity factor

TABLE 1. Parameters Obtined From curve of 1/K' vs [SDS]
r=correlation coefficient , Int.=Intercept

Sample	Acenaphthene	Naphthalene	Acenaphthylene	Biphenyl	Fluorene	Phenanthrene
Code	1	2	3	4	5	6
r	0.99	0.99	0.99	0.99	0.99	0.98
Slope	2.1	0.42	0.37	0.34	0.30	0.30
Int.	0.037	-0.0032	-0.0026	-0.0023	-0.0013	-0.0015
K2	56	-	-	-	-	-

TABLE 2. Parameters Obtined From curve of 1/K'vs [SDS]
r=correlation coefficient, Int.=Intercept

Sample	Anthra cenee	Pyrene	Fluora nthene	Triphe nylene	2-Methyl Anthracen	11H-Benzo aFluorene
Code	7	8	9	10	11	12
r	0.99	0.98	0.99	0.99	0.99	0.98
Slope	0.28	0.26	0.26	0.23	0.22	0.20
Int.	-0.0013	-0.0021	-0.0021	-0.0024	-0.0016	-0.0018
K2	-	-	-	-	-	-

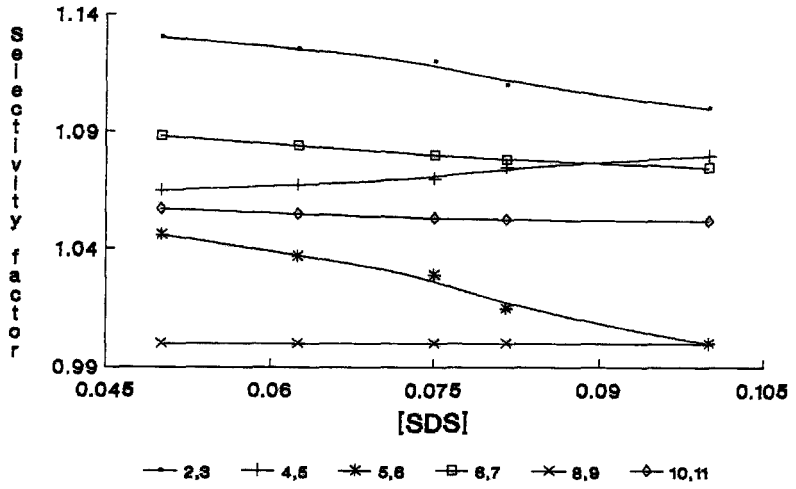


FIGURE 4. Effect of SDS conc. on selectivity factor

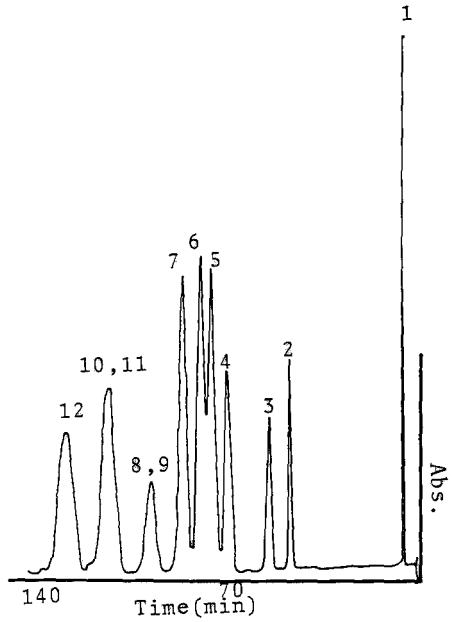


FIGURE 5. Typical chromatogram for separation of PAH compunds .
HPLC conditions : 0.035M SDS,%3 iso-PrOH(v/v) as MP with flow rate of 1 ml/min. , colum (3.9*150 mm) C18 (sample number as shown in tables 1 & 2)

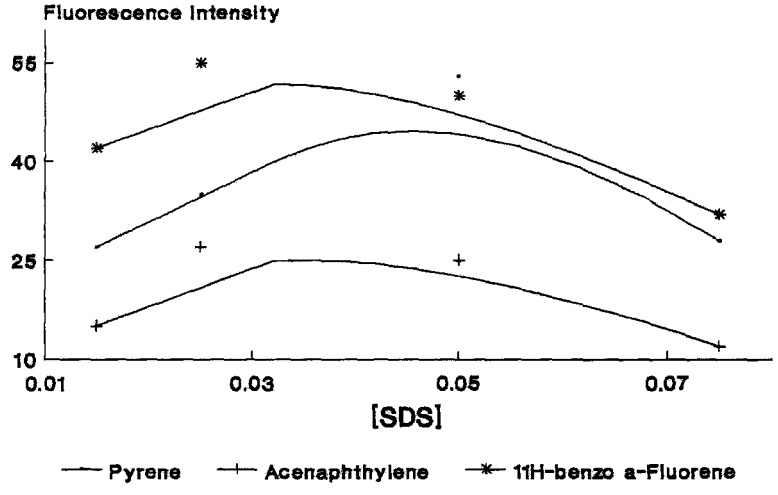


FIGURE 6. Effect of SDS consentration on fluorescence intensity

TABLE 3. The Comparison of LOD for Some PAH Compounds in Micellar and Hydroorganic Mobile Phase

Sample	Em.Filter (nm)	Ex.Filter (nm)	LOD 0.035M SDS	LOD 70/30 H2O/MeOH
Pyrene	400	338	1.74 ng/ml	17.4 ng/ml
Acenaph thylene	425	360	0.1 ppm	0.27 ppm
11 H-Benzo a-Fluorene	425	360	0.27 ppm	0.48 ppm

and 8,9) as the concentration of SDS increases in mobile phase (fig.4). The overall resolution at the lower SDS concentration increases at the expense of higher time of analysis. To improve separation of PAH compounds both SDS concentration and flow rate of mobile phase separately were changed.The experimental results show that the best resolution could be achieved with 0.035M SDS at 1.0 ml/min flow rate (fig.5).

III) The effects of surfactant concentration on fluorescence intensity

Effect of SDS concentration on fluorescence intensity of PAH compounds where their suitable absorption and emmission filter were available were studied . The fluorescence intensity vs SDS concentration as shown in fig.6 passes a maximum point . The lower fluorescence intensity before maximum point is related to the smaller ratio of number of micelles to sample molecules while after maximum it is due to impurities which quench the excited molecules. The maximum fluorescence intensity could occur where the number of micelles and sample are equal. The comparison of LOD in micellar mobile phase with hydroorganic mobile phase for some PAH compounds at same retention time is shown in table 3 . The results show a lower LOD for micellar mobile phase as compared to hydroorganic one.

REFERENCES

1. S.A.Wise, L.C.Sanders, W.E.May, J. of Chrom. 642:329 -349 (1993)

2. J.J. Kirkland , L.R.Snyder , Introduction to modern Liquid Chromatography , John Wiley & Sons , Inc. New York (1979)

3. A.H.Radgers, J.K.Straster , M.G.Khaledi, J.of Chrom. 636:203-212 (1993)

4. D.W.Armstrong, S.L.Henry, J. Liquid Chrom. 3:657-662 (1980)

5. F.Tomassela, L.Cline Love, J.Anal.Chem. 62:1315-1319 (1990)

6. D.W.Armstrong , N.Faruk, J.Anal.Chem. 53:1662-1666 (1981)

7. M.Arumyanart , L.Cline Love , J.Anal.Chem. 56:1557-1560(1984)

8. M.F.Borgerding, F.H.Quina,W.L.Hinze, J.Anal.Chem.60: 2520-2527 (1988)

9. R.Humphry-Baker, J.Am.Chem.Soc. 102:847-848(1980)

10. B.B.Crag, J.Kirkland, A.Rodgers, J. Chem.Phys. Lett. 49:437-440 (1977)

11. M.F.Borgerding , W.L.Hinze , L.D.Stafford ,G.W.Flup, J.Anal.Chem. 61:1353-1358(1988)

12. H.Sing, W.Hinz, Anal.Lett. 15(A3):221-243(1982)

Received: February 1, 1995
Accepted: February 17, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2579-2592 (1995)

SYNTHESIS AND CHARACTERIZATION OF A POLYMERIC FLUOROCARBON-DIAMINE REVERSED PHASE WEAK ANION EXCHANGE SILICA HPLC COLUMN PACKING

N. D. DANIELSON*, J. WANGSA, AND S. A. SHAMSI
Department of Chemistry
Miami University
Oxford, Ohio 45056

ABSTRACT

Polymeric fluorocarbon-diamine silica column packings were synthesized by first reacting a co-polymer of chlorotrifluoroethylene and vinylidene fluoride (Kel-F 800) with piperazine and then reacting this product with aminopropyl silica. This mixed mode reversed phase-weak anion exchange HPLC column and a hydrocarbon (C-8) weak anion exchange silica HPLC column were compared for the separation of aromatic organic acids. Although the fluorocarbon column was generally less retentive than the hydrocarbon one, good resolution and fast analysis of simple mixtures was still possible. Nucleotides were retained longer on the fluorocarbon column. Good alkaline pH stability of the polymeric fluorocarbon-diamine silica column packing was exhibited.

INTRODUCTION

Porous silica modified with polymers for use as HPLC column packings combine the rigidity of a metal oxide with the stability of polymeric materials. A recent review (1) summarizes these chromatographic packings prepared either by physical adsorption of polymers, covalent attachment of polymers, or graft polymerization to silica. Generally amines such as polyN-vinyl pyrrolidone (2), polyethyleneimine (often crosslinked) (3), or poly1-vinyl,2,4 triazole (1) can be effectively adsorbed to porous silica for aqueous weak anion exchange chromatography of proteins. Reaction of polymers such as poly(succinamide)(4) or co-polymers of N-vinyl-pyrrolidone and acryloyl chloride (1) with aminopropyl derivatized silica has been done to prepare hydrophilic weak anion exchange packings with good shielding of the silica surface. Ion exchange of proteins and size exclusion chromatography of biological samples including viruses have been done with these columns. These packings were not considered to have any reversed

phase retention. Graft reactions of vinyl monomers such as styrene or methylmethacrylate with vinyl silyated suspended silica have produced a wide variety of HPLC packings with different polarities (5,6). A review (7) of fluorocarbon bonded phases on silica HPLC packings indicated as a class these reversed phase packings were less retentive than their corresponding hydrocarbon counterparts. Reports of fluorocarbon polymers grafted onto silica HPLC column packing are quite unusual. Sabarov et. al (8) has polymerized tetrafluoroethylene on the surface of silica and characterized these sorbents as having low adsorption activity to most classes of compounds. Effective isolation of t-RNA from proteins and plasmids was demonstrated. Recently, a chlorotrifluoroethylene-vinylidene fluoride co-polymer (Kel-F 800) has been crosslinked to aminopropyl silica to form a fluoropolymeric reversed phase weak anion exchange HPLC column packing. This mixed mode column was particularly useful for the separation of aliphatic anionic surfactants using a naphthalenedisulfonate-acetonitrile mobile phase for indirect photometric detection (9,10). The ion exchange retention was deemed most important for the indirect photometric detection mechanism to be operable.

In this report, we have expanded the versatility of this class of fluorocarbon reversed phase-weak anion exchange packings by modification of the Kel-F 800 first with an organic amine before reaction with the aminopropyl silica. The resultant Kel-F 800 piperazine aminopropyl HPLC column packing was characterized for both ion exchange and reversed phase retention of organic anions. Comparison of this packing to a commercial hydrocarbon based weak anion exchange column was made. Good pH stability of the polymeric fluorocarbon-amine column was found.

EXPERIMENTAL

Instrumentation

The liquid chromatograph was composed of a Model 510 HPLC pump, a Model U6K injector equipped with a 20 μL loop, a Model 490 programmable multiwavelength detector, and a Model 730 data module integrator, all from Waters Chromatography (Milford, MA). A Model LP-121 low pulse dampener from Scientific Systems, Inc (State College, PA) was added to the pump to eliminate baseline noise especially at low detector settings. The HPLC columns used were either the fluorocarbon polymer piperazine (FPP) silica type synthesized in house or the mixed mode C-8 weak anion exchange column purchased from Alltech Associates (Deerfield, IL).

Reagents

Kel-F 800, a copolymer of 78% cholorotrifluoroethylene and 22% vinylidene fluoride, was provided by the 3M Co. (Minneapolis, MN). This polymer (number average M.W. = 29,400; weight

average M.W. = 75,700) (11) is soluble in tetrahydrofuran (THF) but not in acetonitrile or methanol. For the data in Table 1, 10 μm RSil silica from Alltech Associates (Deerfield, IL) was used. For the preparation of the FPP HPLC column packing, 10-14 μm Zorbax diameter silica microspheres provided by the DuPont Co were used and treated as described in reference (12). Basically, the silica was refluxed with 10% HCl and 10% HNO_3 acid overnight, respectively. After the acid treatment, the particles were thoroughly washed with distilled water until pH tested to neutrality, filtered, and then dried at 125 ^{0}C in vacuum overnight. The silica particles were sintered at 850 ^{0}C for one hour to give an optimum surface area of about 40 m^2/g. Then the particles were refluxed in a 75 ppm HF solution overnight. After filtering and a thorough washing with distilled water, the particles were refluxed in distilled water overnight. Finally, the particles were filtered and dried at 125 ^{0}C in a vacuum oven overnight.

The γ-aminopropyltrimethoxysilane, toluene (dry 99%, grade), and piperazine were obtained from Aldrich (Milwaukee, WI). Mobile phases were prepared from HPLC grade acetonitrile (ACN) (EM Science, Morristown, NJ) and distilled water purified with an E-pure water treatment system(Bransted/Thermolyne Corp., Dubuque, IA). The common sample solutes such as the organic acids were obtained from a variety of sources. The biochemical sample components separated in Figures 7 and 8 were received from Sigma Chemical Co (St. Louis, MO).

PROCEDURE

Aminopropyl silica was synthesized in batches by reacting 0.50 g of silica with 120 mL of 2.5% γ-aminopropyltrimethoxysilane in toluene for 18 hours at 80 ^{0}C. The apparatus consisted of a three neck round bottom flask with a thermometer, a N_2 purge line, and a Dean-Stark trap connected to a condenser to remove the ethanol. The resulting particles were filtered and washed 3 times with toluene and then with acetone. Several batches of aminosilica were dried in vacuum at 120^{0}C for 8 hours before use. Reproducibility of the amino silica synthesis for 2 batches was within 0.5% for C and 0.2% for H and N.

The fluorocarbon diamine silica packings were synthesized using a two-step procedure. Kel-F 800 and the crosslinking agent such as piperazine at a 3:1 by weight stoichiometric ratio were reacted with stirring in 150 mL of THF at 57 ^{0}C for 24 hours. Then a known weight of aminosilica was added to the flask and the reaction was allowed to proceed another 46 hours. This two step procedure as opposed to a one step process involving adding the Kel-F 800, crosslinking agent, and aminosilica all together resulted in higher %C, %N and %F from elemental analysis. In the

Table 1 Elemental Analysis Data for Kel-F 800-amine Polymer-Aminosilica (RSil) Reaction Products

Kel-F 800/Amine Polymer	Kel-F 800-amine Polymer/ Amino Silica Reaction Ratio	%C	%H	%N	%F
	0:1	7.96	2.10	2.61	
Kel-F 800/Piperazine	1:1	10.51	2.41	1.85	3.92
Kel-F 800/Piperazine	2.5:1	13.75	3.63	5.52	4.01
Kel-F 800/Piperazine	5:1	7.61	4.73	8.03	4.78
Kel-F 800/N,N' Diethylethylenediamine	5:1	15.63	5.31	7.59	3.93
Kel-F 800/N,N' Dimethyl 1,6-hexanediamine	5:1	29.47	6.73	7.80	4.22

one step process, the %N of the silica product did not increase significantly when a crosslinking agent was added. Initially, small scale reactions were tried; for the 5:1 Kel-F aminosilica packing, 1 g of the Kel-F-piperazine product was reacted with 0.2 g aminosilica. However, this reaction was scaled up to 1 g batch quantities of aminosilica to prepare the column packing. The light orange silica products were washed copiously with THF and light microscopy showed discrete uniform particles with little agglomeration. Elemental analyses were carried out by Microanalysis Inc. (Wilmington, DE). The FPP column (0.40 x 25 cm) was packed from a silica slurry in methanol at 8,000 psi using a Haskel pneumatic pump. The FPP column as well as the Alltech mixed mode column were conditioned with the desired mobile phase for about an hour before use. All separations were carried out at ambient temperature. Retention factors, k', were calculated in the usual way; the solvent front (injection peak) was taken as the retention of an unretained peak.

RESULTS AND DISCUSSION

A characterization of the reaction of Kel-F 800 with diamines to form various silica products was carried out first. Table 1 shows elemental analysis data for a variety of Kel-F 800 aminosilica

reaction products. As the reaction ratio increases from 1:1 to 5:1 for the piperazine product, both the %N and %F increase as expected. Above the reactant ratio of 5:1, agglomeration of particles was observed. Straight chain agents such as N-methyl substituted ethylenediamine and hexanediamine gave similar products as evidenced by the %N and %F data. However agglomeration of the microspheres was evident in these two products.

Based on the fluorescent reaction of aminosilica with o-phthaldehyde and mercaptoethanol, it was estimated about one-third of the aminopropyl groups were unreacted. A schematic of the reaction product in agreement with the proposed mechanism (13) is shown in Figure 1. It is likely that some of the piperazine groups are not crosslinked and secondary amine groups are present. In addition, reaction of both the starting Kel-F polymer and the final amine Kel-F 800 aminosilica product with bromine decolorized both samples indicating some degree of unsaturation is present. Proton NMR also showed a strong band upfield at about 6 ppm for the polymer-silica product indicating double bonds are likely present.

The (FPP) HPLC column packing was based on Zorbax silica with a lower surface area to make this packing more comparable to the C-8 hydrocarbon anion exchange (HAE) column. Elemental analysis data for these two columns are shown in Table 2. The ion exchange capacity from %N for the HAE column can be calculated to be about 0.5 mmole/g (14). For the aminopropyl silica used to make the FPP column, an ion exchange capacity of 0.45 mmole/g was calculated. However, it is likely that many of these aminopropyl groups are not accessible due to steric hindrance by the fluorocarbon polymer. Some of this ion exchange capacity is undoubtedly replaced by the piperazine groups which should be more accessible. However, the increase of %N by 0.35% due to the piperazine is quite modest and it is likely the ion exchange capacity of the FPP column is significantly less than the the HAE column.

A chromatographic comparison of the FPP column was made to the commercial HAE column. The retention factor, k', for a wide variety of analytes was determined as a function of pH on both columns as shown in Figures 2 and 3. However, the mobile phase ionic strength was required to be a factor of 10 greater for the HAE column to get reasonable retention times. For the simple inorganic anion nitrate, retention decreases as the pH increases as expected. Phenol was weakly retained since only reversed phase and no ion exchange interaction was possible. The retention of monoprotic acids increases with mobile phase pH because of their increasing ionization. Many of these organic acids tend to show a maximum retention at pH 5.5 where both the ion exchange and the hydrophobic retention mechanisms should play roles. This maximum retention at pH 5.0 was also observed for short chain organic acids on a C-8 HAE column (15). The retention of benzoic acid and sorbic acid is greater on the HAE column as compared to the FPP column. Both columns showed strong retention for the diprotic aromatic acid, phthalic acid. Note that the k' of phthalic acid (pka_2 = 5.40) is more than double at a pH of 5.5. A further increase in pH decreases

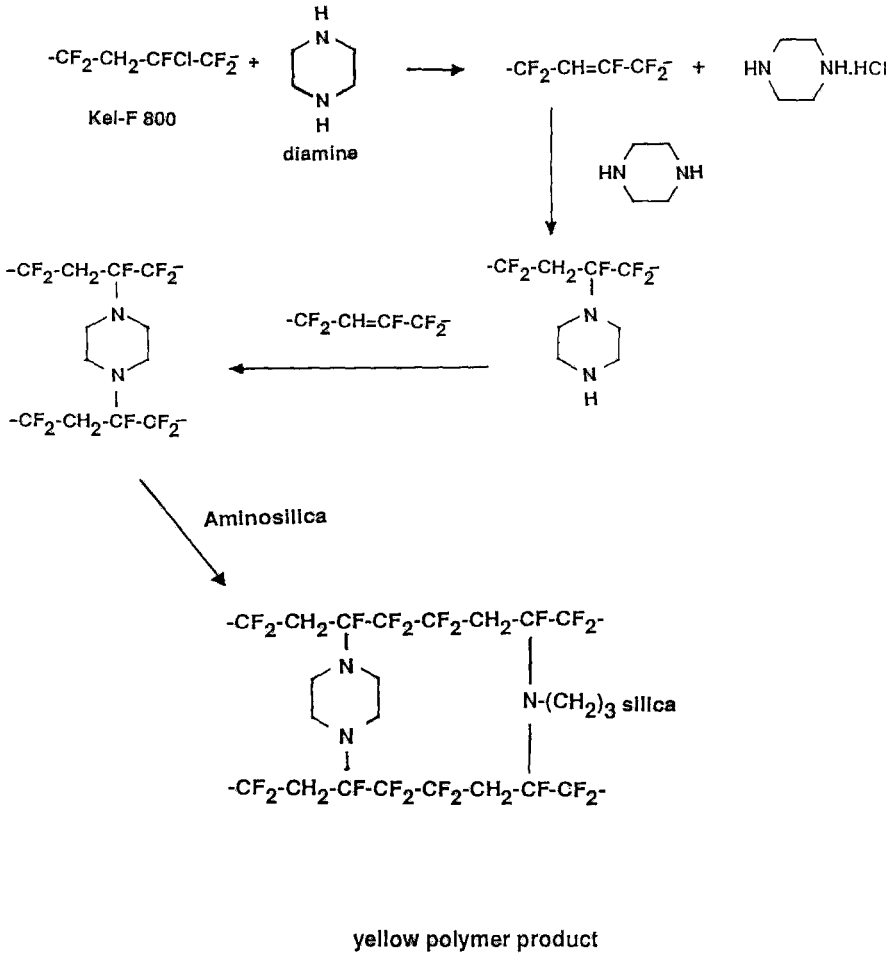
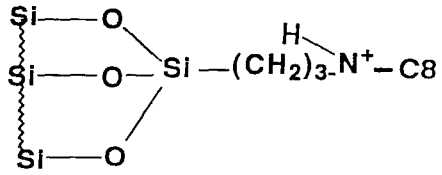


Figure 1: Proposed synthesis of the fluoropolymer-diamine weak anion exchange silica.

Table 2 Elemental Analysis Data for Fluorocarbon Polymer-Piperazine (FPP) / Aminosilica (Zorbax) and the C-8 Hydrocarbon Weak Anion Exchange Packings (Alltech)

Silica Product	%C	%H	%N	%F
Aminopropyl Zorbax	2.55	0.54	0.59	-
FPP(5:1) Zorbax	7.66	1.34	0.94	7.53
C-8 Weak Anion Exchange*	8.20	-	0.60	-

* Proposed structure for C-8 hydrocarbon weak anion exchange packing (Alltech):



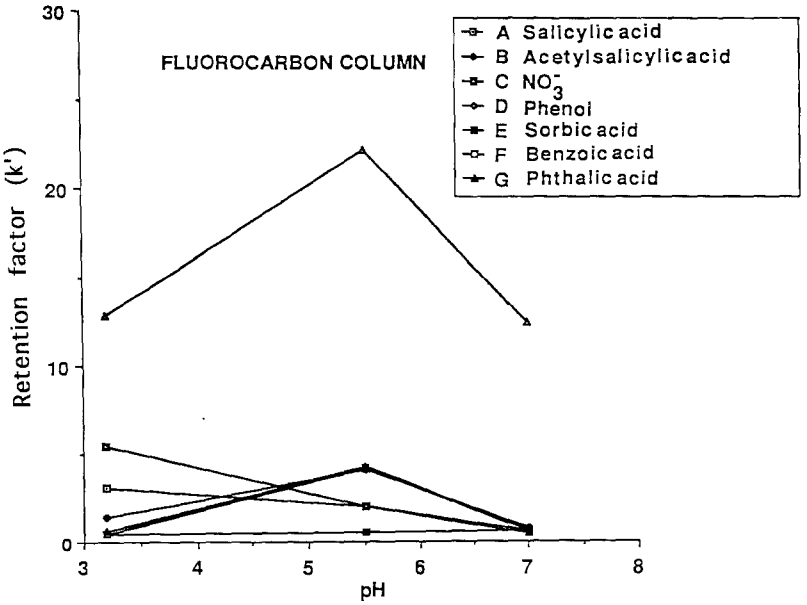


Figure 2: Retention of organic acids as a function of eluent pH on the fluorocarbon column.

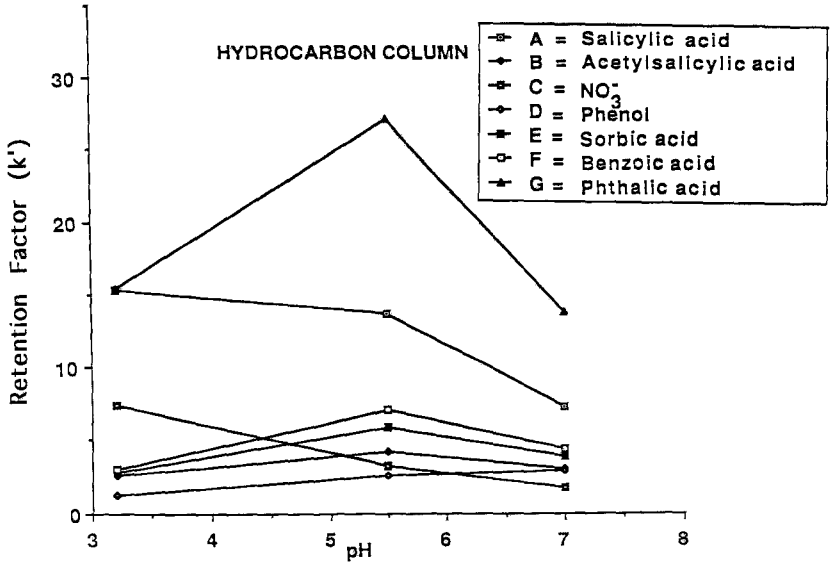


Figure 3: Retention of organic acids as a function of eluent pH on the hydrocarbon column.

the retention as the amine functionalities on both columns become deprotonated. Citric acid being triprotic could not be eluted at this ionic strength on either column. Representative chromatograms for 5 of these solutes are shown in Figure 4. The retention of the first four monoprotic carboxylic acids is about twice as long on the HAE column as that on the FPP column. The diprotic acid, phthalic acid, is eluted at 19 min on the HAE column compared to 14 min on the FPP column. The separation of the isomers maleic acid and fumaric acid isomers with baseline resolution was also possible using 10% methanol-90% acetate buffer (data not shown).

Figure 5 shows the effect of pH on the separation of salicylic acid (pKa= 2.97) and acetylsalicylic acid (pka = 3.49) on three columns: aminosilica, FPP, and HAE. Retention of these salicylates is substantially longer on the aminosilica and HAE columns as compared to the FPP column. Although the concentration of acetate is a factor of 10 higher in the mobile phase for the HAE column, retention of salicylate is still substantial on this column as compared to the aminosilica column. Since the ion exchange capacities of aminosilica and the HAE columns are similar, reversed phase retention must be strong on the HAE column even at 50% ACN. Indeed at pH 3.2, the retention of salicylate is 22 min on the HAE column compared to 12 min on the aminosilica column. The retention of the salicylates on the FPP column is substantially shorter

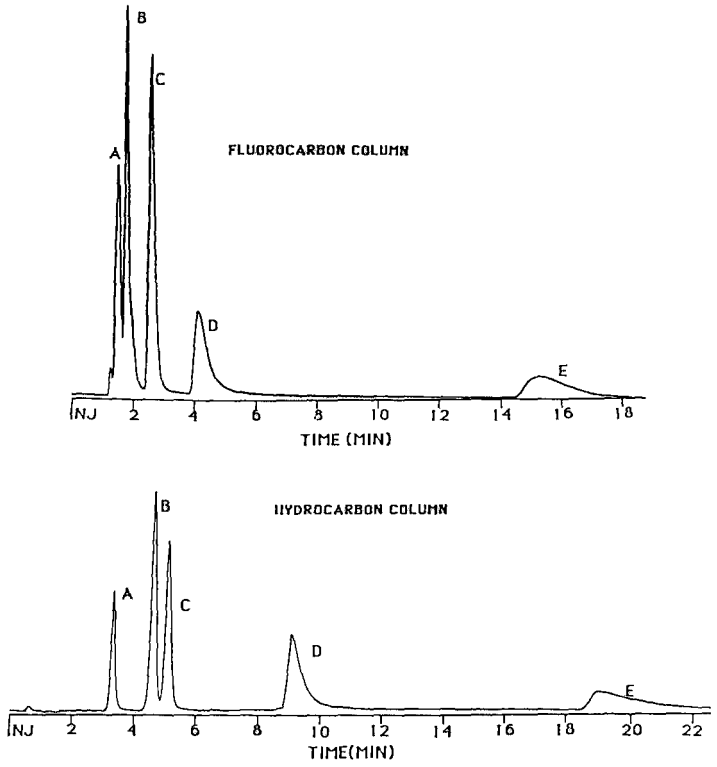


Figure 4: Separation of organic acids. Conditions: flow rate 1.0 mL/min, injection volume 20 μL, UV detection at 254 nm, 0.1 AUFS. Solute concentration: 25 ppm of (A) benzoic acid (B) sorbic acid (E) phthalic acid, and 100 ppm of (C) acetylsalicylic acid and (D) salicylic acid. Mobile phase is 50% 0.010 M sodium acetate/ 50% ACN pH = 3.2 for the fluorocarbon (FPP) column and 50% 0.100 M sodium acetate /50% ACN pH = 7.0 for the hydrocarbon (HAE) column.

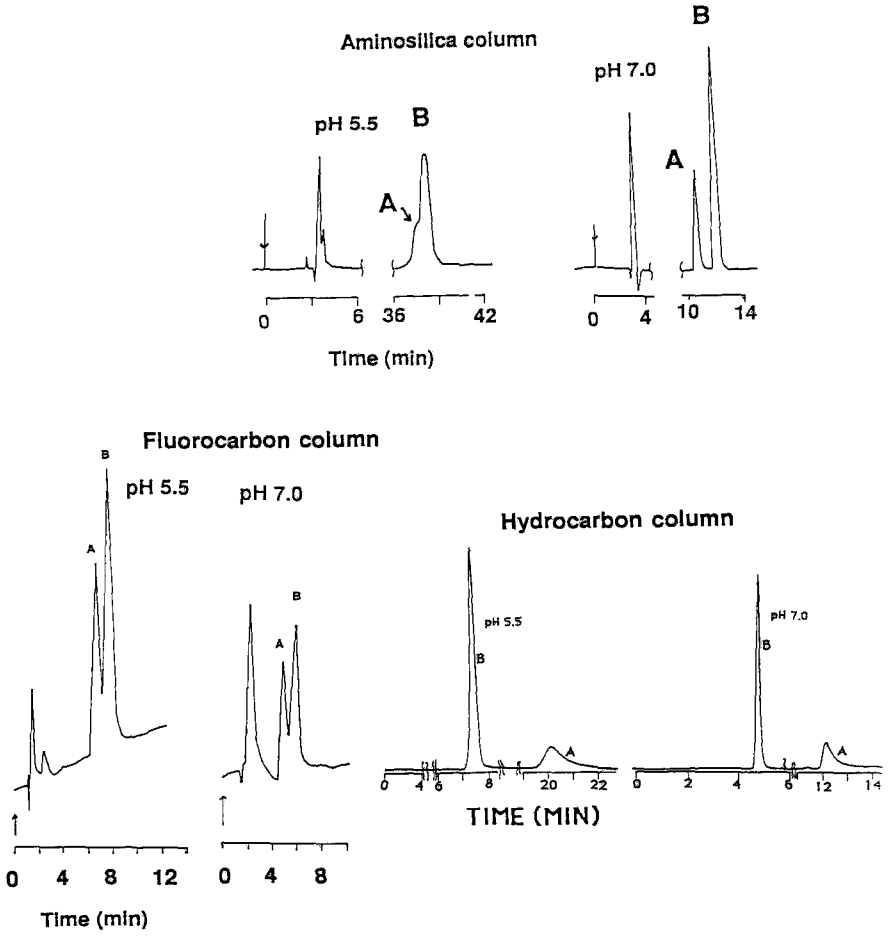


Figure 5: Effect of eluent pH on the retention of salicylate (A) and acetylsalicylate (B) on the aminosilica, fluorocarbon (FPP), and hydrocarbon (HAE) columns. Conditions are similar to those in Figure 4. Mobile phases: 50% 0.010 M sodium acetate, pH = 5.5 or 7.0/ 50% ACN for the aminosilica and the FPP columns; 50% 0.10 M sodium acetate, pH = 5.5 or 7.0/ 50% ACN for the HAE column.

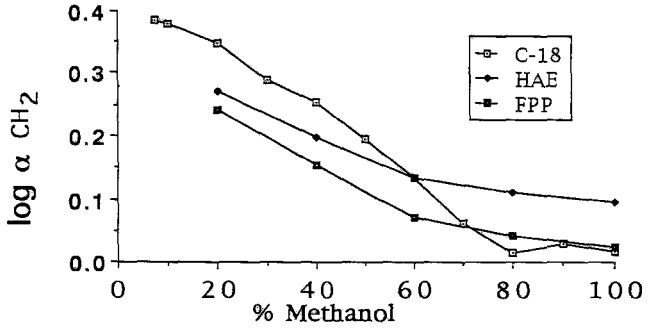


Figure 6: Plot of log α for phenylethyl alcohol and benzyl alcohol as a function of methanol content in the mobile phase for a C-18 silica, the HAE, and FPP columns.

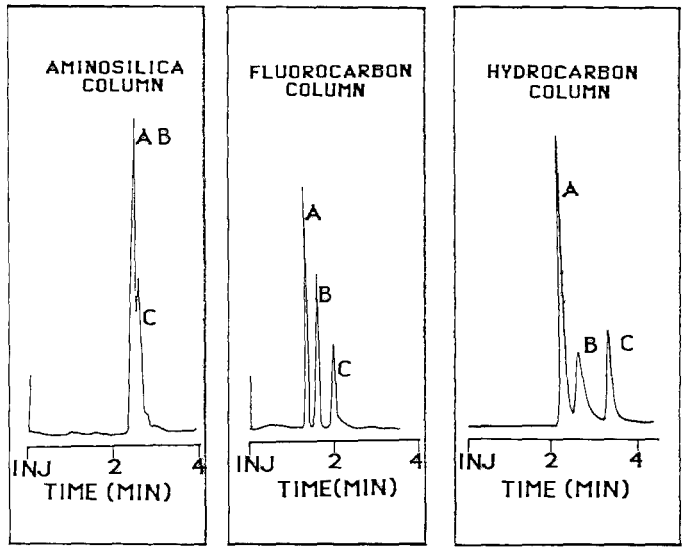


Figure 7: Separation of 10 ppm each of (A) pyridoxamine (B) pyridoxal and (C) pyridoxine. Conditions are the same as in Figure 4 except mobile phase is 20% ACN/80% H_2O on the aminosilica column and the fluorocarbon (FPP) column but 5% ACN/ 95% H_2O, 0.01% CH_3COOH on the hydrocarbon (HAE) column.

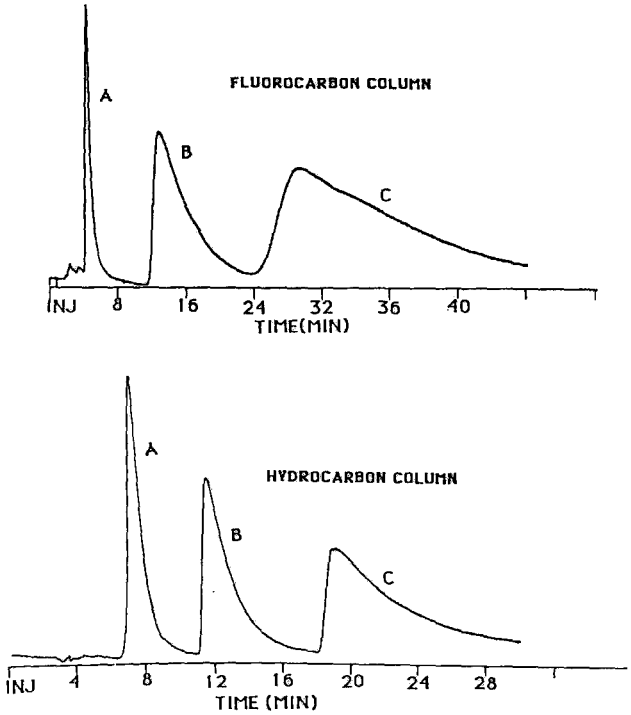


Figure 8: Separation of nucleotides on the fluorocarbon (FPP) and hydrocarbon (HAE) columns. Conditions: flow rate 1.2 mL/min, injection volume 20 μL, UV detection at 254 nm, 0.1 AUFS, mobile phase. 0.200M sodium acetate - 10% ACN. Solute concentration: 25 ppm each of (A) adenosine monophosphate (AMP), (B) adenosine diphosphate (ADP), (C) adenosine triphosphate (ATP).

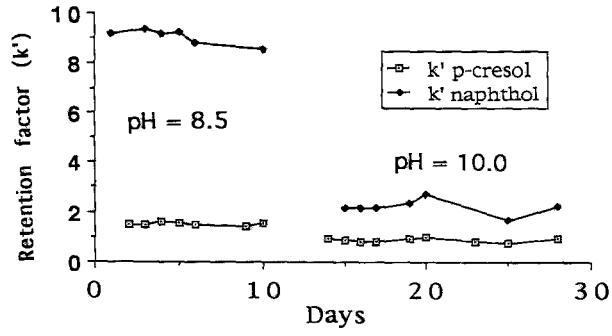


Figure 9: Retention factors for p-cresol and naphthol as a function of time the FPP column was in contact with a basic mobile phase (65% methanol-35% borate buffer, pH 8.5 or 10).

indicating both less retention by ion exchange as compared to aminosilica and less retention by reversed phase as compared to the HAE column. Interestingly, the retention order of the salicylates at pH 3.2 for both the aminosilica and FPP columns switches to that found for the HAE column at any of the three pH values. The retention order difference between the HAE column and the FPP column is unclear but may be due to differences in the type of base silica or in the hydrophobic retention mechanism.

The selectivity for the methylene group ($^{\alpha}CH_2$) when plotted semilogarithamically versus % organic solvent in the mobile phase has been found to be linear to about 75% methanol for reversed phase silica HPLC columns (16). Similar plots for both the reversed phase-ion exchange FPP and HAE columns were compared to that found for a C-18 column (Figure 6). The log α data is consistently ordered C-18 > HAE > FPP to about 70% methanol which is expected considering the relative hydrophobicities of C-18, C-8, and fluorocarbon substituents. Above 70% methanol, the retention of the methylene group for the mixed mode columns tends to plateau out. This may signal a change in the reversed phase retention mechanism. In high organic content mobile phases, retention is thought to be based on attractive dispersive interactions between the solute and the stationary phase not the repulsion of the solute out of the mobile phase to the stationary phase which is more likely in high water content mobile phases (17).

Figure 7 shows the separation of compounds that constitute vitamin B_6 (pyridoxamine, pyridoxine and pyridoxal) on both mixed mode columns. The retention is the reverse of that found for cation exchange chromatography (18). Retention is dominated by hydrophobic effects since little resolution is seen on the hydrophilic aminosilica column. This is consistent with the separation of this same vitamin B_6 mixture on a picolyl Kel-F polymeric HPLC column which is also a mixed mode reversed phase weak anion exchange column (19). Addition of acetic acid to the mobile phase used for the HAE column was necessary to reduce silanol interaction and ensure a narrow peak shape for pyridoxine. Even with 20% ACN- 80% water (no acetic acid), the retention of pyridoxine was about 10 min with a 3 min peak width at half height.

The separation of the highly charged nucleotides, adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP), was carried out on both mixed mode columns (Figure 8). Retention of these solutes was stronger on the FPP column even though the same mobile phase was used for both separations. Fluorocarbon polymers have been previously reported effective for the separation of nucleotides (20).

Finally, stability of the FPP column was found to be good for at least 4 weeks even when operated at pH 8.5 or 10 (Figure 9). At pH = 8.5, the k' for naphthol was about 3 times longer than that at pH = 10. The retention factors for the mixture of p-nitrophenol, cresol, and naphthol remained constant within 10% during each pH trial period.

ACKNOWLEDGEMENT

We thank John Hofferberth for assistance with some of the separations and the E.I. DuPont Company for financial support.

REFERENCES

1. A. E. Ivanov, V. V. Saburov, V. P. Zubov, Advances in Polymer Science., 104: 1-41 (1992).
2. L. Letot, J. Lesec, C. Quivoron, J. Liq. Chromatogr., 4: 1311 (1981).
3. A. Alpert, F. E. Regnier, J. Chromatogr., 185: 375 (1979).
4. A. Alpert, J. Chromatogr., 266: 23 (1983).
5. B. B. Wheals, J. Chromatogr., 107: 402 (1975).
6. M. Hanson, K. K. Unger, Trends. Anal. Chem., 11: 368 (1992).
7. N. D. Danielson, L. G. Beaver, J. Wangsa, J. Chromatogr., 544: 187 (1991).
8. V. V. Saburov, M. R. Muidinov, S. A. Guryanov, A. D. Kataer, S. I. Turkin, V. P. Zubov, Zh. Fiz Khim., 65: 2692 (1991). Chem. Abstr. 115: 274916e.
9. S. A. Maki, J. Wangsa, N. D. Danielson, Anal. Chem., 64: 583 (1992).
10. N. D. Danielson, S. A. Shamsi, S. A. Maki, J. High Resol. Chromatogr., 15: 343 (1992).
11. L. G. Beaver, N. D. Danielson, Polymer Bulletin., 30: 47 (1993).
12. J. Kohler, J. J. Kirkland, J. Chromatogr., 385: 125 (1987).
13. K. L. Paciorek, L. C. Mitchell, C. T. Lenk., J. Polymer Sci., 45: 405 (1960).
14. S. A. Shamsi, N. D. Danielson, J. Chromatogr Sci., in Press.
15. R. Saari-Nordhaus, J. M. Anderson., Anal. Chem., 64: 2283 (1992).
16. B. L. Karger, J. R. Gant, A. Hartkopf, P. H. Weiner, J. Chromatogr., 128: 65 (1976).
17. J. G. Dorsey, W. T. Cooper, Anal. Chem., 66: 857A (1994).
18. R. C. Williams, D. R. Baker, J. A. Schmit, J. Chromatogr Sci., 11: 618 (1973).
19. M. Kruempelman, N. D. Danielson., Anal. Chem., 57: 340 (1985).
20. D. A. Usher, Nucleic Acids Res., 6: 2289 (1979).

Received: February 25, 1995
Accepted: March 7, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2593-2609 (1995)

SOLID-PHASE EXTRACTION STUDY AND RP-HPLC ANALYSIS OF LAMOTRIGINE IN HUMAN BIOLOGICAL FLUIDS AND IN ANTIEPILEPTIC TABLET FORMULATIONS

I. N. PAPADOYANNIS*, A. C. ZOTOU, AND V. F. SAMANIDOU
Laboratory of Analytical Chemistry
Chemistry Department
Aristotle University of Thessaloniki
GR - 54006 Thessaloniki, Greece

ABSTRACT

An efficient off-line solid-phase extraction (SPE) of lamotrigine, 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine (LTG), a new antiepileptic drug, from human serum and urine, prior to high-performance liquid chromatographic analysis, was tested and optimized. High extraction recoveries were achieved from C_8 Bond Elut cartridges (200mg/3ml), using acidic acetonitrile for the elution of LTG and the internal standard, 3,5-diamino-6-(2-methoxyphenyl)-1,2,4-triazine. Isocratic reversed-phase high-performance liquid chromatographic (RP-HPLC) analysis on octylsilica, using a Lichrosorb RP-8, 5μm, 250x4.6 mm i.d. column and a mobile phase consisting of 0.05M acetate buffer pH5.6 and acetonitrile (72:28 v/v) proved to be sensitive and rapid. The identification of LTG was performed by UV detection at 306nm. The method detects approximately 0.9 ng of LTG on-column, using a 20-μl loop, and linearity holds from approximately 0.044 to 7.8 μg/ml in standard solutions. In plasma and urine, the limits of detection are 1.1 and 1.2ng respectively, while linearity holds from approximately 0.087 to 3.49μg/ml. The proposed method was also used for the direct analysis of antiepileptic tablets.

* To whom correspondence and reprint requests should be addressed.

INTRODUCTION

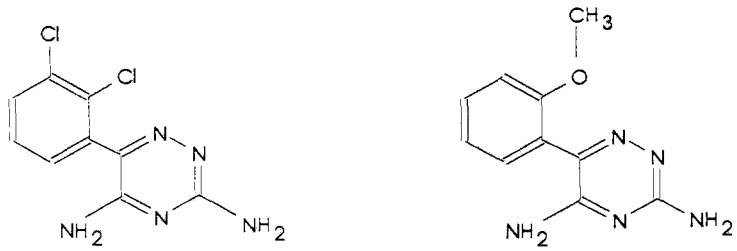
Lamotrigine, 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine (LTG), is a new anticonvulsant, which belongs to the triazine class of compounds and has a cationic character and a pK_a value of 5.5 [1], thus differing from the conventional antiepileptic drugs. It acts by blocking the sensitive sodium channels and inhibiting the release of the excitatory transmitter glutamate [2].

The pharmacokinetics of LTG after single and multiple oral doses, from 30-240 mg, in normal humans show good and rapid absorption. The mean t_{max} is less than 3 hours and after a single oral dose of LTG (120mg), at least 70% on average is excreted in the urine and of that total 90% is the glucuronide metabolite. A linear relationship between dose administered and maximal plasma concentration of LTG has been observed, which indicates that saturation of absorption or elimination mechanisms do not occur in the therapeutic dose range. LTG does not induce its own metabolism and inhibition or saturation of drug-metabolizing enzymes do not occur. The predicted therapeutic plasma concentration of LTG in humans is 1-3 μg/ml [3].

LTG is metabolized by glucuronidation. Its glucuronide is not detected in plasma but appears in urine at a rate equal to the elimination of LTG from plasma. Doig and Clare [4] have elucidated the structure of urinary metabolites of LTG by means of thermospray liquid chromatography-mass spectrometry and found that man produces two N-glucuronides of LTG, of which only the major one (the N-2-glucuronide) is cleaved by the action of β-glucuronidase. They concluded that the most likely structure of the minor metabolite is the N-5-glucuronide.

In literature appears a limited number of publications dedicated to the analysis of LTG, since it is a relatively new antiepileptic drug and they almost exclusively describe HPLC methods. Only two publications describe different methods, namely a radioimmunoassay [5] and an immunofluorimetric assay for the determination of human plasma concentrations of LTG [6].

Normal-phase HPLC methods for the determination of LTG in human plasma and/or urine have been described in clinical reports [1-3,7]. More recent reports describe RP-HPLC methods for the quantitation of LTG in human plasma [8-10] or ion-pairing RP-HPLC methods in guinea pig blood and urine [11]. RP-HPLC is more appropriate for the direct quantitation of the highly polar glucuronide metabolite of LTG, when therapeutic monitoring of the LTG clearance is necessary.



A B

FIGURE 1. Molecular structures of (A) LTG and (B) the internal standard.

Most of the cited methods make use of liquid-liquid extraction for the isolation of LTG from endogenous compounds in the samples prior to HPLC analysis, except for one [11] that uses SPE. The objective of the present work was to find the optimum SPE conditions for achieving the highest possible recovery of LTG from biological matrices, therefore a comparative study of SPE conditions, appearing in the LTG literature for the first time, was performed. The developed RP-HPLC method is rapid, offers higher sensitivity compared to the existing methods and can be applied with high precision and accuracy to the direct analysis of antiepileptic drugs and to the analysis of extracted plasma and urine samples.

EXPERIMENTAL

Materials and Reagents

LTG, 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine, the internal standard, 5-diamino-6-(2-methoxyphenyl)-1,2,4-triazine (BW725C78), (Fig.1) and the LTG pharmaceutical preparations (Lamictal tablets) were obtained from Wellcome Foundation (London, UK). Stock solutions of LTG (109μg/ml), of the internal standard (55μg/ml) and of the tablet homogenate were prepared by dissolving the appropriate amounts in HPLC-grade methanol and their respective dilute solutions were prepared in mobile phase. All solutions were kept refrigerated until use.

HPLC-grade methanol and acetonitrile were obtained from Riedel-de-Haen AG (Hannover, Germany). The analytical-reagent grade glacial acetic acid 100% and NaOH, which were used for the preparation of the 0.05M acetate buffer pH5.6 were obtained from Merck (Darmstadt, Germany). After appropriate dilution of the reagents with double-deionized water, the buffer was prepared by mixing 56.0ml of 1M acetic acid solution with 50.0ml of 1M NaOH solution and diluting to 1 litre with double-deionized water [12]. The buffer was filtered through a 0.2μm filter before use.

The cartridges used for the SPE study were C_{18}, C_8 (200mg/3ml) and PH (100mg/ml) Bond Elut, obtained from Analytichem International a division of Varian (Harbor City, U.S.A.) and C_8 (200mg/3ml) Alltech, obtained from Alltech Associates (Deerfield, IL, U.S.A.).

Apparatus

A Shimadzu isocratic pump, model LC-9A (Kyoto, Japan), equipped with a Rheodyne 7125 injection valve (California, U.S.A.) and a 20-μl loop, an SSI, model 500, variable wavelength UV-visible detector (State College, PA, U.S.A.) and a Hewlett Packard, model HP 3396 II integrator (Hewlett Packard, Avondale, PA, U.S.A.) were employed for the analysis of LTG. The analytical column was a Lichrosorb RP-8, 5μm, 250x4.6mm i.d. column, obtained from MZ-Analysentechnik (Mainz, Germany).

A glass vacuum-filtration apparatus obtained from Alltech Associates, was employed for the filtration of the buffer solution, using 0.2μm membrane filters obtained from Schleicher and Schuell (Dassel, Germany).

A Glass-col, Terre Haute in 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for the pretreatment of biological samples.

The SPE study was carried out on a Vac-Elut system, obtained from Analytichem International, with a 10-cartridge capacity, equipped with Bond Elut luer stopcocks.

Chromatographic Conditions

The analysis of LTG was performed under isocratic conditions at ambient temperatures and at a detection wavelength of 306nm with the detector operating at

0.002 AUFS sensitivity range. The mobile phase consisted of acetate buffer pH5.6 - acetonitrile (72:28v/v) and the flow-rate was 1ml/min.

With the above mentioned conditions the retention times of the internal standard and LTG standard solutions diluted in mobile phase were approximately 3.5min and 6.5min respectively.

LTG Calibration Curve

Appropriate dilutions with mobile phase of the stock methanolic solutions of LTG (109μg/ml) and of the internal standard (55μg/ml) were made separately and from these dilute solutions, LTG working standards of 0.0436, 0.0872, 0.436, 0.872, 1.744, 3.488 and 7.848μg/ml were prepared in 25-ml volumetric flasks with mobile phase, all containing the internal standard at the same fixed concentration of 1.1μg/ml.

The analysis of each standard solution was repeated eight times and the calibration curve was constructed by plotting the mean value of the Peak Area ratios of LTG/internal standard at each concentration level versus the concentration of LTG.

Analysis of LTG Tablets

Ten tablets of 25mg (labelled amount) were weighed and their mean weight was found to be 0.0797g. The tablets were powdered in a porcelain mortar and a 0.0347g amount (equivalent to 0.0109g LTG) of the homogenized sample was transferred into a 100-ml volumetric flask and diluted to volume with methanol. The resulting solution was 109μg/ml in LTG according to labelled amount. The solution was sonicated for 5min and a portion of it was centrifuged at 3500g for 15min. A 5-ml volume of the supernatant was diluted to volume with mobile phase in a 25-ml volumetric flask, to give a 21.8μg/ml solution of LTG and a 10-fold dilution of this solution gave a 2.18μg/ml of LTG according to labelled amount.

Volumes of 1- and 5-ml of the 2.18μg/ml solution and 4-ml of the 21.8μg/ml solution of LTG were transferred into 25-ml volumetric flasks where 5ml of a 5.5μg/ml solution of the internal standard had already been added and the mixtures were diluted to volume with mobile phase to give solutions of 0.0872, 0.436 and 3.488μg/ml in LTG respectively and 1.1μg/ml in internal standard.

Five replicate injections (20μl) onto the HPLC column, of each of the above solutions were made and the mean value of the peak area ratios of LTG/internal

standard were employed for the quantitation of the LTG concentration using the regression equation for standard LTG solutions.

SPE and Determination of LTG in Biological Samples (Plasma, Urine)

The plasma and urine samples were free from LTG and collected from healthy volunteers. The plasma sample employed in the study was a pooled sample derived from the mixing of plasma from ten healthy volunteers and the urine sample was a pooled sample from three volunteers.

To five 40-μl aliquots of spiked plasma and five 100-μl aliquots of spiked urine samples, 80-μl and 200-μl volumes of acetonitrile were added respectively (for protein precipitation) in Eppendorf vials. Each plasma and urine aliquot had been spiked with a 200-μl volume of methanolic solutions containing respectively 0.0872, 0.436, 0.872, 1.744 and 3.488μg/ml of LTG and the same fixed concentration of the internal standard (1.1 μg/ml) before the addition of acetonitrile.

The samples were then centrifuged at 3500g for 15min and the supernatants were quantitatively transferred into clean Eppendorf vials. The organic solvents present were subsequently evaporated in a waterbath at 45°C under a stream of nitrogen and the remaining aqueous phase was applied to preconditioned C_8 Bond Elut cartridges (200mg/3ml) for the isolation of LTG from endogenous compounds by means of SPE. The cartridges and SPE conditions finally employed were selected after a complete optimization SPE study.

The C_8 Bond Elut cartridges, which were selected finally, were preconditioned with 1 volume of methanol followed by 1 volume of double-deionized water with use of vacuum and after application of the samples they were washed with 2 volumes of double-deionized water. The application of samples and the wash step was carried out under atmospheric pressure with no use of vacuum. LTG and the internal standard were finally eluted with 1 volume of acidic acetonitrile (0.01M in HCL) under slight vacuum and the eluates, collected in 3-ml cone vials, were evaporated to dryness at 45°C under nitrogen. The residues were reconstituted with 200μl mobile phase and aliquots of 20μl were analyzed by HPLC.

The SPE procedure was repeated 3 times for the plasma and urine samples at all the above-mentioned concentration levels of LTG and the reconstituted residues from each extraction were then analyzed 5 repeated times each. The mean

concentration values obtained from the 5 replicate analyses, at each concentration level, were calculated for each extraction performed. Subsequently, the concentration overall means, i.e. means of the 3 values obtained from each extraction, were calculated and their RSDs and recoveries were estimated in order to establish a comprehensive study of the SPE precision and accuracy, covering the whole range of the calibration curves. For the calculation of the LTG measured concentrations in plasma and urine, the respective pooled calibration curves were employed. The pooled calibration curves were constructed by plotting the overall mean value of the peak area ratios of eluted LTG to eluted internal standard, at each concentration level versus the concentration of LTG.

SPE Optimization Study - Extraction Recovery

Different cartridges and various washing and elution conditions were tested in order to establish the best extraction conditions for LTG. For this purpose, aliquots of the pooled plasma (40μl) and urine samples were spiked with a methanolic solution containing both LTG (0.872μg/ml) and the internal standard (1.1μg/ml). The samples were then processed as mentioned earlier and subsequently subjected to SPE.

The objective of this study was to find those SPE conditions which yield high extraction recoveries and ensure that LTG and the internal standard are eluted to almost the same extent, when both compounds are present during extraction. This latter criterion must be satisfied if the internal standard is to be used as a recovery one, i.e. added together with LTG from the beginning of sample treatment. If the extraction recoveries of analyte and internal standard differ considerably, erroneously high or low peak area ratios are obtained, which would affect the calibration curve's slope and hence the sensitivity of the method.

The absolute extraction recoveries of LTG and of the internal standard were calculated as percentage ratios of peak areas of the eluted compounds from plasma and urine, to the respective peak areas of the compounds in a non-extracted standard solution. The standard solution, containing the same concentrations of LTG and of the internal standard as the extracted samples, was analyzed alternatively with the samples during the same day.

RESULTS AND DISCUSSION

A chromatogram of a LTG standard solution with its internal standard is shown in Fig.2. The selectivity of the proposed method, with respect to the analysis of pharmaceutical preparations and extracted plasma and urine pooled samples, is shown in the chromatograms of Figs. 2-4, where it can clearly be seen that at the retention time of LTG no interferences from endogenous compounds occur.

In standard solutions the ratios of LTG peak area to internal standard peak area were linearly related to concentrations ranging from 0.0436 to 7.848μg/ml (or 0.872 to 156.96ng injected on-column). The limit of detection (LOD) defined as that quantity which produces a signal of a peak height twice the size of background noise, was found to be approximately 0.9ng (on-column) and the same quantity was found from the statistical treatment of the regression equation data [13]. The limit of quantitation (LOQ), defined as that quantity which gives a signal equal to that of the blank $\pm$ 10SD (based on the calibration curve) [13] was found to be approximately 1.7ng (on-column).

In the processed plasma and urine samples, the peak area ratios of eluted LTG to eluted internal standard were linearly related to LTG concentrations ranging from 0.0872 to 3.488μg/ml. The LOD calculation, based on the statistical treatment of the regression equation data [13], revealed values of approximately 1.1 and 1.2ng for LTG determination in plasma and urine samples respectively. The LOQs, calculated as mentioned above, were approximately 2.8 and 3.0ng of LTG in plasma and urine respectively.

The linear regression equations with their confidence limits at 95% confidence level (for n-2 degrees of freedom) and the correlation coefficients for LTG in standard solutions and spiked plasma and urine samples are presented in Table 1.

The intra- and inter-day precision and accuracy of measurements was assessed by analyzing LTG standard solutions of 0.0872, 0.872 and 3.488μg/ml (or 1.744, 17.44 and 69.76ng injected on-column) eight repeated times each during the same day and five times each per day during a period of twelve days, respectively. During this period the standard solutions were kept refrigerated and no change in LTG concentration was observed. The intra-day Relative Standard Deviations (RSDs) and recoveries ranged from 0.98 to 3.0% and from 100.7 to 101.7% respectively, while the

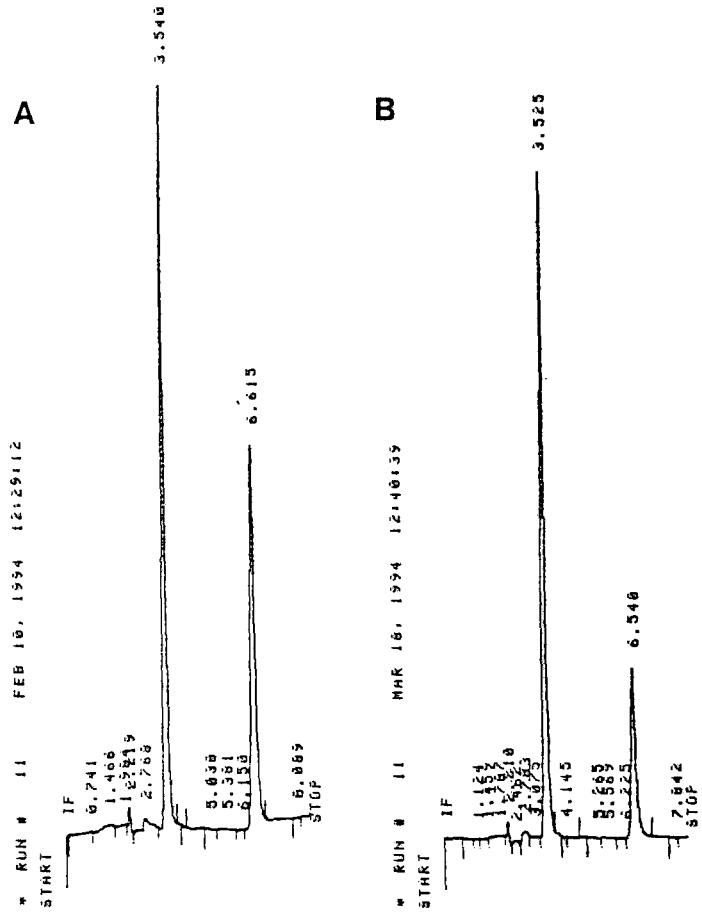


FIGURE 2. Chromatograms of (A) a standard 0.872µg/ml LTG solution and (B) a Lamictal tablet solution containing 0.436µg/ml of LTG (according to labelled amount). Concentration of internal standard 1.1µg/ml. Chromatographic conditions as described in Experimental. Chart speed: 0.5cm/min. Peaks in (A): 3.540min=internal standard, 6.615min=LTG; peaks in (B): 3.525min=internal standard, 6.540min=LTG.

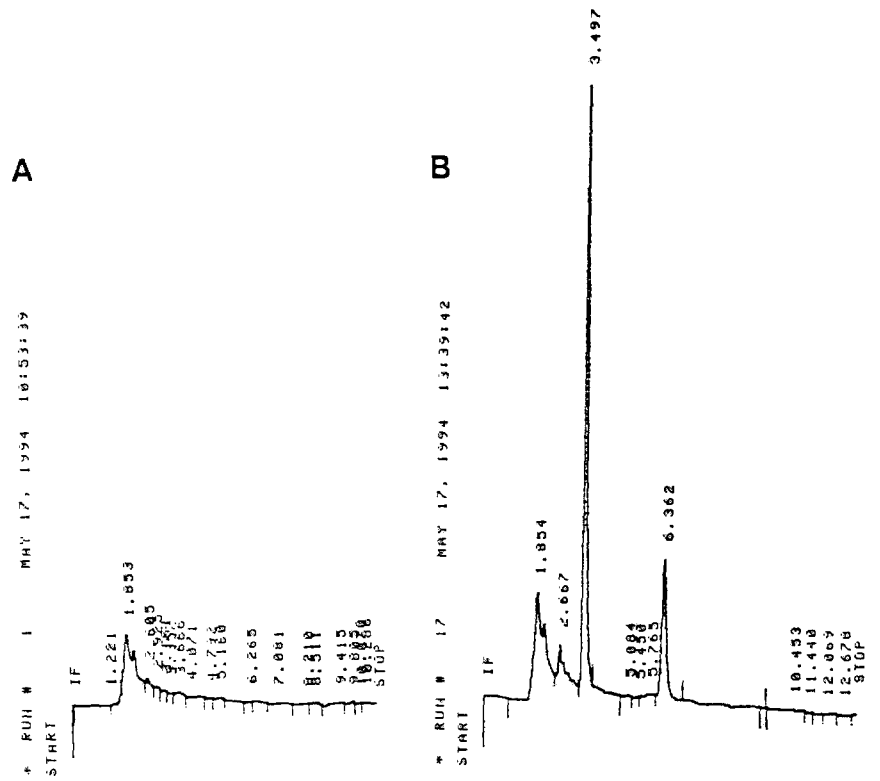


FIGURE 3. Chromatograms of (A) extracted blank plasma sample and (B) extracted plasma sample spiked with 0.436µg/ml of LTG and 1.1µg/ml of the internal standard. Chromatographic conditions as described in Experimental. Chart speed: 0.5cm/min. Peaks in (B): 3.497min=internal standard, 6.362min=LTG.

inter-day RSDs and recoveries ranged from 0.78 to 3.0% and from 94.4 to 102.2% respectively, as can be seen in Table 2.

LTG was determined with highly reproducible and accurate results in Lamictal tablets of 25mg. Since no interference from the tablet extract occured, as can be confirmed by comparing the chromatograms A (of a standard solution) and B (of the tablet extract) in Fig.2, which are identical, there was no need to apply a standard

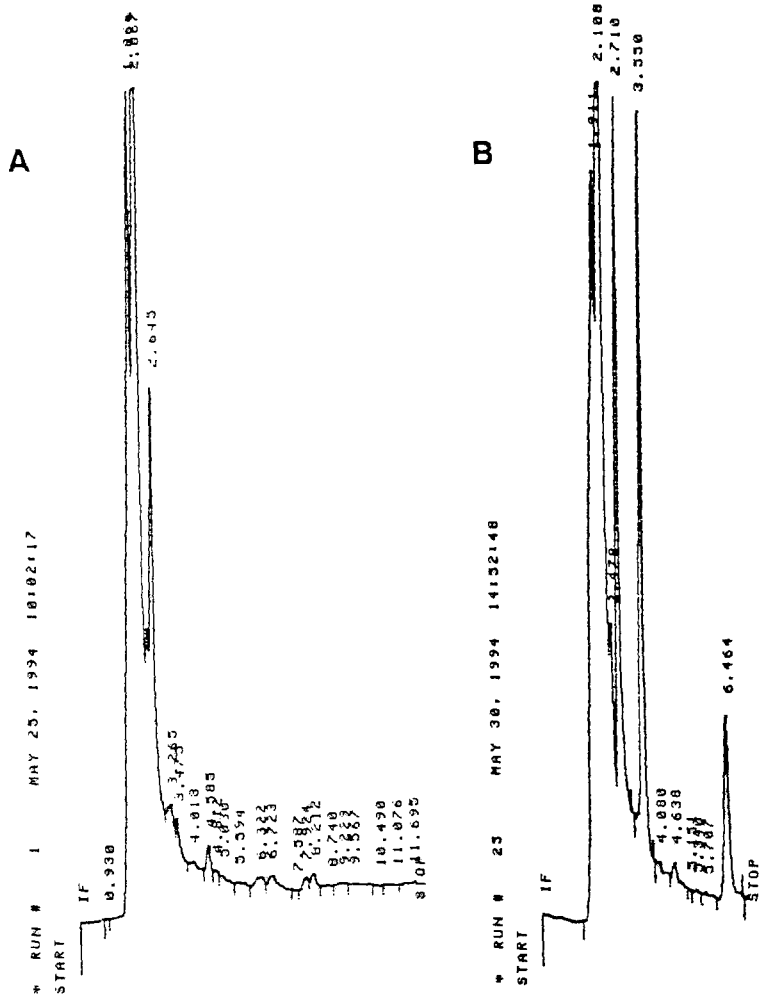


FIGURE 4. Chromatograms of (A) extracted blank urine sample and (B) extracted urine sample spiked with 0.436µg/ml of LTG and 1.1µg/ml of the internal standard. Chromatographic conditions as described in Experimental. Chart speed: 0.5cm/min. Peaks in (B): 3.550min=internal standard, 6.464min=LTG.

TABLE 1

Statistical Evaluation of Regression Equations

LTG Samples	Regression Equation $Y = (a \pm t_a S_a) + (b \pm t_a S_b)X$	Correlation Coefficient
Standard Solutions	$Y^* = (0.02450 \pm 0.01943) + (0.87210 \pm 0.00583)X$	0.99998
Plasma	$Y^{**} = (0.01344 \pm 0.03761) + (0.83361 \pm 0.02092)X$	0.99991
Urine	$Y^{**} = (0.05560 \pm 0.04041) + (0.83509 \pm 0.02247)X$	0.99989

Y=peak area ratio of LTG to internal standard, X=concentration in μg/ml, a=intercept, b=slope, S_a,S_b=standard deviations of intercept and slope respectively, t_a=student's t-test for n-2 degrees of freedom and 95% confidence level.

* Mean value of 8 replicate measurements at each concentration level; based on 7 data points.

**Overall mean: Mean value of 3 extractions; the value obtained for each extraction is the mean of 5 replicate measurements at each concentration level; the pooled calibration curves are based on 5 data points.

TABLE 2

Within-day and Between-day Precision and Accuracy of LTG Determination in Standard Solutions

Injected Quantity (ng)	Within-day			Between-day		
	Mean Measured Quantity[a] ± SD* (ng)	RSD** (%)	Recovery (%)	Mean Measured Quantity[b] ± SD* (ng)	RSD** (%)	Recovery (%)
1.744	1.771±0.054	3.0	101.5 ± 3.1	1.646±0.050	3.0	94.4 ± 2.8
17.44	17.73±0.20	1.1	101.7 ± 1.2	17.83±0.17	0.95	102.2 ± 1.0
69.76	70.24±0.69	0.98	100.7 ± 0.99	70.44±0.55	0.78	101.0 ± 0.79

[a] Mean of values obtained from the linear regression equation for standard solutions for n=8 measurements during the same day.

[b] Mean of values obtained from the linear regression equation for standard solutions for n=5 measurements / day over a period of 12 days.

* Standard Deviation; ** Relative Standard Deviation.

TABLE 3

Precision and Accuracy of LTG Determination in Lamictal Tablets of 25mg

Injected Quantity[a] (ng)	Mean Measured Quantity[b] ± SD* (ng)	Found in Tablets[c] ± SD* (mg)	RSD** (%)	Recovery (%)
1.744	1.707±0.026	24.49±0.37	1.5	98.0 ± 1.5
8.720	8.533±0.184	24.49±0.53	2.2	98.0 ± 2.1
69.76	71.95±0.21	25.81±0.07	0.27	103.2 ± 0.28

[a] According to labelled amount.

[b] Mean of values obtained from the linear regression equation for standard solutions for n=5 measurements.

[c] According to the procedure described under Experimental.

* Standard Deviation; ** Relative Standard Deviation.

TABLE 4

SPE Recoveries of LTG and of the Internal Standard in Plasma using Various Types of Cartridges

Cartridge Type[a]	Absolute Extraction Recovery[b] ± SD* (%)	
	LTG (0.872µg/ml)	Internal Standard (1.1µg/ml)
C_{18} Bond Elut (200mg/3ml)	68.0±2.5	61.3±2.2
C_8 Bond Elut (200mg/3ml)	87.1±3.3	88.4±2.8
C_8 Alltech (200mg/3ml)	67.9±3.3	67.6±2.4
PH Bond Elut (100ml/1ml)	80.1±4.2	61.9±2.4

[a] Preconditioning of cartridges with 1 volume methanol + 1 volume double-deionized water; application of sample; washing of cartridges with 2 volumes double-deionized water; elution of the compounds with 1 volume methanol.

[b] Calculated as described under Experimental; Mean value of three determinations.

* Standard Deviation.

TABLE 5
SPE Recoveries of LTG and of the Internal Standard in Plasma using C_8 Bond Elut Cartridges (200mg/3ml) at Different Conditions

SPE Conditions	Absolute Extraction Recovery[a] ± SD* (%)	
	LTG (0.872μg/ml)	Internal Standard (1.1μg/ml)
(1)	77.5±0.75	84.9±0.79
(2)	78.0±2.3	27.2±1.1
(3)	88.7±0.89	95.6±4.8
(4)	74.0±1.7	8.0±0.78
(5)	89.0±4.6	89.4±1.2
(6)	No-elution	No-elution
(7)	86.8±2.1	95.7±2.1

[a] Calculated as described under Experimental; Mean value of three determinations.
* Standard Deviation
(1) Preconditioning of cartridges with 1 volume methanol + 1 volume double-deionized water; application of sample; washing of cartridges with 2 volumes double-deionized water; elution of the compounds with 1 volume acidic methanol (0.01M in HCl).
(2) Preconditioning with 1 volume methanol + 1 volume acetate buffer pH5.6; application of sample; washing with 1 volume acetate buffer pH5.6 + 1 volume double-deionized water; elution with 1 volume methanol.
(3) As in (2), but elution with 1 volume acidic methanol (0.01M in HCl).
(4) As in (1), but elution with 1 volume acetonitrile.
(5) As in (1), but elution with 1 volume acidic acetonitrile (0.01M in HCl).
(6) As in (2), but elution with 1 volume acetonitrile.
(7) As in (2), but elution with 1 volume acidic acetonitrile (0.01M in HCl).

additions' method here and the calibration curve for standard solutions was employed directly for the quantitation of LTG content in the tablet extract. For obtaining a comprehensive study of the precision and accuracy of LTG determination in Lamictal tablets, three different dilutions of the tablet extract were prepared yielding nominal concentrations of 0.0872, 0.436 and 3.488μg/ml or 1.744, 8.720 and 69.76ng on-column respectively, which cover representative concentrations of the standard calibration curve. LTG was determined with RSDs ranging from 0.27 to 2.2%, while the recoveries ranged from 98.0 to 103.2% of the true value, indicating good agreement with the labelled amount as shown in Table 3.

Tables 4-6 show the extraction recoveries obtained for LTG and the internal standard, when various types of cartridges and different SPE washing and elution conditions are used for spiked plasma and urine samples. The final selection of

TABLE 6

SPE Recoveries of LTG and of the Internal Standard in Urine using Various Types of Cartridges and Different Elution Conditions

Cartridge Type[a]	Absolute Extraction Recovery[b] ± SD* (%)			
	Elution with 1 Volume Methanol		Elution with 1 Volume Acidic Acetonitrile	
	LTG (0.872µg/ml)	Internal Standard (1.1µg/ml)	LTG (0.872µg/ml)	Internal Standard (1.1µg/ml)
C_{18} Bond Elut (200mg/3ml)	71.3±0.29	148.0±3.5	87.4±2.7	71.4±2.3
C_8 Bond Elut (200mg/3ml)	No elution	No elution	96.9±0.28	97.2±1.3
PH Bond Elut (100mg/1ml)	No elution	No elution	109.0±2.5	88.5±2.3

[a] Preconditioning with 1 volume methanol + 1 volume double-deionized water; washing with 2 volumes double-deionized water after application of the sample.

[b]Calculated as described under Experimental; Mean value of three determinations.

* Standard Deviation.

TABLE 7

Precision and Accuracy of SPE in Spiked Plasma and Urine Samples

Injected Quantity (ng)	In Plasma			In Urine		
	Mean Measured Quantity[a] ± SD* (ng)	RSD** (%)	Recovery (%)	Mean Measured Quantity[b] ± SD* (ng)	RSD** (%)	Recovery (%)
1.744	1.785±0.016	0.92	102.4 ± 0.95	1.833±0.006	0.33	105.1 ± 0.35
8.720	8.878±0.039	0.44	101.8 ± 0.44	8.264±0.066	0.80	94.8 ± 0.75
17.44	17.11±0.04	0.23	98.1 ± 0.20	17.46±0.13	0.74	100.1 ± 0.73
34.88	34.41±0.27	0.78	98.6 ± 0.78	35.49±0.48	1.3	101.7 ± 1.4
69.76	70.04±0.81	1.2	100.4 ± 1.2	69.50±1.36	2.0	99.6 ± 1.9

[a,b] Overall mean of values obtained from the pooled regression equations, i.e. mean of values obtained from each of the three extractions performed, which in turn are the means of values obtained from 5 replicate measurements at each concentration level.

* Standard Deviation; **Relative Standard Deviation.

cartridges and SPE conditions was made on the basis of the following criteria: 1) high extraction recovery for both LTG and its internal standard and 2)if possible, same percentage of LTG and the internal standard eluted. As can be seen from Tables 4-6, the use of C_8 Bond Elut (200mg/3ml) cartridges, preconditioned with one volume of methanol plus one volume of double-deionized water and washed with two volumes of double-deionized water after application of the samples, combined with the use of one volume of acidic acetonitrinile (0.01M in HCl) for the elution of the compounds fulfill the criteria mentioned above.

The results of LTG determination in spiked plasma and urine samples are given in Table 7. For concentrations of LTG between 0.0872 and 3.488μg/ml (or 1.744 to 69.76ng on-column respectively) RSDs ranging from 0.23 to 1.2% and recoveries ranging from 98.1 to 102.4% were achieved for the analysis of plasma spiked samples, while for the same concentrations of LTG in urine spiked samples the RSDs ranged from 0.33 to 2.0% and the recoveries from 94.8 to 105.1%.

CONCLUSION

The satisfactory results obtained from the SPE study which were confirmed by the determination of LTG in spiked plasma and urine samples, as well as the simplicity, rapidity, sensitivity and the satisfactory precision and accuracy of the HPLC assay, make the proposed method suitable for the analysis of LTG in pharmaceutical preparations and in extracted human biological fluids.

ACKNOWLEDGEMENTS

The authors of this paper wish to thank Prof. P. Georgakopoulos and Wellcome Foundation Ltd for providing the reagents to carry out this project.

REFERENCES

1. A. Fazio, C. Artesi, M. Russo, R. Trio, G. Oteri and F. Pisani, Ther. Drug Monit.,2 14: 509-512 (1992).

2. M.J. Leach, C.M. Marsden and A.A. Miller, Epilepsia, 27: 490 (1986).

3. A.F. Cohen, G.S. Land, D.D. Breimer, W.C. Yuen, C. Winton and A.W. Peck, Clin. Pharmacol. Ther., 42: 535-541 (1987).

4. M.V. Doig and R.A. Clare, J. Chromatogr., 554: 181-189 (1991).

5. R.A. Biddlecombe, K.L. Dean, C.D. Smith and S.C. Jeal, J. Pharm. Biomed. Anal., 8: 691-694 (1990).

6. J.M. Sailstad and J.W.A. Findlay, Ther. Drug Monit., 13: 433-442 (1991).

7. J. Posner, A.F. Cohen, G. Land, C. Winton and A.W. Peck, Br. J. Clin. Pharmacol., 28: 117-120 (1989).

8. M. Cociglio, R. Alric and O.Bouvier, J. Chromatogr., 572: 269-276 (1991).

9. M. Meyler, M.T. Kelly and M.R. Smyth, Chromatographia, 36: 27-32 (1993).

10. S. Ramachandran, S. Underhill and S.R. Jones, Ther. Drug Monit., 16: 75-82 (1994).

11. M.W. Sinz and R.P. Remmel, J. Chromatogr., 571: 217-230 (1991).

12. C. Long, E.J. King and W.M. Sperry, Biochemists' Handbook, Van Nostrand, New York, 1961, p.31.

13. J.C. Miller and J.N. Miller, Statistics for Analytical Chemistry, Ellis Horwood, England, 1986, pp. 94-100.

Received: April 1, 1995
Accepted: April 13, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2611-2619 (1995)

DIRECT LIQUID CHROMATOGRAPHIC SEPARATION OF 2,3-DIHYDRO-2-ETHYL-BENZOFURAN-2-CARBOXYLIC ACID ENANTIOMERS USING A CELLULOSE CARBAMATE-BASED CHIRAL STATIONARY PHASE COLUMN

SECHOING LIN
Chemical Development Department
Parke-Davis Pharmaceutical Research Division
Warner-Lambert Company
188 Howard Avenue
Holland, Michigan 49424

ABSTRACT

Direct liquid chromatographic separation of 2,3-dihydro-2-ethylbenzofuran-2-carboxylic acid enantiomers on a commercial cellulose carbamate-based chiral stationary phase column is described. The addition of a small amount of organic acid in the mobile phase is required for the elution and separation of both enantiomers. Better peak shapes are obtained when trifluoroacetic acid is used. The method allows the detection of the undesired (+)-enantiomer down to a level of 0.5 % and can be used to measure the enantiomeric purity of (-)-enantiomer after resolution.

INTRODUCTION

Efaroxan, a chiral compound, has been extensively investigated as a potential anti-diabetic agent (1). 2,3-Dihydro-2-ethylbenzofuran-2-carboxylic acid

is used in a synthesis of efaroxan (2). It is convenient and cheaper when a resolution is carried out early in a synthesis. The development of an analytical method to measure the enantiomeric purity of the carboxylic acid is required.

A literature survey indicated that resolution of racemic carboxylic acids can be achieved using HPLC chiral stationary phases after conversion of the acids into either esters or amides (3-8). Derivatization procedures have many drawbacks (7-11). Recent efforts have focused on development of direct resolution of carboxylic acids on various chiral stationary phases and examples have been documented (7,8,11-22).

In this communication, the direct HPLC separation of 2,3-dihydro-2-ethylbenzofuran-2-carboxylic acid (as shown in Figure 1) is described using a chiral stationary phase column and is compared to derivatization.

EXPERIMENTAL

Apparatus

The liquid chromatographic system consisted of a Hitachi L-6200 intelligent pump, a Micromeritics 728 autosampler and a Valco injector with a 20 μl loop, a Kratos Spectroflow 757 variable wavelength UV absorbance detector, and a Hitachi D-2000 Chromatointegrator. The analytical column was a Chiralcel OF (250 x 4.6 mm I.D., 10 micron particle size) column and was purchased from Chiral Technologies, Inc, Exton, PA.

Chemicals

Hexane and 2-propanol (HPLC grades) were obtained from EM Science, Gibbstown, NJ. Formic acid (88%) and acetic acid (reagent grade) were purchased

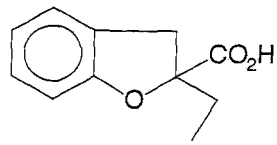


FIGURE 1. The structure of 2,3-dihydro-2-ethylbenzofuran-2-carboxylic acid.

from J.T. Baker Inc., Phillipsburg, NJ. Trifluoroacetic acid (99%) was obtained from Aldrich Chemical Company, Milwaukee, WI. (-)-2,3-Dihydro-2-ethylbenzofuran-2-carboxylic acid, 2,3-dihydro-2-ethylbenzofuran-2-carboxylic acid and the corresponding amide, methyl, 1-propyl, and 1-pentyl esters were prepared at the Parke-Davis Pharmaceutical Research Division, Holland, MI (23).

Chromatographic Conditions

The mobile phase for the amide and esters was hexane/2-propanol. An organic acid modifier, 0.01-0.1 %, was used to separate 2,3-dihydro-2-ethylbenzofuran-2-carboxylic acid enantiomers. These modifiers were trifluoroacetic acid, formic acid or acetic acid. Flow rate was 1.0 mL/min. The UV detection wavelength was 280nm. Sample amount for the enantiomeric purity of (-)-2,3-dihydro-2-ethylbenzofuran-2-carboxylic acid was about 0.11 μmole. Column void volume was determined by injection of tri-t-butylbenzene (15,24).

RESULTS AND DISCUSSION

The enantiomeric separation of the amide and esters of 2,3-dihydro-2-ethylbenzofuran-2-carboxylic acid enantiomers can be achieved using hexane/2-propanol as mobile phase. As shown in Table 1, good separation and resolution were observed. However, this system did not separate the parent acid enantiomers.

TABLE 1. Enantiomeric Separations for 2,3-Dihydro-2-ethylbenzofuran-2-carboxylic Acid Derivatives

Compound	k'_1	α	R_s
methyl ester	1.73	2.08	6.95
1-propyl ester	1.20	2.45	6.92
1-pentyl ester	0.99	2.54	6.92
amide	5.46	1.46	3.72

k'_1 is the capacity factor of first eluted enantiomer; α is the stereoselectivity; R_s is the resolution factor.

HPLC conditions: Mobile phase = 2 % 2-propanol in hexane for esters and 10 % 2-propanol in hexane for amide; flow rate = 1.0 mL/min.; detector wavelength = 280 nm; detector sensitivity = 0.01 AUFS;
column temperature = room temperature.

TABLE 2. Effect of Trifluoroacetic Acid Modifier on Resolution of 2,3-Dihydro-2-ethylbenzofuran-2-carboxylic Acid Enantiomers

% Modifier	k'_1	α	R_s
0.01	0.57	8.99	9.59
0.05	0.55	9.15	9.74
0.10	0.55	9.07	9.81

k'_1 is the capacity factor of first eluted enantiomer; α is the stereoselectivity; R_s is the resolution factor.

HPLC Conditions: Mobile phase = 20 % 2-propanol in hexane with added amount of trifluoroacetic acid modifier; flow rate = 1.0 mL/min.; detector wavelength = 280 nm; detector sensitivity = 0.01 AUFS; column temperature = room temperature.

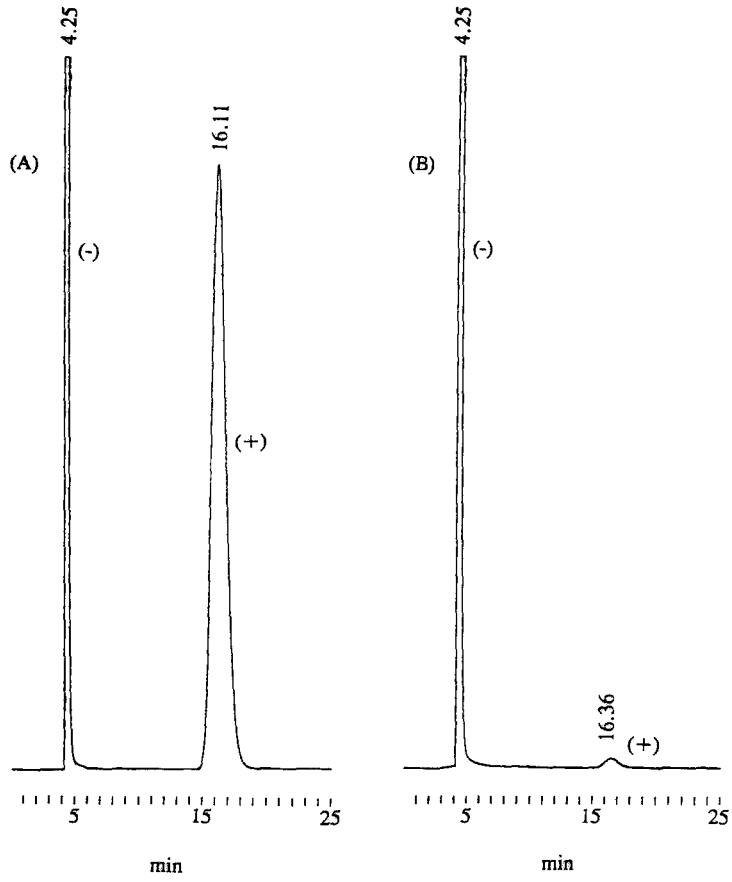


FIGURE 2. Enantiomeric separation of (A) racemic 2,3-dihydro-2-ethyl-benzofuran-2-carboxylic acid and (B) (-)-2,3-dihydro-2-ethyl-benzofuran-2-carboxylic acid after resolution. Mobile phase = 20% 2-propanol in hexane with 0.10 % trifluoroacetic acid added; flow rate = 1.0 mL/min.; detector wavelength = 280 nm; detector sensitivity = 0.01 AUFS; column temperature = room temperature; sample amount injected = 20 μg.

The addition of an organic acid to the mobile phase to effect the elution of carboxylic acid containing compounds from a tris-(3,5-dimethylphenyl carbamate) derivative of cellulose and amylose based chiral columns has been reported earlier (15,19). The same strategy was applied in this study. Acetic acid, formic acid and trifluoroacetic acid were each added as a modifier to the mobile phase. In each case, separation of 2,3-dihydro-2-ethylbenzofuran-2-carboxylic acid enantiomers was reasonably good. Nevertheless, moderate tailing of peak shapes were observed associated with the use of both acetic and formic acid. Much improved peak shapes as well as better separation and resolution (α = 9.07, R_s = 9.81) were seen when the added modifier was trifluoroacetic acid.

The amount of trifluoroacetic acid in the mobile phase was varied to study the concentration effect on the resolution of 2,3-dihydro-2-ethylbenzofuran-2-carboxylic acid enantiomers. The results shown in Table 2 indicate there was not a significant change in resolution between 0.01 and 0.1 % of trifluoroacetic acid.

Finally, chromatograms of a racemic sample and a typical sample after resolution are shown in Figure 2. The enantiomers were readily resolved without derivatization. The method has been used to screen a number of chiral agents for large scale resolution. Moreover, the method allows the detection of the undesired (+)-enantiomer down to a level of 0.5 % and has been used routinely to check the enantiomeric purity of (-)-2,3-dihydro-ethylbenzofuran-2-carboxylic acid after resolution.

ACKNOWLEDGEMENTS

I gratefully thank Drs. Donald E. Butler and William T. Suggs for very useful comments and proofreading, and Ms. Virginia Sloothaak for the typing of this manuscript.

REFERENCES

1. Chan, S.L.F.; Dunne, M.J.; Stillings, M.R.; Morgan,N.G., The α_2-Adrenoceptor Antagonist Efaroxan Modulates K_{ATP}^+ Channels in Insulin-secreting Cells, Eur. J. Pharmacol.,204, 41 (1991).

2. Edwards, C.R.; Readhead, M.J.; Tweddle, N.J. A Practical Synthesis of 2,3-Dihydro-2-benzofuran-carboxylic Acid: A General Route to 2,3-Dihydrobenzofurans, J. Heterocyclic Chem.,24, 495 (1987).

3. Wainer, I.W.; Doyle, T.D., Application of High-Performance Liquid Chromatographic Chiral Stationary Phases to Pharmaceutical Analysis: Structural and Conformational Effects in the Direct Enantiomeric Resolution of α-Methylarylacetic Acid Anti-inflammatory Agents, J. Chromatogr., 284, 117 (1984).

4. McDaniel, D.M.; Snider, B.G., Resolution of α-Arylacetic Acid Enantiomers on Two Chiral Stationary Phases, J. Chromatogr., 404, 123 (1987).

5. Pirkle, W.H.; McCune, J.E., Improved Chiral Stationary Phase for the Separation of the Enantiomers of Chiral Acids as their Anilide Derivatives, J. Chromatogr., 471, 271 (1989).

6. Pirkle, W.H.; Murray, P.G., The Separation of the Enantiomers of A Variety of Non-Steroidal Anti-Inflammatory Drugs(NSAIDS) as their Anilide Derivatives Using A Chiral Stationary Phase, J. Liquid Chromatogr., 13, 2123 (1990).

7. Bojarski, J., Chromatography of Enantiomers of 2-Arylpropionic Acids, J. Liquid Chromatogr., 12, 2685 (1989).

8. Pirkle, W.H.; Welch, C.J., Chromatographic Separation of Underivatized Naproxen Enantiomers, J. Liquid Chromatogr., 14, 3387 (1991).

9. Ader, U; Muschalek, V.; Schneider, M.P.,Chromatographic Resolution of Chiral Intermediates in β-Adrenergic Blocker Synthesis on Chiral Stationary Phases, Chirality, 5, 554 (1993).

10. Castelnovo, P., Determination of the Enantiomeric Purity of 5,6- and 6,7-Dihydroxy-2-aminotetralins by Chiral HPLC, Chirality, 5, 181 (1993).

11. Caccamese, S., Direct High-Performance Liquid Chromatography (HPLC) Separation of Etodolac Enantiomers Using Chiral Stationary Phases, Chirality, 5, 164 (1993).

12. Armstrong, D.W.; Rundlett, K.L.; Chen, J.R., Evaluation of the Macrocyclic Antibiotic Vancomycin as a Chiral Selector for Capillary Electrophoresis, Chirality, 6, 496 (1994).

13. Ishikawa, A.; Shibata, T., Cellulosic Chiral Stationary Phase under Reversed-Phase Condition, J. Liquid Chromatogr., 16, 859 (1993).

14. Hermansson, J.; Eriksson, M., Direct Liquid Chromatographic Resolution of Acidic Drugs using a Chiral α_1-Acid Glycoprotein Column (Enantiopac®), J. Liquid Chromatogr., 9, 621 (1986).

15. Okamoto, Y.; Aburatani, R.; Kaida, Y.; Hatada, K., Direct Optical Resolution of Carboxylic Acids by Chiral HPLC on Tris(3,5-dimethylphenylcarbamate)s of Cellulose and Amylose, Chem. Lett., 1125 (1988).

16. Okamoto, Y.; Aburatani, R.; Kaida, Y.; Hatada, K.; Inotsume, N.; Nakano, M., Direct Chromatographic Separation of 2-Arylpropionic Acid Enantiomers Using Tris(3,5-dimethylphenylcarbamate)s of Cellulose and Amylose As Chiral Stationary Phases, Chirality, 1, 239 (1989).

17. Allenmark, S.; Andersson, S., Optical Resolution of Some Biologically Active Compounds By Chiral Liquid Chromatography on BSA-Silica (Resolvosil) Columns, Chirality, 1, 154 (1989).

18. Miwa, T,; Miyakawa, T.; Kayano, M., Application of an Ovomucoid-conjugated Column for the Optical Resolution of some Pharmaceutically Important Compounds, J. Chromatogr., 408, 316 (1987).

19. Okamoto, Y.; Aburatani, R.; Hatada, K., Chromatographic Resolution XXI*. Direct Optical Resolution of Abscisic Acid By High-Performance Liquid Chromatography on Cellulose Tris(3,5-dimethylphenylcarbamate), J. Chromatogr., 448, 454 (1988).

20. Chan, K.Y.; George, R.C.; Chen, T.M.; Okerholm, R.A., Direct Enantiomeric Separation of Terfenadine and its major Acid Metabolite By High-Performance Liquid Chromatography, and the lack of Stereoselective Terfenadine Enantiomer Biotransformation in Man, J. Chromatogr., 571, 91 (1991).

21. Bettoni, G.; Ferorelli, S.; Loiodice, F.; Tangari, N.; Tortorella, V.; Gasparrini, F.; Misiti, D.; Villani, C., Chiral α-Substituted α-Aryloxy Acetic Acids: Synthesis, Absolute Configuration, Chemical Resolution, and Direct Separation by HPLC, Chirality, 4, 193 (1992).

22. Castellani, L.; Federici, F.; Sinibaldi, M.; Messina, A., Direct Resolution of Organic Acid Enantiomers on a novel Polymer-based Stationary Phase, J. Chromatogr., 602, 21 (1992).

23. The derivative amide and esters were produced using standard reactions by Ms. Punam Giri at Parke-Davis.

24. Pirkle, W.H.; Welch, C.J., A Convenient Void Volume Marker for Several Chiral HPLC Columns, J. Liquid Chromatogr., 14, 1 (1991).

Received: December 9, 1994
Accepted: April 10, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2621-2633 (1995)

QUANTITATIVE DETERMINATION OF OXAPROZIN AND SEVERAL OF ITS RELATED COMPOUNDS BY HIGH-PERFORMANCE REVERSED-PHASE LIQUID CHROMATOGRAPHY

FAYEZ B. IBRAHIM
Ganes Chemicals, Inc.
33 Industrial Park Road
Pennsville, New Jersey 08070

ABSTRACT

An accurate, sensitive, and selective high-performance liquid chromatographic method for the quantitative determination and the separation of oxaprozin from several of its related compounds (including some precursors) has been developed. Reversed-phase ion-pair chromatography, with 1-decanesulfonic acid sodium salt as the ion pairing agent, was used. A μ Bondapak C_{18} column with a mobile phase consisting of 0.01 M KH_2PO_4, methanol, and acetonitrile (2:1:1), pH 4.2, was used at a flow rate of 1 mL/min. Oxaprozin and its related compounds were monitored at 254nm. The method is rapid because no sample extraction is involved. Precision and ruggedness (relative standard deviation) on oxaprozin and several of its related compounds were below 1.0%. Detection limits ranged from 0.05 μg/mL to 0.08 μg/mL depending on the compound. The method is stability-indicating, and has applications in bulk pharmaceutical manufacturing.

INTRODUCTION

Oxaprozin (4,5-diphenyl-2-oxazolepropionic acid) is a non-steroidal anti-inflammatory agent (1,2) used for the treatment of rheumatic and inflammatory conditions.

Several methods for the determination of oxaprozin are cited in the literature. It was separated and identified by thin-layer chromatography (3), determined by gas-liquid chromatography (4), and high-performance liquid chromatography (HPLC) (5-12).

Many of the above methods were used for determining oxaprozin in biological fluids, and are not suited to the problems encountered in bulk pharmaceutical manufacturing such as detecting unreacted starting materials, intermediates, and by-products, as well as completion reactions and kinetic studies.

The present report describes a rapid and sensitive technique to determine oxaprozin and several of its related compounds in pharmaceutical production, including wet cake, mother liquor and reaction completion samples.

EXPERIMENTAL

Apparatus

The system was a Varian 5000 liquid chromatograph with an 8055 autosampler (both by Varian Instrument Division, Walnut Creek, CA) and a 783A programmable absorbance detector by Applied Biosystems, Foster City,

CA. The diode array instrument was a Model 9010 pump, a Model 9095 autosampler and a Model 9065 photodiode array detector (all by Varian Instrument Division). System control was via the Series 9020 LC Star Workstation, Revision C (Varian Instrument Division). Spectral manipulation and comparison were performed using Polyview™ Revision D (Varian Instrument Division). The LC control and spectral processing software functions in a Windows™ environment (Windows Version 3.0, Microsoft Corporation, Redmond, WA) on a 386 PC equipped with a math coprocessor. A 300 x 3.9 mm μ Bondapak C_{18} column (Waters Division of Millipore, Milford, MA) was used.

Reagents

High purity acetonitrile was purchased from Burdick and Jackson (Baxter Healthcare Corporation, Muskegon, MI). Deionized water was from a NANOpure II (Barnstead Thermolyne, Dubuque, IA) water purification system. Benzaldehyde, 99%, benzoic acid, 99% and DSA (1-decanesulfonic acid sodium salt, 99%) were purchased from Aldrich Chemical Company, Milwaukee, WI. WY-23,096 [*N*-(α-phenylphenacyl)succinamic acid], benzoin (2-hydroxy-2-phenylacetophenone), WY-21,879 (benzoin hemisuccinate), WY-23,120 (4,5-diphenylimidazole-2-propionic acid), WY-22,075 (4,5-diphenyl-2-oxazolepropionamide), WY-20,910 (diphenylethanedione), oxaprozin (4,5-diphenyl-2-oxazolepropionic acid, also known as WY-21,743),

oxaprozin ethyl ester, tetraphenylpyrazine, and WY-23,027 (phenanthro[9,10-*d*]oxazole-2-propionic acid) were provided by Wyeth-Ayerst Laboratories, P.O. Box 8299, Philadelphia, PA 19101.

Determination of Oxaprozin

The mobile phase consisted of aqueous solution (0.01M KH_2PO_4 - 0.005M DSA), methanol, and acetonitrile (2:1:1). The pH of the mobile phase was adjusted to 4.2 with phosphoric acid (85%). After mixing and pH adjustment, the mobile phase was filtered through a 0.22-μm Millipore filter and degassed by sonication under vacuum. Oxaprozin standard or sample was prepared to contain about 0.4 mg/mL in the mobile phase. All other related compounds were prepared to contain each about 0.04 mg/mL in the mobile phase. The liquid chromatograph was equipped with a 254 nm detector, and the flow rate was about 1 mL/min. As an example, the retention times for oxaprozin and several of its related compounds are shown in Table 1. The standard preparation was injected several times (10 μL) and the response was measured. The relative standard deviation for six replicate injections of the standard preparation is shown in Table 2. The sample preparation was injected, and the concentrations of oxaprozin and any of its related compounds were determined.

Results and Discussion

Oxaprozin was analyzed and completely separated from its major related compounds and precursors using the above described HPLC method, as shown

TABLE 1

Limit of Detection and Regression Analyses

	Retention Time (min)	Limit of Detection (μg/mL)	Correlation Coefficient (RS)
WY-23,096	5.9	0.060	1.0000
Benzoin	6.8	0.084	0.9997
WY-21,879	9.5	0.084	0.9997
WY-23,120	10.6	0.048	0.9997
WY-22,075	12.5	0.048	0.9999
WY-20,910	17.6	0.078	0.9990
Oxaprozin	19.8	0.054	0.9998
WY-23,027	22.9	0.060	0.9999

TABLE 2

Precision and Ruggedness Analyses

	Precision, RSD (%) n=6		Ruggedness, RSD (%) n=6	
	Day 1	Day 2	Day 1	Day 2
WY-23,096	1.00	0.71	0.24	0.24
Benzoin	0.68	0.39	0.20	0.24
WY-21,879	0.23	0.15	0.25	0.36
WY-23,120	0.34	1.00	0.22	0.37
WY-22,075	0.34	0.44	0.55	0.90
WY-20,910	0.44	0.56	0.15	0.36
Oxaprozin	0.22	0.16	0.38	0.81
WY-23,027	0.44	0.64	0.27	0.61

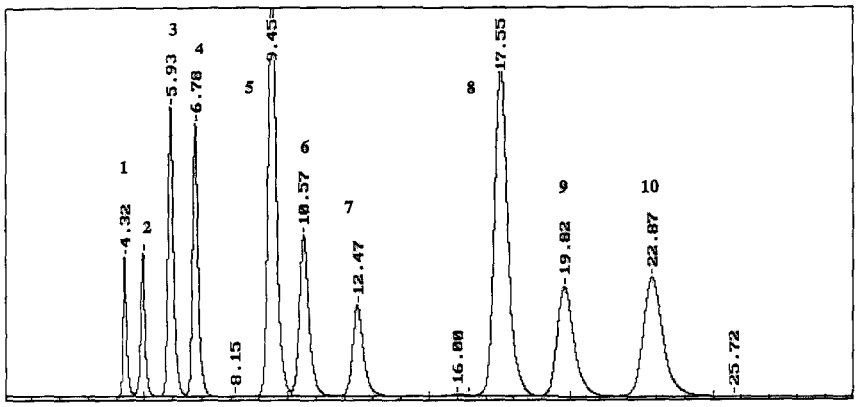


Fig. 1. Separation of oxaprozin from its potential related compounds and precursors: Benzoic acid (1), Benzaldehyde (2), WY-23,096 (3), Benzoin (4), WY-21,879 (5), WY-23,120 (6), WY-22,075 (7), WY-20,910 (8), Oxaprozin (9), and WY-23,027 (10). Column, 300 x 3.9 mm μ Bondapak C_{18}, flow rate 1.0 mL/min, detection at 254 nm.

in Fig 1. Photodiode array detection was used as evidence of the specificity of the method, and to evaluate the homogeneity of the peaks. Chromatographic peak purity at peak heart was determined using wavelength ratio comparison (224 nm vs 283 nm). This technique is applied to resolve coeluting peaks. The plot (Fig 2) showed that oxaprozin exhibited a homogeneous peak with no detectable impurities embedded in it. Also, complete resolution of oxaprozin from its related compounds, with good peak symmetry and no apparent shoulders, was demonstrated as shown in Fig 3.

Method precision and ruggedness were evaluated on oxaprozin and its major related compounds by two different analysts on two different days using different columns; the RSD's from each analyst (n=6) are listed in Table 2.

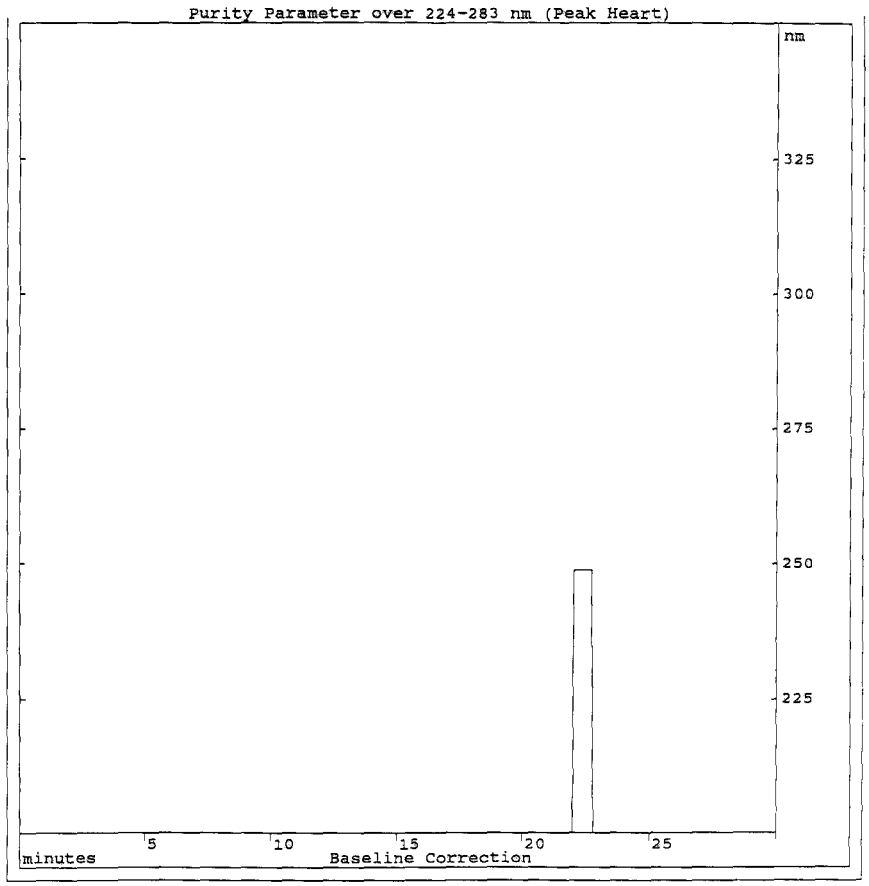


Fig. 2. Peak purity at peak heart for oxaprozin performed over 224 nm - 283 nm using diode array spectrophotometry.

Linearity of response was measured for oxaprozin and its major related compounds at 5 and 6 levels. The area count of each compound plotted against its consecutive concentration produced a straight line through the origin. Regression analysis yielded a correlation coefficient (RS) of 0.999 or better for

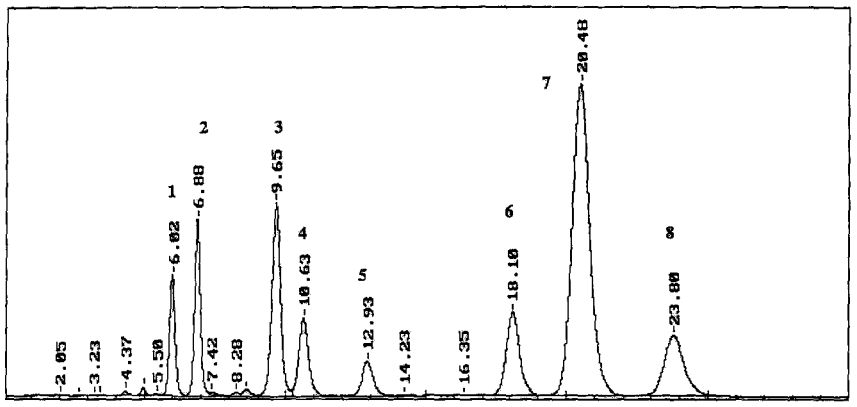


Fig. 3. Chromatogram of WY-23,096 (1), Benzoin (2), WY-21,879 (3), WY-23,120 (4), WY-22,075 (5), WY-20,910 (6), Oxaprozin (7), and WY-23,027 (8). Column, 300 x 3.9 mm μ Bondapak C_{18}, flow rate 1 mL/min, detection at 254 nm.

oxaprozin and its analogs, as can be seen in Table 1. These results showed a high degree of linearity in the range between 1.1 mg/mL to 0.4 mg/mL for oxaprozin, and between 0.08 mg/mL to 0.05 μg/mL for the other analogs.

The limit of detection was about 0.05 μg/mL for oxaprozin, and for its related compounds ranged from 0.05 μg/mL to 0.08 μg/mL (Table 1).

The ethyl ester of oxaprozin was also determined using the above described procedure, and its retention time was about 42 minutes when a flow rate of 1.5 mL/min. was used.

Tetraphenylpyrazine (TPP), which might be formed as a by-product during the synthesis of oxaprozin, is a symmetrical non-polar compound and was not eluted using the above HPLC method. However, using a modified mobile

phase consisting of 0.01 M KH_2PO_4, acetonitrile (1:9), pH 4.2, TPP was eluted and determined in oxaprozin samples. The retention time of TPP was 6.1 minutes, and that of oxaprozin was 3.1 minutes.

Accelerated degradation studies were performed to conform the validity of the method, also to demonstrate its specificity and to show that it can be stability indicating. Two separate solutions of standard oxaprozin (1 mg/mL in mobile phase) were prepared. One solution was exposed to ultraviolet (254 nm and 366 nm) light for 40 hours, and the other was kept at 50°C for two days. The degraded samples were then analyzed using the above HPLC method. Interestingly enough, oxaprozin exposed to UV light was mostly converted to WY-23,027, accompanied by some unidentified decomposition products, as shown in Fig 4. On the other hand, the heated oxaprozin solution showed several decomposition products, including three known related compounds (WY-23,096, WY-23,120 and WY-22,075), but no WY-23,027 was formed, as shown in Fig 5. Both degraded samples (UV and 50°C) were subjected to diode array analysis, and the more interesting photodegraded plot is shown in Fig 6. This plot report showed that oxaprozin (Rt = 22 min) and WY-23,027 (Rt = 26 min) had no detectable impurity peaks embedded in them and are free of co-eluting compounds. Their purity parameter values are not changing over the course of the peak and appear with a flat top. The earlier peaks, on the other hand, are bifurcated or show slope at

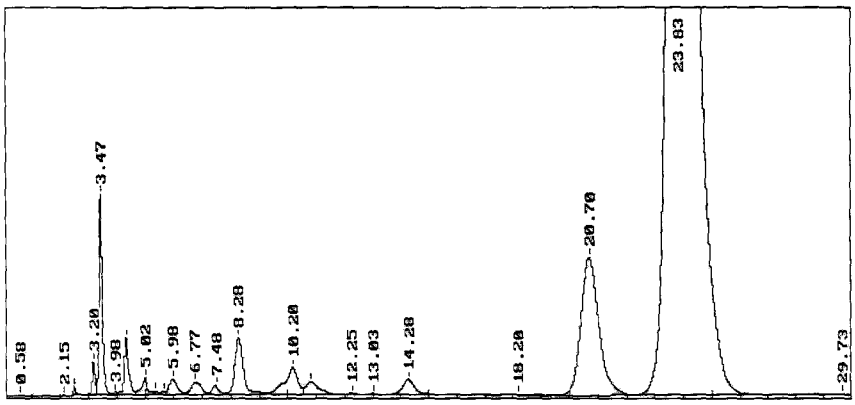


Fig. 4. Chromatogram of a solution of oxaprozin exposed to ultraviolet light. Column 300 x 3.9 mm μ Bondapak C_{18}, 1.0 mL/min, detection at 254 nm.

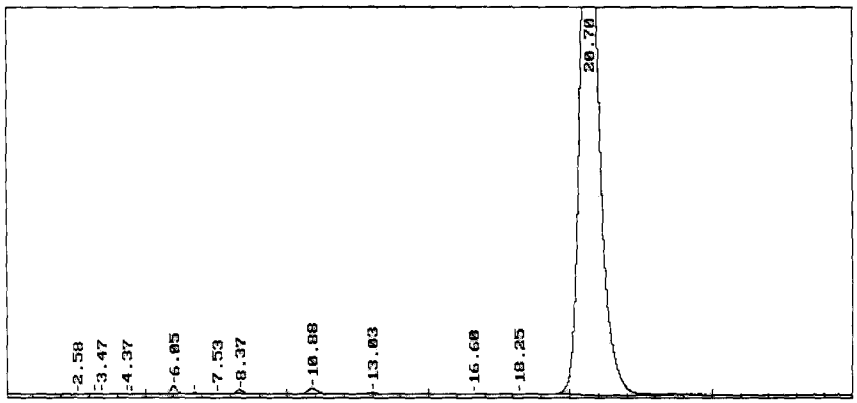


Fig. 5. Chromatogram of a solution of oxaprozin exposed to heat. Column, 300 x 3.9 mm μ Bondapak, C_{18}, flow 1.0 mL/min, detection at 254 nm.

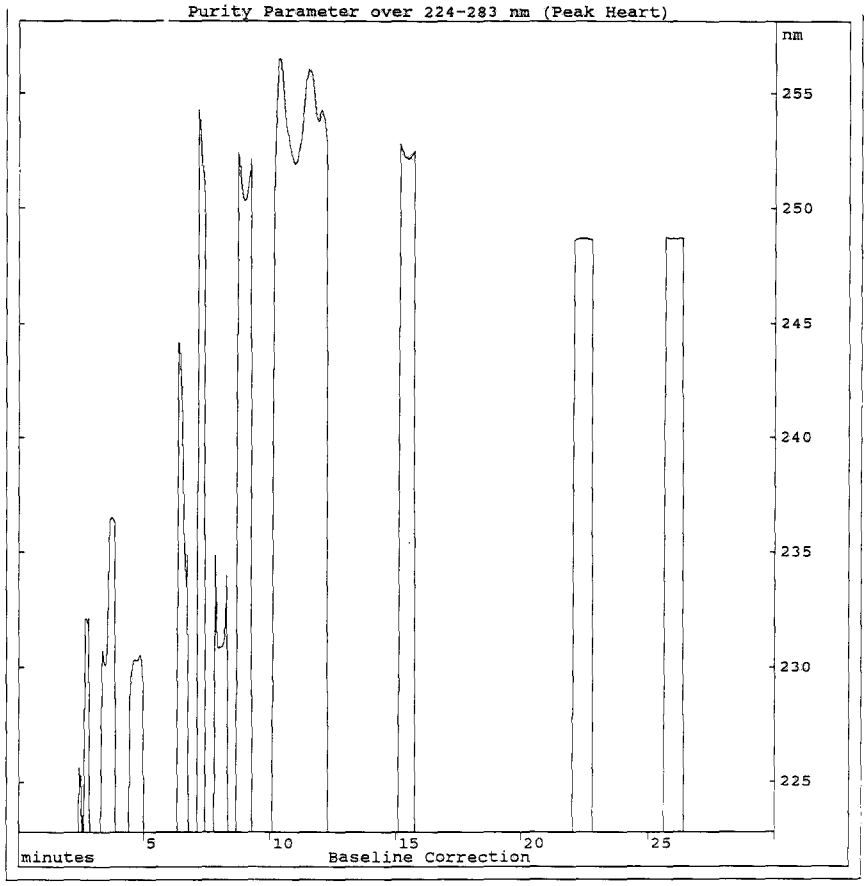


Fig. 6. Peak purity at peak heart for a photodegraded oxaprozin sample performed over 224 nm - 283 nm using diode array spectrophotometry.

top, indicating that their purity parameter values are not consistent across the width of the peak and hense the coelution of two (or more) substances (Fig 6).

This illustrates that the method can be used for determining the stability of oxaprozin in bulk pharmaceuticals. The method is also capable of detecting

intermediates and other related compounds which might be present at trace levels in finished products. Also, kinetic measurements can be carried out by this method to determine completion times for synthetic reactions.

CONCLUSION

The above method provides a simple technique to separate and determine quantitatively oxaprozin from its precursors or possible related compounds in bulk pharmaceuticals. The method was rapid because no extraction was involved. Oxaprozin and several of its related compounds were quantitatively determined simultaneously in a single run. Furthermore, the method is stability-indicating, since several analogs and degradation products which might have been formed under the described stress conditions were completely resolved from oxaprozin (Figs 4 and 5) as shown by the photodiode array technique (Figs 2 and 6).

ACKNOWLEDGEMENTS

The author expresses his gratitude to Orrin Viele III and Daniel Gawiak for their helpful suggestions and for reviewing the manuscript. He also thanks Tim Ryan for running the samples using the diode array instrument and for plotting their graphs.

REFERENCES

1. W.J. Reynolds, S.F. Shaar, A. Bulk, W.J. Lancee, J. Rheumatol., **6**: 345-350 (1979).

2. K. Brown, J.F. Cavalla, D. Green, A.B. Wilson, Nature, **219**: 164 (1968).

3. D.M. Pierce, Xenobiotica, **11**: 857-862 (1981).

4. F.W. Janssen, S.K. Kirkman, J.A. Knowles, H.W. Ruelius, Drug Metab. Dispos., **6**: 465 (1978).

5. M. Kurowski, H. Thabe, Agents Actions, **27**: 458-460 (1989).

6. H.R. Ochs, D.J. Greenblatt, M. Knuchel, Arzneimittelforschung, **36**: 1837-1840 (1986).

7. D.J. Greenblatt, R.M. Arendt, A. Locniskar, Arzneim.-Forsch., **33**: 1671-1673 (1983).

8. S.L. McHugh, S.K. Kirkman, J.A. Knowles, J. Pharm. Sci., **69**: 794-796 (1980).

9. D.J. Greenblatt, R. Matlis, D.R. Abernathy, H.R. Ochs, J. Chromatogr., **275**: 450-457 (1983).

10. D.J. Greenblatt, R. Matlis, J.M. Scavone, G.T. Blyden, J.S. Harmatz, R.I. Shader, J. Clin. Pharmacol., **19**: 373-378 (1985).

11. R. Matlis, D.J. Greenblatt, J. Chromatogr., **12**: 445-449 (1984).

12. S.T. Chiang, J.A. Knowles, J.A. Hubsher, H.W. Ruelius, B.R. Walker, J. Clin. Pharmacol., **24**: 381-385 (1984).

Received: April 12, 1995
Accepted: April 12, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2635-2645 (1995)

A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE SEPARATION OF COCAINE AND SOME OF ITS METABOLITES FROM ACEPROMAZINE, KETAMINE, AND ATROPINE FROM SERUM

J. MUZTAR, G. CHARI, R. BHAT, S. RAMARAO, AND D. VIDYASAGAR
Department of Pediatrics
The University of Illinois at Chicago
Chicago, Illinois 60612

ABSTRACT

A high-performance liquid chromatographic procedure for the separation of cocaine and its metabolites from such non-test drugs as acepromazine, ketamine and atropine in serum is described. The method involves the use of a SemiPermeable Surface (SPS) C8 column with a mobile phase constituted of 3.25% tetrahydrofuran and 96.75% 0.0025 M potassium phosphate buffer, v/v, containing 0.0025% triethylamine, the final pH of the mobile phase being 2.75. The flow rate was 0.5 ml/min. A photodiode array detector equipped with a computer software was used to monitor the analyte peaks. Retention times for cocaine (COC), benzoylecgonine (BE), benzoylnorecgonine (BN), norcocaine (NOR) and bupivacaine (BV) were 15.5, 7.4, 12.25, 21.0 and 24 minutes, respectively. The non-test drugs ketamine and atropine co-eluted at 10.0 minutes while acepromazine eluted with the solvent front (3.8 min.). The sensitivity of this assay for each of COC, BE, BN and NOR, at a signal to noise (S/N) ratio of greater than 6.0, was 1.0 ng/ml while that for BV was 5ng/ml at the same S/N value.

INTRODUCTION

There are numerous published methods for the determination of cocaine (COC) and one or more of its metabolites in biological fluids and tissue, using high-performance liquid chromatography (HPLC) (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11). However, none of these methods have

addressed the issue of separating COC and its metabolites from acepromazine (ACE), ketamine (KET) and atropine (ATR). Experiments investigating the distribution of COC and its metabolites in tissues and biological fluids of larger animal species, particularly the non-human primates (monkeys, baboons, etc.), commonly involve administration of these non-test drugs at frequent intervals before surgery and during sampling in order to sedate the animal and thus permit safe handling (12, 13, 14)..

Due to the basic nature of these drugs and those of COC and its metabolites, all these are expected to demonstrate very similar chromatographic properties and hence , not surprisingly we were not able to separate them using the existing HPLC methods (Muztar et al , unpublished results).

The adverse pharmacological effects of COC and its active metabolite benzoylecgonine (BE) on the fetus of pregnant women abusing the drug are well established (15, 16). Therefore, the need for an analytical method that can distinctly separate COC and its metabolites from the non-test drugs can hardly be overemphasized.

In the present study, we have developed an HPLC method which separates COC and some of its metabolites, norcocaine (NOR) , BE and benzoylnorecgonine (BN) from the non-test drugs (ACE, KET and ATR).

MATERIALS AND METHODS

Reagents and standards

HPLC grade tetrahydrofuran (THF) and reagent grade triethylamine (99%) were purchased from Fisher Scientific (Itasca, Illinois, U.S.A.).

(-) Cocaine hydrochloride, BE, and bupivacaine (BV) were purchased from Sigma (St. Louis, Missouri, U.S.A.). (-) Benzoylnorecgonine hydrochloride and (-) norcocaine were supplied by NIDA, National Institute of Health , Rockville, Md. Benzethonium chloride and benzyl alcohol were purchased from Aldrich Chemicals (Milwaukee, U.S.A). Atropine sulfate was a product of

American Reagent Laboratories (Shirley, NY, U.S.A.), while acepromazine maleate and ketamine hydrochloride were made by Aveco Co. (Fort Dodge, Iowa, U.S.A.) and Parke-Davis (Morris Plains, New Jersey, U.S.A.), respectively.

Stock solutions (1.0 mg/ml) of COC , BN and NOR were made in water. BE was taken in 0.1 M $NaHCO_3$. Appropriate dilution of the stock solutions were made for running HPLC standards and to spike drug-free serum except for BN which was spiked at four times the above concentrations because of its very poor recovery. The internal standard BV was used at a concentration of 0.5, 0.1, 0.2, 0.4, 0.8 and 1.0 μg/ml.

Chromatographic conditions

A Waters 600E multi-solvent HPLC pump was used to provide solvent at a flow rate of 0.5 ml/min. Tetrahydrofuran was used as the organic modifier. A 0.0025 M potassium phosphate buffer mixed with THF (96.75: 3.25, v/v) containing 0.0025% triethylamine with the final pH adjusted to 2.7 to 2.8 with 85% orthophosphoric acid formed the mobile phase.

A SemiPermeable Surface (SPS) C8 column (Regis Chemicals, Morton Grove, IL, U.S.A.) with dimensions of 15-cm length and 4.5-cm i.d. was used for the analysis of the drugs. The HPLC system was equipped with a U6K injector (Water-Millipore, Milford, MA) and a 100-μl loop. The eluting drugs were detected with a Waters 991 photodiode array detector at 4 different wavelengths.

Extraction of the drugs

Solid phase extraction of the drugs form spiked serum samples was carried out using 3-ml Clean Screen Columns (Worldwide Monitoring, Horsham, PA, U.S.A). The cartridges were placed on a 24-station VAC-Elut (Varian, Harbor City, CA, U.S.A.) manifold and vacuum applied from 1 to 5 mmHg or as necessary. The cartridges were first conditioned by washing with methanol (2 x 2 ml), then with water (3 ml) and 0.01 M phosphate buffer, pH 3.0 (3 ml). A 1-ml serum mixed with 0.5 ml of a 0.01 M phosphate buffer, pH 3.0, was then applied to the

extraction cartridge and air dried for about 30 s. This was then washed with 3 ml of phosphate buffer, 3 ml of 100 mM HCL and finally with 3 ml of methanol. The elution of the drugs was performed by using 2 ml of $CHCl_3$- Isopropyl alcohol- NH_4OH (77:20,5:2.5). The extract was evaporated at 30° C by passing N_2 gas using the Meyer N-Evap analytical evaporator (Organomation Assoc., Berlin, MA, U.S.A.) and taken in 100μl of the mobile phase for injection into the HPLC column.

RESULTS

The calibration was performed using seven concentrations (50 ng/ml to 1.0 μg/ml) of COC, BE, NOR in serum. The mean coefficient of variation (CV) was 10.4 %. Linear correlations were done by regression analysis. The correlation coefficients for each standard were > 0.998. Similar correlations were obtained for BN with four times greater concentrations. The intra-assay precisions were done over 5 days for 500 ng/ml and 1μg/ml concentrations. The CVs ranged from 4.2 to 7.6 % for COC, BE, NOR and BN. The inter-assay precision CVs ranged from 4.8 to 7.3 %. Since quantitation of the non-test drugs ACE, KET and ATR was not the focus of this study, the precision of the HPLC measurements for these drugs was not determined.

A standard chromatogram (Figure 1) obtained with a mixture of the test drugs COC and its three metabolites along with the non-test drugs KET, ACE and ATR demonstrated a clear separation of COC and its three metabolites from the non-test drugs. Retention times for BE, BN, COC and NOR were 7.4, 12.25, 15.5 and 21.0 min., respectively and that for the internal standard BV was 24.0 min. Acepromazine eluted at 3.8 min.

Serum was spiked with 10 ng (injected volume 10 μl) of both the test and non-test drugs as well as the internal standard and extracted with Clean Screen column as described in the experimental section. HPLC analysis (Figure 2) showed a clear separation of COC and its three metabolites as well as the internal standard from the non-test drugs. Also noticeable was the fact

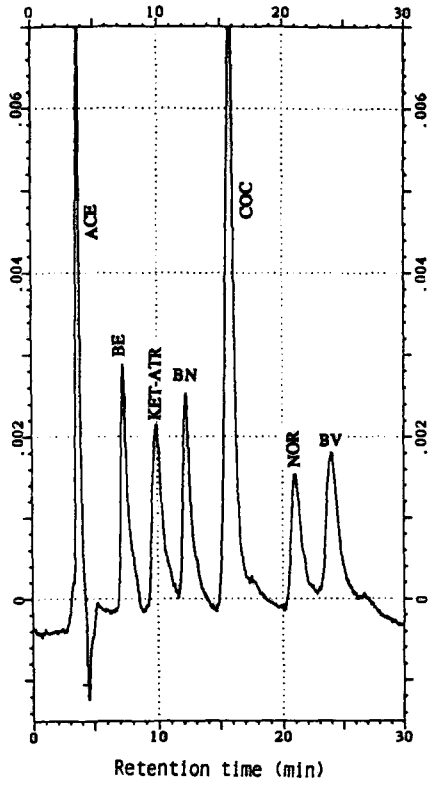


FIGURE 1: Chromatogram showing the separation of a standard mixture of the test drugs COC, BE, BN, NOR and BV from the non-test drugs , ACE, KET and ATR.

that there was no peaks of the additives, benzethonium chloride and benzyl alcohol present in ketamine and atropine. Apparently these were washed out from the SPE cartridge during the sample clean-up steps. Figure 4 shows a chromatogram of a serum sample obtained from a baboon injected with 1 mg/kg of COC.

Comparing differences of areas under the curve of extracted and unextracted samples for the same concentrations, recoveries of BE , COC and NOR were 95, 97 and 96% respectively and of BV was 95%. Recovery of BN was very poor (<2%). Limit of detection for the COC and its

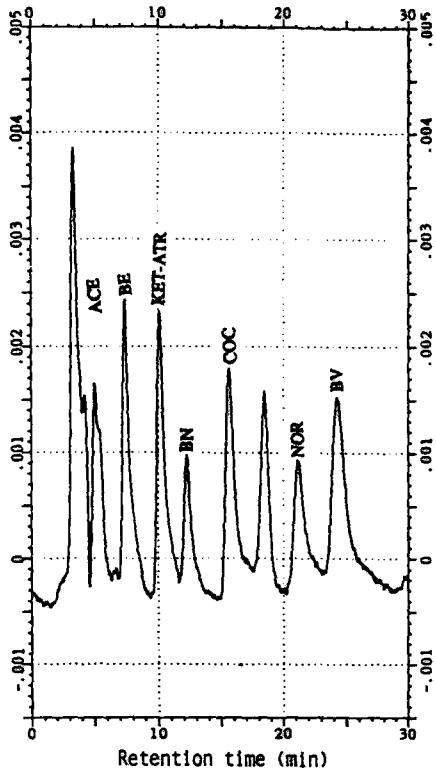


FIGURE 2: Chromatogram showing the separation of a serum extract mixture of 10 ng (10 µl injection) of the test drugs cocaine (COC), benzoylecgonine (BE), benzoylnorecgonine (BN), norcocaine (NOR) with the internal standard bupivacaine (BV) plus the non-test drugs acepromazine (ACE), ketamine (KET) and atropine (ATR).

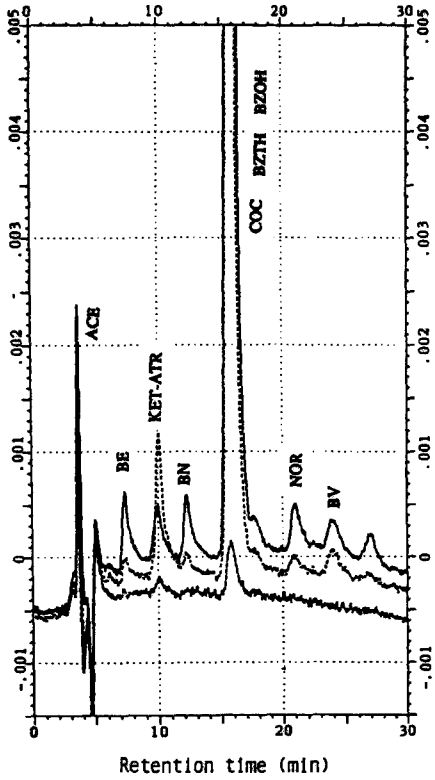


FIGURE 3: Shows the absorbance spectra of a standard mixture of the test drugs and the non-test drugs at three different wavelengths, 235 (------), 255 (------) and 280 nm (.....). BZTH refers to benzethonium chloride and BZOH to benzyl alcohol. For other abbreviations see Figures 1 & 2.

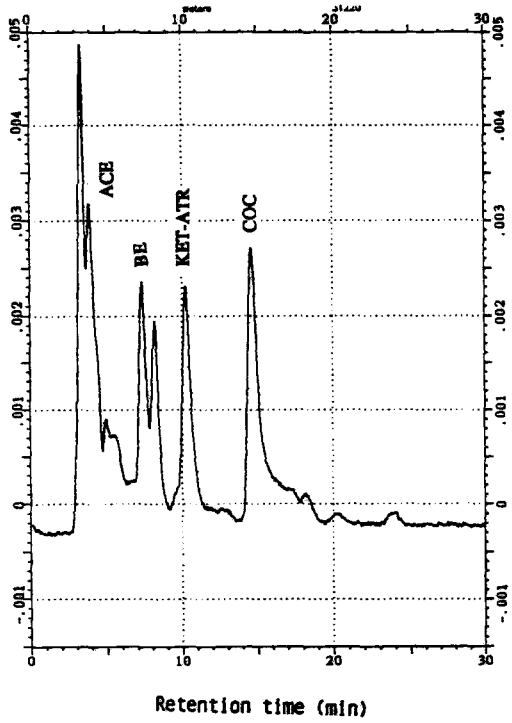


FIGURE 4: Chromatogram obtained from a baboon serum sample injected with 1 mg/kg of COC.

three metabolites was 1.0 ng/ml at a signal to noise (S/N) ratio of greater than 6.0, while for BV was 5.0 ng/ml at the same S/N ratio. Since quantitation of the non-test drugs ACE, KET and ATR was not the focus of this study, recovery data was not obtained.

DISCUSSION

In theory, the non-test drugs can be thought of being eliminated during sample preparation in the solid phase extraction (SPE). However, it is important to note that SPE procedures rely heavily on the gross differences in such physico-chemical parameters as pKa and hydrophobicity,

and thus lack manipulation capability and the subtle selectivity of an analytical method such as HPLC. For example, BE (pKa 2.6), which is far apart from that of COC (pKa 8.6) and the non-test drugs, ACE (pKa 9.3), KET (pKa 7.5) and ATR (pKa 9.9), yet attempts to elute BE alone from the SPE cartridge using an eluent mixture of pH 8.0 to 10.0 results in very poor or no recovery (Muztar et al, unpublished results). On the other hand, when using an eluent mixture of pH $\geq$ 11.5, over 90% of BE and COC are eluted and so are the non-test drugs (ACE, KET and ATR).

Using the existing HPLC procedures we were unable to separate BE and BN in serum samples obtained from animals administered with COC and non-test drugs because of the overlapping retention times. Further, our attempts to accurately quantitate BE and BN in serum by analyzing chromatograms using differential wavelength scanning produced very little success since the non-test drugs, particularly ATR and KET, show significant absorption over the entire absorption wavelength of both BE and BN (Figure 3). For reason of clarity, absorption spectra is shown only for three wavelengths (235, 255 and 280 nm). However, the 200 through 210 nm region causes KET and ATR to absorb as much as BE and BN while the 215 through 235 nm zone demonstrates relatively less absorption. On the other hand, in the 240 through 280 nm region KET and ATR absorb much more than BE and BN with absorption increasing with an increase in the wavelength. In the present separation method the absorption of ACE with respect to the wavelength is not in sequence since it is found to elute with the solvent front. From these results it is apparent that the non-test drugs absorb much less than the test drugs between the wavelengths of 215 through 235 nm.

Initially, a number of mobile phases consisting of various proportions of acetonitrile to varying molarities of phosphate buffer were tried but none separated KET and ATR from BE while certain proportions caused coelution of BN and COC. Finally, acetonitrile was replaced with tetrahydrofuran. Using 0.0025 M potassium phosphate buffer (96.75%) to THF (3.25%), v/v, and 0.0025% triethylamine with the final pH adjusted to 2.7 to 2.8 with 85% orthophosphoric

acid, we were able to clearly separate both BE and BN from ACE, ATR and KET as evident from Figures 1 and 2.

It was also evident from the test chromatogram (Figure 2) that the additives benzethonium chloride and benzyl alcohol, contained in the non-test drugs, are eliminated during the SPE treatment as indicated by the absence of their respective peaks. Absence of these peaks from the chromatogram was confirmed by extracting serum spiked with a known amount of these two compounds. The disappearance of the additive peaks post-extraction is not surprising because these differ substantially in structure from COC and its metabolites as well as the non-test drugs and hence should exhibit significantly different physico-chemical properties during sample clean-up using the Clean Screen solid phase extraction cartridge.

Another important aspect of this separation method is that, it not only distinctly separates the COC and its metabolites from the non-test drugs, but also provides us with a very sensitive HPLC procedure (detection limit of 1.0 ng/ml, S/N ratio > 6.0).

ACKNOWLEDGEMENTS

The authors wish to thank the National Institute on Drug Abuse (Rockville, MD, U.S.A) for providing samples of BN and NOR. This work was supported in part by NIH Grant HHS-DA 06794-02.

REFERENCES

1. R. L. Miller, C.L. DeVane, J. Chromatogr., 570: 412-418 (1991).

2 J. A. Sandberg , G.D. Olsen, J. Chromatorgr., 525: 113-121 (1990).

3. R. R. MaCgregro, J. S. Fowler, A. P. Wolf, J. Chromatogr., 590: 354-358 (1992).

4. C. E. Lau, F. Ma , J. L. Falk, J. Chromatogr., 532: 95-103 (1990)

5. P. Jatlow , H. Nadim, Clin. Chem., 36:1436-1439 (1990).

6. I. R. Tebbett, Q. W. MacCartney, Forensic Sci. Int., 39 287-290 (1988).

7. S. P. Brown, C. M. Moore, J. Scheurer, I.R. Tibbet, B. K. Logan, J. Forensic Sci., 36: 1662-1665 (1991).

8. B. M. Lampert , J. T. Stewart, J. Chromatogr., 495: 153-165 (1989).

9. K. Nakashima, M. Okamoto, K. Yashida, N. Kuroda , S. Akiyama, M. Yamaguchi , J. Chromatogr., 584:275- 279 (1992).

10 S. M. Roberts, J. W. Munson, R. C. James , R. D. Harbison. Anal. Biochem., 202: 256-261(1992).

11. L. J. Murphey, G. D. Olsen and R. J. Konkol, J. Chromatogr., 613: 330-335 (1993).

12. Z. Binienda, J. R. Bailey, H. M. Duhart, W. Slikker, Jr. , M. G. Paule, Drug Met. Dispos. Biol. Fate. Chem ., 21 : 364-368 (1993).

13. M. A. Morgan, S. L. Silavin, M. Randolph, G. G. Payne, Jr., R. E. Sheldon, J. I. Fishburne, Jr., R. A. Wentworth , P. W. Nathanielsz, Am. J. Obstet. Gynecol., 164 :1021-1027 (1991).

14. L. L. Howell , L. D. Byrd, J. Pharmcol. Exp. Ther., 258: 178-185 (1991).

15. B. B. Little, L. M. Snell, V. R. Klein, L. C. Gilstrap III, Obstet. Gynecol., 73 :157-160 (1989).

16. D. A. Roe, B. B. Little, R. E. Bawdon, L. C. Gilstrap III, Am. J. Obstet. Gynecol., 163 715-718 (1990).

Received: February 4, 1995
Accepted: February 17, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2647-2659 (1995)

HPLC DETERMINATION OF ONDANSETRON-ATROPINE AND ONDANSETRON-GLYCOPYRROLATE MIXTURES IN 0.9% SODIUM CHLORIDE INJECTION

T. G. VENKATESHWARAN, D. T. KING,
AND JAMES T. STEWART
Department of Medicinal Chemistry
College of Pharmacy
The University of Georgia
Athens, Georgia 30602-2352

ABSTRACT

High Performance Liquid Chromatography procedures have been developed for the assay of ondansetron-atropine and ondansetron-glycopyrrolate mixtures in 0.9% sodium chloride injection. The separation and quantitation of the ondansetron-atropine mix was achieved on an octylsilane column at ambient temperature using a mobile phase of 60:40 v/v 0.01 M phosphate buffer, pH 4-acetonitrile at a flow rate of 1.0 mL/min with detection of the analytes at 254 nm. The separation is achieved within 15 min. The method showed linearity for ondansetron and atropine in the 266-1332 and 28-138 μg/mL ranges, respectively. Accuracy and precision were in the 0.2-5.6% and 0.4-1.8% ranges, respectively, for both drugs. The limits of detection for ondansetron and atropine were 2.1 ng/mL and 8.6 μg/mL, respectively, based on a signal to noise ratio of 3 and a 20 μL injection. The separation and quantitation of the ondansetron-glycopyrrolate mix was achieved on an octylsilane column at ambient temperature using a mobile phase of 55:45 v/v 0.01 M phosphate buffer, pH 4- acetonitrile at a flow rate of 1.0 mL/min with detection of the analytes at 254 nm. The separation is achieved within 15 min. The method showed linearity for

ondansetron and glycopyrrolate in the 500-2000 and 50-200 μg/mL ranges, respectively. Accuracy and precision were in the 2.5-3.7% and 0.1-1.5% ranges, respectively, for both analytes. The limits of detection for ondansetron and glycopyrrolate were 90 ng/mL and 6.9 μg/mL, respectively, based on a signal to noise ratio of 3 and a 20 μL injection.

INTRODUCTION

Mixtures containing ondansetron-atropine and ondansetron-glycopyrrolate are used as perioperative medications in operating room in U.S. hospitals. Interest in our laboratories in the stability and compatibility of these drug mixtures over time in 0.9% sodium chloride injection required the development of HPLC methods for each mixture. A search of the literature indicated that HPLC methods were not available to assay for the mixtures concurrently in a single injection.

Individually, atropine and glycopyrrolate have been previously analyzed by non-aqueous titrimetry (1,2), spectrophotometry (3), radioimmunoassay (4), and gas (5) and high performance liquid chromatography (2,6). The titrimetric assays were used to assay each respective drug substance in USP 23/NF 18. The spectrophotometric method measured glycopyrrolate in commercial tablets using a bromocresol purple-drug ion pair extracted into chloroform. The radioimmunoassay procedure was used to analyze as low as 2.5 ng/mL levels of atropine in plasma or serum. The GC method provided separation of atropine on a 3% loaded stationary phase in a glass column with flame ionization detection. One HPLC method for atropine injection used an octadecylsilane column with an acetonitrile-acetate buffer

containing tetrabutylammonium hydroxide as an ion-pair reagent. An HPLC method for glycopyrrolate was also reported that utilized an octadecylsilane column and an acetonitrile-methanol-water mobile phase containing sodium 1-pentanesulfonate as an ion-pair reagent.

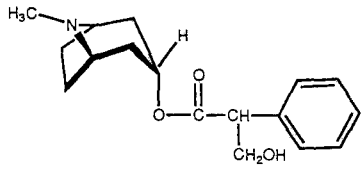
Ondansetron has been assayed by high performance thin-layer chromatography (HPTLC) and HPLC methods (7-9). The HPTLC method was developed especially for plasma samples, but the sample throughput was low and the equipment is not generally available in most laboratories. The HPLC assays used either a silica column with an aqueous-organic mobile phase or a cyanopropyl column operated in the reverse-phase mode.

In this paper, isocratic HPLC assays are presented that will simultaneously analyze for ondansetron-atropine or ondansetron-glycopyrrolate mixtures in 0.9% sodium chloride using a single injection. Each mixture is separated on an octylsilane column using a buffered aqueous-acetonitrile eluent. The separations are achieved within 15 min at ambient temperature with sensitivities in the ng/mL and μg/mL ranges for ondansetron and atropine/glycopyrrolate, respectively.

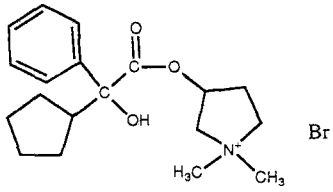
EXPERIMENTAL

Reagents and Chemicals

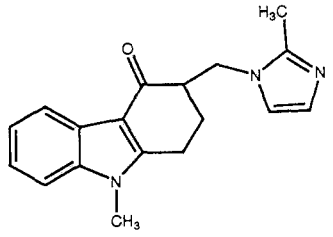
The structure formulae of the compounds studied are shown in Figure 1. Atropine sulfate (Lot K-1) and glycopyrrolate (Lot F-1) were



Atropine



Glycopyrrolate



Ondansetron

Figure 1 - Chemical Structures of Compounds Studied.

purchased from the United States Pharmacopeial Convention (Rockville, MD 20852). Ondansetron hydrochloride (Lots AWS17 or AWS332A) was a gift from Glaxo, Inc. (Research 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 potassium phosphate and potassium hydroxide were J.T. Baker analyzed reagents (Phillipsburg, NJ 08865).

Instrumentation

The chromatographic separations were 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). The ondansetron-atropine separation was accomplished on an octylsilane column (RP-8 Spheri 5, 100 x 4.6 mm id, Applied Biosystems, Foster City, CA 94404). The mobile phase consisted of 60:40 v/v 0.01 M aqueous monobasic potassium phosphate, pH 4.0 (adjusted with 1 N potassium hydroxide)-acetonitrile.

The ondansetron-glycopyrrolate separation was achieved on an octylsilane column (RP-8 Spheri 5, 100 x 4.6 mm id, Applied Biosystems, Foster City, CA 94404). The mobile phase consisted of 55:45 v/v 0.01 M aqueous monobasic potassium phosphate, pH 4.0 (adjusted with 1 N potassium hydroxide)-acetonitrile.

Each 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 on both columns was 1 mL/min and the detector was set at 254 nm for both assays.

Preparation of Standard Solutions

A combined standard solution for ondansetron-atropine was prepared by adding accurately weighed samples of 14.8 mg of ondansetron hydrochloride and 1.65 mg of atropine sulfate into a 10 mL volumetric flask, and adding 0.9% sodium chloride injection to volume. This combined standard solution along with appropriate dilutions of the solution gave solutions containing 266, 666 and 1332 μg/mL of ondansetron and 27.6, 69.1 and 138.2 μg/mL of atropine expressed as free base concentrations. The same process was used to prepare a combined standard solution and dilutions for ondansetron-glycopyrrolate to obtain solutions containing 500, 1000 and 2000 μg/mL for ondansetron and 50, 100 and 200 μg/mL for glycopyrrolate expressed as free base concentrations. Three point calibration curves were constructed for each analyte in their respective mixtures and additional dilutions in sodium chloride injection were prepared to serve as spiked samples to determine accuracy and precision of method for each of the analytes. 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 HPLC assays using isocratic conditions for the analysis of an ondansetron-atropine or ondansetron-glycopyrrolate mixture in 0.9% sodium chloride injection. A stability study of each mixture would require an assay which would detect and quantitate each analyte with reasonable accuracy and precision.

There were no reports in the scientific literature describing separation and quantitation of ondansetron-atropine or ondansetron-glycopyrrolate in a single mixture. Studies to develop a single isocratic HPLC method for each mixture involved the use of underivatized silica, phenyl, octyl, base deactivated octyl and octadecyl columns with various mobile phases containing methanol-aqueous phosphate buffers and/or acetonitrile-aqueous phosphate buffers at 1 mL/min.

The octylsilane column was finally selected for both assays based upon peak separation and run-time parameters versus the actual concentration levels of the analytes in the injection stability study. In addition, there were no interferences from the paraben preservatives contained in the commercial injections. Typical chromatograms showing the separation of the two analytes in each of the two mixtures are shown in Figures 2 and 3. The detection wavelength of 254 nm was selected for both assays since it provided good accuracy and precision data for each two component mix.

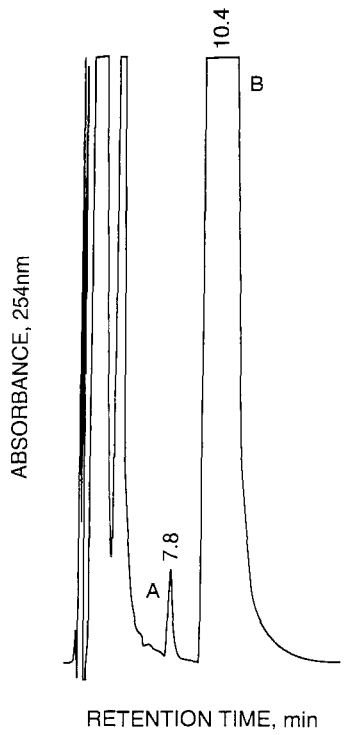


Figure 2 - Typical HPLC chromatogram of atropine (A) and ondansetron (B) on an octylsilane column with an aqueous phosphate buffer pH 4.0 - acetonitrile mobile phase. See Experimental Section for assay conditions.

The HPLC methods showed concentration versus absorbance linearity for the analytes at 254 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 the analytes would interfere with

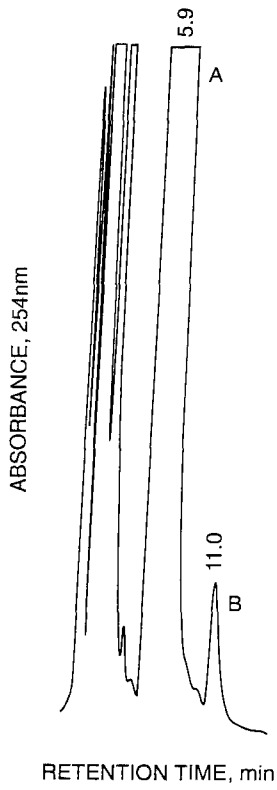


Figure 3 **Typical HPLC chromatogram of ondansetron (A) and glycopyrrolate (B) on an octylsilane column with an aqueous phosphate buffer pH 4.0- acetonitrile mobile phase. See Experimental Section for assay conditions.**

the quantitation of the other drug in the mixture at 254 nm. These experiments were performed on solutions of each drug in 0.9% sodium chloride injection after they had been degraded for 6 hr at 80 ± 1°C in both 1.0 N acid and 1.0 N base.

TABLE 1

Analytical Figures of Merit for Atropine, Glycopyrrolate and Ondansetron in Their Respective Mixtures.

Mixture	r^2	System Suitability	LOD[i]	k[j]	Theoretical Plates[j]	Tailing Factor[k]	Rs
1. Atropine	0.9988[a]	2.26[e]	8.6 μg/mL	4.91	915	1.4	3.4
Ondansetron	0.9939[b]	0.98[f]	2.1 ng/mL	6.99	984	1.8	
2. Glycopyrrolate	0.9989[c]	1.62[g]	6.9 μg/mL	7.94	1060	2.0	3.4
Ondansetron	0.9984[d]	1.62[h]	89.8 ng/mL	3.85	355	1.4	

[a] Range examined from 27.6 - 138.2 μg/mL atropine (n = 9).
[b] Range examined from 266.4 - 1332 μg/mL ondansetron (n = 9).
[c] Range examined from 50 - 200 μg/mL glycopyrrolate (n = 9).
[d] Range examined from 500.0 - 2000 μg/mL ondansetron (n = 9).
[e] Mean RSD% of 6 replicate injections at 69.1 μg/mL atropine,
[f] Mean % RSD of 6 replicate injections at 666 μg/mL ondansetron,
[g] Mean % RSD of 6 replicate injections at 100 μg/mL glycopyrrolate,
[h] Mean % RSD of 6 replicate injections at 1000 μg/mL ondansetron.
[i] Limit of detection, S/N = 3.
[j] Calculated as $N = 16 (t/w)^2$.
[k] Calculated at 5% peak height.

Table 2

Accuracy and Precision Using Samples With Added Drug

Analyte	Concn Added (μg/mL)	Concn Found (μg/mL)[a]	Percent Error	RSD (%)
1. Atropine	46.1	48.7 ± 0.87	5.6	1.8
	82.9	82.6 ± 0.78	0.4	0.9
Ondansetron	444.0	440.9 ± 5.21	0.7	1.2
	799.2	797.9 ± 3.52	0.2	0.4
2. Glycopyrrolate	77.3	79.2 ± 0.74	2.5	0.9
	154.6	160.3 ± 2.36	3.7	1.5
Ondansetron	750.0	776.3 ± 3.41	3.5	0.4
	1500.0	1538.4 ± 1.86	2.6	0.1

[a] Based on n = 3.

Percent error and precision of each method were evaluated using samples containing added analyte. The data shown in Table 2 indicate that both procedures give acceptable accuracy and precision for each analyte in each of the two mixtures.

Intra and inter-day variabilities of the assay for the ondansetron and atropine mixture were 2.14 (n = 6) and 0.98% (n = 15 over 3 days) and 3.33 (n = 6) and 2.26 (n = 15 over 3 days), respectively. Intra- and interday variabilities of the method for the ondansetron and glycopyrrolate mixture were 1.62 (n = 6) and 0.98% (n = 18 over 3 days) and 1.62 (n = 6) and 0.90% (n = 18 over 3 days), respectively.

In summary, an octylsilane column with an 60:40 v/v aqueous phosphate buffer pH 4- acetonitrile mobile phase for the ondansetron-

atropine assay and a 55:45 v/v aqueous phosphate buffer pH 4-acetonitrile mobile phase for the ondansetron-glycopyrrolate assay has been shown to be amenable for the separation and quantitation of each mixture in 0.9% sodium chloride injection. This study suggests that each HPLC method can be used to investigate the chemical stability of each drug in the respective mixtures.

ACKNOWLEDGEMENTS

The authors thank Glaxo Research Institute for financial assistance.

REFERENCES

1. Atropine Monograph, The United States Pharmacopeia 23- National Formulary 18, The United States Pharmacopeial Convention Inc., Rockville, MD, 1995, pp 145-146.

2. Glycopyrrolate Monograph, The United States Pharmacopeia 23 - National Formulary 18, The United States Pharmacopeial Convention Inc., Rockville, MD, 1995, p 716.

3. Glycopyrrolate Tablet Monograph, The United States Pharmacopeia 23- National Formulary 18, The United States Pharmacopeial Convention Inc., Rockville, MD, 1995, p 717.

4. R. Virtanem, J. Kanto, and E. Lisalo, Radioimmunoassay for Atropine and l-Hyoscyamine. Acta Pharmacol. Toxicol., 47, 208 (1980).

5. Atropine Ophthalmic Solution Monograph, The United States Pharmacopeia 23- National Formulary 18, The United States Pharmacopeial Convention Inc., Rockville, MD, 1995, pp 147-148.

6. Atropine Injection Monograph, The United States Pharmacopeia 23- National Formulary 18, The United States Pharmacopeial Convention Inc., Rockville, MD, 1995, pp 146-147

7. P.V. Colthup, in Recent Advances in Thin-Layer Chromatography, F. A. Dallas, H. Read, R.J. Ruane, and I.D. Wilson, Eds., Plenum, New York (1988) pp 179-185.

8. Personal Communication, J. Fox, Glaxo, Inc., Research Triangle Park, NC 27709 (1994).

9. P.V. Colthup, , C.C. Felgate, J.L. Palmer, and N.L. Scully, Determination of Ondansetron in Plasma and Its Pharmacolinetics in the Young and Elderly, J. Pharm. Sci., 80, 868 (1991).

Received: February 17, 1995
Accepted: March 7, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2661-2673 (1995)

TRIACYLGLYCEROLS-PROFILING BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: A TOOL FOR DETECTION OF PORK FAT (LARD) IN PROCESSED FOODS

KHALID A. AL-RASHOOD[1], EZZAT M. ABDEL-MOETY[1], ABDUL RAUF[1]*, RAFIQ R. ABOU-SHAABAN[2], AND KHALIL I. AL-KHAMIS[3]

[1]Department of Pharmaceutical Chemistry
[2]Department of Pharmaceutics
[3]Department of Clinical Pharmacy
College of Pharmacy
King Saud University
P.O. Box 2457
Riyadh-11451, Saudi Arabia

ABSTRACT

A rapid high-performance liquid chromatographic procedure for separation of the triacylglycerols (TG) of animal fats using refractive index detection is described. A LiChrospher-100 RP-18 (5 μm) column was used for the TG-profiling of pork, beef, mutton, chicken and turkey fats. Detection of pork fat in processed foods and lard in fat-admixtures is also discussed. TG-separation and checking genuinity and adulteration was achieved isocratically in $\sim$15 min. by using CH_3CN/CH_2Cl_2 (58:42, v/v) at ambient temperature.

* *Present Address:* Applied Sciences Section, University Polytechnic, Faculty of Engineering and Technology, Aligarh Muslim University, Aligarh 202002, India.

INTRODUCTION

Fraudulent substitution of expensive flesh with cheaper ones in meat products is objectionable to the consumer for different reasons; such as, medical requirements of a person who may have specific food allergies, economics and religious dietary restrictions. Inter-species meat adulteration is common in many parts of the world. Meat of similar pigmentation, beef and mutton or pork and poultry, are difficult to distinguish by eye once they have been frozen or cooked. In order to detect any possible substitution or admixing adulterations and to ensure that processed foods comply with quality restrictions, identification of the component lipid part of the food has become an important area of research to many analysts.

Several methods have been published on detection of lard in processed foods. Saeed *et al.* (1) selected the presence of 11,14-eicosadienoic acid as an indicator for lard. However, this acid has been claimed in beef and mutton samples (2). Pork fat has mainly 2-palmityl acylglycerols (3). Palmitic acid enrichment factor (PAEF), i.e. percent ratio of palmitic acid in the 2-monoacylglycerols to that of original triacylglycerols has been taken as a detection merit for lard (3,4). However, positional distribution affected by randomization. The use of enzyme-linked immunosorbent assay (ELISA) in food analysis has been also reported (5).

Reversed phase high-performance liquid chromatography (RP-HPLC) is a good separation technique and has application to the analysis of animal and vegetable fats and oils (6-10). Detection of TG is complicated as the ester bond absorbs at low wavelength which limits the selection of suitable mobile phase for UV-detection. Generally, refractive index (RI), (11), ultraviolet (UV) (12), for highly unsaturated TG,

and light-scattering detectors (13) are used for the analysis of TG. Refractive index detection is generally adopted becuase it doesn't have the drawback of low sensitivity and of different responses towards saturated and unsaturated TG like in case of UV-detection. HPLC-profiling of derivatized TG have been reported for the detection of pork in beef (14), but major disadvantage of the method is lengthy and needs tedius sample preparation.

This report describes a rapid RP-HPLC screening procedure for the profiling of the component TG of animal fat extracted from unprocessed and processed meat products. Also, TG-profiling of fat-admixtures are included. Such profiling characterization is of value for the genuinity testing and checking the adulteration in fatty products.

EXPERIMENTAL

Chemicals

All solvents were HPLC-grade (Merck, Darmstadt-Germany) and were filtered through a 0.22 μm Millipore filter. Standard simple and mixed TG ($\sim$99% purity), namely, trilaurin, tirpalmitin, tristearin, triolein and trilinolein were obtained from NU CHEK PREP, INC (Elysian, MN-USA) and 1,2-dioleoyl-3-palmitoyl-*rac*-glycerol (OPO), & 1-palmitoyl -2-oleoyl-3-stearoyl-*rac*-glycerol (OSP) were purchased from Sigma Chemical Co. (St. Louis, MO-USA).

Samples

Genuine and processed pork were obtained from the Amon Factories for pork and their processed foods, Shoubra, Cairo-Egypt, Some of the luncheon meat and

sausages were imported from USA and Bahrain. Processed samples of beef, mutton, chicken and turkey from various origin were obtained from the local markets. Genuine samples of beef and mutton fats have been freshly prepared from the underskin fat's caracass and adipose tissues (various parts) of freshly slaughtered animals at local slaughter house. For chicken and turkey fats, their skin and visible fats were removed from the flesh and homogenised. In all cases, lipids were extracted from the ground samples by modified Folch procedure (15) using $CHCl_3/CH_3OH$ (2:1, v/v) as the solvent. Fat extraction and storage were performed under nitrogen atmosphere to prevent oxidation of the unsaturated fatty acids.

Isolation of Triacylglycerols

TG were isolated from fats by column chromatography. Small chromatographic column (14 cm × 2 cm, ϕ) was packed with a slurry of 6 gm of silica gel 100 (70-230 mesh) in n-C_6H_{14}. After settle down of the silica, 0.5 g of the dired, melted fat in 0.5 ml of n-C_6H_{14} was introduced carefully into the top of the column and sample was allowed to adsorb on the surface of silica. Column was eluted with 20 ml of n-C_6H_{14} followed by 5% $(C_2H_5)_2O$ in n-C_6H_{14} till TG were completely eluted. Fractions, 20-ml each, were collected and the solvent was removed under vacuum. The purity of TG-fraction was checked on TLC-plate utilizing n-$C_6H_{14}/(C_2H_5)_2O/CH_3COOH$ (90:10:1, by volumes) as the developing system. Visualization of the spots was made by charring the plates after spraying with 20% $HClO_4$ (aq.)

If isolated fats from the processed foods containing only traces of free fatty acids, mono- and diacylglycerols and/or coloring matter, it is advisblae to use fat directly for analysis.

RP-HPLC/RI Analysis

The liquid chromatographic analysis of TG was performed on LiChrospher 100RP-18 (5 μm) column (12.5 cm $\times$ 4mm, ϕ, E. Merck, Darmstadt-Germany) and a refractive index detector (Waters-differential refractrometer R401). The mobile phase contained CH_3CN/CH_2Cl_2 (58:42, v/v) was used as an isocratic mixture at a flow rate of 1 ml/min^{-1} at ambient temperature. HPLC-system of Shimadzu (Kyoto, Japan), consisting of a solvent delivery module LC-10AD with a double plunger reciprocating pump, a DGU-3A degasser, an injector valve (20-μl loop), column embeded in a CTO-10A column oven and C-R4A Chromatopac multi-functional data processor, was used.

RESULTS AND DISCUSSION

Figure 1 shows the HPLC profile for a reference mixture containing four triacylglycerol standards, while, figure 2 represents the TG-separattion of genuine animal meat fats (beef, lard, mutton and chicken turkey). The complete elution of all the molecular species of genuine animal fat samples was under 15 minutes. The reproducibility of the retention times of the eluted peaks was very good for standard and other lipid samples.

Few molecular species of animal fats were characterized by comparing retention times of sample to that of analyzed standard. If three stereospeific position on glycerol molecule were assumed to be equivalent and isomers are not separated, then for seven fatty acids, the total possible molecular species was calculated to be 84 by using the formula $(n^3 + 3n^2 + 2n)/6$; (where n = number of fatty acids) (16). However, practically observed value for TG is always less than calculated number (17).

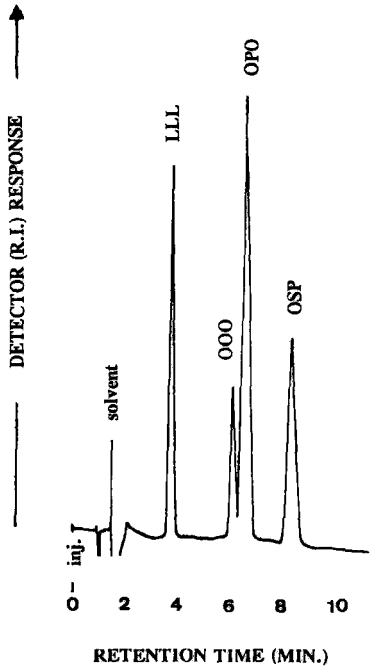


Figure 1: Separation of simple and mixed standard triacylglycerols.

The TG-profile of lard (figure 2) shows two most characteristic peaks with retention times, t_R-values, of about 5.60 and 8.66 min. Clear differences were observed in the overall profiles of beef, mutton; chicken and turkey which differentiate them from lard. Such fingerprints can be quickly used to check the presence or absence of lard (pork fat) in meat and meat products, even without making effort to obtain quantitative data.

TG-profile of admixed fats may depends principally on the mixing proportions of each fat component and follows the additivity rules. In order to check the validity

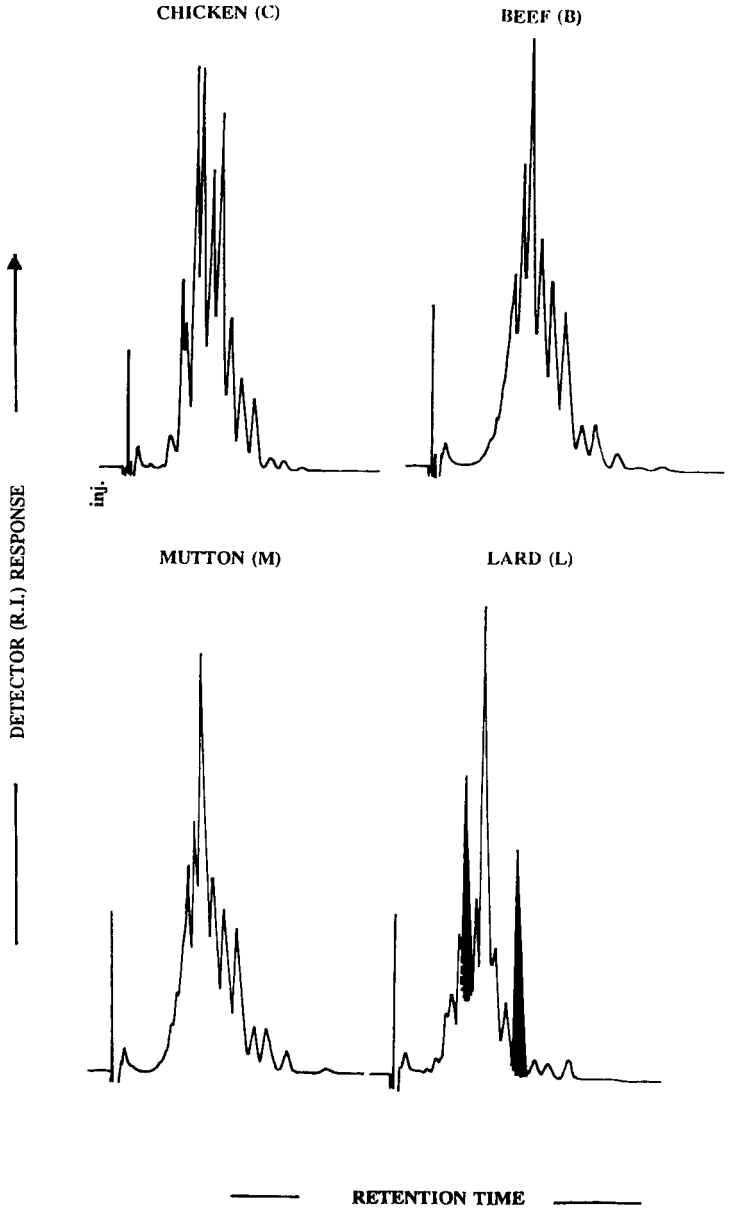


Figure 2: Component triacylglycerols of genuine beef, mutton, chicken and lard.

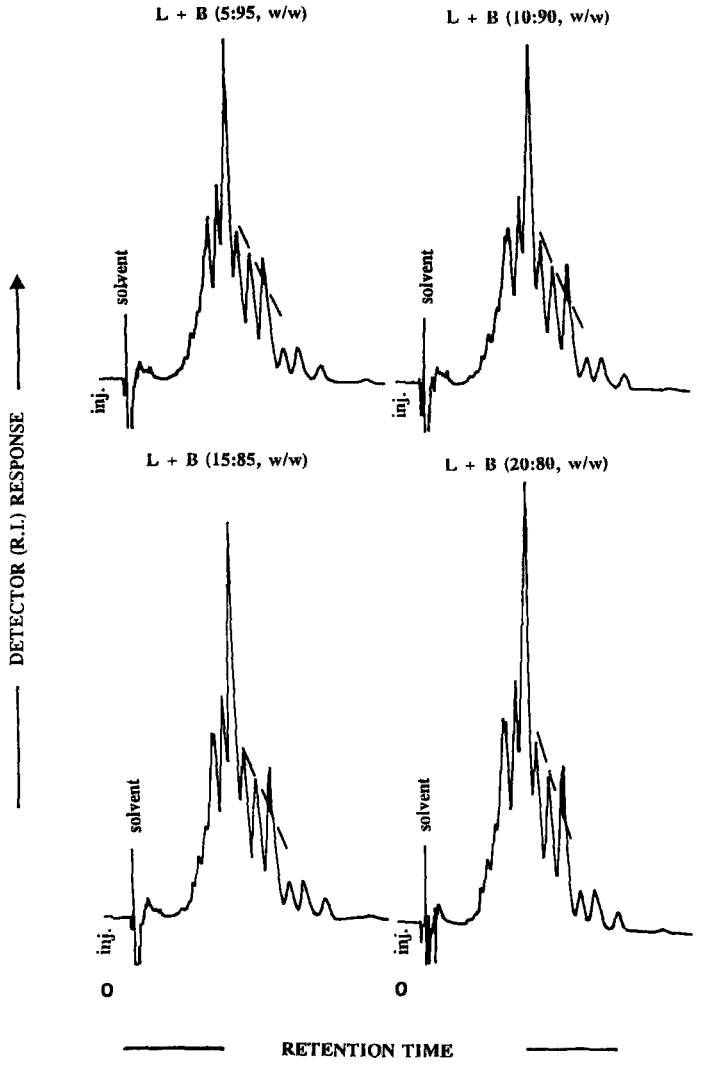


Figure 3: TG-profilings of different lard (L) beef (B) admixtures (5-20%, w/w).

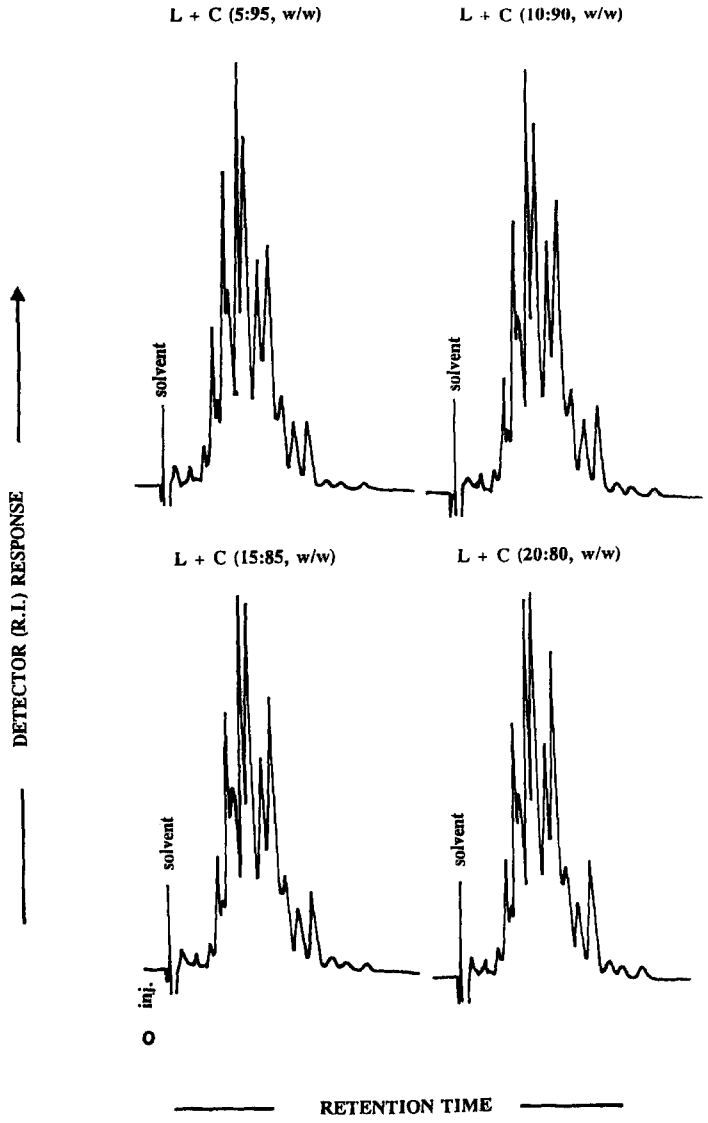


Figure 4: TG-profilings of different lard (L) chicken (C) admixtures (5-20%, w/w).

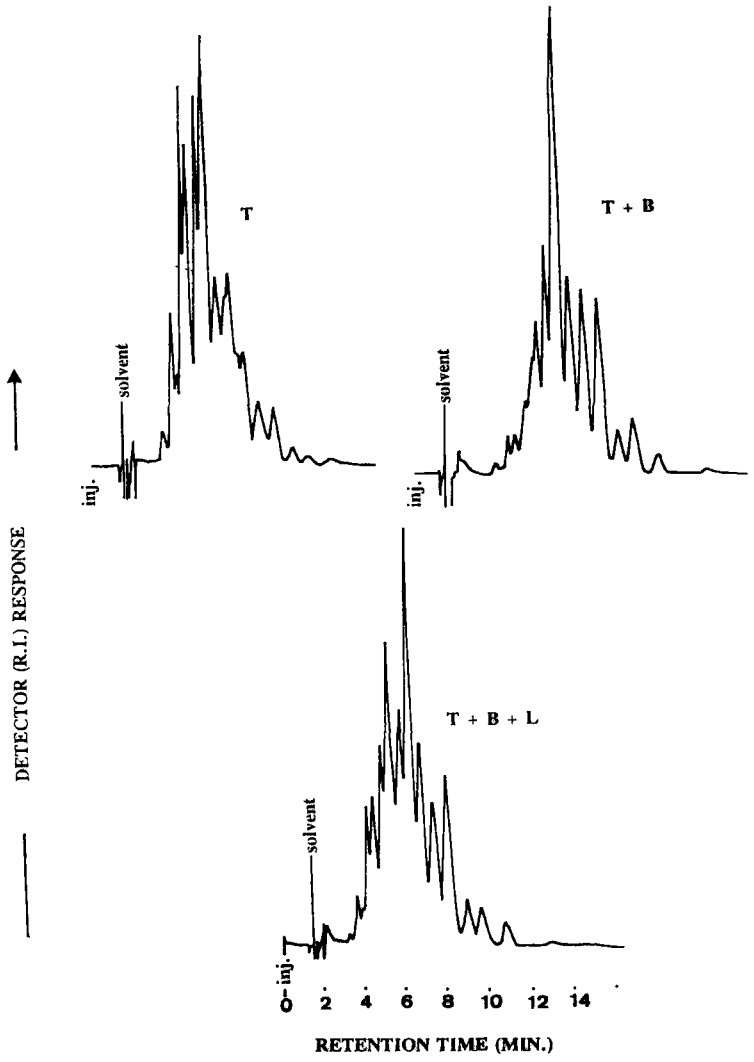


Figure 5: TG-profiling of genuine turkey fat (T), turkey fat + beef (B), and ternary admixture of turkey fat, beef and lard (L).

of the additivity measures in case of fat-admixtures containing lard and beef, binary labroatory admixtures of the individual genuine fats containing 5 to 20% lard and beef (w/w) have been prepared. The TG-profilings of such laboratory admixed fats at different proportions are represented in figure 3. Proportional increase in the peak intensity and net area count of that characteristic peaks of lard, *i.e.* dark shaded, can be clearly noticed. The indicative value of the justified peak for lard detection if admixed with beef is of great value and the additivity proportionality is quite rectilinear. Similarly, figure 4 illustrates the resolved triacylglycerols of laboratory prepared lard-chicken fat admixtures, where obvious differences could be identified. It is clear that the additivity rule is obeyed clearly on suggestive increasing of the lard portions added to chicken. Furthermore, it is clear that the addition of lard is easily detectable by observing the proportional increases in the intensity of the peak characteristic for it (figure 5).

The investigated TG-profiling method was also applied to triacylglycerols isolated from the processed meat products (salamy, sausages, luncheon) containing or free from pork of different origins. Clear detection of lard/pork can be easily concluded just by matching the obtained TG-profiles.

CONCLUSION

TG-profiling of animal fats and fats extracted from genuine meat specimens and from processed fatty foods is of great value for the detection of pork in processed foods samples. The detection limit for pork fat was observed to be about 5% admixtures based on the fat basis. The method is easy, simple, rapid and needs no derivatization,

and it allows also no artifact formation and confirms the presence of lard singly or admixed in food samples.

Acknowledgement

The authors are indebted to King Abdul-Aziz City for Science and Technology (KACST), Riyadh for financial support (Project No. AR-12-53).

REFERENCES

(1) SAEED T., ABU-DAGGA F., RAHMAN H.A., *J. Assoc. Off. Anal. Chem.* **69,** 999 (1986) .

(2) FIRESTONE D., *J. Assoc. Off. Anal. Chem.* **71,** 76 (1988).

(3) YOUSSEF M.K.E., OMAR M.B., SKULBERG A., RASHWAN M., *Food Chem.* **30,** 167 (1988).

(4) THIELEMANS A., MASSART D.L., DE-BRABANDER H., *Belg. J. Food Chem. Biotechnol.* **44,** 42 (1989).

(5) SAMARAJEEWA U., WEI C.I., HUANG T.S., MARSHALL M.R., *Critical Reviews in Fd. Sci. and Nutr.* **29,** 401 (1991).

(6) CHRISTIE W.W., *"High-Performance Liquid Chromatography and Lipids"*, Pergamon Press (1987).

(7) WOJITUSIK M.J., BROWN P.R., TURCOTTE J., *Chem. Rev.* **89,** 397 (1989).

(8) BARRON L.J.R., SANTA-MARIA G., *Rev. Agroquim. Tecnol. Aliment.* **31,** 66 (1991).

(9) AITZETMÜLLER K., GRÖNHEIM M., *Fat Sci. Technol.* **95,** 164 (1993).

(10) LETTER W.S., *J. Liq. Chromatogr.* **16,** 225 (1993).

(11) BLAND J.M., CONKERTON E.J., ABRAHAM G., *J. Am. Oil Chemist's Soc.* **68,** 840 (1991).

(12) AITZETMÜLLER K., GRÖNHEIM M., *J. High Resolution Chromatography* **15,** 219 (1992).

(13) CHRISTIE W.W. (eds) *"Advances in Lipid Methodology-One"*, The Oily Press, Ayr, Scotland, 239 (1992).

(14) SAEED T., ALI S.G., RAHMAN H.A., SAWAYA W.N., *J. Assoc. Off. Anal. Chem.* **72,** 921 (1989).

(15) CHRISTIE W.W. *"Gas Chromatography and Lipids"*, The Oily Press, Ayr, Scotland, 27 (1989).

(16) GUNSTONE F.D., NORRIS F.A., *"Lipids in Foods, Chemistry, Biochemistry and Technology"* Pergamon Press, NY, 19 (1983).

(17) HIERRO M.T.G., TOMAS M.C., FERNANDEZ-MARTIN F., SANTA-MARIA G., *J. Chromatogr.* **607,** 329 (1992).

Received: February 18, 1995
Accepted: March 6, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2675-2682 (1995)

DETERMINATION OF TOLFENAMIC ACID IN HUMAN PLASMA BY HPLC

I. NIOPAS AND M. GEORGARAKIS
Department of Pharmacy
Aristotle University of Thessaloniki
Thessaloniki 540 06, Greece

ABSTRACT

Tolfenamic acid is a potent prostaglandin synthetase inhibitor used clinically as non-steroidal anti-inflammatory and analgesic-antipyretic agent. A simple, sensitive, accurate, and precise reverse phase high performance liquid chromatographic method has been developed and validated for the quantitative determination of tolfenamic acid in small volumes of human plasma. The chromatographic separation of tolfenamic acid and the internal standard (phenylbutazone) was performed on a reversed phase, 5-μm C18 column (250 x 4 mm) using acetonitrile-10 mM phosphoric acid (60:40, v/v) as mobile phase with a flow rate of 1.1 ml/min and the chromatographic peaks were detected at 280 nm. Plasma was deproteinized with acetonitrile, the supernatant fraction was evaporated to dryness and the resulting residue was reconstituted in the mobile phase and injected into the HPLC system. Calibration curves were linear in the range 0.2-5.0 μg/ml with a squared correlation coefficient (r^2) of 0.999 or better and the detection limit was 50 ng/ml for 100-μl plasma samples. The method was not interfered with by other endogenous compounds or metabolites and one assay can be completed in 12 min. The within-day and between-day assay variation for three different concentrations was found to be less than 6% and the accuracy was nearly 100%.

INTRODUCTION

Tolfenamic acid, N-(2-methyl-3-chlorophenyl)anthranilic acid, is a potent non-steroidal anti-inflammatory agent with analgesic and antipyretic activities (1,2).

Like other fenamates tolfenamic acid is a prostaglandin synthetase inhibitor and its clinical efficacy has been documented in rheumatoid arthritis, dysmenorrhea, and migraine (3-5). Recently, tolfenamic acid has been introduced as a veterinary treatment and it is also used to dope horses (6).

To study the pharmacokinetics of tolfenamic acid in neonatal and pediatric patients, it is important to minimize the total volume of plasma required for the analysis. For the determination of tolfenamic acid in human plasma high performance liquid chromatographic (HPLC) methods have been previously reported (7-10). However, these methods require relatively large plasma samples, time-consuming or expensive sample preparation techniques, or use commonly used compounds as internal standards (e.g. caffeine) which may interfere with the assay, and are not directly applicable to the determination of plasma levels of tolfenamic acid in pediatric patients.

The present study was undertaken to develop a simple, sensitive, and reliable isocratic reversed phase HPLC assay for the determination of tolfenamic acid in small volumes of human plasma, with advantages over previously published methods (7-10). The method has been applied to the pharmacokinetic study of pediatric patients receiving oral administration of tolfenamic acid.

MATERIALS AND METHODS

Chemicals and reagents

HPLC-grade acetonitrile and analytical reagent grade 85% phosphoric acid were obtained from Merck (Darmstadt, Germany). Tolfenamic acid (batch 10818) was kindly supplied by ELPEN (Athens, Greece). The internal standard, phenylbutazone was purchased from Sigma (St. Louis, MO, USA).

HPLC instrumentation

The liquid chromatographic system (Varian, Palo Alto, CA, USA) consisted of a 2510 high-pressure solvent delivery pump, a 2550 variable-wavelength UV-Vis detector set at 280 nm, and a 7125 manual injector with a 20-µl fixed loop (Rheodyne, Cotati, CA, USA). The analysis was performed using a LiChrospher 100 RP-18, 5-µm particle size, analytical column (250 x 4 mm, ID) (Merck, Darmstadt, Germany) proceeded by a guard column (30 x 4.6 mm, ID) dry packed with C18 ODS (37-53 µm). The chromatograms were recorded on a 4290 integrator (Varian, Palo Alto, CA, USA), and peak heights were reported.

Chromatographic conditions

The mobile phase consisted of acetonitrile-10 mM phosphoric acid solution (60:40, v/v) and the resulting pH was approximately 2.6. The mobile phase was prepared fresh on the day of analysis and was filtered through a 0.45-µm pore size nylon filter (Alltech, Deerfield, IL, USA) and degassed by ultrasonic treatment before use. The HPLC system was allowed to equilibrate isocratically at a flow rate of 1.1 ml/min resulting in an inlet pressure of approximately 1700 psi. All chromatographic separations were carried out at ambient temperature and the effluent was monitored at 280 nm.

Preparation of standard solutions

Stock standard solutions of tolfenamic acid and the internal standard, phenylbutazone (0.1 mg/ml) were prepared in acetonitrile and stored at -20^{0}C. An aliquot of tolfenamic acid solution was diluted 1:10 with acetonitrile to produce

fresh working standard solution (0.01 mg/ml). Internal standard solution consisting of 4.0 μg/ml of phenylbutazone was prepared in acetonitrile and stored at -20°C. Calibration standards were prepared using 1.5-ml conical polypropylene tubes (Eppendorf, Hamburg, Germany) in 100 μl of drug-free plasma spiked with appropriate volumes of tolfenamic acid working solution and 250 μl of internal standard solution to prepare standards with concentrations of 0.2, 0.5, 1.0, 2.0, 3.0, and 5.0 μg/ml. Aliquots of acetonitrile were added to each tube to make the final volumes equal (400 μl).

Sample preparation

The sample preparation employed was as previously described (11). A 100 μl sample of plasma was pipetted into a 1.5 ml conical polypropylene Eppendorf tube. A 250-μl aliquot of internal standard solution and 50 μl of acetonitrile were added, mixed on a vortex mixer for 60 sec and centrifuged at 9000 g for 3 min. An aliquot of the supernatant fraction (250 μl) was transferred into a new Eppendorf tube and evaporated to dryness in a SC110A Savant SpeedVac concentrator (Farmingdale, NY, USA). The residue was then reconstituted with 50 μl of mobile phase, mixed on a vortex agitator and a 20-μl aliquot was injected into the HPLC system.

Quantitation

Tolfenamic acid concentrations in plasma samples were calculated by interpolation from the linear least-squares regression line of the standard curve plot of peak height ratio tolfenamic acid/phenylbutazone, *versus* tolfenamic acid concentration in the calibration standards. The concentrations of tolfenamic acid

standards were chosen to cover the range of tolfenamic acid concentrations in children following oral administration with this drug.

RESULTS AND DISCUSSION

The retention times for phenylbutazone (internal standard) and tolfenamic acid were 7.9 and 11.5 min respectively. The retention times were found to be reproducible and the coefficients of variation were less than 0.3%. Typical chromatograms obtained from human plasma samples are shown in Fig. 1. Fig. 1A shows the chromatogram from the analysis of drug-free human plasma. No interfering peaks due to endogenous compounds or metabolites were observed near the retention time corresponding to phenylbutazone or tolfenamic acid. Fig. 1B shows the chromatogram of a plasma sample obtained from a 22-month hospitalized feverish child who had received a 1 mg/kg oral administration of tolfenamic acid (syrup). The plasma concentration of this patient 4 h after administration was 0.6 μg/ml.

A least-squares linear regression analysis was used to calculate the equation relating the peak height ratio tolfenamic acid/internal standard (Y) *versus* tolfenamic acid concentration (μg/ml) in spiked plasma samples (X). The calibration curves were linear (r^2>0.999) in the range 0.2-5.0 μg/ml tolfenamic acid concentration and the intercepts did not differ significantly from the origin. A typical equation calculated from a calibration curve with six points was: $Y = 0.005 + 0.200X$ with a squared correlation coefficient (r^2) of 0.9997. The lower limit of detection for tolfenamic acid was 50 ng/ml. At this concentration the signal to noise ratio was greater than 5:1.

Table 1 summarizes the within-day and between-day precision and accuracy data of the method at three different concentrations in spiked plasma samples, 0.5,

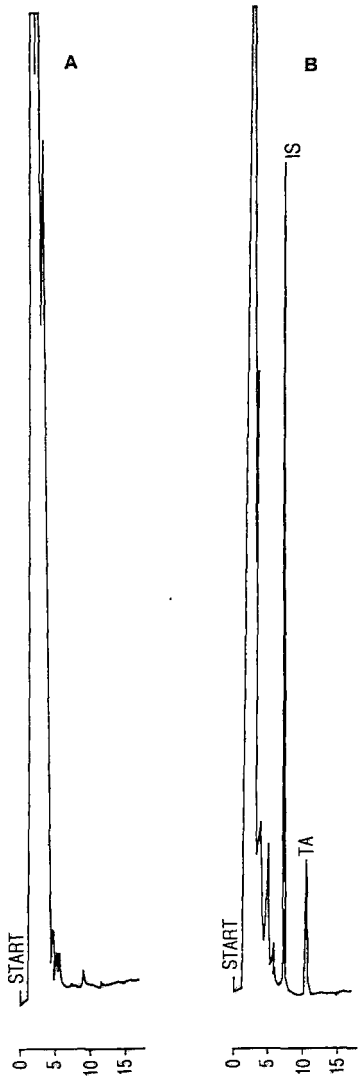


FIGURE 1. HPLC of plasma samples: (A) drug-free plasma; (B) sample obtained from a child 4 h after a single 1 mg/ml oral dose of tolfenamic acid containing 0.6 μg/ml of the drug. Peaks: IS = phenylbutazone (internal standard); TA = tolfenamic acid.

TABLE 1

Within-day and Betwen-day Precision and Accuracy Data of the Determination of Tolfenamic Acid in Human Plasma.

Concentration added (μg/ml)	Concentration found (μg/ml)	Precision CV (%) (n=6)	Accuracy Concentration found/added (%)
Within-day			
0.5	0.48	4.7	96
2.0	1.94	3.9	97
5.0	5.05	4.6	101
Between-day			
0.5	0.47	5.9	94
2.0	1.96	5.1	98
5.0	5.15	4.9	103

2.0, and 5.0 μg/ml for tolfenamic acid. The within-day precision of the method has been evaluated by calculating the coefficient of variation for six determinations at each concentration and was found to be less than 5%. The between-day precision of the method was evaluated by determining the coefficient of variation for six samples at each concentration analyzed on three different days and was found to be less than 6%. The accuracy of the method has been quantified as the percent of the estimated concentration divided by the nominal concentration and was found to be nearly 100%. These data indicate that the method developed provides good accuracy and precision.

In conclusion, the assay utilized a simple, rapid and inexpensive sample preparation procedure required a minimal volume of human plasma (100 μl) from which proteins were removed by acetonitrile precipitation. The HPLC method developed for the quantitation of tolfenamic acid in human plasma is easy, simple,

sensitive, precise, and accurate and seems well suited to characterize tolfenamic acid pharmacokinetics in pediatric patients.

REFERENCES

1. I.B. Linden, J. Parantainen, H. Vapaatalo, Scand. J. Rheumatol., 5: 129-132 (1976).

2. A. Kajander, J. Martio, O. Murtu, G. Gothoni, Scand. J. Rheumatol., 5: 158-160 (1976).

3. V. Rejholec, H. Vapaatalo, O. Tokola, G. Gothoni, Scand. J. Rheumatol. (Suppl.) 24: 13-16 (1979).

4. A. Kauppila, O. Ylikorkala, Eur. J. Obstet. Gynecol. Reprod. Biol., 7: 59-64 (1977).

5. H. Hakkarainen, H. Vapaatalo, G. Gothoni, J. Parantainen, Lancet 2: 326-328 (1979).

6. P. Jaussaud, D. Guieu, D. Courtot, B. Barbier, Y. Bonnaire, J. Chromatogr., 573: 136-140 (1992).

7. S.B. Pedersen, B. Aldhede, O. Buchardt, J. Moller, K. Bock, Arzeim.-Forsch./Drug Res., 31: 1944-1948 (1981).

8. P.J. Pentikaeinen, P.J. Neuvonen, C. Backman, Eur. J. Clin. Pharmacol., 19: 359-365 (1981).

9. S.H. Hansen, S.B. Pedersen, J. Pharm. Biomed. Analysis, 4: 69-82 (1986).

10. I. Papadoyannis, M. Georgarakis, V.Samanidou, A. Zotou, J. Liq. Chromatogr., 14: 2951-2967 (1991).

11. I. Niopas, K. Mamzoridi, J. Chromatogr., 656: 447-450 (1994).

Received: February 5, 1995
Accepted: April 2, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2683-2693 (1995)

HPLC METHOD DEVELOPMENT AND VALIDATION FOR FORMALDEHYDE IN ENTERIC COATING OF HARD GELATIN CAPSULES

E. PINA, A. T. SOUSA, AND A. P. BROJO
Laboratory of Pharmaceutical Technology
University of Coimbra
3000 Coimbra, Portugal

ABSTRACT

A reversed-phase high-performance liquid chromatography (HPLC) method for determination of formaldehyde in enteric coating of hard gelatin capsules is described and fully validated. This method entails the separation of formaldehyde as its 2,4-dinitrophenylhydrazone derivative using isocratic solvent eluition and its quantification with appropriate internal standard and ultraviolet detection. The results for selectivity, linearity, precision, accuracy and recovery were in agreement with validation parameters.

INTRODUCTION

The method to prepare hard gelatin capsules, which are resistant to gastric juice by crosslinking with formaldehyde and their stabilisation, succintly includes, for determined time, different stages such as: immersion of capsules in hidroalcoholic solutions of formaldehyde, first drying followed by "washing" and second drying. So the quantification of this aldehyde is very important besides other requirements (1).

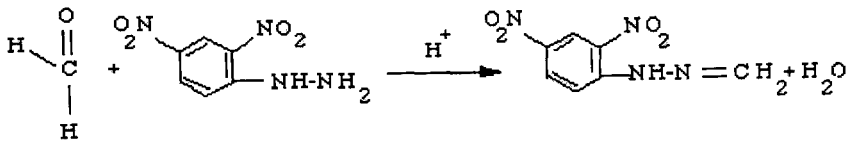


FIGURE 1. Chemical reaction of formaldehyde with 2,4-dinitrophenylhydrazine.

For the analysis of formaldehyde, colorimetric determination has generally been used (2, 3, 4, 5). However the colorimetric assay is not necessarilly reliable because the samples are sometimes coloured or the excipients (e.g. lactose) can give different reactions with formation of colour (6, 7).

So, we attempted to develop a precise high-pressure liquid chromatography (HPLC) method based on the formation of the derivative formaldehyde 2,4-dinitrophenylhydrazone by reaction of the formaldehyde its carbonyl group with 2,4-dinitrophenylhydrazine under acidic conditions (Figure 1).

After optimization and validation, the method was used to determine the amount of spent and extracted formaldehyde by analysing respectively "coating" and "washing" solutions applied to prepare the gastro-intestinal tract resistant gelatin capsules. It is also possible to assay the residual formalin content immediately after preparation and during storage time of gelatin capsules. As formaldehyde is the crosslinking agent of gelatin, this methodology is very important for a rigorous and reproducible control of the process.

MATERIALS AND METHODS

Chemicals

Water (HPLC degree, Milli-Q). Methanol (Lichrosorb, Merck). Formaldehyde (36,6 % w/w), hydrochloric acid, n-hexane, methylene chloride and 2,4-dinitrophenylhydrazine, all proanalyse (Merck).

2,4-Dinitrophenylhydrazine reagent

Dissolve powdered 2,4-dinitrophenylhydrazine (0,025g) in 6NHCl and bring the volume to 10 ml. This solution should be freshly prepared on the day of use.

Instrumentation

The HPLC apparatus consisted of a Hewlett Packard model 1050, a injector with a 20μl loop, a variable wavelenght UV/Vis detector set a 350 nm with a sensitivity range of 0,016 AUFS and an Hewlett Packard model 3396A. Recorder/integrator using chart speed at 0,5 cm min^{-1}.

The melting points were determined on a Buchi model 512 apparatus.

The proton nuclear magnetic resonance (^{1}HNMR) spectra were recorded on a Varian XL-Spectrometer.

Chromatographic conditions

A reversed-phase column Rp-18 Lichrosorb (200x4,6 mm) 5 μm was used at ambient temperature. The mobile phase was methanol: water (70:30) filtered using 0,20 μm membrane filters (Schleicher & Schuell) and degassed prior to use.

Injection volume was 10 μl and flow rate 1,4 ml/min.

2,4-Diniitrophenylhydrazone standards

Formaldehyde 2,4-dinitrophenylhydrazone and acetone 2,4-dinitrophenylhydrazone (internal standard) were prepared by standard procedure described by Shriner et al.(8) and used by Papa and Turner (9). They were purified by different recristallizations from ethanol to a constant melting point. The purities were checked by this method (HPLC) and methanolic solutions showed 100 by area

per cent. The standards were also characterized by proton nuclear resonance magnetic (^{1}HMNR) (10-12).

Formaldehydde 2,4-dinitrophenylhydrazone:

MP = 165 - 166 °C (ethanol/water) (13)
^{1}H-NMR - (p p m, δ) - 6,739 (d; J 10,8 Hz, 1H, -N = C<u>H</u>) 7,149 (d, J 10,8 Hz, 1H, -N = C<u>H</u>); 7,981 (d, J 10,5 Hz, 1H; 6 -H$_{arom.}$); 8,362 (q; J$_{ortho}$ 10,5 Hz; J$_{meta}$ 2,4 Hz, 1H; 5 -H$_{arom.}$); 9,151 (d, J 2,4 Hz, 1H, 3 -H$_{arom}$); 11,025 (s, 1H, -N<u>H</u> -)

Acetone 2,4-dinitrophenylhydrazone:

MP = 128 - 129 °C (ethanol/water) (13)
^{1}H-NMR - (p p m, δ) - 2,089 (s, 3H, C<u>H</u>$_3$); 2,183 (s, 3H, C<u>H</u>$_3$); 7,960 (d, J 6,5 Hz, 1H, 6 H$_{arom.}$); 8,295 (q; J$_{ortho}$ 6,5 Hz; J$_{meta}$ 2,6 Hz, 1H; 5-H$_{arom.}$); 9,190 (d, J 2,6 Hz, 1H; 3- H$_{arom.}$); 11,026 (s, 1H, -N<u>H</u> -)

Stock solutions of these standards (100 μg ml^{-1}) were prepared by their dissolution in a mixture n-hexane: methylene chloride (70:30 v/v) and preserved from light.

<u>Calibration curve</u>

Six standard solutions of formaldehyde 2,4-dinitrophenylhydrazone (2,5-10 μg ml^{-1}) corresponding to formaldehyde (0,357-1,428 μg ml^{-1}) containing 20 μg ml^{-1} of acetone 2,4-dinitrophenylhydrazone (internal standard) were prepared according to Table 1. A 10 μl volume was then injected in the chromatograph and the calibration curve was calculated by linear regression of the peak-area ratios of formaldehyde 2,4-dinitrophenylhydrazone to internal standard versus concentrations. Unknown formaldehyde concentrations were determined from the regression equation.

TABLE 1
Standard and Sample Preparation

Standard	Sample
- 2 ml of distilled water	-1 ml of distilled water
-different volumes of formaldehyde 2,4-dinitrophenylhydrazone solution	-0,1 ml of 2,4-dinitrophenylhydrazine reagent
-1 ml of internal standard (100 μg ml $^{-1}$)	-1 ml of assay solution
-5 ml of organic mixture	-wait 5 min
	-1ml of internal standard (100 μg ml-1)
	-5 ml of organic mixture
. Stirring for 20 min. . Removing of aqueous phase . Washing of organic phase . Separation of organic extract . Drying of organic phase . Recovery of residue . Filtration . Injection	

Sample preparations

Samples were prepared in parallel to the standards according to Table 1. The solutions to analyse were derived from solutions used in enteric coating of hard gelatin capsules and obtained by three manners: [1] Before and after immersion (with stirring) of hard gelatin capsules in a clean vial well stoppered, 1 ml of coating solution was withdrawn and used for determine spent formaldehyde. [2] Twelve formalin treated capsules were placed in a clean vial containing 100 g of

hidroalcoholic solution. After closing with rubber stoppers the vial was stirred on a mechanical agitation apparatus, 1 ml was withdrawn and used for determination of extracted formaldehyde. [3] Two formalin treated capsules were placed in a clean vial containing 20 ml of distilled water. After closing the vial was stirred on a mechanical agitation apparatus for two hours. Subsquently 1 ml was used to analyse residual formalin content.

RESULTS AND DISCUSSION

Optimization of the derivatization reaction

To each of six Erlenmeyer flasks, 1 ml of water, 0,1 ml of the 2,4-dinitrophenylhydrazine reagent and 1 ml of formaldehyde solution (5 μg) was added. After five minutes, 5 ml of the mixture n-hexane: methylene chloride (70:30 v/v) and 1 ml of acetone 2,4-dinitrophenylhydrazone (internal standard) were added. The mixtures were stirred by mechanical agitation for 5, 10, 15, 20, 25 and 30 minutes. After these intervals, aqueous phase was recoverd and organic extract washed with deionized water to remove the acid and most of the unreacted 2,4-dinitrophenylhydrazine reagent.

The results from Figure 2 indicate that formaldehyde was quantitatively converted to its 2,4-dinitrophenylhydrazone in 20 minutes, which is in accordance with Selim (14) in a analogous study with propionaldehyde.

Selectivity

In Figure 3A and B typical chromatograms can be seen from blank solution containing 20 μg ml^{-1} of acetone 2,4-dinitrophenylhydrazone (internal standard) and from standard solution containing 5 μg ml^{-1} of formaldehyde 2,4-dinitrophenylhydrazone and 20 μg ml^{-1} of internal standard respectively. In Figure 3C a typical chromatogram from sample can be seen.

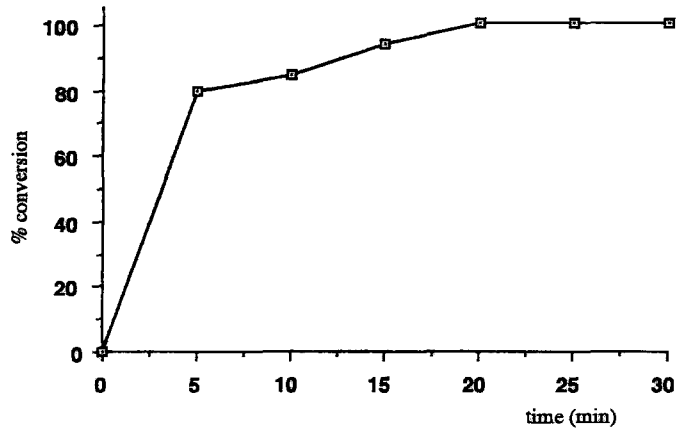


FIGURE 2. Rate of conversion of formaldehyde to its 2,4-dinitrophenylhydrazone.

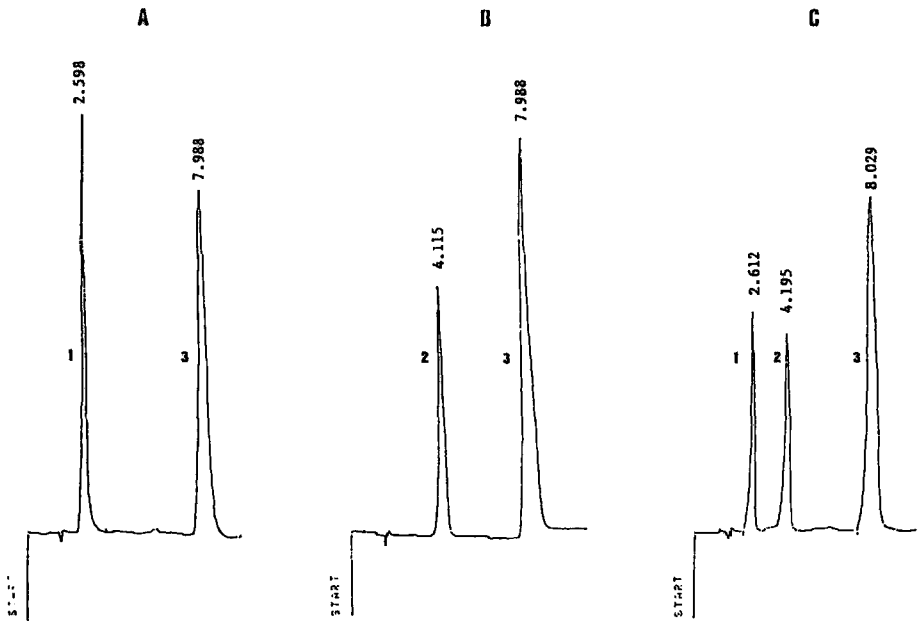


FIGURE 3. Chromatograms: (A) blank solution containing 20 μg ml^{-1} of internal standard; (B) standard solution containing 5 μg ml^{-1} of formaldehyde 2,4-dinitrophenylhydrazone and 20 μg ml^{-1} of internal standard; (C) sample solution.

(1) 2,4-dinitrophenylhydrazine reagent
(2) formaldehyde 2,4-dinitrophenylhydrazone
(3) internal standard

No interference of formaldehyde 2,4-dinitrophenylhydrazone with internal standard or with 2,4-dinitrotrophenylhydrazine reagent were observed.

The retention times of 2,4-dinitrophenylhydrazine reagent, formaldehyde 2,4-dinitrophenylhydrazone and internal standard were 2,6, 4,1 and 8,0 minutes, respectively. It can be concluded that the proposed method is selective for formaldehyde.

Linearity

The regression equation obtained was : A = 0,532 C - 0, 0094325 where A-peak-area ratios and C-formaldehyde concentrations $\mu g\ ml^{-1}$. The coefficient of variation of calibration curve of 2,45 % and the correlation coefficient of 0.999511 (N = 6), proved excellent linearity between peak-area ratios and concentration.

Precision

Run variation within day (repeatability) and run by run (reproducibility) on three different days were calculated for known formaldehyde contents. Coefficient of variation for the repetability test was 1,79 (N=5). For the reproducibility the coefficient of variation was 1,88% (N=9). The low results proved that this analytical method had acceptable precision for formaldehyde quantification.

Accuracy

For determination of the accurancy we calculated the closeness of agreement between the value accepted as the conventional true value and the value found applying the Student' s test (N=8) for a 0,05 probability. The results were $t_{theoretical} = 2,37$ and $t_{experimental} = 2,39$. So we can affirm the exactness of the analytical method.

Recovery

The recovery of known formaldehyde concentration added to the blank subjected to sample treatment was analysed. The results varied between 96% and 100,6% which indicates good effectiveness.

Detection Limits

Detection limits are of the order of a few nanograms (15).

CONCLUSIONS

It was demonstrated that the procedure developed is simple, sensitive precise and accurate, making it important for assay of formaldehyde during coating of hard gelatin capsules (16-23).

The selectivity of this procedure make it likely that this assay can also be utilised to analyse formaldehyde in a wide variety of other pharmaceutical samples .

REFERENCES

1. Pina, E., Gastro-resistência, Estabilidade e Liodisponibilidade de Cápsulas Gelatinosas Formiladas. Ph. D.Thesis , Faculdade de Farmácia, Coimbra, 1994, pp. 189.

2. Altshuller, A. P., Miller, D. L. .and Slevas, S. F., Determination of formaldehyde in gas mixtures by the chromotropic acid method. Anal. Chem., 33 : 621-625 (1961)

3. Houle, M. J., Long, D. E. and Smette, D., A simplex optimized colorimetric method for formaldehyde. Anal. Lett., 3 : 401-409 (1970) .

4. Hennebert, P., La stérilization au formaldéhyde gaseaux. Ph. D. Thesis, Université Catholique, Louvain, 1986, pp. 49-51.

5. Hennebert, P., Gillard, D. and Roland, M., A new method for gaseous formaldehyde stérilization. S. T. P. Pharma., 2: 536-542 (1986).

6. Stevens, H. M., "Colour tests". in Clarke's isolation and identification of drugs, 2nd Edn.,The Pharmaceutical Press, London , 1986, pp. 128-147.

7. Noda, H., Minemoto, M., Noda,A., and Ushio, T., High-perfomance liquid chromatographic determination of formaldehyde accomplished using hydralazine. Chem. Pharm. Bull. 34 : 3499-3501 (1986).

8. Shriner, R. L., Fusonon, R. C. and Curtin, D. Y., The systematic identification of organic compounds., 5th Edn., Wiley, New York, 1965.

9. Papa, L. J. and Turner, L. P., Chromatographic determination of carbonly compounds as their 2,4-dinitrophenylhidrazones II. High pressure liquid chromatography. J. Chromatogr. Sci., 10: 747-750 (1972).

10. Karabatsos, G. J. and Krumel, K. L., Structural studies by nuclear magnetic resonance - XII. Conformations and configurations of N-methylphenylhydrazones. Tetrahedron, 23: 1097-1105 (1967)

11. Karabatsos, G. J. and Taller, R. A., Structural studies by nuclear magnetic resonance V. Phenylhydrazones. J. Amer. Chem. Soc ., 85 : 3624-3629(1963).

12. Karabatsos, G. L. and Taller, R. A., Structural studies by nuclear magnetic resonance - XIX N, N-Dimethylhydrazones and general comments on configurational and conformational isomerism. Tetrahedron., 24: 3923-3937 (1968).

13. Terence, C. and Owen, JR., Characterization of organic compounds by chemical methods: an introductory laboratory textbook., Marcel Dekker, New York, 1969.

14. Selim, S., Separation and quantitative determination of carbonyl compounds as their 2,4-dinitrophenylhydrazones by high-pressure liquid chromatography. J. Chromatogr., 136: 271-277 (1977).

15 Fung, K. and Grosjean, D., Determination of nanogram amounts of carbonyls as 2,4-dinitrophenylhydrazones by high-perfomance liquid chromatography. Anal. Chem., 53: 168-171 (1981) .

16. Lecompte, D., Validation d'une méthode de dosage par chromatographie liquide. S. T. P. Pharma., 2: 843-849 (1986).

17. Caporal, J., Nivet, J. M., Baschung-Bertrand, M., Beautemps, R., Boutin, R., Brohon, J., Bruna, E., Coulon, J., Gadefait, A., Gallo, A. M., Grelet, P., Guilloteau, M., Laubignat, M. C., Loiseau, J. B., Michaud, S., Pellerin, F., Polge, P., Philippon, F., Plantefeve, J., C., Sorraing, F., Suedt, J., Touchon, M. and Willenot, F., Validation Analytique Commentaires sur la note explicative européene et exemple d' application. Rapport d' une commission SFSTP. S. T. P. Pharma., 6 : 588-594 (1990) .

18. Campmany, A. C. , Ferrer, E. and Lastra, C., Validación de los métodos analíticos. Farm. Clin., 7: 749-758 (1990) .

19. US Pharmacopeia XXII, US Pharmacopeial Convention, Rockville, MD, 1990.

20. Karnes, H. , Shiu, G. and Shah, V., Validation of bioanalytical methods. Pharm. Res., 8 : 421-426 (1991).

21. Shah, V. P., Midha, K. K., Dighe, S., Mcgilveray, I. J., Skelly, J. P., Yacobi, A., Layoff, T., Viswanathan, C. T., Cook, C. T., Mcdowall, R. D., Pittnan, K., A. and Spector, S., Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies. J. Pharm. Sci. 81 : 309-312 (1992).

22. Caporal- Gautier, J., Nivet, J.M., Algranti, P., Guilloteau, M., Histe, M., Lallier,M., N' Guyen-Huu, J. J., and Russotto, R., Guide de validation analytique. Rapport d' une comission SFSTP I. Méthodologie. S. T. P. Pharma Prat., 2 : 205-226 (1992).

23. Bourquin T., Bordi, D., Bounine, J. P., Compagnon, P. A., Drouin, J. E., Fatras, A., Lecointre, L., Lepabic, C., Mottu, P., Penchinat, L., Richard, F. and Verneyre, N., Les tests de conformité en chromatographie en phase liquide. Rapport d´une comission SFSTP. STP. Pharma Prat., 4: 170-176 (1994)

Received: January 11, 1995
Accepted: April 12, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2695-2702 (1995)

DETERMINATION OF DIHYDROSTREPTOMYCIN SULFATE IN MILK BY HPLC USING ION-PAIR AND POSTCOLUMN DERIVATIZATION

VÍCTOR HORMAZÁBAL AND MAGNE YNDESTAD
Division of Food Hygiene
Norwegian College of Veterinary Medicine
P.O. Box 8146-Dep.
N-0033 Oslo 1, Norway

ABSTRACT

An isocratic, paired-ion reversed-phase high-performance liquid chromatographic method for the determination of dihydrostreptomycin sulfate in milk by postcolumn derivatization has been developed. The milk was treated with 85% trichloroacetic acid to precipitate proteins, and the supernatant clean-up was performed using a tC18 pretreatment column. The correlation coefficients were 0.999. The lower limit of quantification was 25 ng/ml and the limit of detection close to 15 ng/ml. The recovery of dihydrostreptomycin sulfate varying from 82.6 to 82.8%.

INTRODUCTION

Dihydrostreptomycin (DHS) is a clinically useful aminoglycoside antibiotic with high potency against a wide range of Gram-negative and same Gram-positive bacteria in cattle, pigs, sheep and humans, (1 - 4).

In veterinary medicine the combination of penicillin and DHS is widely used in the treatment of different systemic and local infections e.g. mastitis in dairy cows. This represents a potential hazard to consumers due to persistence of residues in the milk (5-7). DHS is produced by the catalytic hydrogenation of streptomycin (8). Numerous chemical and physical methods have been reported for the analysis of streptomycin and DHS, including paper, thin-layer, and column chromatography, electrophoresis, spectrophotometry and colorimetry, titrimetry, and polarography (9). These methods are time-consuming and have poor specificity, sensivity, and precision.
A post-column derivatization system with ninhydrin as the fluorigenic reagent in the mobile phase, has also been developed for the fluorimetric determination of guanidino compounds by HPLC (10). A procedure for HPLC determination of streptomycin in serum (11), and a method for the determination of streptomycin in meat by HPLC has been published (12). More recently a method for the determination of both streptomycin and DHS in pork and bovine muscle and kidney has also been published(13). However, none of the published methods appeared to be applicable to DHS in milk.

MATERIALS AND METHODS

Materials and Reagens

Whole milk (pasteurized, 3.8% fat) was purchased from local grocery stores, for use as control material and for spiking with DHS to conduct recovery experiments.
1-Heptanesulfonic acid and 1-octanesulfonic acid were obtained from Supelco Inc.Supelco Park, Bellefonte. USA.
DHS and triethylamine were supplied by Sigma Co. (St. Louis, MO, USA).
All chemicals and solvents were of analytical and HPLC grade. DHS stock solution and working standards were prepared by dilution with solution A consisting of 0.02 M 1-heptanesulfonic acid (sodium salt) and 0.01 M di-sodium hydrogenphosphate-2-hydrate,(Ferax, Berlin, Germany), made by dissolving 4.45 g/l heptane sulfonate and 1.8 g/l di-sodium hydrogenphosphate in approx.750 ml of water when making 1 litre of solution. The pH was then adjusted to c. 6.3 with 5 M phosphoric acid and to 6.0 with 1 M phosphoric acid, and the solution made up to volume (1 l) with water and the pH again adjusted to 6.0 with 1 M phosphoric acid. The stock solution and

working standards were stored in a refrigerator.
1,2-ethanedisulfonic acid (disodium salt) was supplied by Tokyo Kasei Kogyo (Tokyo, Japan).
Ninhydrin was obtained from Riedel-de Haën, (Germany), while ortho-phosphoric acid 85% was supplied by Merk (Germany), and trichloroacetic acid (TCA) by Ferax (Laborat GMBH, Berlin, Germany).
Sep-Pak Vac cartridges for solid phase extraction, tC18 Vac (trifunctional) 6cc (1g), were supplied by Waters Division of Millipore (34 Maple Street, Milford, Massachusetts 01757 U.S.A.).

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 410 Bio solvent delivery system, and a ISS 100 sampling system equipped with a Lauda RMT6 cooler (14 °C) from Messgueräte Werk Lauda, (Lauda Köningshafen, Germany). The integration was carried out using the software programme Omega-2 (Perkin-Elmer), wich was operated on an Olivetti M300 personal computer connected to a Bj-330 printer (Canon). The analytical column (operated at room temperature), (stainless steel, 150 x 4.6 mm I.D.) and guard column (stainless steel, 2.0 cm x 4.6 mm I.D.) were packed with 5-µm particles of Supelcosil LC-ABZ (Supelco, Bellefonte, PA, USA).
The mobile phase was a mixture of three solutions B, C, and D (63:19:18). Solution B was 0.04 M 1-octanesulfonic acid Na salt, 0.02 M 1,2-ethanedisulfonic acid disodium salt, and 0.005 M ninhydrin, made by dissolving 8.65 g/l 1-octanesulfonate and 4.68 g/l 1,2-ethanedisulfonic acid in ca. 750 ml of water when making 1 litre of solution. The pH was then adjusted to 3.2 with acetic acid, and 0.891 g/l ninhydrin added and dissolved. The solution made up to volume with water and the pH again adjusted to 3.2 with acetic acid. Solution C was acetonitrile with 0.3% triethylamine (freshly prepared every day), while solution D was methanol. The flow rate was 1.4 ml/min. The column effluent was introduced into a vortex mixer from a heater system for HPLC post column reactions (PCRS 520 - Kratos) equipped with a heat exchanger (vortex mixer is not a mixing tee, but a low volume (1.2 µl) mixing device for two fluid streams).
A Series 10 Liquid Chromatograf (Perkin-Elmer), (included pulse-dampened pump) with a mobile phase of 0.3 M NaOH at a flow rate of 0.5 ml/min. was coupled to the vortex mixer, and a reaction coil (Beam Boost

Photochemical Reactor Unit, PTFE 10 m x 0.3 mm I.D.-ITC Handelsgesellschaft m. b. H. Frankfurt, Germany). The reaction temp. was 80 °C. The solvent stream was then cooled using a heat exchanger (operated at room temperature), to prevent gas emission in the detector, which was a LS 240 fluorescence detector (Perkin-Elmer, Norwalk, Conn., USA). The detector was operated at an excitation wavelength of 305 nm and emission wavelength of 500 nm, with a response of 5 and a factor of 256. The samples were injected at intervals of 10 min. Aliquots of 75 µl were injected onto the column for the determination of DHS.

Sample pretreatment

To 6 ml whole milk, were added 0.5 ml solution A (or standard) and 1.5 ml 85% TCA in water. The sample was shaken vigorously for 10 s. followed by centrifugation for approximately 3 min. (4000rpm). After adding 2 ml dichloromethane, the sample was mixed for 5 s. and then centrifuged for 5 min. (4000 rpm).
Six ml of the supernatant (corresponding 4.5 ml milk) was pipetted into a graduated glass-stoppered centrifuge tube, together with 2.5 ml 4 M NaOH. The sample was mixed for 2 s. The homogenate was then centrifuged for 10 min. (4000 rpm). The upper layer were transferred to a clean tube, 2.5 ml of 0.5 M phosphoric acid added, and the pH adjusted to six (+- 0.03) with 1 M NaOH or 0.5 M phosphoric acid. After 1.5 ml solution A had been added, the sample was mixed and the solution loaded onto a conditioned tC18 column.

Clean-up on SPE-column. The column was activated with 2x5 ml methanol, 2x5 ml water, 3x5ml methanol, 3x5 ml water and 2 ml solution A, prior to extract of the milk. The aqueous milk extract was applied onto the column and washed with 1 ml solution A, then with 1 ml 3% methanol in water, and finally with 16 ml 30% methanol in water without sucking, and eluted with 3.5 ml 20% formic acid in methanol with full vacuum. The collected eluates were evaporated to dryness under a stream of nitrogen (60 °C), and dissolved in 300 µl of solution A, after which 200 µl chloroform were added. The extract was mixed vigorously for 10 s. followed by centrifugation for approximately 3 min. Aliquots of the aqueous layer (75 µl) were injected onto the column for the determination of DHS.

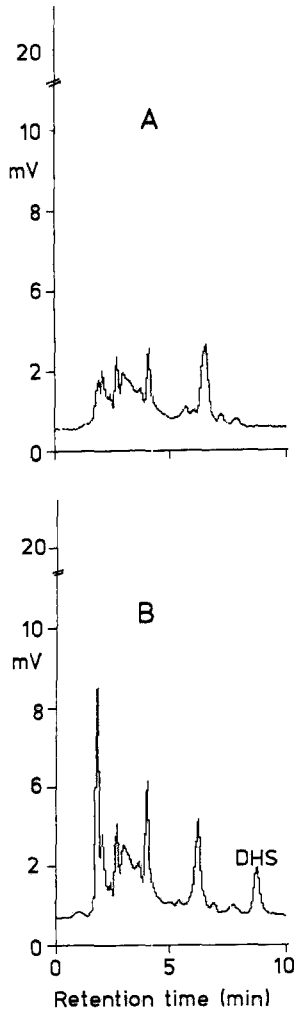


FIGURE 1
Chromatograms of extracts from milk.
A: drug-free milk, **B**: Milk spiked with DHS (400 ng/ml).

TABLE 1

Recovery and Repeatability for Dihydrostreptomysin Sulfate from Spiked Samples of Milk.

Sample	No. of samples	Amount of DHS in spiked samples (µg/ml)	Recovery % DHS Mean	SD*
Wole Milk (6 ml)	8	0.1	82.6	0.7
	8	0.4	82.8	1.2

*SD = relative standard deviation

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for DHS were determined by spiking milk samples with standard solutions to yield 25, 30, 50, 100, 200, 300 and 400 ng DHS per ml of sample, respectively. Duplicate samples were used. The recovery rates were determined by comparing analysis of spiked milk, with those of pure standard solutions.
The linearity of the standard curves for DHS in milk was calculated using peak height measurements.

RESULTS AND DISCUSSION

Chromatograms of clean milk samples and spiked samples with DHS are shown in Figure 1.
The standard curves were linear in the investigated area 25 - 400 ng/ml for DHS in milk, while the corresponding correlation coefficients were r=0.999.
Table 1 shows the recovery and repeatabilities of DHS from milk.
The average recovery over the concentration range of the standard curve varied from 82.6 to 82.8%. The precision of these recovery studies varied from 0.7 to 1.2%.
Ninhydrin forms high intensity fluorophors with guanidino compounds in alkaline media (13). DHS, which has two guanidino groups, yields similar fluorophors.
In our preliminary studies, non-polar sorbent materials

such as C18, C18OH, envirelut PAH, C8, C2 from Varian and C18 and tC18 SPE-cartridges from Waters were tested and acceptable recovery for DHS were obtained for DHS in milk pretreated on tC18 from Waters. We recommend however to test each lot of tC18 before use.
The eluting agents were tested, the eluting solvents methanol/formic acid (8:2, v/v) giving 100% recovery. It appeared, however, necessary to reduce the acidity of the eluate before injection onto the analytical column. The eluate therefore had to be evaporated to dryness in a stream of nitrogen gas and reconstituted in the solution A.
The chromatographic system appeared to be efficient for the determination of DHS in milk, the limit of quantification being 25 ppb and the limit of detection close to 15 ppb. No interference was seen during analysis, when calibrating the curves, or when performing recovery studies. The method presented in this paper is selective, robust, sensitive and accurate. This method can also be used for the determination of streptomycin in milk.

ACKNOWLEDGEMENTS

We are grateful to the Agricultural Research Council of Norway for financial support.

REFERENCES

1 D. A. Cox, K. Richardson and B. C. Ross, in P. G. Sammes (Editor), Topics in Antibiotics Chemistry, Vol. 1, Ellis Horwood, Chichester, 1977, pp. 5-15, 32, 41-43, 51.

2 AMA Drug Evaluations, Fourth Edition. American Medical Association/American Society for Clinical Pharmacology and Therapeutics, Publishing Sciences Group, Litleton, MA, 1980, pp. 767-768, 803-804, 963-964.

3 U. Hollstein, in M.E. Wolff (Editor), Burger`s Medicinal Chemistry. Part II, Wiley, New York, Chichester. Brisbane, Toronto, 4 th ed., 1979, pp. 219-222.

4 P. Sensi and G. Gialdroni-Grassi, in M.E. Wolff (Editor), Burger`s Medicinal Chemistry, Part II, Wiley, New York, Chichester, Brisbane, Toronto, 4 th ed., 1979, pp. 311-315.

5 G. Ziv, F. G. Sulman, Res. Vet. Sci. 1974, 17, 68-74.

6 C. Jayachandrani, M. K. Singh, S. D. Singh, N. C. Banerjee, Vet. Res. Commun. 1987, 11, 353-358.

7 J. G. Manners, R. Stewart, Aust. Vet. J. 1982, 58, 203-204.

8 R. L. Peck, C. E. Hoffhine, Jr. and K. Folkers, J. Amer. Chem. Soc., 68 (1946) 1390.

9 D. W. Hughes, A. Vilim and W. L. Wilson, Can. J. Pharm. Sci., 13 (1978) 21.

10 Y. Kobayashi, H. Kubo and T. Kinoshita. J. of Chromatogr., 400 (1987) 113-121.

11 H. Kubo, H. Li, Y. Kobayashi and T. Kinoshita. Anal. Biochemistry 162 (1987) 219-223.

12 A. Okayama, Y. Kitada, Y. Aoki, S. Umesako, H. Ono, Y. Nishii and H. Kubo. Bunseki Kagaku Vol. 37 (1988). Nara Prefectural Institute of Public Health, 57-6, Ohmori-cho, Nara-shi, Nara 630; School of Pharm.Sciences, Kitasato University, 5-9-1, Shirokane, Minato-ku, Tokyo 108.

13 G. C. Gerhardt, C. D. C. Salisbury and J. D. MacNeil. Journal of AOAC International Vol. 77, No. 2, (1994) 334-337.

14 F. Faure, and P. Blanquet, (1961) Bull. Soc. Chem. Biol. 43, 953-967.

Received: March 17, 1995
Accepted: April 2, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2703 (1995)

ERRATUM

corrected 29 AUG 39/AS

S. Chen, Y. Liu, D. W. Armstrong, J. I. Borrell, B. Martinez-Taipel, J. L. Matallana: "Enantioresolution of Substituted 2-Methoxy-6-oxo-1,4,5,6-tetrahydropyridine-3-carbonitriles on Macrocyclic Antibiotic and Cyclodextrin Stationary Phases," which was published in Journal of Liquid Chromatography, **18**, 1495-1507 (1995)

The separation data for compound L_{M1} in Table 1 of the above cited paper should be as follows:

$$\alpha = 1.20, R_S = 0.6$$

instead of:

$$\alpha = 2.19, R_S = 5.40$$

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2705 (1995)

ANNOUNCEMENT

BASIC PRINCIPLES OF HPLC
and
HPLC SYSTEM TROUBLESHOOTING

One-Day & Two-Day
In-House Training Courses

The courses, which are offered for presentation at corporate laboratories, are aimed at chemists and technicians who work with HPLC. They cover HPLC fundamentals and method development, as well as systematic diagnosis and solution of HPLC module and system problems.

The following topics are covered in depth:

- Introduction to HPLC Theory
- Modes of HPLC Separation
- Developing and Controlling Resolution
- Mobile Phase Selection & Optimization
- Ion-Pairing Principles
- Gradient Elution Techniques
- Calibration & Quantitation
- Logical HPLC Troubleshooting

The instructor for the courses, Dr. Jack Cazes, is Editor-in-Chief of the Journal of Liquid Chromatography, of Instrumentation Science & Technology journal, and of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 30 years; he pioneered the development of modern HPLC technology. Dr. Cazes was also Professor-in-Charge of the ACS short course and the ACS audio course on Gel Permeation Chromatography for many years.

Details of these in-house courses may be obtained from Dr. Jack Cazes, Post Office Box 2180, Cherry Hill, NJ 08034-0162, USA; Tel: (609) 424-3505; FAX: (609) 751-8724.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2707-2712 (1995)

ANNOUNCEMENTS

BIOTECHNOLOGY LAW
for the
NON-LAWYER

A One-Day Conference

Monday, Oct. 30, 1995 - San Francisco
Wednesday, Nov. 1, 1995 - Research Triangle
Friday, Nov. 3, 1995 - Boston

These timely and informative conferences will cover the intellectual property aspects of biotechnology law, from initial identification & reporting through licensing. The conferences will clearly and, in *jargon-free language*, explain the rules, regulations and procedures typically encountered by researchers in the development of their innovations.

Designed specifically for the non-legal audience, ***Biotechnology Law for the Non-Lawyer*** is essential for industrial scientists, engineers and managers in R&D facilities who must understand the fundamentals of evolving law relating to developments in the field of biotechnology.

The conferences will include

* Identifying, Evaluating and Reporting Innovative Research Developments
* Choosing the Form of Legal Protection
* Preparing the Patent Application
* Inventorship in the Research Laboratory
* Ownership of Laboratory Discoveries & Work Product
* Patent Infringement in Biotechnology

* Intellectual Property Policies, Research Agreements & Conflicts of Interest
* Technology Transfer and Commercialization

The lecturers, Gale R. Peterson (Cox & Smith, Inc., San Antonio, Texas) and Robert A. Armitage (Vinson & Elkins L.L.P., Washington, DC) are eminently qualified to lead these conferences.

Further details and registration information may be obtained from Marcel Dekker Professional Conferences, 270 Madison Avenue, New York, NY 10016; Tel: (800) 203-8782; FAX: (212) 685-4540.

ACCOUNTING & FINANCE FOR ENGINEERS

A One-Day Conference

Monday, Jan. 8, 1996 - San Francisco
Wednesday, Jan. 10, 1996 - Atlanta
Friday, Jan. 12, 1996 - Saddle Brook, NJ

This premier conference stresses the basic principles and applications of accounting and finance in a manner that is particularly well suited to the needs of non-accountants. Fundamental concepts and techniques of the subject will be discussed in detail.

The conference is aimed at scientists and engineers, in managerial and non-managerial positions who need to understand cost concepts and financial management of the work they do for their companies.

The conference will include

* Understanding Financial Statements
* Cost Concepts
* Cost Behavior
* Cost Accounting
* Cost-Volume-Profit Analysis and Leverage
* Applying the Time Value of Money
* Evaluating Capital Expenditure Projects

The lecturer, Dr. Jae K. Shim, is Professor of Accounting & Finance at the California State University in Long Beach. As an outstanding educator and consultant, he has published numerous articles in academic and professional journals. He has more than thirty college and professional books to his credit, including *Managerial Accounting; Financial Accounting; The Vest-Pocket CPA; The Vest Pocket CFO; Barron's Accounting Handbook; Encyclopedia of Accounting and Finance;* and others.

Further details and registration information may be obtained from Marcel Dekker Professional Conferences, 270 Madison Avenue, New York, NY 10016; Tel.: (800) 203-8782; FAX: (212) 685-4540.

ISO-9000
PRINCIPLES & PRACTICE

A One-Day Conference

Monday, Jan. 29, 1996 - Los Angeles
Wednesday, Jan. 31, 1996 - Chicago
Friday, Feb 2, 1996 - Saddle Brook, NJ

Stressing the basic principles and application of the International Standards Organization (ISO) 9000 registration, this cutting-edge conference answers the most asked questions regarding the organization, documentation and implementation of a quality assurance system based on the ISO-9000 (ANSI/ASQC) series of quality standards.

This conference is designed specifically for executive officers, directors, administrators and managers in industry who are responsible for selecting and setting a quality standards system that will enable their companies to compete in world markets and achieve quality in a cost-effective manner.

The conference will include

* General Introduction to Quality Standards
* Quality System Requirements

* Third Party Assessments; Implementation Strategy; Documentation Overview
* ISO in the Service, Software & Environmental Industries
* ISO and BS 7750; ISO and Other Quality Systems
* The Future of ISO

The lecturer, Dr. D. H. Stamatis, is President of Contemporary Consultants, Southgate, Michigan. He has over 25 years of experience in management and quality training/consulting in the private sector, government and academia and has written and lectured extensively in the field.

Further details and registration information may be obtained from Marcel Dekker Professional Conferences, 270 Madison Avenue, New York, NY 10016; Tel: (800) 203-8782; FAX: (212) 685-4540.

QUALITY AUDITING
for the
INDUSTRIAL LABORATORY

A One-Day Conference

Monday, Feb. 5, 1996 - Saddle Brook, NJ
Wednesday, Feb. 7, 1996 - Chicago
Friday, Feb. 9, 1996 - San Francisco

This timely conference will provide practical guidance for the quality auditing of chemical and biological laboratories, detailing how to develop and improve quality control and quality assurance programs to meet certification standards and requirements, and to easily pass external-source audits.

The conference is aimed at laboratory directors, administrators, managers, supervisors and analysts, as well as quality auditors responsible for QC and QA at analytical and microbiological laboratories serving pharmaceutical, food, cosmetic and biotechnology industries.

The conference will include

* The Need for QC and QA in the Laboratory
* The Regulated Industries
* Goals of a Laboratory Quality Audit
* Working Knowledge & Audit Documents
* Parameters for Evaluation
* Laboratory Instrumentation: Calibration & Preventable Maintenance
* Final Report, Followup & Frequency of Audits

The lecturers, Donald C. Singer (SmithKline Beecham Pharmaceuticals, King of Prussia, PA) and Ronald P. Upton (Independent Consultant, Newton, NC) are eminently qualified to lead this conference. Mr. Singer is Chairman of the ASQC's Technical Media Committee and he has over 15 years experience in establishing and maintaining laboratory QC practices for various industries; Dr. Upton has had more than 20 years experience in quality control and reliability assurance in the pharmaceutical industry.

Further details may be obtained from Marcel Dekker Professional Conferences, 270 Madison Avenue, New York, NY 10016; Tel.: (800) 203-8782; FAX: (212) 685-4540.

SYSTEM VALIDATION CONCERNS
and the
U.S. FOOD & DRUG ADMINISTRATION

A One-Day Conference

Monday, March 18, 1996 - Philadelphia
Wednesday, March 20, 1996 - Chicago
Friday, March 22, 1996 - San Francisco

Control is the key issue in the regulated world of pharmaceuticals. Validation is the ability to demonstrate that control through the ability to access appropriate records of tests, procedures and the data underlying results.

This cutting-edge conference is aimed at scientists, engineers, managers, administrators in the pharmaceutical industries who need to understand and comply with government regulation of the industry.

The seminar will focus on

* System Validation Principles
* USFDA Requirements and Guidelines
* Background to the Validation Debate
* Definitions and Terminology
* System Life Cycle for the Regulated System
* Testing the System: Principles, Terms & Strategies
* Case Study

The lecturer, Dr. Sandy Weinberg is Chairman of Weinberg, Spelton & Sax, Inc., and is responsible for Research and Development and Regulatory Affairs Publications. He has worked with a number of world-class companies. Dr. Weinberg is Research Professor of Management Information Systems, Director of the Center for Computing in Health Care and a member of the Institute for Biomedical Engineering & Science at Drexel University. He has authored seven books and more than 100 academic and professional articles, and he writes regular columns for several magazines and journals.

Further details and registration information may be obtained from Marcel Dekker Professional Conferences, 270 Madison Avenue, New York, NY 10016; Tel.: (800) 203-8782; FAX: (212) 685-4540.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(13), 2713-2721 (1995)

LIQUID CHROMATOGRAPHY CALENDAR

1995

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

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

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

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

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

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

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

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

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

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

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

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

OCTOBER 30: Biotechnology for the Non-Lawyer - A One-Day Seminar, San Francisco, California. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

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

NOVEMBER 1: Biotechnology for the Non-Lawyer - A One-Day Seminar, Research Triangle Park, North Carolina. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

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

NOVEMBER 2: Anachem Symposium, Dearborn, Michigan. Contact: Prof. C. Evans, University of Michigan, Chem Dept, 4807 Chemistry Bldg, Ann Arbor, MI 48109-1055, USA.

NOVEMBER 3: Biotechnology Law for the Non-Lawyer - AS One-Day Seminar, Boston, Massachusetts. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

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

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

NOVEMBER 14 - 16: Kemia'95: Finnish Chemical Congress, Helsinki Fair Center, Helsinki, Finland. Contact: The Associatioon of Finnish Chemical Societies, Hietaniemenkatu 2, FIN-00100 Helsinki, Finland.

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

JANUARY 8: Accounting & Finance for Engineers - A One-Day Seminar, San Francisco, California. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

JANUARY 10: Accounting & Finance for Engineers - A One-Day Seminar, Atlanta, Goergia. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

JANUARY 12: Accounting & Finance for Engineers - A One-Day Seminar, Saddle Brook, New Jersey (New York Area), Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

JANUARY 29: ISO-9000: Principles & Practice - A One-Day Seminar, Los Angeles, California. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

JANUARY 31: ISO-9000: Principles & Practice - A One-Day Seminar, Chicago, Illinois. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

FEBRUARY 2: ISO-9000: Principles & Practice - A One-Day Seminar, Saddle Brook, New Jersey (New York Area). Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

FEBRUARY 5 - 7: PrepTech'96, Sheraton Meadowlands Hotel, East Rutherford, New Jersey. Contact: Dr. R. Stevenson, 3338 Carlyle Terrace, Lafayette, CA 94549, USA.

FEBRUARY 5 : Quality Auditing for the Industrila Laboratory - A One-Day Seminar, Saddle Brook, New Jersey. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

FEBRUARY 7: Quality Auditing for the Industrial Laboratory - A One-Day Seminar, Chicago, Illinois. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

FEBRUARY 9: Quality Auditing for the Industrial Laboratory - A One Day Seminar, San Francisco, California. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

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

MARCH 3 - 8: 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 18: System Validation Concerns and the U. S. Food & Drug Administration, Philadelphia, Pennsylvania. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

MARCH 20: System Validation Concerns and the U. S. Food & Drug Administration - A One-Day Seminar, Schaumburg, Illinois (Chicago Area). Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

MARCH 22: System Validation Concerns and the U. S. Food & Drug Administration - A One-Day Seminar, San Francisco, California. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

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

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

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

MAY 7 - 9: VIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques and

Applications in Chromatography and Capillary Electrophoresis, Monte Carlo, Monaco. Contact: Prof. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Harelbekestraat 72, B-9000 Ghent, Belgium.

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

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

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

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

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

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

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

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

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

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

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

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

1997

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

APRIL 14 - 19: Genes and Gen Families in Medical, Agricultural and Biological Research: 9th International Congress on Isozymes, sponsored by the Southwest Foundation for Biomedical Research, Hilton Palacio del Rio, San Antonio, Texas. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, 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.

The **Journal of Liquid Chromatography** will publish, at no charge, announcements of interest to scientists in every issue of the journal. To be listed in Meetings & Symposia, we will need to know:

a) Name of the meeting or symposium,

b) Sponsoring organization,

c) When and where it will be held, and

d) Whom to contact for additional details.

Incomplete information will not be published. You are invited to send announcements to **Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P.O. Box 2180, Cherry Hill, NJ 08034-0162, USA.**

Create polymer products that have an optimal molecular weight distribution with the...

Handbook of Size Exclusion Chromatography

(Chromatographic Science Series/69)

edited by **CHI-SAN WU**
International Specialty Products, Wayne, New Jersey

January, 1995 / 472 pages, illustrated / $175.00

This outstanding single-source reference details the **practical** use of size exclusion chromatography (SEC) in characterizing the molecular weight distribution of important polymeric materials—addressing problems encountered in the SEC of specific substances, including copolymers, polyamides, polyvinyl alcohol and acetate, lignin derivatives, proteins, and starch.

Written by more than **25** internationally renowned authorities in their respective fields, the ***Handbook of Size Exclusion Chromatography***

- delineates the employment of semirigid polymer gels and modified silica-based packing materials for SEC
- discusses recently developed molecular weight-sensitive detectors
- examines column technology in SEC
- covers materials seldom considered in SEC surveys such as rubber and asphalt
- describes how to determine the absolute molecular distribution of polymers by SEC without calibration
- and more!

Furnishing over **1000** citations to key sources in the literature as well as more than **275** useful tables, drawings, and equations, the ***Handbook of Size Exclusion Chromatography*** is an invaluable resource for chromatographers; analytical, polymer, organic, and physical chemists; biochemists; quality assurance managers; and upper-level undergraduate and graduate students in these disciplines.

Marcel Dekker, Inc.
270 Madison Avenue, New York, NY 10016
(212) 696-9000
Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland
Tel. 061-261-8482

Contents

ISBN: 0—8247—9288—2

This book is printed on acid-free paper

Stay at the forefront of your field by incorporating the latest applications and theoretical developments into your work with...

Contains over **200** drawings, photographs, tables, and equations!

Advances in Chromatography

VOLUME

35

edited by

PHYLLIS R. BROWN, *University of Rhode Island, Kingston*

ELI GRUSHKA, *Hebrew University of Jerusalem, Israel*

January, 1995 / 448 pages, illustrated / $165.00

Reviewer praise for previous editions...

"...reflects the high standards expected from this respected series." —***Clinical Chemistry***

"...the articles [are] of high scientific standard, up to date, very well written, and interesting to read...well presented and edited." —***Journal of Chromatography***

"...a valuable contribution to the chromatography literature... belongs in every library used by chromatographers." —***Liquid Chromatography***

"...maintains the high quality that chromatographers have come to expect from this invaluable series." —***Journal of Pharmaceutical Sciences***

The rapidly expanding growth of the literature on chromatography, capillary electrophoresis, field flow fractionation, and other separation techniques makes it difficult for any individual to maintain a coherent view of progress in the field. Rather than attempt to read the avalanche of original research papers, investigators trying to preserve even a modest awareness of advances must rely upon **authoritative surveys**.

Featuring reliable, **up-to-the-minute reviews** of major developments in chromatography and other separation techniques, this critically praised series separates the most important advances from an overabundance of supplementary materials.

Internationally acknowledged experts analyze the most current innovations in their areas of specialization!

Providing more than **1000** bibliographic citations, allowing for further, in-depth study of recent trends in research, **Volume 35** examines timely subjects such as

- performance requirements, detection modes, and ancillary techniques for optical detectors in capillary electrophoresis
- instrumentation, limitations, and applications of capillary electrophoresis combined with mass spectrometry
- experimental variables that influence the optimization of separation parameters in capillary electrophoresis
- capillary electrophoresis in pharmaceutical analyses, including a comparison with high performance liquid chromatography
- methods for the chromatographic characterization of gasoline
- and much more!

Advances in Chromatography is an indispensable resource for all researchers who need to use separation methods effectively—especially analytical, organic, inorganic, clinical, and physical chemists; chromatographers; biochemists and biological chemists; agrochemists; chemical, materials, pollution, and quality control engineers; biotechnologists; toxicologists; pharmacologists; pharmacists; physiologists; zoologists; botanists; food, cosmetic, polymer, and environmental scientists; microbiologists; virologists; oceanographers; research and quality control scientists in academia, government, hospitals, and industry; and upper-level undergraduate and graduate students in these disciplines.

Contents

ISBN: 0—8247—9361—7

This book is printed on acid-free paper.

Marcel Dekker, Inc.

270 Madison Avenue, New York, NY 10016
(212) 696-9000

Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland
Tel. 061-261-8482

Employ the latest analytical techniques in the solute partitioning between immiscible aqueous phases with...

Provides over **150** phase diagrams for a variety of aqueous polymer systems—more than **70** of which *have never before been published!*

Aqueous Two-Phase Partitioning

BORIS Y. ZASLAVSKY
KV Pharmaceutical Company
St. Louis, Missouri

November, 1994 / 688 pages, illustrated / $195.00

Physical Chemistry and Bioanalytical Applications

This useful reference offers **in-depth coverage** of the fundamental principles of solute partitioning in aqueous two-phase systems, explains their important practical features, **and** furnishes methods of characterization.

Examines*—for the first time in a book of this kind—*the information provided by the partition behavior of a solute in an aqueous two-phase system!

Integrating experimental results and theoretical concepts from a wide range of fields such as drug design, bioseparation, physical organic chemistry, and water soluble polymers, ***Aqueous Two-Phase Partitioning***

- describes the phase separation of two polymers in an aqueous medium and the role of the solvent in this phenomenon
- compares the physicochemical properties of the aqueous polymer phases governing solute partitioning with those of water-organic systems
- supplies a definitive model for the partitioning of solutes in aqueous two-phase systems that accounts for **all** experimental data available
- demonstrates the advantages of the partition technique over other methods for studying the hydrophobicity of synthetic and biological substances
- delineates the role of hydrophobicity as a general structure descriptor in quantitative structure-activity relationship analyses for drugs and biopharmaceuticals
- gives hands-on recommendations for the development of timesaving analytical and separation procedures using aqueous two-phase systems
- illustrates **new technical applications** of the partition technique as a bioanalytical tool for quality control in recombinant products, medical diagnostics, toxicology, and other areas
- and much more!

With over **1000** literature citations, figures, and equations, ***Aqueous Two-Phase Partitioning*** is an incomparable resource for biochemists; biophysicists; chemical and biochemical engineers; physical, biophysical, analytical, medicinal, and clinical chemists; bioprocess technologists; pharmacologists and biopharmacologists; soil scientists; microbiologists and virologists; pathologists; toxicologists; and graduate-level students in these disciplines.

Contents

ISBN: 0—8247—9461—3

This book is printed on acid-free paper.

Marcel Dekker, Inc.

270 Madison Avenue, New York, NY 10016
(212) 696-9000

Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland
Tel. 061-261-8482

ELECTRONIC MANUSCRIPT SUBMISSION

Effective immediately, manuscripts will be accepted on computer diskettes. A printed manuscript must accompany the diskette. For approximately one year, the diskettes will be used, on an experimental basis, to produce typeset-quality papers for publication in the Journal of Liquid Chromatography. Diskettes must be in an IBM-compatible format with MS-DOS Version 3.0 or greater. The following word processing formats can be accommodated:

ASCII	DisplayWrite Native
EBCDIC	Enable 1.0, 2.0, 2.15
Framework III 1.0, 1.1	IBM Writing Assistant
Microsoft Word 3.0, 3.1, 4.0, 5.0	Multimate 3.3
Multimate Advantage 3.6	Multimate Advantage II 3.7
Navy DIF	Office Writer 4.0, 5.0, 6.0, 6.1
PeachText 5000 2.12	PFS:First Choice 1.0, 2.0
PFS:Write Ver C	Professional Write 1.0, 2.0, 2.1
Q&A Write 3.0	RapidFile (Memo Writer) 1.2
Samna Word IV & IV+ 1.0, 2.0	Total Word 1.2, 1.3
Volkswriter 3, 4	Volkswriter Deluxe 2.2
Wang PC Ver 3	WordPerfect 4.1, 4.2, 5.0, 5.1*
WordStar 3.3, 3.31, 3.45, 4.0, 5.0, 5.5, 6.0	XyWrite III
	XyWrite III+

* The **preferred** word processor is **WordPerfect 5.1.**

Manuscripts and diskettes should be prepared in accordance with the **Instructions for Authors** given at the back of this issue of the Journal. They should be sent to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography
P. O. Box 2180
Cherry Hill, NJ 08034

INSTRUCTIONS TO AUTHORS

Journal of Liquid Chromatography is published in the English language for the rapid communication of research in liquid chromatography and its related sciences and technologies.

Directions for Submission

One typewritten manuscript, suitable for direct reproduction, and two (2) clear copies with figures must be submitted. Since the Journal is produced by direct photography of the manuscripts, typing and format instructions must be strictly followed. Non-compliance will result in return of the manuscript to the author and will delay its publication. To avoid creasing, manuscripts should be placed between heavy cardboards before mailing.

Manuscripts may also be submitted on **computer diskettes**. A printed manuscript must also be submitted with diskettes because, at the present time, we are experimenting with manuscripts on diskettes. Diskettes must be readable with an IBM-compatible computer (Macintosh or other type not acceptable) and must be formatted with MS-DOS 3.1 or greater. Be sure to indicate the word processing software that was used to prepare the manuscript diskette.

Manuscripts and computer diskettes should be mailed to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography
P. O. Box 2180
Cherry Hill, NJ 08034

Reprints

Due to the short production time for papers in this journal, it is essential to order reprints immediately upon receiving notification of acceptance of the manuscript. A reprint order form will be sent to the author with the letter of acceptance for the manuscript. Reprints are available in quantities of 100 and multiples thereof. Twenty (20) free reprints will be included with orders of 100 or more reprints.

Format of the Manuscript

1. The general format of the manuscript should be:
   Title
   Author(s)' names and full addresses
   Abstract
   Text Discussion
   References

2. **Title & Authors:** The entire title should be in capital letters and centered within the width of the typing area, located at least 2 inches (5.1 cm) from the top of the page. This should be followed by 3 lines of space, then by the names and addresses of the authors, also centered, in the following manner:

A SEMI-AUTOMATIC TECHNIQUE FOR THE SEPARATION AND DETERMINATION OF BARIUM AND STRONTIUM IN WATER BY ION EXCHANGE CHROMATOGRAPHY AND ATOMIC EMISSION SPECTROMETRY

F. D. Pierce and H. R. Brown
Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

3. **Abstract:** The title **ABSTRACT** should be typed, capitalized and centered, 3 lines below the addresses. This should be followed by a **single-spaced**, concise abstract. Allow 3 lines of space below the abstract before beginning the text of the manuscript.

4. **Text Discussion:** Whenever possible, the text discussion should be divided into major sections such as

INTRODUCTION
MATERIALS
METHODS
RESULTS
DISCUSSION
ACKNOWLEDGEMENTS
REFERENCES

These **major headings** should be separated from the text by two lines of space above and one line of space below. Each major heading should be typed in capital letters, centered and underlined.

Secondary headings, if any, should be placed flush with the left margin, underlined and have the first letter of main words capitalized. Leave two lines of space above and one line of space below secondary headings.

5. The first word of each **paragraph** within the body of the text should be indented five spaces.

6. **Acknowledgements**, sources of research funds and address changes for authors should be listed in a separate section at the end of the manuscript, immediately preceding the references.

7. **References** should be numbered consecutively and placed in a separate section at the end of the manuscript. They should be typed single-spaced, with one line space between each reference. Each reference should contain names of all authors (with initials of their first and middle names); do not use *et al.* for a list of authors. Abbreviations of journal titles will follow the American Chemical Society's Chemical Abstracts List of Periodicals. The word **REFERENCES** should be capitalized and centered above the reference list.

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

8. Each page of manuscript should be numbered lightly, with a light blue pencil, at the bottom of the page.

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

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

Additional Typing Instructions

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

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

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

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

5. **Figures (drawings, graphs, etc.)** should be professionally drawn in **black** India ink on separate sheets of **white** paper, and should be placed at the end of the text. They should not be inserted in the body of the text. They should not be reduced to a small size. Preferred size for figures is from 5 inches x 7 inches (12 7 cm x 17.8 cm) to 8½ inches by 11 inches (21.6 cm x 27.9 cm). **Photographs** should be professionally prepared *glossy* prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn.

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

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