JOURNAL OF LIQUID CHROMATOGRAPHY

VOLUME 18 NUMBER 17 1995

Editor: DR. JACK CAZES
Associate Editors: DR. HALEEM J. ISSAQ
DR. STEVEN H. WONG

JOURNAL OF LIQUID CHROMATOGRAPHY

October 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	Airmail to Europe	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(17) i–iv, 3373–3556 (1995)
ISSN: 0148-3919

Printed in the U.S.A.

Subscribe Today!

Use the cards below to subscribe to the *Journal of Liquid Chromatography* or to recommend the journal to your library for acquisition.

Order Form

Journal of Liquid Chromatography

Please enter my subscription to Vol. 18, 20 Numbers, 1995 at the ☐ institutional rate of $1450.00; ☐ individual rate of $725.00. *Individual subscriptions must be prepaid in American currency by personal check or credit card. Please add $3.50 per issue (number) for shipping outside the U.S. For airmail to Europe, add $5.50 per issue; to Asia, add $6.50 per issue. Canadian customers please add 7% GST.*

____ Please send me a proforma invoice.
____ Check enclosed made payable to Marcel Dekker, Inc.
____ Charge my: ☐ MasterCard ☐ Visa ☐ American Express

Card No._____ Exp. Date_____

Signature_____

Name_____

Address_____

City/State/Zip_____

Does your library subscribe to the ***Journal of Liquid Chromatography***? Just complete this card and submit it to your librarian or department head.

Attention: Librarian/Department Head: I have examined the ***Journal of Liquid Chromatography*** and would like to recommend the journal for acquisition.

Signature_____ Date_____

Name_____ Department _____

Journal of Liquid Chromatography
Volume 18, 20 Numbers, 1995: $1450.00
ISSN: 0148—3919 CODEN: JLCHD8

Sample copy and proforma invoice available upon request.

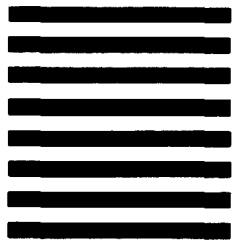
Please contact the Promotion Department at:

Marcel Dekker, Inc.
270 Madison Avenue
New York, NY 10016
(212) 696-9000 phone
(212) 685-4540 fax

Subscribe Today!

Use the cards below to subscribe to the *Journal of Liquid Chromatography* or to recommend the journal to your library for acquisition.

NO POSTAGE
NECESSARY
IF MAILED
IN THE
UNITED STATES



BUSINESS REPLY MAIL
FIRST CLASS PERMIT NO. 2863 NEW YORK, NY

POSTAGE WILL BE PAID BY ADDRESSEE

Promotion Department
MARCEL DEKKER, INC.
270 Madison Avenue
New York, NY 10016-0601

Journal of Liquid Chromatography

Editor: JACK CAZES
Cherry Hill, New Jersey

The *Journal of Liquid Chromatography* contains an outstanding selection of critical, analytical, and preparative papers involving the application of liquid chromatography to the solution of problems in all areas of science and technology, as well as papers that deal specifically with liquid chromatography as a science within itself. The coverage spans such areas as paper and thin layer chromatography and all modes of liquid column chromatography, including classical and HPLC. On a regular basis, entire issues are devoted to special topics in liquid chromatography, including an annual directory of LC manufacturers, suppliers, and services. In addition, each issue offers book reviews, liquid chromatography news, and a calendar of meetings and exhibitions.

JOURNAL OF LIQUID CHROMATOGRAPHY

Editor:
DR. JACK CAZES

Editorial Secretary:
ELEANOR CAZES

P.O. Box 2180
Cherry Hill, New Jersey 08034

Associate Editors:

DR. HALEEM J. ISSAQ
NCI-Frederick Cancer Research & Development Center
Frederick, Maryland

DR. STEVEN H. WONG
Medical College of Wisconsin
Department of Pathology
8700 West Wisconsin Ave.
Milwaukee, WI 53226

Editorial Board

H.Y. ABOUL-ENEIN, *King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia*
V.K. AGARWAL, *Bayer Corporation, West Haven, Connecticut*
J.G. ALVAREZ, *Harvard University, Boston, Massachusetts*
D.W. ARMSTRONG, *University of Missouri, Rolla, Missouri*
A. BERTHOD, *Université Claude Bernard-Lyon 1, Villeurbanne, France*
U.A.TH. BRINKMAN, *The Free University, Amsterdam, The Netherlands*
P.R. BROWN, *University of Rhode Island, Kingston, Rhode Island*
W.B. CALDWELL, *Princeton Chromatography, Inc., Cranbury, New Jersey*
R. DEMURO, *Shimadzu Scientific Instruments, Inc., Columbia, Maryland*
J.G. DORSEY, *Florida State University, Tallahassee, Florida*
Z. EL RASSI, *Oklahoma State University, Stillwater, Oklahoma*
J.C. GIDDINGS, *University of Utah, Salt Lake City, Utah*
E. GRUSHKA, *The Hebrew University, Jerusalem, Israel*
G. GUIOCHON, *University of Tennessee, Knoxville, Tennessee*
N.A. GUZMAN, *R.W. Johnson Pharm. Res. Inst., Raritan, New Jersey*
S. HARA, *Tokyo College of Pharmacy, Tokyo, Japan*
G.L. HAWK, *The Cardinal Instrument Co., Inc., Bristol, Pennsylvania*
W.L. HINZE, *Wake Forest University, Winston-Salem, North Carolina*

(continued)

JOURNAL OF LIQUID CHROMATOGRAPHY

Editorial Board *(continued)*

C. HORVATH, *Yale University, New Haven, Connecticut*
W.J. HURST, *Hershey Foods Technical Center, Hershey, Pennsylvania*
J. JANCA, *Université de la Rochelle, La Rochelle, France*
G.M. JANINI, *NCI-Frederick Cancer R&D Center, Frederick, Maryland*
M. JARONIEC, *Kent State University, Kent, Ohio*
K. JINNO, *Toyohashi University of Technology, Toyohashi, Japan*
P.T. KISSINGER, *Purdue University, West Lafayette, Indiana*
J. LESEC, *Ecole Superieure de Physique et de Chemie, Paris, France*
F. LYABAYA, *Shimadzu Scientific Instruments, Inc., Columbia, Maryland*
H.M. MC NAIR, *Virginia Polytechnic Institute, Blacksburg, Virginia*
R.B. MILLER, *Fujisawa USA, Inc., Melrose Park, Illinois*
S. MORI, *Mie University, Tsu, Mie, Japan*
M. MOSKOVITZ, *Consultant, Atlanta, Georgia*
I.N. PAPADOYANNIS, *Aristotelian University of Thessaloniki, Thessaloniki, Greece*
L.A. PAPAZIAN, *Consultant, Cranbury, New Jersey*
W.H. PIRKLE, *University of Illinois, Urbana, Illinois*
F.M. RABEL, *E-M Separations, Inc., Gibbstown, New Jersey*
D.A. ROSTON, *Searle Research & Development, Skokie, Illinois*
C.G. SCOTT, *Retired, East Stroudsburg, Pennsylvania*
R.P.W. SCOTT, *Consultant, Avon, Connecticut*
Z.K. SHIHABI, *Bowman Gray School of Medicine, Winston, Salem, North Carolina*
J.H.M. van den BERG, *Solvay Duphar BV, Weesp, The Netherlands*
R. WEINBERGER, *CE Technologies, Chappaqua, New York*

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 of Scientific and Technical Information of the Russian Academy of Sciences ■ Alerts ■ Aluminium Industry Abstracts ■ Analytical Abstracts ■ ASCA ■ Berichte Pathologie ■ CAB Abstracts ■ Cambridge Scientific Abstracts ■ Chemical Abstracts ■ Chemical Reactions Documentation Service ■ Current Awareness in Biological Sciences ■ Current Contents/Life Sciences ■ Current Contents/Physical and Chemical Sciences ■ Current Opinion ■ Engineered Materials Abstracts ■ Engineering Index ■ Excerpta Medica ■ Metals Abstracts ■ Reference Update ■ Saltykov-Shchedrin State Public Library ■ Science Citation Index ■ Tobacco Abstracts

Manuscript Preparation and Submission. See end of issue.

Copyright © 1995 by Marcel Dekker, Inc. All rights reserved. Neither this work nor any part may be reproduced or transmitted in any form or by any means, electronic or mechanical, microfilming and recording, or by any information storage and retrieval systems without permission in writing from the publisher.

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.

Authorization to photocopy items for internal or personal use, or the internal or personal use of specific clients, is granted by Marcel Dekker, Inc., for users registered with the Copyright Clearance Center (CCC) Transactional Reporting Service, provided that the fee of $10.00 per article is paid directly to CCC, 222 Rosewood Drive, Danvers, MA 01923. For those organizations that have been granted a photocopy license by CCC, a separate system of payment has been arranged.

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, 18(17), 3373-3396 (1995)

HIGH PERFORMANCE MICELLAR LIQUID CHROMATOGRAPHY WITH SILICA MICROPARTICLES HAVING SURFACE-BOUND CATIONIC SURFACTANT MOIETIES. I. COMPARISON WITH OCTADECYLSILICA AND APPLICATIONS TO THE SEPARATION OF DANSYL AMINO ACIDS, HERBICIDES, AND CATECHOLAMINES

YING ZHANG AND ZIAD EL RASSI
Department of Chemistry
Oklahoma State University
Stillwater, Oklahoma 74078-0447

ABSTRACT

The usefulness of silica microparticles having surface-bound cationic surfactant ligands in reversed phase chromatography (RPC) and micellar liquid chromatography (MLC) was examined, and the influence of surfactant type on retention and separation selectivity was studied. Different selectivities were observed by using silica microparticles having surface-bound cationic surfactant ligands, such as n-octadecyldimethyl(propylsilyl)ammonium groups ($C_{18}N^+(Me)_2Pr$) when compared to a C_{18}-silica column in the separation of dansyl amino acids (Dns-AA), herbicides and catecholamines by RPC and MLC. The presence of surface-bound charged moieties affected the amount of surfactant adsorbed onto the surface of the stationary phase from a given micellar mobile phase, which in turn influenced the retention behavior of neutral and charged species, thus leading to a unique chromatographic selectivity.

INTRODUCTION

Reversed phase chromatography (RPC) using alkyl bonded phases is the most frequently used technique for the separation of non-volatile compounds [1]. The use of

Copyright © 1995 by Marcel Dekker, Inc.

secondary chemical equilibria in RPC including micellar liquid chromatography (MLC) has greatly enlarged the scope of applications of RPC. In fact, MLC has proved to be a powerful analytical tool [2-6] since its introduction by Armstrong and Henry [2] in 1980.

Thus far, the majority of MLC separations have been carried out on traditional C_{18}-silica stationary phases [7-9] and to a lesser extent on the less hydrophobic cyanopropyl-silica [10] and phenyl bonded stationary phases [11]. Although it is widely recognized that in all modes of HPLC the nature of the stationary phase largely affects retention and selectivity, little or no attempts have been made to introduce novel stationary phases specially designed for MLC. In addition, since under most circumstances the stationary phases used in MLC are dynamically coated by the surfactant thus forming *in situ* surfactant-modified stationary phases, it becomes obvious that the nature of the stationary phase has a major role in determining selectivity and resolution among the separated analytes.

Thus, the aim of this paper is to introduce a novel stationary phase and to report on its utility in the HPLC separation of species of biological and environmental interests, including dansyl amino acids, catecholamines and some representative herbicides. The stationary phase described here is based on silica whose surface is chemically bonded with a cationic surfactant moiety, namely an octadecyldimethyl(propylsilyl)ammonium function. The results are compared to those obtained on the traditional C_{18}-silica stationary phase under otherwise identical conditions.

EXPERIMENTAL

Reagents

n-Octadecyldimethyl[3-(trimethoxysilyl)propyl]ammonium chloride, n-octadecyldimethylchlorosilane were obtained from Hüls America (Bristol, PA, U.S.A.). Analytical grade sodium phosphate monobasic was from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Isopropanol (HPLC grade) used to make the mobile phase solution was from

Table 1. Structure, CMC and nature of surfactants used in this study.

surfactant	structure	CMC (mM)	nature
SDS	$CH_3(CH_2)_{11}-O-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-O^-N_a^+$	8.2[a]	anionic
Em	$CH_3(CH_2)_{11}-\overset{\overset{CH_3}{\|}}{\underset{\underset{CH_3}{\|}}{N^+}}-CH_2-COO^-$	1.8[b]	zwittergent, pH > 6 cationic, pH < 6

a. 25 °C.
b. 23 °C.

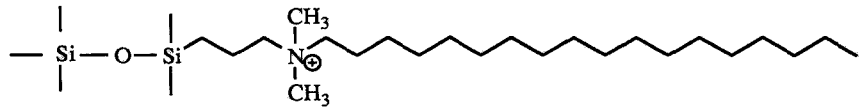
Fisher Scientific or Baxter (McGaw Park, IL, U.S.A.). Reagent grade isopropanol used in column packing was from EM Science (Cherry Hill, NJ, U.S.A.). The surfactants sodium dodecyl sulfate (SDS) and Empigen BB (N-dodecyl-N,N-dimethyl-glycine) (Em) were purchased from Calbiochem (LaJolla, CA, U.S.A.). The structure, CMC and nature of each surfactant is shown in Table 1. Dansyl-L-amino acids (Dns-AA) and catecholamines were obtained from Sigma (St. Louis, MO, U.S.A.). Herbicides were form Chem service (West Chester, PA, U.S.A.).

Apparatus

The chromatograph was assembled from an LDC-Milton Roy (Riviera Beach, FL, U.S.A.), Model CM4000 solvent delivery pump with a variable wavelength detector SpectroMonitor 3100, a Rheodyne (Cotati, CA, U.S.A.) Model 7125 sampling valve with a 20-µl sample loop, and a C-R5A Chromatopac integrator from Shimadzu (Columbia, MD, U.S.A.). The detection wavelength was set at 230 nm for herbicides, 245 nm for urea herbicides and 254 nm for all the other solutes. Home-made 100 x 4.6 mm I.D. C_{18} and $C_{18}N^+(Me)_2Pr$ columns were used.

Preparation of Stationary Phases

Typically, 5.0 g of Zorbax microspherical silica of 4.6 μm mean particle diameter and 150 Å mean pore diameter were suspended in 50 mL of dry DMF in a round-bottom flask. To this mixture, 7.0 mL of n-octadecyldimethyl[3-(trimethoxysilyl)propyl]ammonium chloride were added, and the suspension was stirred with a paddle stirrer. The reaction was performed at 120°C for 27 hours. The structure of the resulting stationary phase is given by



The C_{18}-silica stationary phase was prepared in a similar manner using 2.6 g of n-octadeclyldimethylchlorosilane, 5.0 g silica and 50 mL toluene. The suspension was heated at 125°C and stirred for 72 hours. In both cases, the resulting stationary phase was washed with acetone and methanol several times and then air dried at room temperature.

Elemental Analysis and Surface Coverage of Stationary Phases

The % C, H and N for C_{18}-silica and $C_{18}N^{+}(Me)_2Pr$-silica stationary phases were determined by elemental analysis at Galbraith Laboratories, Inc. (Knoxville, TN, USA). The % C and H for C_{18}-silica were 7.28 and 1.38, respectively, while the % C, H and N for $C_{18}N^{+}(Me)_2Pr$-silica were found to be 8.64, 1.71 and 0.53, respectively. These amounts when converted to surface coverage yielded 2.1 μmoles ligands/m^2 of silica for both silica bonded stationary phases.

Column Packing

The above stationary phases were packed from an isopropanol slurry at 8000 psi using a Shandon column packer (Keystone scientific, Bellefonte, PA, U.S.A.).

Isopropanol was used as the solvent for stationary phase suspension and as the packing solvent. All columns were 100 x 4.6 mm I.D., No. 316 stainless steel tubes (Alltech Associates, Deerfield, IL, U.S.A.). Column end fittings were also No. 316 stainless steel fitted with 0.5-μm frits and distributor disks from Alltech Associates.

Procedures

Micellar mobile phases were prepared by dissolving the appropriate amounts of surfactants and NaH_2PO_4 in water and then adjusting the pH to the desired value. For gradient elution runs, two mobile phases, A and B, were prepared for each set of experiments. They contained the same amounts of sodium phosphate buffer and surfactant, but solvent B contained 50% (v/v) isopropanol (2-PrOH). Throughout this study, two different gradient profiles were utilized. Gradient I consisted of 15 min at linearly increasing 2-PrOH concentration from 20% solvent B (i.e., 10% v/v 2-PrOH) to 100% solvent B (i.e., 50% v/v 2-PrOH) while gradient II was performed for 15 min at linearly increasing 2-PrOH concentration from 30% solvent B (i.e., 15% v/v 2-PrOH) to 50% solvent B (i.e., 25% v/v 2-PrOH). All mobile phase solutions were filtered through a S/P™ filter paper grade 360, qualitative from Baxter (McGaw Park, IL, U.S.A.).

RESULTS AND DISCUSSION

Since different types of interactions (such as electrostatic and hydrophobic) and competing equilibria are operating in MLC, it is obvious that the nature and concentration of the surfactant and the type of the stationary phase, as well as the concentration and type of the organic modifier in the mobile phase have profound effects on retention and selectivity [12, 13].

The retention behavior of dansyl amino acids (Dns-AA), catecholamines and herbicides on a $C_{18}N^+(Me)_2Pr$ column were examined under various conditions using

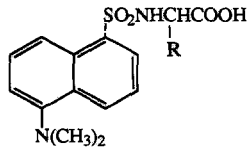
hydro-organic eluents with or without micellar phases. In the following sections, the results of these studies are discussed and compared to those obtained on a C_{18} column under the same operating conditions.

Comparison of C_{18} and $C_{18}N^+(Me)_2Pr$ Stationary Phases

a) Hydro-organic eluents. Dns-AA, and typical herbicides were used as the test solutes to compare the stationary phase having surface-bound cationic surfactant ($C_{18}N^+(Me)_2Pr$) to the traditional C_{18} sorbent in terms of retention, selectivity and the overall elution pattern. As shown in Experimental, both sorbents had the same surface coverage in terms of ligands, i. e., 2.1 μmole ligands/m^2 of silica.

Figure 1a and b shows chromatograms of Dns-AA obtained on $C_{18}N^+(Me)_2Pr$ and C_{18} columns, respectively, by using hydro-organic eluents at pH 2.5. Different elution orders of the solutes, i.e., change in selectivity, can be observed on the $C_{18}N^+(Me)_2Pr$ column when compared to the C_{18} column under otherwise identical elution conditions. In addition, the $C_{18}N^+(Me)_2Pr$ column afforded higher peak capacity than the traditional C_{18}-silica column, meaning that the number of resolved peaks is higher. This may be due in part to the fact that with the $C_{18}N^+(Me)_2Pr$ column, electrostatic interaction are superimposed on hydrophobic interaction.

The general chemical structure of the Dns-AA is given by



where R is the side chain group. According to studies on the ionization of Dns-AA [14, 15], the pK_a value of the dimethylamino group of Dns-AA is between 3.0 and 4.0 regardless of the ionic properties of the side chain group of the amino acids. The amino group adjacent to the sulfonyl group of the dansyl moiety has a pK_a value of 11.7, which

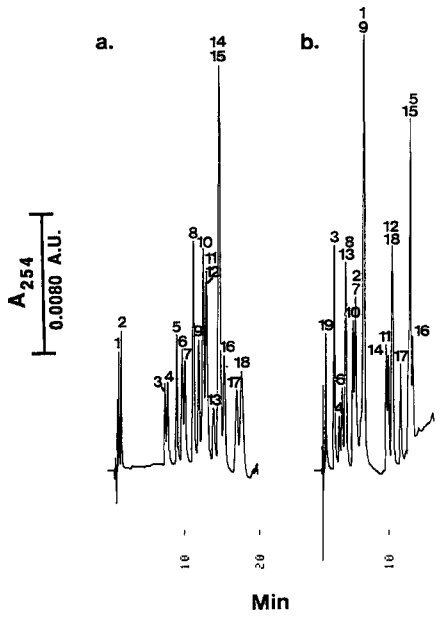
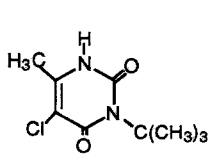


FIGURE 1. Chromatograms of Dns-AA obtained on $C_{18}N^{+}(Me)_2Pr$ column (a) and C_{18} column (b). Columns, 100 x 4.6 mm ID. 15 min linear gradient from 10 to 50% (v/v) 2-PrOH in 20.0 mM sodium phosphate, pH 2.5. Flow rate, 1.0 mL/min. Solutes: 1, arginine; 2, lysine; 3, asparagine; 4, glutamine; 5, tyrosine; 6, serine; 7, threonine; 8, glutamic acid; 9, alanine; 10, glycine; 11, proline; 12, valine; 13, aspartic acid; 14, methionine; 15, isoleucine; 16, leucine; 17, phenylalanine; 18, tryptophan; 19, cysteic acid.

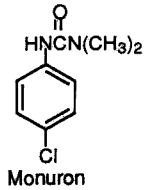
will become negatively charged only at an extreme alkaline pH. The pK_a value of the α-carboxyl group of each Dns-AA is around 4.8 [15]. At the pH of the experiment (i. e., pH = 2.5), the dimethyl amino group is positively charged (fully protonated), the amino group adjacent to the sulfonyl group is uncharged and the α-carboxyl group of the derivative is neutral. As expected, on the C_{18} column (see Fig. 1b), the Dns-AA with nonpolar side chains were eluted after polar amino acids and they emerged out of the column in the order of increasing hydrophobic character of the side chain, i.e., glycine < alanine < valine < leucine. The elution order of the Dns-AA with polar side chains was influenced by the polarity of the solute. Less hydrophobic and charged amino acids, e.g., cysteic acid,

eluted first. Asparagine and glutamine each carrying a side chain acetamido group (polar groups) and a short alkyl chain eluted thereafter. Although lysine and arginine are doubly positively charged, they were more retarded due to their relatively stronger hydrophobic character.

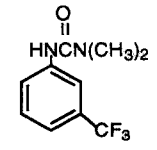
In the case of the $C_{18}N^{+}(Me)_2Pr$ column (see Fig. 1a), the surface of which is positively charged, cysteic acid whose net charge is zero, but it has a negatively charged group (a sulfonic acid group) at the pH of the experiment could not be eluted because of strong electrostatic interaction with the positively charged stationary phase. The doubly positively charged solutes, i.e., arginine and lysine, eluted first due to their stronger electrostatic repulsion from the positively charged stationary phase. In summary , on the $C_{18}N^{+}(Me)_2Pr$ column under investigation, most solutes exhibited longer retention time



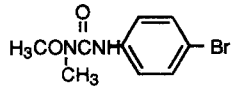
Terbacil



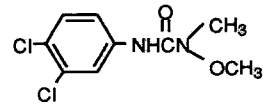
Monuron



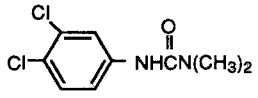
Fluometuron



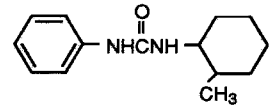
Metobromuron



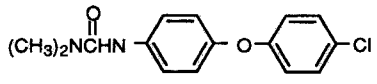
Linuron



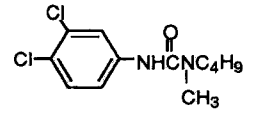
Diuron



Siduron



Chloroxuron



Neburon

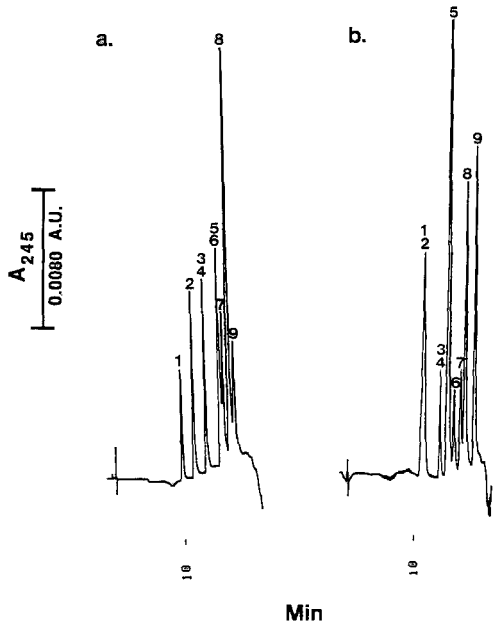
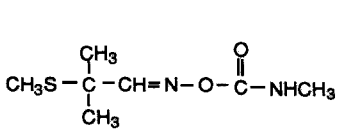


FIGURE 2. Chromatograms of urea herbicides obtained on $C_{18}N^+(Me)_2Pr$ column (a) and C_{18} column (b). Mobile phase pH is 4.0. Other conditions as in Fig. 1. Solute: 1, terbacil; 2, monuron; 3, fluometuron; 4, metobromuron; 5, diuron; 6, linuron; 7, siduron; 8, chloroxuron; 9, neburon.

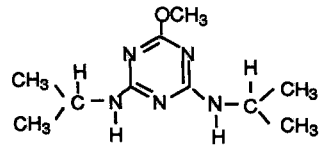
and in turn better separation than on the C_{18} column. In addition, the $C_{18}N^+(Me)_2Pr$ column exhibited a unique selectivity toward the Dns-AA.

Figure 2a and b shows the chromatograms of 9 urea herbicides, namely terbacil, monuron, fluometuron, metobromuron, diuron, linuron, chloroxuron and neburon (for structures, see below), obtained on both $C_{18}N^+(Me)_2Pr$ and C_{18} columns at pH 4.0, respectively. It can be seen that the analysis time is shorter on the $C_{18}N^+(Me)_2Pr$ column because of the weaker interaction between these nonionic species and the mildly hydrophobic $C_{18}N^+(Me)_2Pr$ stationary phase. In addition, different selectivities were observed between terbacil and monuron, and between linuron and diuron.

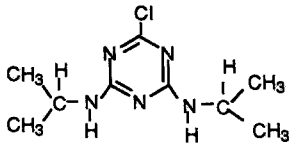
In another set of experiments, seven herbicides including aldicarb, prometon, propazine, prometryne, diazinon, parathion and 2,4-D butyl ester (for structures and pK_a values, see below) can be separated with baseline resolution on the $C_{18}N^+(Me)_2Pr$ column (Fig. 3a), while only five solutes can be resolved on the C_{18} column with different elution order and selectivity (Fig. 3b). It should be noted that prometon and 2,4-D butyl ester, prometryne and diazinon coeluted on the C_{18} column. On the $C_{18}N^+(Me)_2Pr$ column, with the exception of 2,4-D butyl ester which has larger retention time, all other solutes moved down the column much faster than on the C_{18} column, especially prometryne and prometon, because these solutes are slightly positively charged at pH 4.0, thus undergoing repulsion from the $C_{18}N^+(Me)_2Pr$ stationary phase.



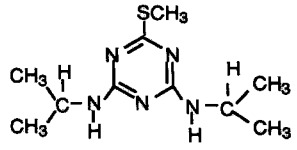
Aldicarb



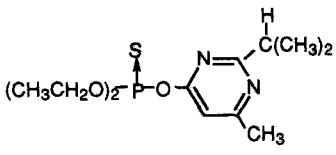
Prometon
pK_a = 4.2



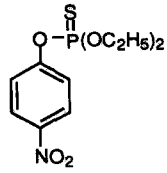
Propazine
pK_a = 1.85



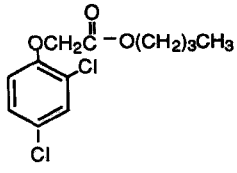
Prometryne
pK_a = 4.05



Diazinon



Parathion



2,4-D butyl ester

In summary, the $C_{18}N^+(Me)_2Pr$ stationary phase under investigation has different interactions with solutes than the C_{18} column, thus providing a method to improve

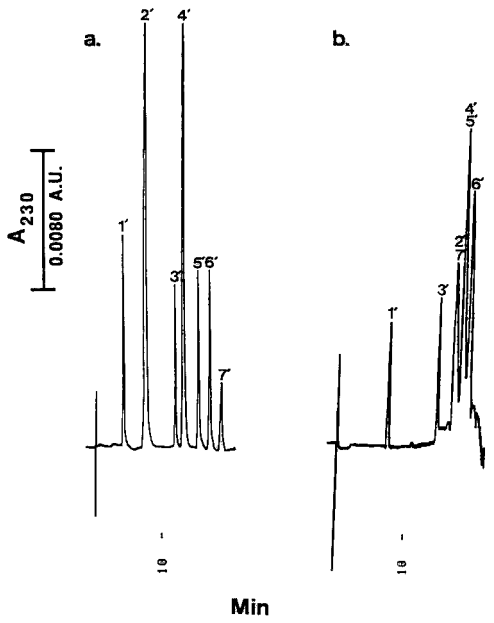


FIGURE 3. Chromatograms of herbicides obtained on $C_{18}N^{+}(Me)_2Pr$ column (a) and C_{18} column (b). Conditions as in Fig. 2. Solutes: 1', aldicarb; 2', prometon; 3', propazine; 4', prometryne; 5', diazonin; 6', parathion; 7', 2,4-D butyl ester.

separation and selectivity of some ionic and nonionic compounds. The difference in selectivity exhibited by the $C_{18}N^{+}(Me)_2Pr$ column may be attributed to the superimposition of polar interactions over nonpolar association of the solutes with the positively charged, hydrophobic ligand of the stationary phase.

b) Micellar hydro-organic (i. e., hybrid) eluents. When Empigen (Em) was added to the hydro-organic mobile phase at pH 2.5, the retention of various Dns-AA solutes under investigation decreased slightly on the $C_{18}N^{+}(Me)_2Pr$ column when compared to the retention observed with the hydro-organic mobile phase but without the surfactant (i. e., without Em); compare Fig. 4a to Fig. 1a. This reduction in retention is the result of electrostatic repulsion between equally charged solute and surfactant-modified stationary

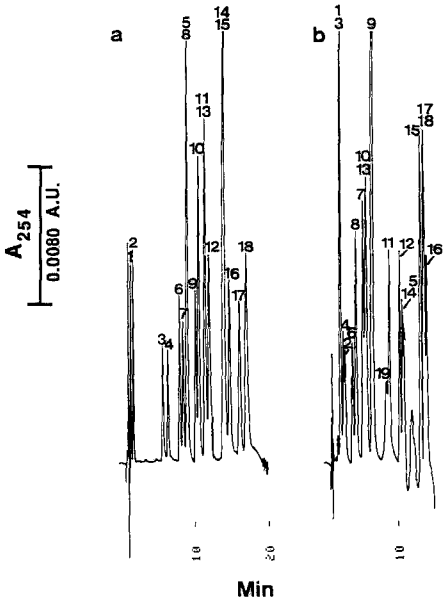


FIGURE 4. Chromatograms of Dns-AA obtained on $C_{18}N^{+}(Me)_2Pr$ column (a) and C_{18} column (b). Columns, 100 x 4.6 mm ID. 15 min linear gradient from 10 to 50% (v/v) 2-PrOH in 20.0 mM sodium phosphate containing 5.0 mM Empigen, pH 2.5. Flow rate, 1.0 mL/min. Solutes: 1, arginine; 2, lysine; 3, asparagine; 4, glutamine; 5, tyrosine; 6, serine; 7, threonine; 8, glutamic acid; 9, alanine; 10, glycine; 11, proline; 12, valine; 13, aspartic acid; 14, methionine; 15, isoleucine; 16, leucine; 17, phenylalanine; 18, tryptophan; 19, cysteic acid.

phase, i.e., a stationary phase with adsorbed surfactant molecules on its surface. At pH 2.5, both the surfactant and the Dns-AA are positively charged. The degree of reduction in retention varied among the various solutes and was largely dependent on the hydrophilic-hydrophobic balance of the solute. The retention of Dns-AA solutes of relatively large hydrophobicity such as tryptophan was not as much affected as that of weakly hydrophobic solutes such as serine and asparagine. The difference in the degree of repulsion/hydrophobic interaction may explain why the elution order of valine/aspartic acid and serine/tyrosine were reversed when the Em surfactant was added to the mobile phase (compare Fig. 4a to Fig. 1a).

Using the same hydro-organic mobile phase (as in the preceding experiment) containing Em but raising the pH to 6.0, all Dns-AA with nonpolar side chains could not be eluted and only some of the Dns-AA with polar side chains, i.e., serine, threonine, asparagine, glutamine, tyrosine, lysine and arginine, were eluted. For the amino acids that eluted at pH 6.0, their retention times were longer than at pH 2.5. This indicates that the electrostatic interaction between each dansyl amino acid and the stationary phase is stronger at higher pH. At higher pH values, i.e., pH 6.0, the carboxyl group of Dns-AA is negatively charged and has a stronger electrostatic interaction with the surfactant modified-stationary phase. Under this condition, the hydrophobic Dns-AA with nonpolar side chains underwent both hydrophobic and electrostatic interactions, and as a result they were retarded longer. This dual interaction mechanism provided even stronger interaction when the solute possessed two carboxyl groups (e.g., aspartic and glutamic acids) and as a result they could not be eluted. At a lower pH, i.e., pH 2.5, however, where the Dns-AA are positively charged, electrostatic repulsion from the modified-stationary phase overshadowed hydrophobic interaction, and were eluted in shorter time.

Figure 4b illustrates the chromatogram of Dns-AA obtained on a C_{18} column under the same mobile phase conditions as in Fig. 4a. With the C_{18} column, the surfactant (i.e., Em) is adsorbed onto the stationary phase, thus forming a surfactant modified-stationary phase. Under this condition, and comparing to a C_{18} column that was used with a hydro-organic mobile phase (Fig. 1b), the doubly positively charged solutes, such as lysine and arginine eluted earlier because of the electrostatic repulsion from the surfactant modified-stationary phase. Also, tyrosine, glutamine and asparagine eluted earlier due to their repulsion and weak hydrophobicity. On the other hand, hydrophobic amino acids such as phenylalaline and tryptophan were retained more (Fig. 4b). When comparing to a $C_{18}N^+(Me)_2Pr$ column that was eluted with an Em micellar mobile phase (Fig. 4a), most of the Dns-AA yielded reduced retention time on the C_{18} column except tyrosine and lysine. Cysteic acid which can not be eluted on a $C_{18}N^+(Me)_2Pr$ column could be eluted here. As can be seen in Fig. 4, the $C_{18}N^+(Me)_2Pr$ column provided a better overall

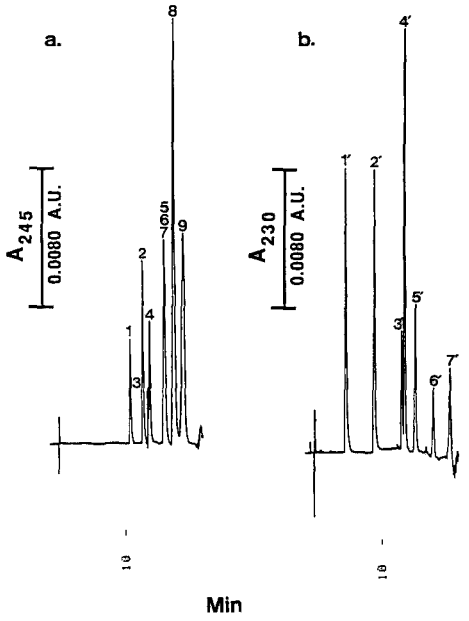


FIGURE 5. Chromatograms of urea herbicides (a) and other herbicides (b) obtained on $C_{18}N^+(Me)_2Pr$ column. Mobile phase pH is 4.0. Other conditions are as in Fig. 4. Solutes in (a): 1, terbacil; 2, monuron; 3, fluometuron; 4, metobromuron; 5, diuron; 6, linuron; 7, siduron; 8, chloroxuron; 9, neburon. Solutes in (b): 1', aldicarb; 2', prometon; 3', propazine; 4', prometryne; 5', diazonin; 6', parathion; 7', 2,4-D butyl ester.

resolution between the various solutes. In fact, 15 Dns-AA were baseline resolved on the $C_{18}N^+(Me)_2Pr$ column as opposed to only 14 Dns-AA partially resolved on the C_{18} column. This may be attributed to the higher coating of the C_{18}-silica surface with the Em surfactant as compared to the $C_{18}N^+(Me)_2Pr$-silica surface, thus rendering the former column more repulsive than the latter. The higher binding of the Em surfactant to the C_{18}-silica surface may be explained by the absence of similarly charged moieties as in the case of the $C_{18}N^+(Me)_2Pr$ column, since both stationary phases (i.e., C_{18} and $C_{18}N^+(Me)_2Pr$) possess the same surface coverage with ligands.

Figure 5a and b displays the chromatograms of herbicides obtained on a $C_{18}N^+(Me)_2Pr$ column using hydro-organic eluents containing Em at pH 4.0. When

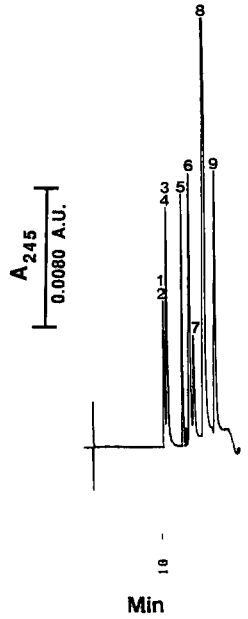


FIGURE 6. Chromatogram of urea herbicides obtained on C_{18} column. Mobile phase pH is 4.0. Other conditions are as in Fig. 4. Solutes: 1, terbacil; 2, monuron; 3, fluometuron; 4, metobromuron; 5, diuron; 6, linuron; 7, siduron; 8, chloroxuron; 9, neburon.

compared to Fig. 2a, the retention of urea herbicides shown in Fig. 5a was little or not affected since these solutes are nonionic. Also, using the same column and mobile phase, the herbicides shown in Fig. 5b and 3a behaved similarly regardless of the presence or absence of Em in the hydro-organic eluent. At this pH, the weakly basic herbicides (prometon and prometryne) were only slightly ionized and did not undergo extensive electrostatic repulsion with the micelle and/or the surfactant modified-stationary phase. On the C_{18} column and using Em as the mobile phase micelles (see Fig. 6), all urea herbicides were less retained when compared to the case of the C_{18} column without the Em surfactant present in the mobile phase. However, the surfactant has no effect on the elution order (compare Fig. 6 to Fig. 2b). When compared to the case of the $C_{18}N^{+}(Me)_2Pr$ column,

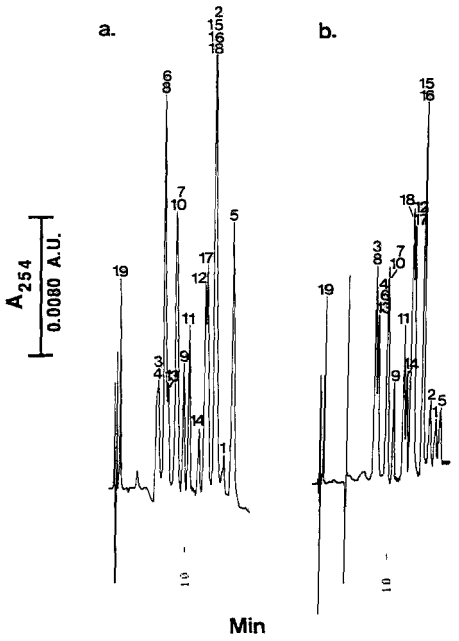


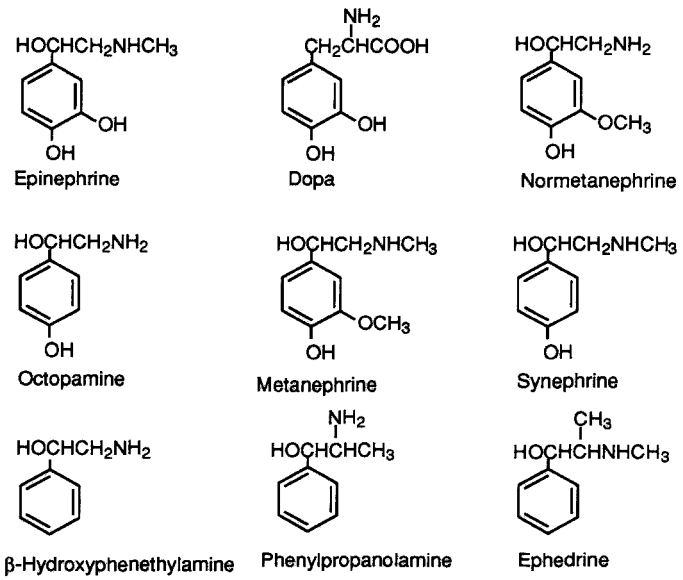
FIGURE 7. Chromatograms of Dns-AA obtained on $C_{18}N^+(Me)_2Pr$ column (a) and C_{18} column (b). Columns, 100 x 4.6 mm ID. 15 min linear gradient from 10 to 50% (v/v) 2-PrOH in 20.0 mM sodium phosphate containing 20.0 mM SDS, pH 2.5. Flow rate, 1.0 mL/min. Solutes: 1, arginine; 2, lysine; 3, asparagine; 4, glutamine; 5, tyrosine; 6, serine; 7, threonine; 8, glutamic acid; 9, alanine; 10, glycine; 11, proline; 12, valine; 13, aspartic acid; 14, methionine; 15, isoleucine; 16, leucine; 17, phenylalanine; 18, tryptophan; 19, cysteic acid.

the presence of the Em surfactant has different effect on the retention and selectivity of urea herbicides on the C_{18} column (compare Fig. 6 to Fig. 5a).

On the other hand, polar and highly positively charged catecholamines (structures are shown below) could not be retained on the $C_{18}N^+(Me)_2Pr$ column.

With a $C_{18}N^+(Me)_2Pr$ column and adding SDS instead of Em to the mobile phase, the Dns-AA behaved differently (see Fig. 7a). Under this condition, the positively charged quaternary ammonium groups of the stationary phase form ion-pairs with the oppositely charged SDS. In addition, the surfactant is adsorbed onto the stationary phase thus

yielding an SDS-modified stationary phase. At pH 2.5, dansyl amino acids with strong side chain acidic groups will repulse from the negatively charged SDS-modified stationary phase, and therefore their retention will be reduced. This is the case of cysteic acid whose strong sulfonic acid group is ionized at any pH. On the other hand, the retention of the doubly positively charged Dns-AA such as lysine and arginine increased and they were retarded much longer in the presence than in the absence of the SDS micellar phase, compare Fig. 7a to Fig. 1a.



On a C_{18} column, and in the presence of SDS in the hydro-organic eluent, the elution order is almost the same as with a $C_{18}N^+(Me)_2Pr$ column but with different selectivity. An exception is that tryptophan eluted earlier on a C_{18} column (see Fig. 7b).

Using a $C_{18}N^+(Me)_2Pr$ column, urea herbicides terbacil and fluometuron eluted faster while siduron and neburon eluted slower when SDS was present as compared to the absence of SDS, so that fluometuron was separated from metobromuron and the elution order of siduron and chloroxuron was reversed (compare Fig. 8a to Fig. 2b). Prometon

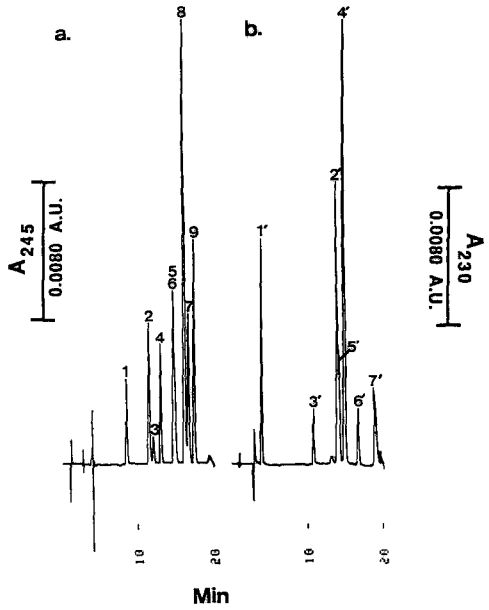


FIGURE 8. Chromatograms of urea herbicides (a) and other herbicides (b) obtained on $C_{18}N^{+}(Me)_2Pr$ column. Column, 100 x 4.6 mm ID. 15 min linear gradient from 10 to 50% (v/v) 2-PrOH in 20.0 mM sodium phosphate containing 20.0 mM SDS, pH 2.5. Flow rate, 1.0 mL/min. Solutes in (a): 1, terbacil; 2, monuron; 3, fluometuron; 4, metobromuron; 5, diuron; 6, linuron; 7, siduron; 8, chloroxuron; 9, neburon. Solutes in (b): 1', aldicarb; 2', prometon; 3', propazine; 4', prometryne; 5', diazonin; 6', parathion; 7', 2,4-D butyl ester.

and prometryne solutes each carrying a positive charge migrated much slower due to the electrostatic attraction to the surfactant-modified stationary phase and eluted after propazine and diazinon, respectively (Fig. 8b). The overall separation of 16 herbicides is illustrated in Fig. 9 whereby 12 of them were resolved.

On a C_{18} column, nine urea herbicides were completely separated using SDS as the micellar mobile phase (see Fig. 10a). As can be seen not only the retention time of each urea herbicides was reduced, but also monuron and metobromuron were separated from terbacil and fluometuron, respectively, when compared to the case without SDS (see Fig.

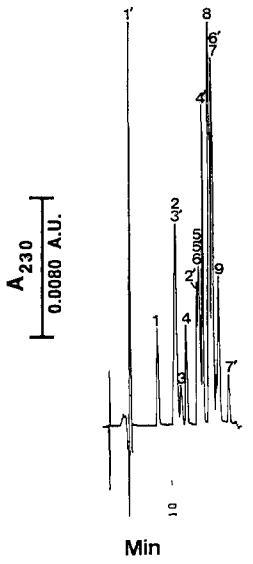


FIGURE 9. Chromatogram of all herbicides obtained on $C_{18}N^+(Me)_2Pr$ column. Conditions are as in Fig. 8. Solutes : 1, terbacil; 1', aldicarb; 2, monuron; 2', prometon; 3, fluometuron; 3', propazine; 4, metobromuron; 4', prometryne; 5, diuron; 5', diazonin; 6, linuron; 6', parathion; 7, siduron; 7', 2,4-D butyl ester; 8, chloroxuron; 9, neburon.

2b). When comparing to a $C_{18}N^+(Me)_2Pr$ with an SDS micellar mobile phase, the retention of diuron and chloroxuron were reduced even more, so that diuron was separated from linuron and chloroxuron was separated from siduron, respectively.

Another set of seven herbicides (namely aldicarb, prometon, propazine, prometryne, diazinon, parathion and 2,4-D butyl ester) was chromatographed on a C_{18} column (Fig. 10b) in the presence of an SDS micellar mobile phase. The weakly ionized cationic herbicides prometon and prometryne were retained for longer time because they underwent electrostatic interactions with the oppositely charged surfactant-modified stationary phase. When compared to the result on a $C_{18}N^+(Me)_2Pr$ column (Fig. 8b), the selectivity was completely different. All solutes were more retained except 2,4-D butyl ester, and in addition, the $C_{18}N^+(Me)_2Pr$ column afforded better overall separation.

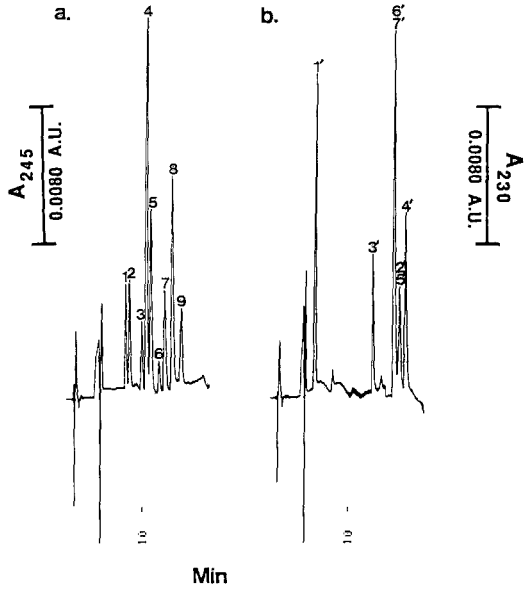


FIGURE 10. Chromatograms of urea herbicides (a) and other herbicides (b) obtained on C_{18} column. Conditions are as in Fig. 7. Solutes in (a): 1, terbacil; 2, monuron; 3, fluometuron; 4, metobromuron; 5, diuron; 6, linuron; 7, siduron; 8, chloroxuron; 9, neburon. Solutes in (b): 1', aldicarb; 2', prometon; 3', propazine; 4', prometryne; 5', diazonin; 6', parathion; 7', 2,4-D butyl ester.

However, when all the 16 herbicides were chromatographed on a C_{18} column, the overall resolution was better than on a $C_{18}N^{+}(Me)_2Pr$ column (see Fig. 11) since the C_{18} column seems to better segregate the urea herbicides from the other herbicides, so that less overlapping of peaks occurred.

In another instance where the C_{18} column under investigation seems to afford superior separation is with polar catecholamines (see Fig. 12). This reflects the larger hydrophobicity of the C_{18} column. In fact, in the presence of SDS as the micellar mobile phase, catecholamines including epinephrine, dopa, normetanephrine, octopamine, metanephrine, synephrine, β-hydroxyphenethylamine, phenylpropanolamine and ephedrine, can be retained and separated using gradient elution (see Fig. 12b). The elution

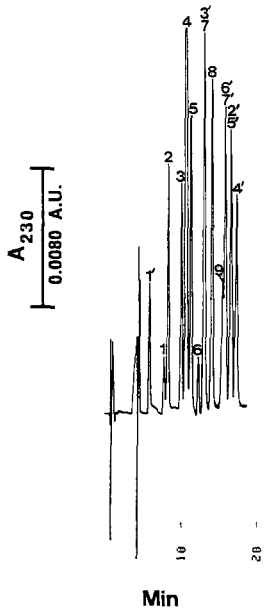


FIGURE 11. Chromatogram of all herbicides obtained on C_{18} column. Conditions are as in Fig. 7. Solutes : 1, terbacil; 1', aldicarb; 2, monuron; 2', prometon; 3, fluometuron; 3', propazine; 4, metobromuron; 4', prometryne; 5, diuron; 5', diazonin; 6, linuron; 6', parathion; 7, siduron; 7', 2,4-D butyl ester; 8, chloroxuron; 9, neburon.

order depends on the hydrophobic character of the solutes. The solutes with two polar hydroxyl groups on their benzene ring, i.e., epinephrine and dopa, eluted first. The solutes without hydroxyl group on their benzene rings, i.e., phenylpropanolamine and ephedrine, eluted last. Catecholamines which could not be retained on a $C_{18}N^+(Me)_2Pr$ column in the absence of SDS in the mobile phase, they were retained on this column in the presence of SDS as the micellar mobile phase (see Fig. 12a). However, since the $C_{18}N^+(Me)_2Pr$ column is less hydrophobic than the C_{18} column, the overall resolution among the catecholamine solutes was less satisfactory on the former column than on the latter.

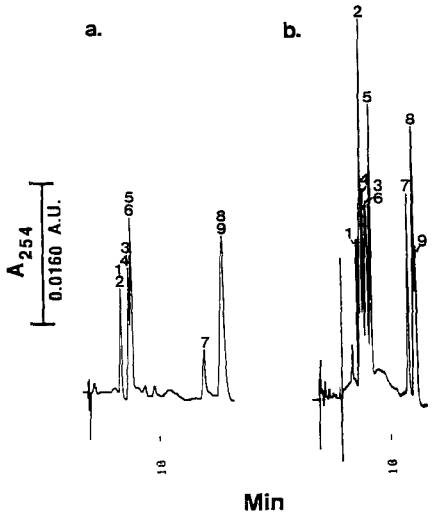


FIGURE 12. Chromatograms of catecholamines on $C_{18}N^+(Me)_2Pr$ column (a) and C_{18} column (b). Columns, 100 x 4.6 mm ID. 15 min linear gradient from 15 to 25% (v/v) 2-PrOH in 20.0 mM sodium phosphate containing 20.0 mM SDS, pH 2.5. Flow rate, 1.0 mL/min. Solutes: 1, dopa; 2, epinephrine; 3, normetanephrine; 4, octopamine; 5, metanephrine; 6, synephrine; 7, β-hydroxyphenethylamine; 8, phenylpropanolamine; 9, ephedrine.

CONCLUSIONS

The possibility of using silica microparticles having surface bound cationic surfactant ligands has been examined. Better separation can be obtained on the $C_{18}N^+(Me)_2Pr$ column for Dns-AA and herbicides. The presence of micelles in the mobile phase has a great influence on the chromatographic selectivity depending on the nature of the micelle. For the Dns-AA, the Em surfactant seems to give a better separation selectivity than the SDS surfactant on the $C_{18}N^+(Me)_2Pr$ column. Conversely, for urea herbicides, it seems that SDS yielded a better separation than Em on the C_{18} column .

ACKNOWLEDGMENTS

This material is based upon work supported in part by the Cooperative State Research Service, U.S. Department of Agriculture, under Agreement No. 94-37102-0989.

REFERENCES

1. W.R. Melander and Cs. Horváth in High Performance Liquid Chromatography, Advances and Perspectives, Cs. Horváth, ed., Academic Press, New York, Vol. 2, 1980.
2. D. W. Armstrong and S. J. Henry, J. Liq. Chromatogr., 3, 657, 1980.
3. M. G. Khaledi, Trends Anal. Chem., 7, 293, 1988.
4. D. W. Armstrong and F. Nome, Anal. Chem., 53, 1662, 1981.
5. D. W. Armstrong, Sep. Purif. Methods, 14 , 213, 1985.
6. J. G. Dorsey, Adv. Chromatogr., 27 , 167, 1987.
7. M.G. Khaledi, J.K. Strasters, A.H. Gogers, E.D. Breyer, Anal. Chem., 62, 130, 1990.
8. M.A. Garcia, S. Vera, M. Bombín and M.L. Marina, J. Chromatogr., 646, 297, 1993.
9. E. Pramauro and E. Pelizzetti, Anal. Chim. Acta, 154 , 153, 1983.
10. L.J. Cline-Love, S. Zibas, J. Noroski and M. Arunynanart, J. Pharm. Biomed. Anal., 3 , 511, 1985.
11. C.M. Kirkman, C. Zu-Ben, P.C. Uden and W.J. Stratton, J. Chromatogr., 317, 569, 1984.
12. M. G. Khaledi, J. K. Strasters, A. H. Rodgers and E. D. Breyer, Anal. Chem., 62, 130, 1990.
13. M. G. Khaledi, Anal. Chem., 60 , 876, 1988.

14. I. M. Klotz, H. A. Fiess, Biochim. Biophys. Acta, 38 , 57, 1960.

15. G. B. Gavioli, G. Grandi, L. Menabue, G. C. Pellacani, M. Sola, J. Chem. Soc. Dalton Trans., 2363, 1985.

Received: April 20, 1995
Accepted: May 9, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(17), 3397-3408 (1995)

ANALYSIS OF 9-HYDROXY ELLIPTICINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

P. FORMISYN[1], A. M. DANNA[2], AND J. BOURGOIS[1]
[1]*Laboratory of Ingénierie de l'Environnement*
Centre Simade
[2]*Laboratory of Génie Enzymatique*
Centre Spin
Ecole des Mines
158, cours Fauriel
F 42023 Saint-Etienne Cedex 2, France

ABSTRACT

9-hydroxyl derivative of ellipticine, compound of current interest known for its antitumor activity, shows a degradation in a reverse-phase column during High-Performance Liquid Chromatography analyses. In this paper, a chemical explanation of this phenomenon is proposed with the help of a degradation mechanism previously plubished, to understand the appearance of additionnal peaks corresponding to other products.

INTRODUCTION

Ellipticine, a natural indolic alkaloid first extracted from plant Ochrosia elliptica Labill. (1) is known for its antitumor activity (2-5).

Numerous derivatives of this alkaloïd have been synthetized such as 9-hydroxy, 9-methoxy derivatives and N-quaternized compounds. They all display significant anticancerous activity (6,7). Clinical trials (8-10) have shown the efficiency of this type of compounds in the treatment of breast, uterus and lung cancers, sarcomas and cerebral tumours.

The synthesis of such compounds, and particularly 9-hydroxy and 9-methoxy ellipticine, can be realized either by chemical routes (11 and mentionned references), or by biological way (12,13). Whatever the choice of the route, it is necessary to determine the quantity of synthetized compounds. High Performance Liquid Chromatography was classically employed to quantify ellipticine and its derivatives (12,14-17). But this method, the conditions of which are described in

Copyright © 1995 by Marcel Dekker, Inc.

literature, needs the stabilization of the ellipticine derivatives molecules, with for example sodium phosphate buffer, to prevent their instability in solution (15,16).

Despite these operating conditions, we show in this paper that instability of 9-hydroxylated ellipticine is a problem in HPLC. This compound undergoes a degradation mechanism, which occurs within the reverse-phase column, and which explains the appearance of additionnal peaks when it is injected alone.

MATERIALS

Apparatus

The chromatographic system consisted of a Waters® system (Milford, MA, USA) with two high-pressure pumps (Waters, Model 501), a mixing chamber and an injection device (type U6K). Ultraviolet detector (Waters, Model 481) and an integrator (Waters, type 745) complete the system. A Waters μBondapak C_{18} reversed-phase column (300 x 3.9 mm I.D; particle size 10 μm) was eluted isocratically at room temperature. Chromatograms were traced on the integrator at a speed of 1 $cm.mn^{-1}$.
Ultraviolet and visible absorption spectra were recorded on a Kontron (Uvikon 860) spectrophotometer.

Chemicals and reagents

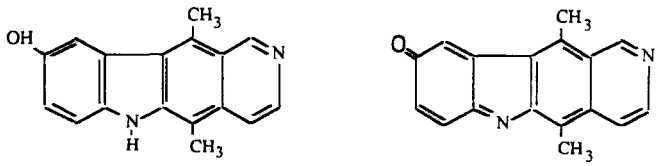
Analytical grade chemicals were employed. 9-hydroxy ellipticine **1** (262 $g.mol^{-1}$) (Figure 1) was obtained as gift from Sanofi (Paris, France). Water, methanol, ammonium acetate and acetic acid were chromatographic grade and were provided by Carlo Erba® (Italy). All mobile phase was filtered through a 0.22 μm Millipore-filter, and renewed every day.

Synthesis of 9-oxo ellipticine **2** (260 $g.mol^{-1}$) (Figure 1) was carried out as following : 320 mg of manganese dioxide was added to a suspension of 200 mg of **1** in 500 ml of chloroform. After 30 minutes of stirring at room temperature, the mixture was filtered. The filtrate was dried and concentrated, the solid obtained was purified by column chromatography (silicagel, benzene/chloroform/ethanol 3/2/1,v/v/v). After removing the solvent under vacuum, **2** was obtained (18). The 91% yield and the NMR spectrum are in agreement with the results existing in the literature (19,20).

METHODS

Procedure

The operating conditions for analyses were experimentally determined. This study was performed using the methanol-water (75/25, v/v) mobile phase containing 5.10^{-2} M ammonium



1 : 9-hydroxy ellipticine **2** : 9-oxo ellipticine

FIGURE 1 : Structure of 9-hydroxy ellipticine **1** and 9-oxo ellipticine **2**

acetate and adjusted to pH 6.0 with glacial acetic acid, at a 1.8 $ml.min^{-1}$ flow-rate. The detection was carried out by reading absorbance at 293 nm, which corresponds to the maximum of absorbance of the 9-hydroxy ellipticine and at 493 nm. Other wavelenghts (λ = 254, 308 nm) mentionned in literature (15,16,21) were tried to compare absorbance. 10 µl of the sample, prepared as described below, were injected within the column for each analysis.

Preparation of samples

Samples were solubilized in methanol, previously degazed under vacuum and different solutions were prepared to obtain 9-hydroxy ellipticine final concentrations ranging from 1 to 10 $\mu g.l^{-1}$.

A guard-column was not used for the "preparation" of samples. It is used usually to avoid too fast a degradation of the column (after few measurements, a lost of concentration of the introduced substance of about 25% was established using a guard-column). It was sufficient to clean regularly the filter of the column head.

RESULTS

High-Performance Liquid Chromatography analysis of the 9-hydroxy ellipticine for quantitative determination can give rise to uncertainties based on the appearance of many peaks during the analysis of a solution containing that molecule. Indeed, although operating conditions described in the literature were used (12,15,17) concerning especially the column type, the temperature and the composition of the mobile phase (presence of ammonium acetate buffer in order to stabilize the 9-hydroxy ellipticine), appearance of additional peaks was observed related to a modification and/or a degradation of the 9-hydroxy ellipticine injected.

The detection of 9-hydroxy ellipticine has been carried out at λ = 293 nm (Table 1) which corresponds to its maximum of absorbance in the ultraviolet range. Tables 2 and 3 present all the retention times and areas as a function of the detection wavelenght for these analyses of 9-hydroxy

TABLE 1 : Retention time and area from peaks detected at $\lambda = 293$ nm.
(Results in bold type indicate the major peaks)

Compound	Concentration ($\mu g.ml^{-1}$)	Retention time (min)	Peak Area (%)	Total (%)
9-hydroxy ellipticine	2.5	2.01 **3.13** 4.01 4.96	5.2 **91.3** 1.5 1.8	99.8
	5	1.99 2.35 **3.13** 4.0 4.96 7.56	3.4 1.9 **91** 1.5 1.1 1	99.9
9-oxo ellipticine	2.5	1.94 **3.16** 6.07 10.82	7 **72.5** 17.2 3.2	99.9
	5	1.97 **3.15** 6.01 10.72	5.4 **81** 6.5 7	99.9
	7.5	1.95 **3.14** 3.96 6.04 10.48	2.3 **80.3** 6 7.2 3.6	99.4
	10	1.91 2.29 **3.09** 3.72 3.96 6.0 10.46	2.5 5.6 **63.9** 4.1 10.2 9.1 3.7	99.1

ellipticine. In order to make sure that 9-hydroxy ellipticine was determinable by HPLC at 293 nm for all concentrations, the linearity of the detector responses was verified as a function of the concentrations of this compound injected from 1 to 10 $\mu g.ml^{-1}$. Area/concentration ratio, if we only consider the major peak (retention time of 3.1 min.), is correctly respected up to 7.5.$\mu g.ml^{-1}$ but the variation is not linear beyond this value (Figure 2). The literature mentions however other wavelenghts ($\lambda = 254$ ou 308 nm) of detection (12,15).Calibration curves were also plotted on Figure 2.

It was noted that when the detection had been carried out in the Ultraviolet range, a few collected fractions showed an absorbance in the visible range. It is known that 9-hydroxy ellipticine oxidizes rapidly and easily into 9-oxo ellipticine in liquid phase (21,22) and that 9-oxo ellipticine is a red compound which absorbs in the visible range at 493 nm (ε=10500) (22).

TABLE 2 : Retention time and area from peaks detected at $\lambda = 254$ nm.
(Results in bold type indicate the major peaks)

Compound	Concentration ($\mu g.ml^{-1}$)	Retention time (min)	Peak Area (%)	Total (%)
9-hydroxy ellipticine	2.5	1.99 2.4 **3.22** 4.13	9.9 7.4 **65.3** 17.3	99.9
	5	2.01 2.44 2.82 **3.25** 4.15 8.59	3.7 4.3 1.6 **65** 14.8 10.5	99.9
	10	2.02 2.44 **3.21** 4.11 8.33 11.53	1.6 1.3 **60.3** 23.5 8.3 4.9	99.9
9-oxo ellipticine	2.5	2.01 **3.28** 4.12 10.93	7 **64.5** 4 24.5	100
	5	1.93 **3.25** 4.09 6.42 8.31 10.93	2.6 **42** 2.4 1.7 37.6 13.6	99.9
	10	1.9 **3.24** 4.07 5.05 10.34 10.85	1.1 **77.9** 6.1 2.4 7.4 4.9	99.8

Therefore we wanted to know if it was possible to detect peaks issued from the 9-hydroxy ellipticine at this wavelenght (Figure 2 showed also the calibration curve of the 9-hydroxy ellipticine at $\lambda = 493$ nm). Figure 3A and 3B present the peaks obtained at $\lambda = 493$ nm for the 9-hydroxy ellipticine and the 9-oxo ellipticine at a concentration of 5 $\mu g.ml^{-1}$.

At last, it was interesting to plot a calibration curve of the 9-oxo ellipticine **2** at all the wavelenght previously mentionned, 254, 293, 308 and 493 nm (Figure 4). Tables 1 to 4 present retention times and areas obtained for 9-hydroxy ellipticine and for 9-oxo ellipticine as a function of the wavelenght of detection in ultraviolet and visible range.

TABLE 3 : Retention time and area from peaks detected at $\lambda = 308$ nm.
(Results in bold type indicate the major peaks)

Compound	Concentration ($\mu g.ml^{-1}$)	Retention time (min)	Peak Area (%)	Total (%)
9-hydroxy ellipticine	2.5	1.91 2.27 **3.01** 3.83 7.13	9.7 6.3 **58.5** 1.8 23.5	99.8
	5	1.97 **3.16** 4.98	5.3 **92.5** 2.1	99.9
	10	1.91 2.23 **3.1** 3.91 4.94	2.5 5 **74** 11.8 6.3	99.6
9-oxo ellipticine	2.5	1.97 **3.14** 3.93	9.3 **87.3** 3.3	99.9
	5	1.94 **3.17** 4.02 10.76	22.4 **52** 1.5 24	99.9
	10	1.98 **3.24** 5.06 10.85 13.15	3.4 **86.8** 3 3.6 3.2	100

DISCUSSION

The examination of the results of 9-hydroxy ellipticine chromatographic analysis at 293, 254 or 308 nm shows the appearance of many peaks. If we only consider the major peak (retention time of 3.1 min., area of 90%) this compound presents a correct linearity for a concentration range from 1 to 7.5 $\mu g.ml^{-1}$ (Figure 2). At $\lambda = 254$ or 308 nm, the linearity area/concentration of the major peak was respected for concentrations of 9-hydroxy ellipticine higher than those which were detected at $\lambda = 293$ nm, but the slopes were much weaker (Figure 2) and consequently, the sensitivity and the accuracy of the measurements carried out at these wavelenghts were much lower. The 9-hydroxy ellipticine is therefore, *a priori*, determinable by high-performance liquid chromatography.

To explain the appearance of additional peaks, we can remember that numerous authors have mentionned that the 9-hydroxy ellipticine, like all indolic compounds (19-23), can be oxidized in liquid phase (21) and that an equilibrium exists between 9-hydroxy ellipticine and 9-oxo ellipticine (19). As the 9-hydroxy ellipticine cannot react, in sufficient quantity, at the

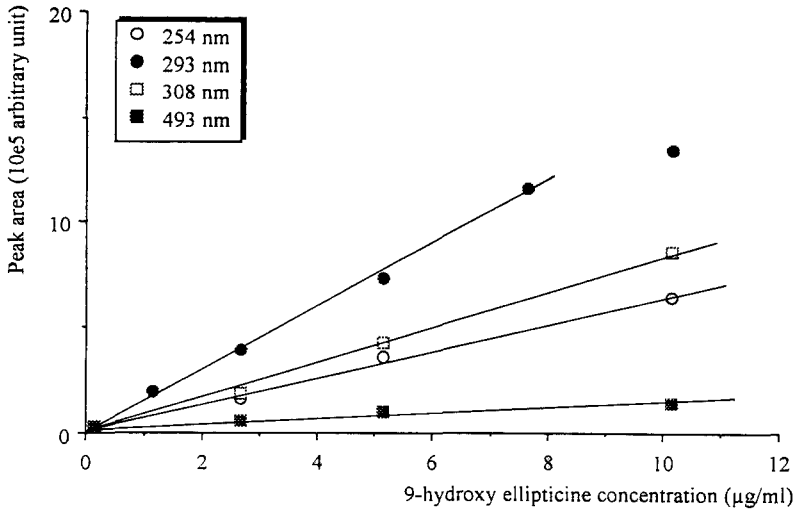
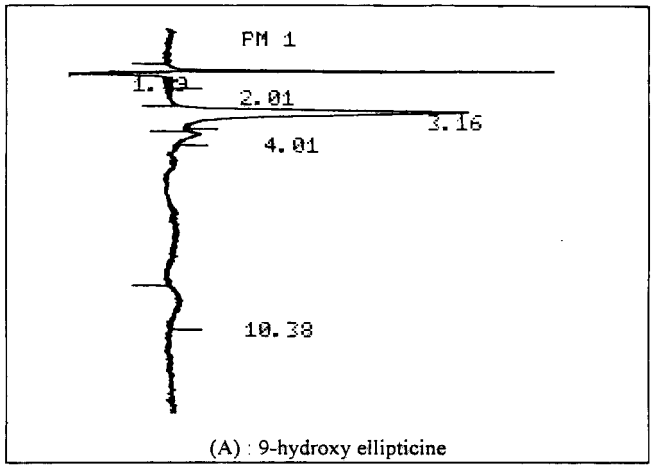


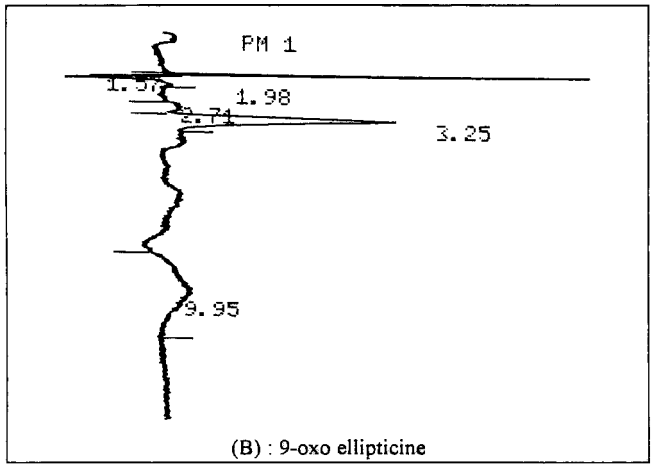
FIGURE 2 : Peaks areas expressed in arbitrary units versus 9-hydroxy ellipticine concentration ($\mu g.ml^{-1}$) at $\lambda = 293$ nm and compared absorbance of this 9-hydroxy ellipticine at three different wavelenght : $\lambda = 254$ nm, $\lambda = 308$ nm and $\lambda = 493$ nm.

analysis temperature (room temperature) with mobile phase compounds, the degradation products must be, therefore, generated either by reaction with the residual oxygen of the mobile phase or of the solution, or with the stationnary phase within the column since its oxidizing nature is known owing to the grafted silica constitutives. The first hypothesis is not very plausible since the solvent and the mobile phase were systematically degazed and saturated with nitrogen in order to avoid or to limit the possible oxidation by oxygen during the elution and the results have always shown the appearance of additionnal peaks in equal number and quantity. For these reasons, it is logical to think that the compounds which appear during the analysis must proceed of an oxidation of 9-hydroxy ellipticine within the column.

In order to verify this hypothesis, the iminoquinone **2** has been synthetized according to the operating conditions previously described and analyzed by HPLC using the operating conditions mentionned above. It is reasonable to think that the peak which is attributable to the 9-oxo ellipticine is the major peak, the retention time of which is close to 3.3. Indeed, the 9-hydroxy ellipticine is colourless in solution and cannot, consequently, be detected in the visible range. Tables 1 to 4 show that most of the peaks which have been detected in the ultraviolet range are equally detected in the visible range (equal or very close retention time) : the detected compounds must be therefore highly coloured. However, the areas of the corresponding peaks are different, which shows that these compounds do not absorb in an equivalent way in the two ranges of the



(A) : 9-hydroxy ellipticine



(B) : 9-oxo ellipticine

FIGURE 3 : Chromatograms of 9-hydroxy ellipticine (A) and 9-oxo ellipticine (B). Compounds are injected alone at a concentration of 5µg.ml^{-1} in a mobile phase methanol-water (75/25 v/v) containing 5.10^{-2} mol.l^{-1} ammonium acetate and are detected at λ = 493 nm.

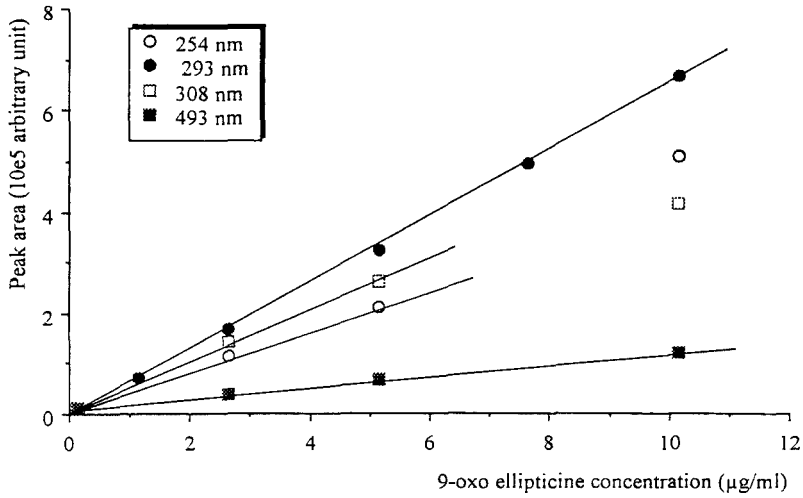


FIGURE 4 : Peaks areas expressed in arbitrary units versus 9-oxo ellipticine concentration ($\mu g.ml^{-1}$) at four different wavelenght (λ = 254, 293, 308 and 493 nm).

TABLE 4 : Retention time and area from peaks detected at λ = 493 nm.
(Results in bold type indicate the major peaks)

Compound	Concentration ($\mu g.ml^{-1}$)	Retention time (min)	Peak Area (%)	Total (%)
9-hydroxy ellipticine	2.5	2.02 **3.19**	36.2 **63.8**	100
	5	2.01 **3.16** 4.01 10.38	23.7 **64.3** 2.9 8.9	99.8
	10	1.99 **3.16** 4.04 12.1	12.3 **60.4** 6.9 20.3	99.9
9-oxo ellipticine	2.5	1.75 2.03 **3.3**	6.8 31.8 **61.3**	99.9
	5	1.98 2.71 **3.25** 9.95	12.4 3.1 **41** 43.4	99.9
	10	1.97 **3.3** 7.21	5.1 **61.1** 33.7	99.9

electronic absorption. In all cases (different concentrations of the injected solutions), the detected peaks in the visible range were found again in the ultraviolet range, especially that attributable to the 9-oxo ellipticine which absorbs in the ultraviolet range because of its aromaticity. However, a few very minor peaks one of which is attributable to the 9-hydroxy ellipticine, were not observed. The 9-hydroxy ellipticine therefore is transformed in totality during the elution using the operating conditions described above.

The presence of minor peaks indicates the formation of other compounds issued from the 9-oxo ellipticine or from the 9-hydroxy ellipticine. In a previous publication, the mechanism was precisely stated of the oxidation of the 9-hydroxy ellipticine in a methanol solution (22) :

- Oxidation of the 9-hydroxy ellipticine into 9-oxo ellipticine **2** (red) : the first step of this mechanism explains exactly the relative stabilizating power of an acidification of the mobile phase by adding ammonium acetate. Indeed, in an acidic medium, the phenolate intermediate ion is formed with much difficulty. But, the pH cannot be too acidic, because in this case, a destruction of the aromaticity, and, consequently, of the molecule to measure occurs (24). However, this addition of salts is not sufficient to avoid completely the appeareance of additionnal peaks and stop the oxidation of the 9-hydroxy ellipticine.
- Oxidation of **2** which yields the 9,10-dioxo ellipticine **3** (yellow).
- Formation of 9-hydroxy,10-methoxy ellipticine **4** (pink) by Michael addition of methanol on the cetoimine **2**.
- Formation of 9-oxo,10-methoxy ellipticine **5** (purple) by oxidation of **4**.
- Formation of 9-oxo,10,10-dimethoxy ellipticine **6** (orange) by Michael addition of methanol on **5**.

It is not surprising to find the additional peaks caused by the products issued of an oxidation of the 9-oxo ellipticine or of a Michael addition on the resulting products. The analyses were carried out with solutions of 9-hydroxy ellipticine or 9-oxo ellipticine with different concentrations and the variability of the results (number of peaks, areas) can be attributed to the modification of the oxidation power of the column and/or to the displacements of the redox equilibria and/or to the addition reactions, which are a function of the concentrations. At the three detection wavelenghts in the ultraviolet range that were used, the results of the analyses are quite different. This is not surprising since, for the compounds **2** to **6**, the aspect of the curves and their maxima of absorption in the ultraviolet range cannot be identical because of the different substitutes existing on the heterocyclic system of the ellipticine.

It is possible to determine quantitatively the 9-hydroxy ellipticine by HPLC with the condition to take into account the peak issued from the 9-oxo ellipticine, but also the peaks caused by the degradation of the 9-hydroxy ellipticine (determinable in the UV range) and the reaction products of the 9-oxo ellipticine (determinable in the UV and visible range). Taking into account all the peaks permits the obtention of the total mass of the products. However, for a comparative determination only one peak can be taken because the ε must be different according to the products. This result is corroborated by the analyses that were carried out on the same solutions of 9-hydroxy ellipticine or 9-oxo ellipticine by a method of colour measurement (22).

A 'column phenomenon' occurs during the determination of the 9-hydroxy ellipticine. The 9-hydroxy ellipticine oxidation during its chromatographic analysis is indeed impossible to avoid by the conventional means of stabilization : decreasing the pH, presence of nitrogen. The quantitative determination of the 9-hydroxy ellipticine is, in fact, a determination of 9-oxo ellipticine in solution.

This study can be concluded with the following points:

* The 9-hydroxy ellipticine is a compound of which the quantitative determination is impossible due to its aptitude to be oxidized within the chromatographic column.
* The stabilization with buffer salts which make the mobile phase acidic is not adapted to stabilize the 9-hydroxy ellipticine well enough.
* In return, if all the eluted peaks are taken into account, the quantitative determination can be realized.
* The major product eluted is the 9-oxo ellipticine.

ACKNOWLEDGEMENTS

The authors thank the Sanofi group for its financial support and for the gift of 9-hydroxy ellipticine derivative.

REFERENCES

1. S. Goodwin, A.F.Smith, E.C. Horning, J. Am. Chem. Soc., 81: 1903-1908 (1959)
2. L.K.Dalton, S. Demerac, B.C. Lelmes, J.W. Loder, T. Teitei, J.M. Swan, Austr. J. Chem., 20: 2715-2727 (1967)
3. J.B. Le Pecq, C. Gosse, N. Dat-Xuong, C. Paoletti, C. R. Acad. Sci. Paris, série D, 277: 2289-2291 (1973)
4. J. Gilbert, D. Rousselle, C. Gansser, C. Viel, J. Heterocycl. Chem., 16: 7-11 (1979)
5. K.W. Kohn, W.E. Ross, D. Glaubiger, Antibiotics. F.E.Hahn Ed., Berlin, Springer-Verlag© 1979, pp. 195-213.
6. P. Juret, A. Tanguy, A. Girard, J.Y. Le Talaer, J.S. Abbatucci, N. Dat-Xuong, J.B. Le Pecq, C. Paoletti, Eur. J. Cancer, 14: 205-206 (1978)
7. C. Paoletti, J.B. Le Pecq, N. Dat-Xuong, P. Juret, H. Garnier, J.L. Amiel, J. Rouesse, Recent Results Cancer Res., 74: 107-123 (1980).
8. C.L. Arteaga, D.L. Kisner, A. Goodman, D.D. Von Hoff, Eur. J. Cancer Clin. Oncol., 23: 1621-1626 (1987)
9. C. Lestienne, Fondamental, 41: 12-27 (1988)
10. G. Anderson, M. Clavel, J. Smyth, G. Giaccone, M. Garcia, A. Planting, O. Dalesio, A. Kirkpatrick, G. McVie, Eur. J. Cancer Clin. Oncol., 25: 909-910 (1989)

11. J. Bourgois, M. Bourgois, P. Formisyn, Trends in Heterocycl. Chem., 2: 205-232 (1991)
12. M.M. Chien, J.P. Rosazza, Appl. Environ. Microbiol., 40: 741-745 (1980)
13. P. Formisyn, These de Doctorat, Lyon-I, France, 1990, 214 p.
14. P. Lesca, B. Monsarrat, S. Cros, C. Paoletti, J. Natl. Cancer Inst., 67: 871-876 (1981)
15. G. Bykadi, K.P. Flora, J.C. Cradock, G.K. Poochikian, J. Chromatogr., 231: 137-144 (1982)
16. B. Meunier, C. Auclair, J. Bernadou, G. Meunier, S. Cros, M. Maftouh, Dev. Pharmacol., 3: 149-181 (1983)
17. P. Bellon, P. Canal, J. Bernadou, G. Soula, J. Chromatogr., 309: 170-176 (1984)
18. R. Sundaramoorthi, V.K. Kansal, B.L. Das, P. Potier, J. Chem. Soc. Chem. Comm., 5: 371-372 (1986)
19. G. Meunier, B. Meunier, J. Biol. Chem., 260 (19): 10576-10582 (1985)
20. C. Auclair, Arch. Biochem. Biophys., 259: 1-14 (1987)
21. C. Auclair, K. Hyland, C. Paoletti, J. Med. Chem., 26: 1438-1444 (1983)
22. P. Formisyn, F. Pautet, C. Tran-Minh, J. Bourgois, J. Pharm. Biomed. Anal., 10 (6): 427-436 (1992)
23. G. Meunier, D. De Montauzon, J. Bernadou, G. Grassy, M. Bonnafous, S. Cros, B. Meunier, Mol. Pharmacol., 33: 93-102 (1988)
24. N. Verbiese-genard, J.M. Kaufmann, M. Hanocq, L. Molle, J. Electroanal. Chem., 170: 243-254 (1987)

Received: April 5, 1995
Accepted: April 22, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(17), 3409-3422 (1995)

MODELLING RETENTION IN REVERSED PHASE LIQUID CHROMATOGRAPHY IN RELATION TO TEMPERATURE AND SOLVENT COMPOSITION. APPLICATION TO THE SEPARATION OF SEVEN P-HYDROXYBENZOIC ESTERS

Y. GUILLAUME AND C. GUINCHARD
Laboratoire de Chimie Analytique
Faculté des Sciences Médicales et Pharmaceutiques
25030 Besançon Cedex, France

ABSTRACT

The retention of seven closely related p-hydroxybenzoic esters in reversed phase liquid chromatography using a different volume fraction θ of methanol in the methanol-water mixture was studied with the determination of the separation factor α at different temperatures. Gibbs Helmholtz parameters $\Delta(\Delta H)$ and $\Delta(\Delta S)$ of two adjacent compounds on a chromatogram were obtained from $\ln\alpha$ versus $\frac{1}{T}$ plots. In this chromatographic system, as θ increases, $\Delta(\Delta H)$ and $\Delta(\Delta S)$ decrease and were found to vary quadratically with θ. A temperature dependent reversal of the elution order was studied and the mobile phase composition and column temperature were optimized to obtain the best separation conditions. A column temperature of 25°C with a volume fraction $\theta = 0.52$ gave the best separation conditions.

Copyright © 1995 by Marcel Dekker, Inc.

INTRODUCTION

Retention in reversed phase liquid chromatography (RPLC) is still not fully understood and is a matter of debate in the chromatographic litterature.

The retention mechanism has been studied in RPLC by measuring thermodynamic parameters [1-9].

This paper, investigate over a wide range of volume fractions of methanol θ in the methanol/water mixture, the thermodynamic behavior of seven p-hydroxybenzoic esters. A temperature dependent reversal of the elution order and a method to separate these compounds were evaluated.

MATERIALS AND METHODS

Reagents

The chromatographed compounds were p-hydroxybenzoic esters. The straight chain esters, methyl to butyl (MeR, EtR, PrR, BuR) were purchased from Interchim (Montluçon, France). The branch chain esters isopropyl, isobutyl and secbutyl (IpR, IbuR, SbuR) were synthetized in our laboratory by an esterification reaction [10]. Methanol was analytical grade HPLC. All the compounds were diluted in methanol with a concentration of 10 mg/mL.

Apparatus

The HPLC system consisted of a HPLC Waters pump 6000 A (Saint-Quentin en Yvelines, France) an Interchim Rheodyne injection valve Model 7125 (Montluçon,

France) fitted with a 20 μl sample loop and a Merck 4500 diode array detector (Nogent-sur-Marne, France). A Merck, RP 18 column (Lichrocart 50943, 119 mm x 4 mm I.D., 5 μm particle size) (Nogent-sur-Marne, France) was used with temperature controlled by an Interchim oven TM N°701 (Montluçon, France). Overall temperature control was maintained within $\pm$ 1°C with a variation from 26°C to 50°C. The mobile phase with a flow rate of 0.90 mL/min was a methanol water mixture with fraction of methanol θ varying from 0.4 to 0.8. Weaker percentages were not used because of the excessively high column pressure obtained with $\theta = 0.4$.

Methods

Thermodynamic relationships: valuable information can be gained by examining the temperature dependence of the separation factor α which is given by the equation:

$$\ln\alpha = - \frac{\Delta(\Delta H)}{RT} + \frac{\Delta(\Delta S)}{R} \quad (1)$$

where α is the separation factor between two compounds i and i+1 on the chromatogram

$$\alpha = \frac{k_{i+1}}{k_i} \quad (2)$$

k_{i+1} (respectively k_i) is the retention factor of compound i+1 (respectively i), $\Delta(\Delta H)$ (respectively $\Delta(\Delta S)$) the difference in the dissolution enthalpy (respectively dissolution entropy) between two compounds i and i+1, R the gas constant and T the column temperature. These differences in the dissolution enthalpy $\Delta(\Delta H)$ and entropy

$\Delta(\Delta S)$ are respectively a measure of polar and configuration effects. The difference in the dissolution free energy $\Delta(\Delta G)$ is given by:

$$\Delta(\Delta G) = \Delta(\Delta H) - T\,\Delta(\Delta S) \qquad (3)$$

RESULTS AND DISCUSSION

The experimental α values were calculated from the chromatograms for each adjacent compound pair noted (i, i+1). The results were processed by a computer and $\ln\alpha$ versus $\frac{1}{T}$ were plotted. The correlation coefficients were higher than 0.991. The typical standard deviation of the intercept and slope were respectively 0.005 and 0.03. Since these data were linear it was possible to calculate $\Delta(\Delta H)$ and $\Delta(\Delta S)$ for this system. Table 1 contains a complete list of the $\Delta(\Delta H)$ and $\Delta(\Delta S)$ values obtained for all solutes with different volume fractions of methanol θ in the methanol-water mixture. From these experimental data, it would appear that $\Delta(\Delta H)$ and $\Delta(\Delta S)$ vary quadrically with θ :

$$\Delta(\Delta H) = \epsilon_1 \theta^2 + \beta_1 \theta + \gamma_1 \qquad (4)$$

$$\Delta(\Delta S) = \epsilon_2 \theta^2 + \beta_2 \theta + \gamma_2 \qquad (5)$$

Combining equations 1, 4, 5 the following was obtained:

$$RT\ln\alpha = \epsilon_1 (-\theta^2) + \beta_1 (-\theta) + \gamma_1 (-1) + \epsilon_2 (T\theta^2) + \beta_2 (T\theta) + \gamma_2 (T) \qquad (6)$$

where α is the separation factor between two compounds for a couple (θ, T).

The values of ϵ_1, β_1, γ_1 ϵ_2, β_2, γ_2, were determined from a matrix product:

$$\underline{RT\ln\alpha} = M.\,\underline{p} \qquad (7)$$

where

$$RT\ln\alpha = \begin{vmatrix} RT\ln\alpha_1 \\ RT\ln\alpha_2 \\ . \\ . \\ . \\ . \\ . \\ RT\ln\alpha_n \end{vmatrix} \qquad P = \begin{vmatrix} \epsilon_1 \\ \beta_1 \\ \gamma_1 \\ \epsilon_2 \\ \beta_2 \\ \gamma_2 \end{vmatrix}$$

$$M = \begin{vmatrix} -\theta_1^2 & -\theta_1 & -1 & T\theta_1^2 & T\theta_1 & T \\ -\theta_2^2 & -\theta_2 & -1 & T\theta_2^2 & T\theta_2 & T \\ . & . & . & . & . & . \\ . & . & . & . & . & . \\ -\theta_i^2 & -\theta_i & -1 & T\theta_i^2 & T\theta_i & T \\ . & . & . & . & . & . \\ . & . & . & . & . & . \\ -\theta_n^2 & -\theta_n & -1 & T\theta_n^2 & T\theta_n & T \end{vmatrix}$$

where α_1, $\alpha_2 \ldots \alpha_n$ are the experimental values of the α recorded for each pair of temperature and volume fraction of methanol (T, θ).

$\underline{P}$ was calculated as:

$$\underline{P} = (M^t M)^{-1} (M^t \underline{RT\ln\alpha}) \qquad (8)$$

where M^t is the transposed matrix of M. The coefficients α_1, β_1, γ_1 and α_2, β_2, γ_2 are given in Table 2. The coefficient determination for these fits was always superior or

Table 1: Values of $\Delta(\Delta H)$ (kJ/mol), $\Delta(\Delta S)$ (J/mol/K) for different mobile phase compositions

Volume Fraction θ of methanol								
	0.50		0.55		0.60		0.65	
peak pair[a]	$\Delta(\Delta H)$	$\Delta(\Delta S)$	$\Delta(\Delta H)$	$\Delta(\Delta S)$	$\Delta(\Delta H)$	$\Delta(\Delta S)$	$\Delta(\Delta H)$	$\Delta(\Delta S)$
(1,2)	-1.40	+6.63	-1.48	+6.08	-1.59	+5.60	-1.60	+5.30
(2,3)	-5.95	-2.56	-6.86	-6.61	-7.66	-10.17	-8.37	-13.21
(3,4)	-7.50	-9.44	-8.29	-13.55	-8.88	-17.52	-9.64	-20.53
(4,5)	-3.21	-6.77	-3.48	-7.89	-3.72	-8.99	-3.93	-9.79
(5,6)	-4.77	-10.96	-5.26	-13.27	-5.71	-15.40	-6.11	-17.34
(6,7)	-8.17	-24.12	-8.96	-27.73	-9.79	-30.86	-10.43	-33.55

Volume Fraction θ of methanol						
	0.70		0.75		0.80	
peak pair[a]	$\Delta(\Delta H)$	$\Delta(\Delta S)$	$\Delta(\Delta H)$	$\Delta(\Delta S)$	$\Delta(\Delta H)$	$\Delta(\Delta S)$
(1,2)	-1.78	+4.80	-1.82	+4.40	-1.91	4.24
(2,3)	-8.97	-15.77	-9.48	-17.82	-9.87	-19.37
(3,4)	-10.20	-23.46	-10.99	-26.00	-11.09	-29.94
(4,5)	-4.11	-10.57	-4.26	-11.24	-4.39	-11.79
(5,6)	-6.47	-19.09	-6.77	-20.66	-7.04	-22.04
(6,7)	-11.03	-35.79	-11.51	-37.59	-11.89	-39.95

[a]: N° in peak pairs correspond to :

1 Methyl p-hydroxybenzoic ester
2 Ethyl p-hydroxybenzoic ester
3 Isopropyl p-hydroxybenzoic ester
4 Propyl p-hydroxybenzoic ester
5 Secbutyl p-hydroxybenzoic ester
6 Isobutyl p-hydroxybenzoic ester
7 Butyl p-hydroxybenzoic ester

Table 2: Values of polynomial coefficients (equations 4 and 5) for $\Delta(\Delta H)$ and $\Delta(\Delta S)$

	$\Delta(\Delta H)$			$\Delta(\Delta S)$		
Compound pair[a]	α_1	β_1	γ_1	α_2	β_2	γ_2
(1,2)	2700	-5400	675	11.36	-22.72	15.15
(2,3)	20234	-39412	8700	99.92	-185.94	65.43
(3,4)	15000	-31500	4500	75.00	-160.00	52.32
(4,5)	5500	-11000	900	22.36	-45.82	10.54
(5,6)	9205	-19544	2700	37.34	-85.48	22.44
(6,7)	19161	-37312	5698	88.92	-164.94	36.09

[a]: see Table 1

equal to 0.995. Table 3 shows experimental and calculated values for $\Delta(\Delta H)$ and $\Delta(\Delta S)$ with $\theta = 0.60$.

For all compound pairs, $\Delta(\Delta H)$, $\Delta(\Delta S)$ and the separation factor α, decreased when θ increased. This proves that the polar and configuration effects also decrease when solvent polarity decreases. Using equation 3, the particular temperature T at which $\Delta(\Delta G)$ is nil with no resolution between the two adjacent compounds was determined. This temperature is expressed by:

$$\bar{T} = \frac{\Delta(\Delta H)}{\Delta(\Delta S)} \quad (9)$$

Combining equations 4, 5, 9:

$$\bar{T} = \frac{\epsilon_1\theta^2 + \beta_1\theta + \gamma_1}{\epsilon_2\theta^2 + \beta_2\theta + \gamma_2} \quad (10)$$

For a temperature higher than $\bar{T}$, the two compound elution orders should have been reversed. In our case, within the temperature range [25°C-50°C], the elution reversal was not detected. Therefore, whatever the factor variation, all the compounds were arranged on the chromatogram in the same order.

Nevertheless, the disappearance of resolution was observed at a temperature close to the calculated temperature $\bar{T} = 40°C$ for the peak pair (IbuR, BuR) with $\theta = 0.65$. When equation 6 is rewritten, the separation factor α relating to column temperature T and the volume fraction of methanol θ, is given by:

$$R\ln\alpha = \theta^2\left(\epsilon_2 - \frac{\epsilon_1}{T}\right) + \theta\left(\beta_2 - \frac{\beta_1}{T}\right) + \left(\gamma_2 - \frac{\gamma_1}{T}\right) \quad (11)$$

Table 3: Experimental and calculated values of $\Delta(\Delta H)$ (kJ/mol) and $\Delta(\Delta S)$ (J/mol/K) for $\theta = 0.60$

peak pairs[a]	$\Delta(\Delta H)_{exp}$	$\Delta(\Delta H)_{cal}$	e[b]	$\Delta(\Delta S)_{exp}$	$\Delta(\Delta S)_{cal}$	e (%)
(1.2)	-1.67	-1.59	4.79	+5.38	+5.60	3.92
(2.3)	-7.44	-7.66	2.87	-9.75	-10.16	4.04
(3.4)	-8.56	-9.00	4.88	-16.90	-17.23	1.92
(4.5)	-3.64	-3.72	2.15	-8.59	-8.99	4.44
(5.6)	-5.50	-5.71	3.67	-15.00	-15.40	2.60
(6.7)	-9.31	-9.79	4.90	-29.40	-30.86	4.73

[a]see Table 1

e: relative difference between calculated and experimental results

Table 4: Results of the simplex process

Temperature (°C)	Volume fraction of methanol θ	Separation factor α
48.00	0.80	1.00
45.00	0.79	1.00
47.10	0.75	1.01
45.10	0.73	1.02
45.20	0.70	1.03
47.40	0.65	1.04
48.00	0.60	1.06
50.00	0.57	1.07
45.94	0.57	1.13
47.96	0.59	1.10
43.00	0.60	1.14
40.10	0.61	1.24
35.00	0.57	1.30
31.41	0.52	1.32
30.95	0.50	1.33
27.00	0.50	1.34
26.00	0.52	1.37
25.00	0.52	1.37
27.00	0.51	1.35
25.00	0.50	1.36

An increase in the separation factor α with an increase in mobile phase polarity for all compound pairs explains that the separation conditions were obtained with low values of the methanol volume fraction in the methanol water mixture. To obtained, the most efficient separation conditions, a simplex method was used. This optimization process has been used in RPLC. Berridge [9] used the simplex algorithm for the optimization of mobile phase parameters in the reversed phase and ion pairing chromatography. Wang et al. [11] employed a mixture design simplex method for the computer-assisted optimization of the mobile phase, pH and/or ion concentrations. Guillaume [10, 12], used this process for the optimization of the mobile phase composition, column temperature and mobile phase flow rate in RPLC. Matsuda [13,

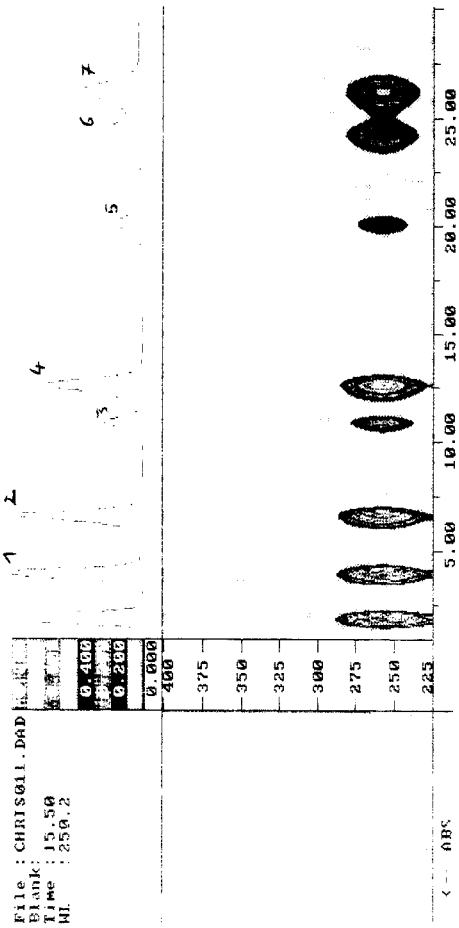


Figure 1: p-hydroxybenzoic esters chromatogram in the optimum conditions: methanol (52% (v/v) in a methanol water mixture), column temperature: 25°C, flow rate: 0.90 mL/min. Number above peaks refers to the seven compounds. See Table 1.

14] demonstrated the advantages of a "function of mutual information (FUMI)" when used with the simplex algorithm for the optimization of mobile phase composition in RPLC.

For the simplex method developed in this experiment, the highest separation factor α was used as the separation criterion. In this procedure, α was calculated for three different starting conditions of the column temperature and mobile phase composition using equation 11.

The point corresponding to the lowest value of α is reflected in relation to the curve defined by the two other points to give a fourth set of starting conditions. Once again, the point with the lowest α is reflected and the process repeated until the same mobile phase composition and column temperature continue to be selected. The maximum α (1.37), the highest separation factor for the worst separated pair of peaks (IbuR, BuR) was determined after 19 operations made by the computer (Table 4). It can be noted that for $\theta \leq 0.50$, even if α increases and θ decreases, the computer stops running because the column efficiency decreases rapidly, the peak widths were too high and the analysis time was too long. The optimum conditions were obtained with an 0.52 volume fraction of water and a column temperature of 25°C. The chromatogram is given in Figure 1.

CONCLUSION

The retention of seven esters in HPLC was studied. Thermodynamic data were determined in a wide range of volume fractions of methanol θ in the methanol/water mixture. Experimental values were in agreement with those calculated by suitable

retention models. These mathematical models associated with a simplex method separated the p-hydroxybenzoic esters and did not require any further experiments.

REFERENCES

[1] Wu C.Y., Scheng J., Zang R., Chromatographia, 35, 33, (1993).

[2] Watter L., Zielinski Jr., Freeman H.D. Anal. Chem., 42, 33, (1970).

[3] Witkiewicz Z., Szulc J., Ziolek A., Dabrowski R., Dziaduszek J. J. Chromatogr., 246, 37, (1982).

[4] Chu Yong J., Haishong Ge., Linxian S., Guo pin S., Fenxi Huaxue, 14, 801, (1994).

[5] Jung M., Schmalzing D., Shurig V., J. Chromatogr., 552, 43, (1991).

[6] Berthod A., Armstrong D.W., Anal. Chem., 64, 873, (1992).

[7] Guillaume Y., Guinchard C., J. Liq. Chromatogr., 17 (13), 2809, (1994).

[8] Guillaume Y., Guinchard C., Chromatographia, 39, 438, (1994).

[9] Berridge J.C., J. Chromatogr., 485, 3, (1989).

[10] Guillaume Y., Guinchard C., J. Chromatogr. Sci., in press

[11] Wang Q., Gao R., Yan B., J. Liq. Chromatogr., 14, 3111, (1991).

[12] Guillaume Y., Guinchard C., J. Liq. Chromatogr., 16, 3457, (1993).

[13] Matsuda R., Hayashi Y., Ishibashi M., Takeda Y., Anal. Chim. Acta, 301, 222, (1989).

[14] Matsuda R., Hayashi Y., Suzuki J., Saito Y. J. Chromatogr., 585, 187, (1991).

Received: April 20, 1995
Accepted: May 9, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(17), 3423-3433 (1995)

HPLC METHOD FOR ANTIGEN-ANTIBODY INTERACTION STUDY. APPLICATION TO ANTI HIV gp120 ANTIBODY. PROPOSITION OF TREATMENT TO IMPROVE THE EFFICIENCY OF THE IMMUNE RESPONSE

GERARD BERGER
Section de Bioénergétique
Département de Biologie Cellulaire et Moléculaire
CEN Saclay
91191 Gif sur Yvette Cedex, France

ABSTRACT

Gel filtration on HPLC columns can be a convenient means for the separation of soluble immune complexes from the mixture of human serum albumin (HSA) - monoclonal serum albumin antibody (Mab anti HSA). Owing to the rapidity of the method, the equilibrium is not modified, as it may be during the salting-out separation.
This method has been applied to the study of the interaction between HSA and a monoclonal antibody against human immunodeficiency virus envelope protein (Mab anti HIV gp120). Supported by the fact that HIV proteins share numerous epitopes with human proteins, a treatment to improve the specific immune response against this pathogen is proposed : it is based on the blocking of the epitopes of the self antigens, expressed in the thymus, which are in common with the virus. Repeated injections within the thymus, of neutralizing antibodies against the pathogen, obtained from a sufficiently distant animal species and purified by affinity chromatography, would prevent the T cells of the host recognizing these common epitopes from negative selection, and would improve the immune response. Moreover, the unfavourable effect of mutations within the virus genome could also be minimized by this treatment.

INTRODUCTION

Liquid chromatography and especially HPLC have been rarely used in immunology. This technique has been involved in a few studies, such as purification of monoclonal

Copyright © 1995 by Marcel Dekker, Inc.

antibodies on hydroxyapatite columns (1, 2), separation of immune complexes on Sephadex columns (3), or study of the topology of the epitopes at the surface of a protein (4).

We describe here the separation, by size exclusion chromatography, of human serum albumin (HSA) and one of its monoclonal antibody, from their immune complexe. We have then applied this method to the study of the interaction between HSA and a particular anti HIV gp120 antibody.

A new kind of therapy is proposed, applicable to AIDS, in order to improve the efficiency of the immune response.

MATERIALS AND METHODS

HPLC was applied with a Waters Ass. liquid chromatograph composed of a 510 pump, a U6K injector and a 996 photodiode array detector with the Millenium 2010 Chromatography Manager (Millipore).

The separations were performed on gel filtration TSK G 3000 SW columns (Beckman), in Tris sulfate buffer 0.1 M pH 8.0, at the rate of 1 ml/min.

Monoclonal antibody against HSA (Mab anti HSA A 6684) was purchased from Sigma Immunochemicals, monoclonal antibody against HIV gp120 (Mab anti gp120 1 C 1) from ICN Biomedicals and human serum albumin (HSA) from Sigma. They were repurified on TSK G 3000 SW column, in order to eliminate high molecular weight impurities or aggregates, interfering with the immune complexes.

RESULTS

a) HSA-Mab anti HSA system

HSA and monoclonal antibody anti HSA are eluted approximately at the same volume on TSK G 3000 SW with Tris sulfate buffer as eluent, and cannot be separated from each other. However they are well separated from another peak, eluting close after the void volume of the column, and visible when the two components have been incubated together before injection (Fig. 1). This peak represents the immune complex formed in the conditions used for the incubation, and the amount can be evaluated by integration, assuming in a first approximation, that HSA, its antibody and their complex have the same extinction coefficient.

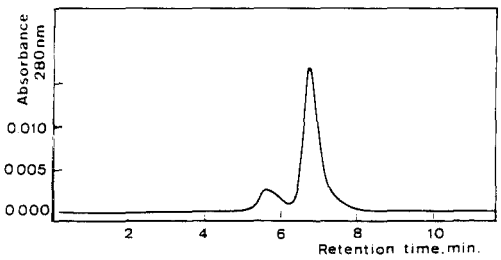


FIGURE 1 : Chromatography of HSA-Mab anti HSA mixture.
TSK G 3000 SW column, Tris sulfate 0.1M pH 8.0 buffer, rate 1ml/min, injected HSA : 5.3 µg, injected Mab anti HSA : 9.0 µg, time of incubation : 5 min. The first eluted peak is the immune complex.

This HPLC separation is rapid, the immune complex is eluted from the column in less than 6 min, far less than with the salting out method (5) (30 min). One must then expect that the equilibrium will be practically not disturbed during the chromatography : the proportion of the immune complex peak is actually independent of the duration of the separation as shown on Fig. 2. It has been verified that the fraction of the immune complex separated by this method does not depend on the amount of the injected mixture : Fig. 3 shows that the surface of the first peak is proportional to the injection volume. On the other hand, the equilibrium between the components of the incubated mixture is reached in less than 2 min, as can be seen on Fig. 4. The time of incubation has been fixed to 5 min for all the samples. Therefore, this method is able to give immediate quantitative results, by integration of the chromatographic peak of the immune complex. However it cannot give information about the composition of these complexes, unless the contribution of the antigen could be specifically measured.

When increasing quantities of HSA are added to a constant amount of monoclonal antibody, the immune complexe increases and reaches a plateau (Fig. 5). In the situation where HSA is constant and the added antibody increases, the same kind of curve is observed (Fig. 6). The level of the plateau is determined by the amount of the limiting component, and no decrease is observed when the other component is added in large excess. However the value of the maximum is lower than expected, on the basis of the formation of a divalent antibody-monovalent antigen complex. This discrepancy could be due to an heterogeneity of the HSA, with regard to the epitope which is recognized by the antibody.

It must be noted that the monoclonal antibody used in this study is specific of the human albumin : the level of the immune complex reached with bovine serum albumin is only 6 % of that obtained with HSA.

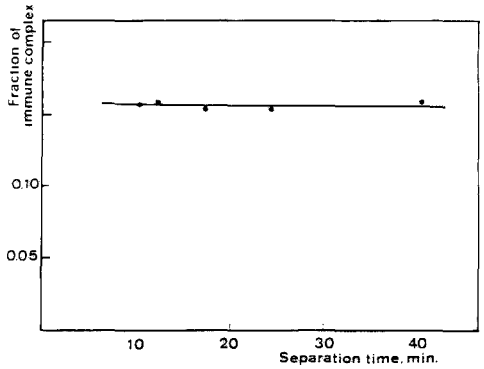


FIGURE 2 : Influence of the time of chromatography on the fraction of the separated immune complex.
Injected HSA : 10.7 µg, injected Mab anti HSA : 9.0 µg. Other conditions as in figure 1.

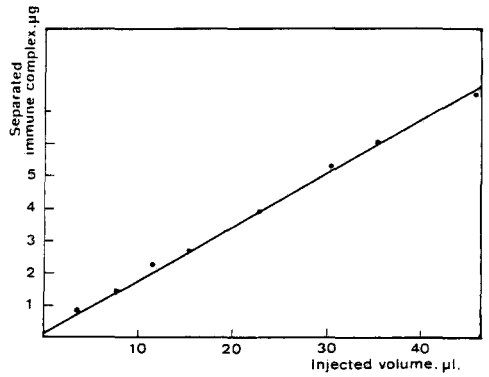


FIGURE 3 : Influence of the injected volume of the incubated mixture on the amount of the separated immune complex.
Rate 1ml/min. Same incubated mixture and other conditions as in figure 2.

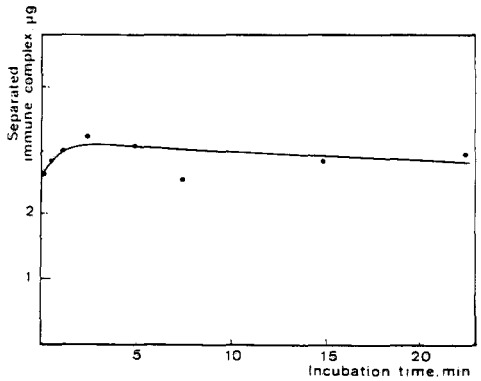


FIGURE 4 : Influence of the incubation time on the amount of the separated complex. Same conditions as in figure 2, except for incubation time.

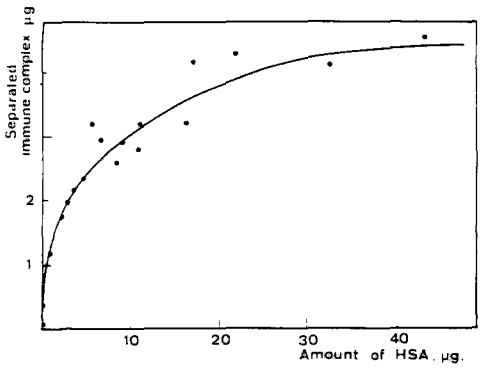


FIGURE 5 : Variation of the amount of the separated immune complex versus HSA concentration, at constant Mab anti HSA concentration.
Injected Mab anti HSA : 9.0 µg.

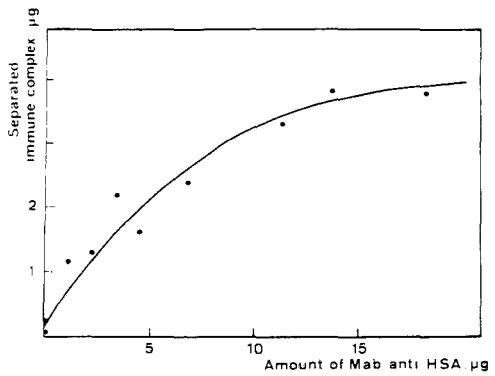


FIGURE 6 : Variation of the amount of the separated immune complex versus Mab anti HSA concentration, at constant HSA concentration.
Injected HSA : 10.7 µg.

b) HSA-Monoclonal anti HIV gp120 system

Among the different theories concerning the mechanism of T cell destruction after HIV infection, autoimmunity has been evoked by several authors. This hypothesis is supported by the presence in the sera of HIV infected individuals of antilymphocytes antibodies, antibodies to Interleukin 2, to peptides from MHC II molecules, to platelets and neutrophils and to partially sialyated form of CD 43 on normal thymocytes (6). It has also been found T lymphocytes that bind to autologous immunoglobulin (7), and cytotoxic T lymphocytes that can lyse uninfected $CD4^+$ T cells (8). These responses are thought to be due to the occurrence of regions of homology between HIV envelope proteins (gp120 and gp41) and different human proteins (MHC class I and class II proteins, Interleukin 2 etc...) (9, 10).

In this work, we have used the HPLC separation of the immune complexes, in order to determine whether other human proteins would possibly cross react with HIV envelope proteins. The system used for this purpose was HSA-Mab anti HIV gp 120. As can been seen on Fig. 7, a high molecular weight complex is separated from a HSA-Monoclonal antibody anti HIV gp120 incubated mixture. Fig. 8 shows the variation of the amount of this immune complex when the antigen increases, the other component being kept constant. Here also, the level determined by the limiting component (Mab anti HIV gp 120) is lower than expected, but the discrepancy could be attributed to heterogeneity of the antibody.

Nevertheless, from these results, one can conclude that HSA, the most abundant human serum protein, shares epitopes with the virus envelope protein gp120, like the other above mentioned proteins.

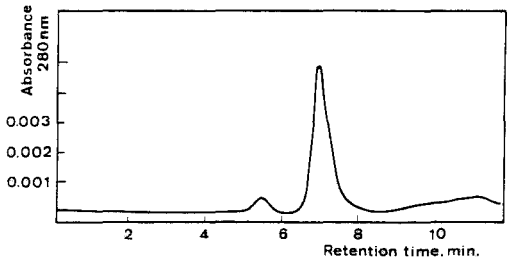


FIGURE 7 : Chromatography of HSA-Mab anti HIV gp120 mixture.
Injected HSA : 3.6 µg, injected Mab anti HIV gp120 : 2.0 µg. Other conditions as in figure 1.

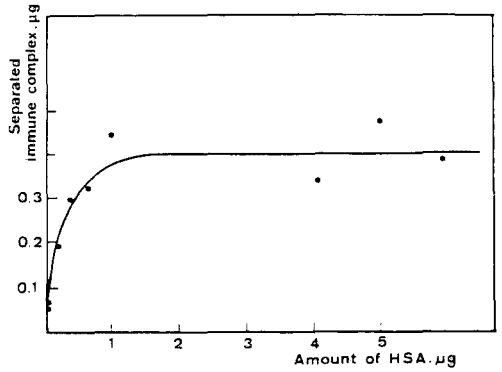


FIGURE 8 : Variation of the amount of the separated immune complex with HSA concentration, at constant Mab HIV anti gp 120.
Injected Mab anti gp120 : 2.0 µg.

PROPOSITION OF TREATMENT TO IMPROVE THE IMMUNE RESPONSE

The difficulty to develop an effective vaccine against certain pathogens can be due to the similarity existing between their antigenic determinants and those of the self antigens of the host. The T cells with receptors recognizing the epitopes common to the two species have been deleted during their maturation in the thymus (negative selection) and there is only a weak immune response following infection, corresponding to the determinants which are recognized as foreign. This response is sometimes insufficient to eradicate the pathogen.

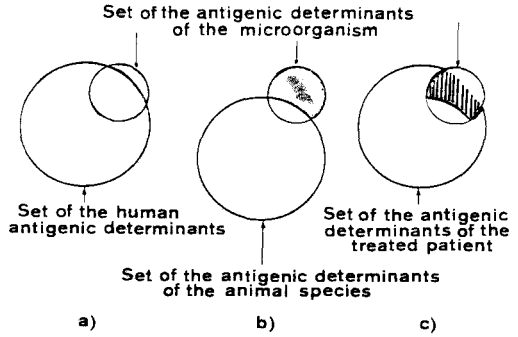


FIGURE 9 : Proposition to improve the immune response.
a) When a pathogen shares the most of its antigenic determinants with those of humans, a small number of antibodies are produced (corresponding to the foreign part, in grey). b) The animal species which is chosen produces a great number of different antibodies (corresponding to the part in grey). They are purified by affinity chromatography and injected into the thymus. c) The set of the self antigenic determinants of the patient is reduced by blocking with the antibodies of the animal species (hachured part). A greater number of determinants of the pathogen are recognized as foreign and elicit antibodies (in grey).

However, there is production of neutralizing antibodies when the same pathogen is injected to another species, sufficiently distant from humans, since many of its determinants are then recognized as foreign. These specific antibodies can easily be purified by chromatography, using an affinity column, with the pathogen bound covalently to the matrix.

In order to improve the efficiency of the human immune response, we propose to block, making use of these specific antibodies, a part of the determinants of the host shared with the pathogen, which are expressed in the thymus, on the MHC antigens, and are responsible for the T cell deletion (Fig. 9). By repeated injections into the thymus, of the specific antibodies prepared and purified as above, one might attempt that the new maturating T cells, with receptors corresponding to the pathogen protein epitopes, will escape deletion and that the immune response will be enhanced.

The choice of the animal species used for the preparation of these antibodies will be important : it must be sufficiently distant from humans, to induce in the animal numerous different antibodies against the pathogen, but not too much distant, to avoid important immune response against the xenoantibodies injected to the patient. It is also necessary to choose animals of great size, in order to obtain sufficient amounts of antibodies. Monkeys, but also Sheeps, Goats, Horses, Pigs could be suitable.

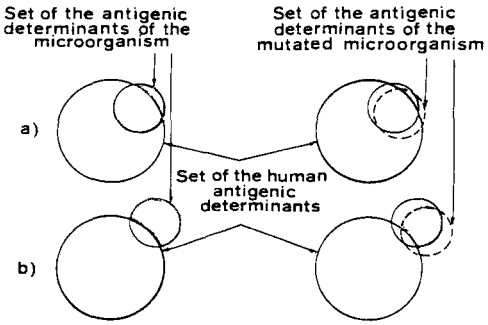


FIGURE 10 : Influence of mutations on the efficacy of a vaccine.
a) The pathogen shares the most of its antigenic determinants with the host. Few determinants elicit antibodies (in grey) and the vaccine prepared with a strain is inefficacious against a mutant. b) Many antigenic determinants of the pathogen are foreign to the host and elicit multiple antibodies (in grey). Even after mutations, a sufficient number of determinants remain unchanged and react with the vaccine prepared with the initial strain.

This reasoning can be applied to the virus of the human immunodeficiency HIV, since its envelope proteins share epitopes with numerous human proteins. By injection of xenoantibodies anti-virus into the thymus, we attempt to make the virus antigenically foreign to the host, by complexing a part of the MHC antigen epitopes which are in common with the virus and are responsible for the deletion of the corresponding T cells. By this means it is expected that the titer of neutralizing antibodies against HIV, which is low or completely missing (11-13), would be enhanced.

One additional difficulty in the preparation of a vaccine against HIV is that the virus undergoes constant antigenic variation, especially in its envelope proteins, from an isolate to the other, even for the same individual (14). In fact, the antibodies are produced against a restricted number of determinants, only those which are recognized as foreign. So that a limited number of mutations can affect these determinants, making the vaccine inefficacious. However, the same number of mutations would be practically without effect if a greater number of epitopes were implied, as it would be the case after the treatment we propose (Fig. 10).

Careful attention must be paid during this treatment to the immune response against the xenoantibodies. In this respect, the choice of the species producing the specific antibodies is essential. When the treatment is stopped, human circulating antibodies against HIV may progressively replace xenoantibodies from their binding sites in the

thymus. A constant watching of the titer of neutralizing antibodies against HIV must be ensured during a sufficient time.

Obviously, a vaccine therapy cannot avoid transmission of the virus by direct cell to cell contact, nor monocyte infection by phagocytose of the immune complexes. However, the validity of the proposed treatment can easily and rapidly be tested with simian immunodeficiency virus SIV infecting Baboons or Rhesus Monkeys.

ACKNOWLEDGEMENTS

I am grateful to all my family for their support and help in the writing of this paper.

REFERENCES

1) Aoyama, K., Chiba, J., J. Immunol. Meth. 162 : 201-210. (1993).

2) Yamakawa, Y., Chiba, J., J. Liquid. Chrom. 11 : 665-681. (1988).

3) Bach J. F., « Réactions antigènes-anticorps. Introduction à la sérologie », in Traité d'Immunologie, Bach J. F. Ed., Médecine Sciences. Flammarion, Paris, pp 407-437 (1993).

4) Stevens, F. J., Carperos, W.E., Monafo, W. J., Greenspan, N.S. J. Immunol. Meth. 108 : 271-278 (1988).

5) Farr, R.S., J. Infect. Dis 103 : 239-262. (1958).

6) Rosenberg, Z. F., Fauci, A. S., « Immunology of HIV Infection », in Fundamental Immunology, Third Edition, Paul, W. E. Ed.,Raven Press Ldt., New York, pp 1375-1397. (1993).

7) Ardman, B., Mayer, K., Bristol, J., Ryan, M., Settles, M., Levy E., Clin. Immunol. Immunopathol. 56 : 249-258. (1990).

8) Zarling, J. M., Ledbetter, J. A., Sias, J., Fultz, P., Eichberg, J., Gjerset, G., Moran, P.A., J. Immunol. 144 : 2992-2998. (1990).

9) Grassi, F., Meneveri, R., Gullberg, M., Lopalco, L., Rossi, G. B., Lanza, P., De Santis, C., Brattsand G., Butto, S., Ginelli, E., Beretta, A., Siccardi, A. G., J. Exp. Med. 174 : 53-62. (1991).

10) Golding, H., Rober, F. A., Gates, F. T. III, Linder, W., Beining, P. R., Hofman, T., Golding, B., J. Exp. Med. 167 : 914-923. (1988).

11) Seligmann, M., Pinching, A.J., Rosen, F. S., Fahey, J. L., Khaitov, R. M., Klatzmann, D., Koenig, S., Luo, N., Ngu, J., Reithmüller, G., Spira, T. J., Ann. Intern. Med. 107 : 234-242. (1987).

12) Weiss, R. A., Clapham, P. R., Cheingsong-Popov, R., Dalgleish, A. G., Carne, C. A., Weller, I. V. D., Tedder, R. S. Nature. 316 : 69-72. (1985).

13) Robert-Guroff, M., Brown, M., Gallo, R. C., Nature. 316 : 72-74. (1985).

14) Fisher, A. G., Ensoli, B., Looney, D., Rose, A., Gallo, R. C., Saag, M. S., Shaw, G. M., Hahn, B. H., Wong-Staal, F., Nature. 334 : 444-447. (1988).

Received: April 20, 1995
Accepted: May 9, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(17), 3435-3443 (1995)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF 5-DODECYLSALICYLALDOXIME AND 3-CHLORO-5-DODECYLSALICYLALDOXIME, TWO LIPOPHILIC ANALOGUES OF SALICYLALDOXIME

M. NIKUPAAVO, M. KOSKINEN, J.-P. HIMBERG, AND H. ELO*
Division of Chemistry
Department of Pharmacy
University of Helsinki
P.O. Box 15 (Fabianinkatu 35)
FIN-00014 University of Helsinki
Helsinki, Finland

ABSTRACT

High-performance liquid chromatographic analyses are reported for 5-dodecylsalicylaldoxime (5-DO-SAO) and 3-chloro-5-dodecylsalicylaldoxime (3-Cl-5-DO-SAO), two lipophilic analogues of salicylaldoxime. A reversed-phase column (Supelcosil LC-18, 150 x 4.6 mm, 5 µm mean particle size) was used as the stationary phase and a mixture of tetrahydrofurane and water as the mobile phase (volume ratio 70:30 for 5-DO-SAO and 72:28 for 3-Cl-5-DO-SAO). UV absorption at 226 nm was used for detection. The retention times of the analytes were 6.0 and 5.8 min, respectively, with a flow rate of 0.5 ml/min. For both compounds, the limit of detection was 0.5 µg/ml. The methods were linear up to 400 and 500 µg/ml, respectively.

Copyright © 1995 by Marcel Dekker, Inc.

INTRODUCTION

Trans-bis(salicylaldoximato)copper(II) ($CuSAO_2$), i.e. the copper(II) chelate of salicylaldoxime (2-hydroxybenzaldoxime; SAO), is a potent antiproliferative agent that essentially totally prevents tumor cell proliferation *in vitro* in very low concentrations and also has potent, even curative, antitumor activity *in vivo* [1-3]. The very low aqueous solubility of the chelate, however, is a serious disadvantage concerning practical therapeutic applications. In order to obtain more soluble analogues, the corresponding copper(II) chelates of certain di- and trihydroxybenzaldoximes were synthesized [1,2] but even these compounds were found to be almost insoluble in water. The immunomodulating properties [4] and, especially, the organ selectivity of this class of antineoplastic compounds (a nearly specific affinity for the pancreas, with almost 1000-fold accumulation in this organ as compared to the liver, kidneys and heart) [5] have further increased the interest in the agents. Therefore, studies are in progress in our laboratory in order to find out novel water-soluble or fat-soluble, yet antineoplastic, analogues of the potentially highly valuable but difficult-to-use agents. In this connection, fat-soluble analogues of SAO are of great potential

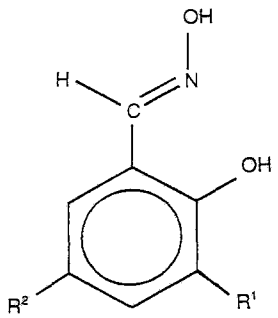


FIGURE 1. The structures of SAO ($R^1 = R^2 = H$), 5-DO-SAO ($R^1 = H$, $R^2 = n$-dodecyl) and 3-Cl-5-DO-SAO ($R^1 = Cl$, $R^2 = n$-dodecyl).

interest. In the present paper, we report high-performance liquid chromatographic analyses for 5-dodecylsalicylaldoxime (5-DO-SAO) and 3-chloro-5-dodecylsalicylaldoxime (3-Cl-5-DO-SAO), two fat-soluble analogues of SAO bearing long aliphatic side chains (see Fig. 1 for structures of SAO and the analogues now studied). The present compounds are commercially used in the mining industry. If they are to be used as starting materials in the synthesis of analogues of $CuSAO_2$ for antitumor screening purposes or for potential therapeutic applications, analytical methods are needed for them.

EXPERIMENTAL

Chemicals

Samples of 5-DO-SAO and 3-Cl-5-DO-SAO were kindly donated by Henkel Corporation, Tucson, AZ, U.S.A. HPLC grade tetrahydrofurane (THF) was obtained from Rathburn (Walkerburn, Scotland). Water was purified by using a Millipore Alpha Q water purification system.

Chromatographic Apparatus and Conditions

All chromatographic measurements were carried out using a high-performance liquid chromatographic system consisting of two LC-10AD liquid chromatograph solvent delivery systems and an SPD-M6A photodiode-array UV-VIS detector (Shimadzu Corporation, Kyoto, Japan). A Hyundai Super 386N Plus computer equipped with a 120 MB hard disk and 4 MB extended memory was used for data acquisition and processing in this system, employing Shimadzu LC workstation [Class-LC10 version 1]. The disk operating system was MS-DOS version 5.0. Injection was done using a Shimadzu SIL-6B auto injector, controlled by a SCL-6B system controller.

A Supelcosil LC-18 column (catalog no. 5-8230, 150 x 4.6 mm I.D., 5μm mean particle size), obtained from Supelco, Inc., Supelco Park, Bellefonte, PA, U.S.A., was employed. A LiChroCART 4-4 (Cat. 50957) precolumn containing LiChrospher 100 RP-18 packing material (particle size 5 μm) was used and was obtained from E. Merck (Darmstadt, Germany).

Chromatographic separations were carried out at room temperature using isocratic systems with a THF-water mixture as the mobile phase, the volume ratio of the solvents being 70:30 in the case of 5-DO-SAO and 72:28 in the case of 3-Cl-5-DO-SAO. The components of the eluents were degassed with the aid of helium. The injection volume was 20 μl, the analytes being dissolved in a mixture of water and THF (volume ratio 1:1). The analytes can also be dissolved in 100 % THF but in that case, a prominent extra peak appears in the chromatograms. A constant flow rate of 0.5 ml/min was used. Detection was based on UV absorption at 226 nm, measured with the diode array detector.

RESULTS AND DISCUSSION

Just as in the case of the HPLC analysis of SAO and 2,4-dihydroxybenzaldoxime (β-resorcylaldoxime) [6], mixtures of THF and water were found to be suitable as the mobile phase also for the lipophilic SAO analogues now studied, although a clearly higher concentration of THF was found to be necessary in the case of the latter compounds. When THF-water mixtures were employed as the eluent, the best results for samples of commercial 5-DO-SAO were obtained with a THF/water volume ratio 70:30, while in the case of the 3-chloro-5-dodecyl congener, a slightly higher THF content gave optimal results (volume ratio 72:28). Typical chromatograms obtained using these conditions are shown in Figs. 2 and 3, respectively. In the chromatogram of 5-DO-SAO, an intense peak was observed at 6.0 min and a very small one at 5.0 min. In the chromatogram of 3-Cl-5-DO-SAO, an intense peak was analogously

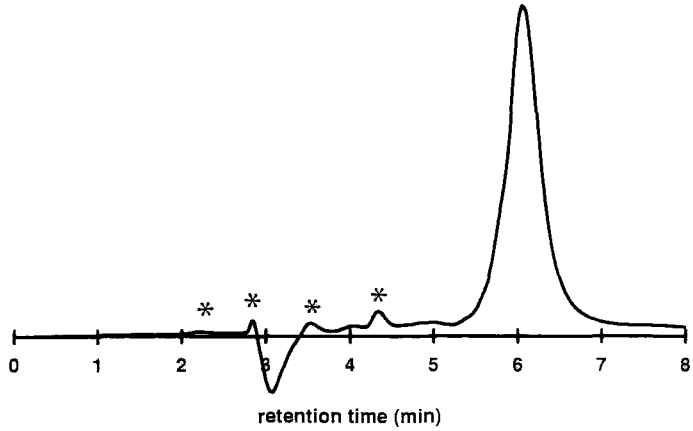


FIGURE 2. Chromatogram of a sample of commercial 5-DO-SAO dissolved in a 1:1 mixture of water and THF (99 µg of 5-DO-SAO/ml; for conditions, see Experimental). * = peaks that appear also in the chromatogram of the mere solvent (water plus THF 1:1).

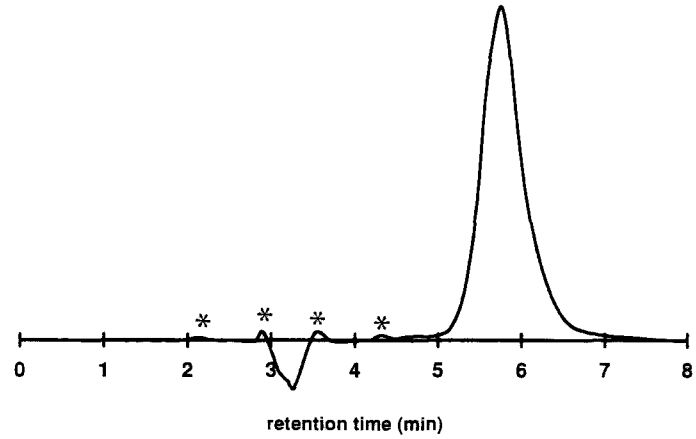


FIGURE 3. Chromatogram of a sample of commercial 3-Cl-5-DO-SAO dissolved in a 1:1 mixture of water and THF (98 µg of 3-Cl-5-DO-SAO/ml; for conditions, see Experimental). * = peaks that appear also in the chromatogram of the mere solvent (water plus THF 1:1).

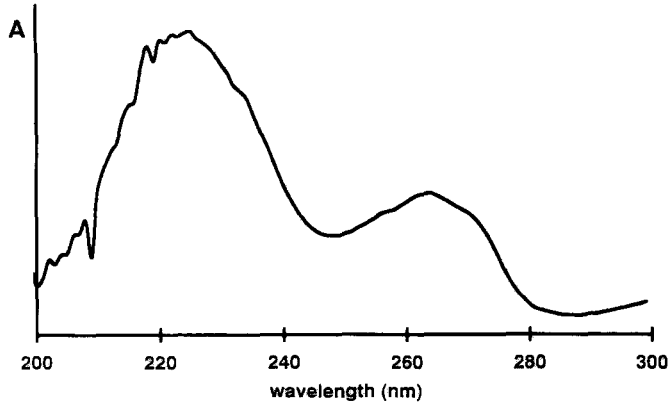


FIGURE 4. UV spectrum of 5-DO-SAO (recorded with the aid of the diode array detector at the top of the peak appearing at 6.0 min in the chromatogram of a sample whose concentration was 95 μg/ml).

observed at 5.8 min and a very small one at 4.7 min. With higher THF concentrations, the above mentioned small peaks that were observed under the conditions described above were no more separated from the intense peaks. When lower THF concentrations were used, the analyte peaks were either broadened or no peaks could be observed.

In the case of both analytes, UV spectra recorded at the intense peaks were largely similar to the spectrum of pure authentic SAO (see Figs. 4-6). In the case of the two compounds studied, spectra that were recorded at the above mentioned small peaks were largely similar to each other but totally different from the spectrum of SAO and from the spectra recorded at the intense peaks. Thus, the intense peaks at 6.0 and 5.8 min could be identified as the analyte peaks. Peak purity indexes obtained from spectra recorded at the peak top, the up slope and the down slope of the intense peaks indicated that, in the case of both compounds, the analyte peak is obviously due to one compound only. The small peaks observed at 5.0 and 4.7 min are obviously due to impurities, whose identity

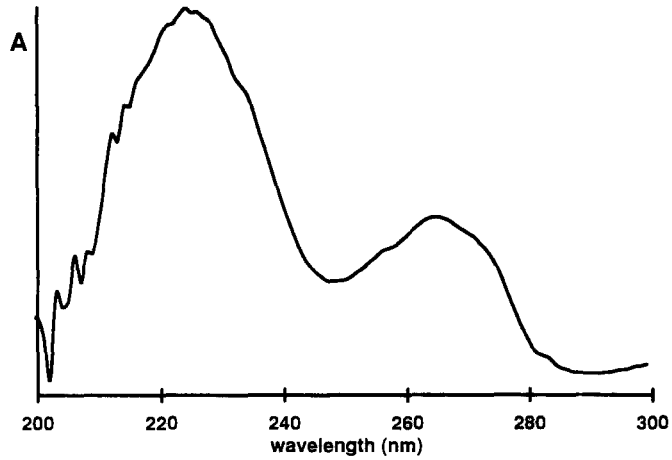


FIGURE 5. UV spectrum of 3-Cl-5-DO-SAO (recorded with the aid of the diode array detector at the top of the peak appearing at 5.8 min in the chromatogram of a sample whose concentration was 98 μg/ml).

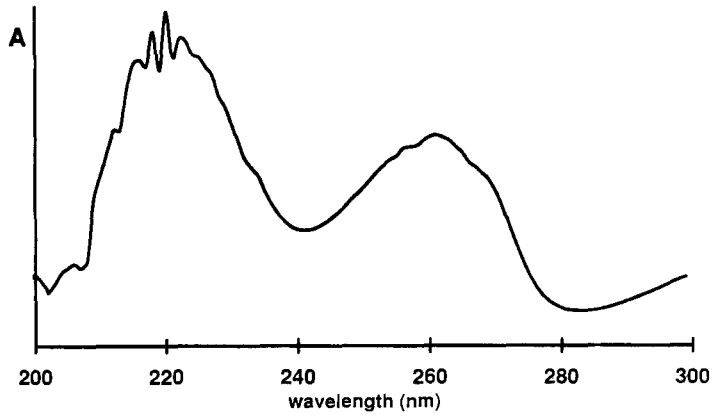


FIGURE 6. UV spectrum of SAO. The spectrum was recorded with the aid of the diode array detector at the top of the SAO peak of a chromatogram of pure authentic SAO. The chromatographic apparatus and conditions were those described in [6].

TABLE 1

Intra-Day Repeatabilities of the Methods for Standard Samples at Three Concentrations

Compound	n	Concentration (μg/ml)	Coefficient of Variation (% of Mean Peak Area)	Coefficient of Variation (% of Mean Peak Height)
5-DO-SAO	6	10	3.7	4.2
3-Cl-5-DO-SAO	6	11	1.2	2.1
5-DO-SAO	6	99	1.4	2.1
3-Cl-5-DO-SAO	6	98	2.4	1.8
5-DO-SAO	6	198	1.8	1.3
3-Cl-5-DO-SAO	6	194	2.0	2.0

TABLE 2

Inter-Day Repeatabilities of the Methods for Standard Samples at Three Concentrations

Compound	n	Concentration (μg/ml)	Coefficient of Variation (% of Mean Peak Area)	Coefficient of Variation (% of Mean Peak Height)
5-DO-SAO	15	10	5.3	5.8
3-Cl-5-DO-SAO	15	11	2.9	3.2
5-DO-SAO	15	99	3.5	3.0
3-Cl-5-DO-SAO	15	98	1.8	2.0
5-DO-SAO	15	198	3.8	3.3
3-Cl-5-DO-SAO	15	194	2.5	2.5

remains to be studied. Also the low UV absorption intensity of the small peaks, as compared to SAO and 2,4-dihydroxybenzaldoxime [6], indicates that these peaks are not due to the SAO analogues studied.

In the chromatogram of 5-DO-SAO, one further very small peak could be observed at 4.0. min. The UV spectrum of this peak was largely similar to that of the obvious analyte peak, but its very low intensity indicates that the peak must be due to an impurity only. The spectrum suggests that this impurity is probably a structural analogue (possibly an isomer) of 5-DO-SAO, perhaps 3-dodecyl-SAO that would be expected to be formed in the synthesis of the analyte.

For both analytes, the limit of detection was 0.5 µg/ml. The methods were linear up to 400 µg/ml for 5-DO-SAO (r= 0.9998 as based on peak areas at a total of 5 different concentrations, and 0.999994 as based on peak heights) and 500 µg/ml for 3-Cl-5-DO-SAO (r= 0.9997 and 0.9996, respectively, as based on 6 different concentrations). The intra-day and inter-day repeatabilities of the methods developed are good (see Tables 1 and 2).

REFERENCES

1. H. Elo, P. Lumme, Inorg. Chim. Acta, 136: 149-153 (1987)
2. H. Elo, P. Lumme, Z. Naturforsch., 41c: 951-955 (1986)
3. H. Elo, P. Lumme, Cancer Treat. Rep., 69: 1021-1022 (1985)
4. H. Elo, Cancer Lett., 36: 333-339 (1987)
5. H. Elo, I. Sunila, Naturwissenschaften, 74: 245-246 (1987)
6. J.-P. Himberg, M. Koskinen, M.-L. Riekkola, H. Elo, J. Liq. Chromatogr., 17: 3519-3529 (1994)

Received: April 15, 1995
Accepted: May 9, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(17), 3445-3463 (1995)

LINEAR SOLVATION ENERGY RELATIONSHIPS IN REVERSED-PHASE LIQUID CHROMATOGRAPHY. PREDICTION OF RETENTION OF SEVERAL QUINOLONES

J. BARBOSA*, R. BERGÉS, AND V. SANZ-NEBOT
Department of Analytical Chemistry
University of Barcelona
Avda. Diagonal, 647
08028 Barcelona, Spain

ABSTRACT

In this study the proportion of the organic modifier and the pH of the hydroorganic mobile phase were optimized in order to separate four important and widely used quinolones: ciprofloxacin, norfloxacin, ofloxacin and pipemidic acid. The Linear Solvation Energy Relationships (LSER) method based on the multiparameter scale developed by Kamlet and Taft can be succesfully used to select the right composition of the eluent and it has been shown that plots of log k' *versus* the Reichardt's E_T^N parameter of the mobile phase are linearly correlated for the solutes studied. Moreover, pH measurements in acetonitrile-water mixtures used as mobile phases have been made, taking into account the reference pH values previously established, in order to optimize the pH of the mobile phase for the chromatographic separation of the four quinolones studied.

INTRODUCTION

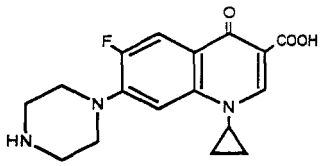
Quinolones comprise a widely used group of antibiotics whose bacterial action is based on their anti-DNA gyrase activity [1]. Owing to their favourable

Copyright © 1995 by Marcel Dekker, Inc.

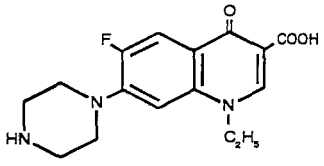
antibacterial and pharmacokinetic profile, quinolones have been used for treatment of systemic infections as well as urinary tract infections. The wide use of quinolones may lead to patients receiving multiple antimicrobials and microbiologically active metabolites of these agents may sometimes be present in biological fluids. The aim of the present study is to select the optimum eluent in order to separate four important and widely used quinolones: ciprofloxacin, norfloxacin, ofloxacin and pipemidic acid, Figure 1 [2-5]. For this purpose the proportion of the organic modifier and the pH of the hydroorganic mobile phase were optimized.

Although the structure and composition of the stationary phase plays an active role in the separation process [6] most researchers have focused attention on mobile phase optimization and with good reason since this is the easiest way to control retention and selectivity in RPLC [7]. Recently, the E_T^N scale of mobile phase polarity, proposed by Dimroth and Reichardt [8], and the multiparameter scale, developed by Kamlet and Taft [9,10], were succesfully used to study retention in RPLC. Dorsey [11-13] has shown that plots of logarithm of capacity factor, log k', *versus* the mobile phases' E_T^N solvatochromic parameter are very often more linear than plots of log k' *versus* volume fraction of organic modifier. Therefore, suitable prediction of the retention for a specific solute can be achieved from the E_T^N solvatochromic parameter values of the mobile phase and a few experimental data. However, Cheong and Carr [14] concluded that good correlations between retention and this single solvent parameter can be obtained only over a narrow range of solvent composition.

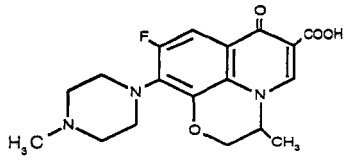
The method of Linear Solvation Energy Relationships (LSER) based on the Kamlet-Taft multiparameter scale has also been used succesfully to study retention in RPLC [15-17]. The LSER approach, when applied to chromatographic processes, correlates retention parameters of solutes to characteristic properties of mobile phases measured by the solvatochromic parameters π^*, α and β. The π^* parameter is used to evaluate solvent dipolarity/polarizability [18], and α and β scales evaluate solvent hydrogen-bond acidity [19] and solvent hydrogen-bond basicity [20] respectively.



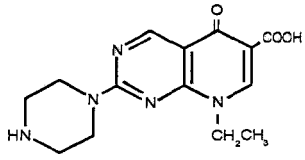
Ciprofloxacin



Norfloxacin



Ofloxacin



Pipemidic acid

FIGURE 1. Structural formula of the quinolones studied.

These approaches only allow the prediction of retention at different mobile phase compositions, but provide no information about the pH of the mobile phase, which is also critical for optimizing selectivity in RPLC. Usually, the operational pH in mixed aqueous-organic solvents is measured assuming that the mobile phase pH is the same as that of the aqueous fraction, in which case errors due to the medium effects contribute to uncertainty as to the true pH [21]. In acetonitrile-water mixtures the influence of the co-solvent on the pH is remarkable [22-24], but from the point of view of practical chromatography, it is possible to measure the activity of the hydronium ion in acetonitrile-water mixtures taking into account the reference pH values of buffer solutions in these solvents, pH_{PS}, assigned in previous works [25-29], which are used for the standardization of potentiometric sensors. Thus, pH measurements in a mixed solvent can be performed as easily as in water, using the operational definition of pH [30-32]:

$$pH_X = pH_{PS} + \frac{E_{PS} - E_X}{g} \quad (1)$$

where E_X and E_{PS} denote the electromotive force (emf) measurements in cell A on the sample solution at unknown pH_X and on the standard reference solution at known pH_{PS} respectively, and $g=(\ln 10)RT/F$.

reference electrode	salt bridge	sample solution at pH_X or standard buffer solution at pH_{PS} in solvent s	H^+ sensing electrode	(A)

In this study the proportion of the organic modifier and the pH of the hydroorganic mobile phase were optimized in order to separate four important and widely used quinolones: ciprofloxacin, norfloxacin, ofloxacin and pipemidic acid. The LSER method, based either on the multiparameter π^*, α and β scale or the single solvent parameter E_T^N and their relationships with log k', have been applied to the optimization of the mobile phase composition and to the prediction of the chromatographic behaviour of the quinolones studied. Moreover, the pH measurements in the acetonitrile-water mixtures used as mobile phases and their correlation with k' have been used in the optimization of the mobile phase pH for the separation required.

EXPERIMENTAL

Apparatus

The chromatographic equipment used consisted of an ISCO Model 2350 pump with an injection valve with a 10 μl sample loop and a variable-wavelength V^4 absorbance detector (ISCO) operating at 280 nm or at 295 nm for ofloxacin. The

chromatographic system was controlled by Chemresearch Chromatographic Data Management System Controller software (ISCO) running on a Peceman AT Supermicro personal computer. A Merck LiChrospher 100 RP-18 (5μm) column, 250 x 4 mm I.D., was used at ambient temperature. The emf values used to evaluate the pH of the mobile phase were measured with a CRISON 2002 potentiometer ($\pm$0.1 mV) using an Orion 8102 ROSS combination pH electrode. All solutions were thermostatted externally at 25$\pm$0.1° C. The electrodes were stabilized in the appropriate acetonitrile-water mixtures previous to the emf measurements, and the measurements were performed in triplicate to ensure potentiometric system stability.

Reagents

All reagents were of analytical grade. Acetonitrile (Merck) and water were of HPLC grade. The eluents were passed through a 0.22 μm nylon filter (MSI) and degassed ultrasonically before use. The quinolones (Figure 1) were obtained from various pharmaceutical firms: ciprofloxacin (Lasa), norfloxacin (Boral Química), ofloxacin (Hoescht Ibérica) and pipemidic acid (Almirall and Prodesfarma). Stock solutions of the quinolones were prepared in acetonitrile-water mixture (10:90, v/v), the concentration of these solutions was 100 $mg \cdot L^{-1}$. The mixture of the four quinolones studied was prepared by diluting 5 ml of the ciprofloxacin, norfloxacin and ofloxacin solutions, and 1 ml of the pipemidic acid solution to 25 ml with acetonitrile-water mixture (10:90, v/v). The samples were passed through a 0.45 μm nylon filter (MSI).

Chromatographic Procedure

The solution used for the optimization of the mobile phase composition was 25 mM phosphoric acid adjusted to pH 3 with 0.1 M tetrabutylammonium

hydroxide [33-36] at different acetonitrile percentages, up to 30% (v/v). The flow-rate of the mobile phase was maintained at 1 ml/min. The hold-up time, t_o, was measured for every mobile phase composition by injection of 0.01% potassium bromide solution [37]. The retention times and the capacity factors for the solutes were determined from three different injections at every mobile phase composition considered.

The mobile phase used was adjusted to different pH values, between 2.5 and 6, with 0.1 M tetrabutylammonium hydroxide in order to study the influence of the eluent pH in the chromatographic separation. Quinolones have been chromatographed on reversed-phase systems by ion-pairing the acidic part of the molecule with tetrabutylammonium salts. However, k' values remain constant when the tetrabutylammonium concentration in the mobile phase is between 10 and 20 mM and , therefore, retention is not affected when tetrabutylammonium hidroxide concentration is changed to achieve higher pH values. The pH was measured in the mixed mobile phase, where the chromatographic separation takes place, taking into account the reference pH values of primary standard buffer solutions, pH_{PS}, for the standardization of potentiometric sensors in acetonitrile-water mixtures assigned in previous works [25-29] in accordance with IUPAC rules [30,38] and on the basis of the multiprimary standard scale according to the National Institute of Standard and Technology (NIST) [31,39]. In this study we have used potassium hydrogen phthalate (0.05 mol/Kg) as primary standard buffer reference solution in the acetonitrile-water mixtures studied and a combination pH electrode [32].

RESULTS AND DISCUSSION

The capacity factor values, k', for the quinolones studied were obtained at different mobile phases as shown in Table 1. Mobile phases assayed were acetonitrile-water mixtures in ratio of (5:95), (7:93), (10:90), (12:88), (15:85),

TABLE 1

Values of the Capacity Factor for the Quinolones Studied at Various Percentages of Acetonitrile in the Mobile Phase

	k'			
% Acetonitrile	Ciprofloxacin	Norfloxacin	Ofloxacin	Pipemidic acid
5	11.66	10.42	6.79	3.19
7	6.46	5.54	3.95	1.87
10	3.23	2.79	2.06	1.10
12	1.99	1.74	1.33	0.74
15	1.23	1.17	1.02	0.55
20	0.67	0.62	0.55	0.42
25	0.45	0.42	0.40	0.34
30	0.36	0.34	0.33	0.28

(20:80), (25:75) and (30:70). To optimize the composition of the mobile phase the Linear Solvation Energy Relationships (LSER) based on the Kamlet-Taft multiparameter scale were used [15-17]. The LSER approach, when applied to chromatographic processes, correlates a general solute property, such as a logarithmic capacity factor, with feature parameters of solute and both mobile and stationary phases [40]:

$$\log k' = SP_0 + M(\delta_s^2 - \delta_m^2)\overline{V}_2/100 + S(\pi_s^* - \pi_m^*)\pi_2^* + A(\beta_s - \beta_m)\alpha_2 + B(\alpha_s - \alpha_m)\beta_2 \quad (2)$$

Here, k' is the chromatographic capacity factor, SP_0 is the intercept of the regression equation, $\overline{V}_2$ is the molar volume of the solute, and δ^2 is the square of the Hildebrand solubility parameter (a measure of the work required to produce a cavity of unit volume in the solvent) and π^*, α and β are the Kamlet-Taft

solvatochromic parameters. Subscripts s and m refer to the stationary and the mobile phases respectively, and subscript 2 refers to the solute properties. The values M, S, A and B are the coefficients for this equation, they are independent of the solutes, and if the model were rigorously correct, they should be independent of the phases [14].

When a system with a fixed pair of solute and stationary phase is considered and assuming the invariance of the properties of the stationary phase with the change in the mobile phase composition [14,15] and the linear relationship between δ_m^2 and the Kamlet-Taft solvatochromic parameters of the mobile phase [14,32], equation 2 can be simplified to:

$$\log k' = (\log k')_0 + s\pi_m^* + a\alpha_m + b\beta_m \qquad (3)$$

where $(\log k')_0$ depends on the parameters of the stationary and the mobile phases, s, a and b are the correlation coefficients which depend on the solute parameters, and π_m^*, α_m and β_m are the Kamlet-Taft solvatochromic parameters of the mobile phase. Values of α [41], β [42] and π^* [43] solvatochromic parameters, together with the E_T^N values [11] for all the acetonitrile-water mixtures studied were determined by interpolating literature values as shown in Table 2.

As a result of the application of the LSER method to log k' values determined in this work, equation 3, the relationships shown in Table 3 were obtained. It can be observed that log k' correlates well with the solvatochromic parameters π^*, α and β, since the average correlation coefficient value (r) was 0.999 using simple linear regression.

The relationship obtained between the chromatographic parameter log k' and the properties of the eluent mixtures π^*, α and β, allow us to predict chromatographic retention of quinolones studied for any composition of the eluent system. For this purpose the capacity factor and the separation factor values for the quinolones studied were calculated for the different compositions of the eluent system using the LSER relationships obtained. In Figure 2, plots of k' values

TABLE 2

Solvatochromic Parameters Values for the Acetonitrile-Water Mixtures Studied

% Acetonitrile	E_T^N	α	β	π^*
5	0.97	1.21	0.41	1.16
7	0.96	1.19	0.39	1.16
10	0.95	1.16	0.37	1.15
12	0.94	1.14	0.36	1.15
15	0.92	1.12	0.35	1.14
20	0.90	1.08	0.34	1.11
25	0.88	1.04	0.34	1.09
30	0.86	1.01	0.35	1.06

TABLE 3

Relationships Between log k' for the Quinolones and π^*, α and β Solvatochromic Parameters of the Eluent System in the Interval Studied Using the LSER Approach

SUBSTANCE	MULTIPARAMETER RELATION	r
Ciprofloxacin	$\log k' = -7.19 - 1.99\,\pi^* + 6.57\,\alpha + 6.32\,\beta$	0.9998
Norfloxacin	$\log k' = -7.56 - 1.03\,\pi^* + 5.63\,\alpha + 7.15\,\beta$	0.9998
Ofloxacin	$\log k' = -7.14 - 0.32\,\pi^* + 4.56\,\alpha + 6.84\,\beta$	0.9993
Pipemidic acid	$\log k' = -1.54 - 7.28\,\pi^* + 7.92\,\alpha + 2.09\,\beta$	0.9988

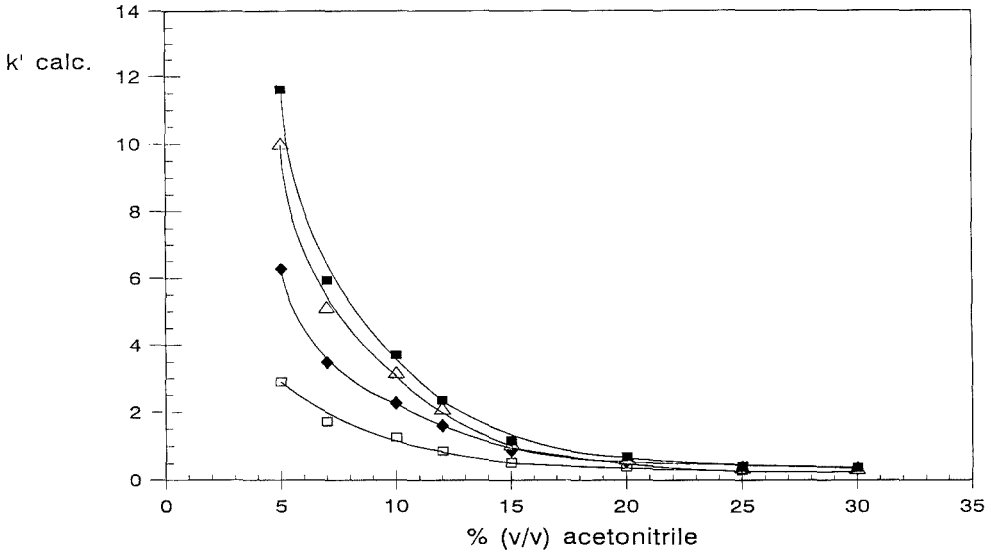


FIGURE 2. Plots of k' values calculated using LSER relationships for the quinolones studied *versus* % (v/v) of acetonitrile in the mobile phase. Ciprofloxacin (■), norfloxacin (△), ofloxacin (◆) and pipemidic acid (□).

calculated using LSER relationships *versus* % (v/v) acetonitrile are shown. From these values we can predict that the best chromatographic separation, in which the separation factor values for the four quinolones studied are higher, takes place when the acetonitrile contents in the mobile phase is 5 to 7 % (v/v).

Further reduction of equation 3 is not directly possible because the remaining Kamlet-Taft solvatochromic parameters measure different solvent effects, and linear correlations between them have not been demostrated. However, the structural features of acetonitrile-water mixtures shows three regions [44]. On the water-rich side there is a region in which the structure of the water remains more or less intact, the acetonitrile molecules gradually occupy the cavities between them with little disruption of the water structure. The limit of acetonitrile molar

fraction, x_{AN}, beyond which the acetonitrile molecules can no be longer accommodated within the cavities of the water structure varies with the method applied, but is ≥ 0.10. In the middle range of compositions, the acetonitrile-water mixtures show microheterogeneity; thus, there is a preference of a given water molecule for other water molecules rather than acetonitrile molecules. The same can be said to the preference of acetonitrile molecules for being in the vicinity of a given acetonitrile molecule. At $x_{AN} \geq 0.75$ the number of water clusters is low and water-acetonitrile interactions, which could be discounted in the middle range, now become important.

The difference in β values between water and acetonitrile is small [42,44]. Moreover, β values are constant over most of the composition range, which includes the microheterogeneity regions but extends beyond it on both sides [44]. Therefore, the β_m term in equation 3 can be included in the independent term. Thus, taking into acount the observed correlation: $E_T^N = 0.009 + 0.415\pi^* + 0.465\alpha$ [45], equation 3 can be reduced to:

$$\log k' = C + eE_T^N \qquad (4)$$

Log k' values of the quinolones studied *versus* the E_T^N parameter values of the acetonitrile-aqueous phase eluent system are shown in Figure 3. From Figure 3, it can be observed that the two parameters correlate linearly over the whole experimental range of acetonitrile contents studied, but there are two straight lines with different slopes, which intersect roughly at acetonitrile percentages of 15 % (v/v). All the quinolones have shown a similar elution profile. These two straight lines could be explained taking into account the two regions of acetonitrile-water mixtures studied. The slope of the plots changes in the region where acetonitrile-water mixtures show microheterogeneity. The use of equation 4 implies an important reduction of experimental work. Figure 3 indicates that good chromatographic separation can be obtained for the quinolones studied when the

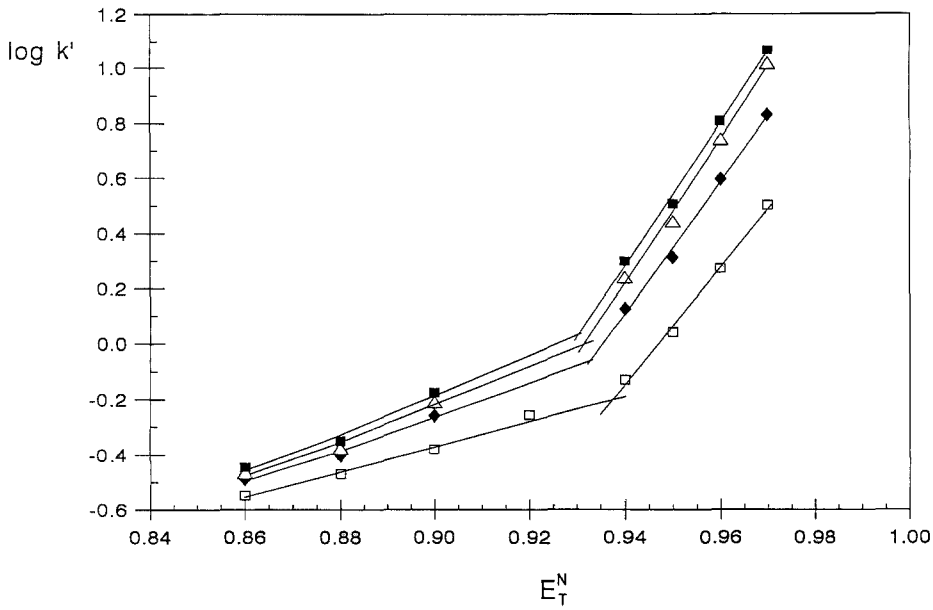


FIGURE 3. Plots of the log k' values of the quinolones *versus* the E_T^N parameters of the eluent systems. Ciprofloxacin (■), norfloxacin (△), ofloxacin (◆) and pipemidic acid (□).

acetonitrile contents in the mobile phase is 5 to 7 % (v/v), where the separation factor values are higher. We chose a composition of 7 % (v/v) of acetonitrile because of the shorter retention time.

The apparently contradictory results of Dorsey [12,13,46] and Cheong and Carr [14] could be explained taking into account that the E_T^N single parameter scale is an accurate descriptor of strength of mobile phase in RPLC only if all of the above conditions are true, and then equation 4 can be used. The results of Dorsey *et. al.* [12,13,46] were obtained with a large number of solutes, but practically all were in the microheterogeneity region of acetonitrile-water mixtures ($0.1 \leq x \leq 0.75$). Thus, over this range of compositions the cavity term (solvent/solvent interactions) and the solute/solvent interaction may covary and

TABLE 4

Values of the Capacity Factor for the Quinolones Studied at Various pH of the Mobile Phase

	k'			
pH	Ciprofloxacin	Norfloxacin	Ofloxacin	Pipemidic acid
2.59	5.51	4.76	3.45	1.66
3.11	6.46	5.54	3.95	1.87
4.14	6.54	5.80	4.83	1.92
5.19	10.16	8.87	15.63	2.64
6.18	22.29	18.98	-	3.81

a single parameter might be valid. The same was observed in previous papers [47,48] where the chromatographic behaviour of a series of steroids was studied in a range of acetonitrile-water mixtures from 40 to 70% (v/v). Plots of log k' values of steroids studied there *versus* the E_T^N values showed one straight line because all the data were obtained in one of the structural regions of acetonitrile-water mixtures, the microheterogeneity region.

In contrast, Cheong and Carr have studied the relations between log k' and $E_T(30)$ using acetonitrile-water systems with a wide range of acetonitrile contents, and their results were obtained in two different structural regions. Thus, correlations between measures of solvent/solute interactions and solvent/solvent interactions can change if molecular structure changes. The same can be said of our results in this work and in a recent study [49] where series of peptides have been considered and where the composition range of acetonitrile-water mixtures studied was from 5 to 40% (v/v), in both cases two different structural regions are studied. The study of higher percentages of acetonitrile in the case of quinolones and peptides is not of practical interest since the resultant k' values are subject to high errors due to the low retention, and there are difficulties in defining the column void volume.

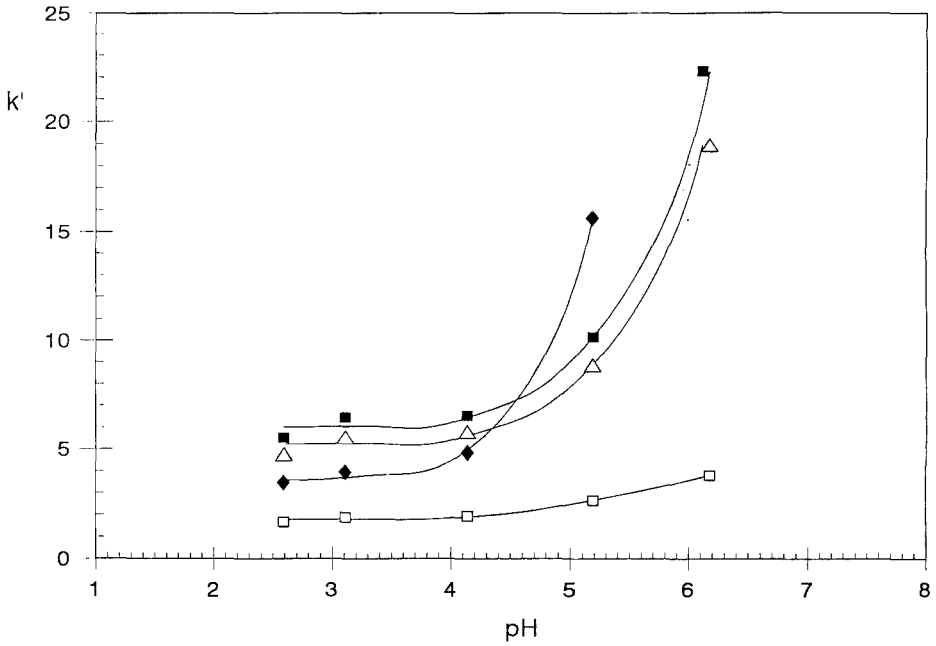


FIGURE 4. Plots of k' values of the quinolones *versus* the pH of the eluent systems. Ciprofloxacin (■), norfloxacin (△), ofloxacin (◆) and pipemidic acid (□).

It is very useful to check the linearity of the log k' values *versus* the E_T^N parameter of the mobile phase for different series of subtances in the practical range of acetonitrile-water mixtures since suitable prediction of the retention for a specific solute can be achieved from E_T^N and a few experimental data. Therefore, the optimization of the chromatographic separation for other related substances can be easily performed.

In order to study the influence of the pH of the mobile phase on the chromatographic retention, k' values for the quinolones studied at different pH of the mobile phase were determined from three different injections at every mobile phase pH considered as shown in Table 4. The National Institute of Standard and

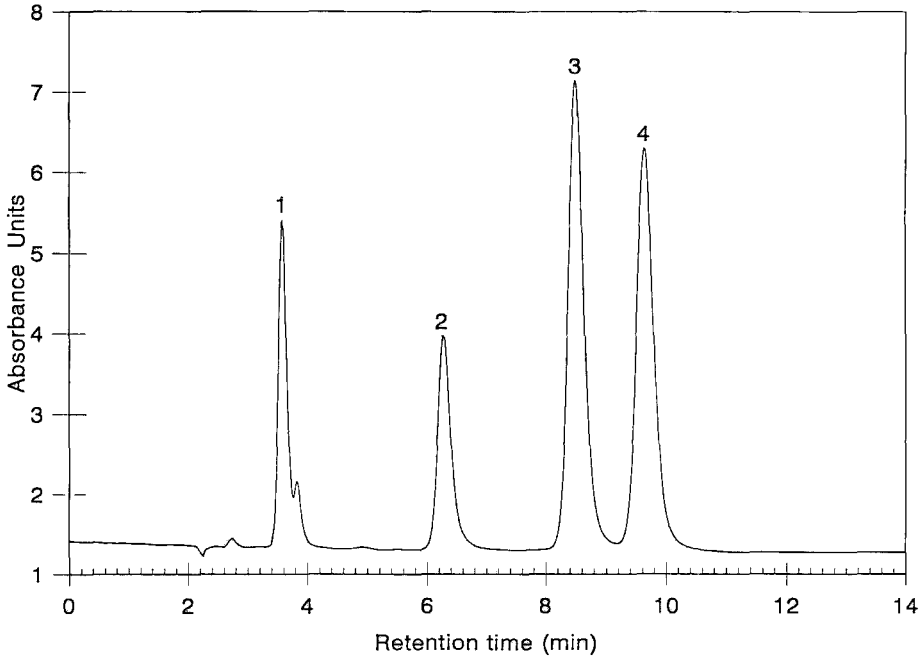


FIGURE 5. Separation of ciprofloxacin (4), norfloxacin (3), ofloxacin (2) and pipemidic acid (1) with an eluent consisting on acetonitrile-25 mM phosphoric acid adjusted to pH 3.09.

Technology (NIST) recommends choosing a standard reference solution with a pH_{PS} value as close as possible to the unknown pH_X [29,31,39]. We have used as reference solution a standard reference solution of potassium hydrogen phthalate and a commercial combination pH electrode, because, as we have shown in a previous works [32], good accuracy and precision are obtained for pH measurements in acetonitrile-water mixtures with pH values up to 7, and quick stabilization of the potentiometric system was observed. pH measurements in the hydroorganic mobile phase used permit the interpretation of chromatographic results without extrapolations of pH values from aqueous solutions. pH and pK_a values show deviations from linear dependence on the composition variations of

the mixture because of preferential solvation [24,28]. If a solute interacts with one of the solvents more strongly than with the other, then the solute is preferentially solvated by the former. On the other hand, pH measurements in the hydroorganic mixture used as mobile phase also permit the determination of pK_a values for the substances studied [49].

Plots of the k' values for the quinolones studied *versus* pH of the acetonitrile-aqueous phase eluent system are shown in Figure 4. The nonpolar stationary bonded phase used, octadecylsilica (ODS), may only be used in the pH range between 2 and 8, thus it was not possible to study the retention of quinolones as typical ampholytes because correlations between k' and the pH of the mobile phase cannot be obtained over the entire range of pH. However, k' values increase with pH, Figure 4, suggesting that the intermediate form of quinolones does not exist appreciably in zwitterion form [50]. Although it has not been shown, if zwitterion formation for the intermediate species did not occur, then a maximum in k' would be expected [50,51]. As can be shown in Figure 4, the best chromatographic separation for the quinolones studied can be performed at a pH of the mobile phase between 3 and 4. The best separation that can be obtained in these conditions was achieved at a pH of 3.09. Figure 5 shows an excellent separation of ciprofloxacin, norfloxacin, ofloxacin and pipemidic acid with an acetonitrile-aqueous phase system (7:93, v/v) adjusted to pH 3.09.

REFERENCES

1. J.P. Monk, D.M. Campoli-Richards, Drugs, 33: 346 (1987).

2. G. Montay, J.P. Tassel, J. Chromatogr., 339: 214-218 (1985).

3. S. Horh, C. Yasuoka, M. Matsumoto, J. Chromatogr., 388: 459-461 (1987).

4. M. Horie, K. Saito, Y. Hoshino, N. Nose, E. Mochizuki, H. Nakazawa, J. Chromatogr., 402: 301-308 (1987).

5. Y. Ikai, H. Oka, N. Kawamura, M. Yamada, K. Harada, M. Suzuki, H. Nakazawa, J. Chromatogr., 477: 397-406 (1989).

6. K.B. Sentell, J.G. Dorsey, J. Chromatogr., 461: 193-207 (1989).

7. S. Reeves, U. Ullmann, High Performance Liquid Chromatography in Medical Microbiology, Gustav Fischer Verlag, New York, 1986.

8. C. Reichardt, Solvent and Solvent Effects in Organic Chemistry, VCH Verlagsgellschaft, Weinheim, 1988.

9. M.J. Kamlet, J.M.L. Abboud, M.H. Abraham, R.W. Taft, J. Org. Chem., 48: 2877-2887 (1983).

10. M.J. Kamlet, R.M. Doherty, M.H. Abraham, Y. Marcus, R.M. Taft, J. Phys. Chem., 92: 5244-5255 (1988).

11. J.G. Dorsey, B.P. Johnson, Chimicaoggi, 11: 23-27 (1986).

12. B.P. Johnson, M.G. Khaledi, J.G. Dorsey, Anal. Chem., 58: 2354-2365 (1986).

13. J.J. Michels, J.G. Dorsey, J. Chromatogr., 457: 85-98 (1988).

14. W.J. Cheong, P.W. Carr, Anal. Chem., 61: 1524-1529 (1989).

15. P.C. Sadek, P.W. Carr, R.M. Doherty, M.J. Kamlet, M.H. Abraham, Anal. Chem., 57: 2971-2978 (1985).

16. P.W. Carr, R.M. Doherty, M.J. Kamlet, R.W. Taft, W. Melander, C. Horváth, Anal. Chem., 58: 2674-2680 (1986).

17. J.H. Park, P.W. Carr, M.H. Abraham, R.W Taft, R.M. Doherty, M.J. Kamlet, Chromatographia, 25: 373-381 (1988).

18. M.J. Kamlet, J.L. Abboud, R.W. Taft, J. Am. Soc., 99: 6027-6038 (1977).

19. R.W. Taft, M.J. Kamlet, J. Am. Chem. Soc., 98: 2886-2894 (1976).

20. M.J. Kamlet, R.W. Taft, J. Am. Chem. Soc., 98: 377-383 (1976).

21. T. Mussini, F. Mazza, Electrochim. Acta, 32: 855-862 (1987).

22. S. Rondinini, A. Nese, Electrochim. Acta, 32: 1499-1505 (1987).

23. T. Mussini, A.K. Covington, P. Longhi, S. Rondinini, S. Tettamanti, Anal. Chim. Acta, 174: 331-337 (1985).

24. J. Barbosa, J.L. Beltrán, V. Sanz-Nebot, Anal. Chim. Acta, 288: 271-278 (1994).

25. J. Barbosa, V. Sanz-Nebot, J. Pharm. Biomed. Anal., 10: 1047-1051 (1992).

26. J. Barbosa, S. Butí, V. Sanz-Nebot, Talanta, 41: 825-831 (1994).

27. J. Barbosa, V. Sanz-Nebot, Michrokim. Acta, 116: 131-141 (1994).

28. J. Barbosa, V. Sanz-Nebot, J. Chem. Soc. Faraday Trans., 90: 3287-3292 (1994).

29. J. Barbosa, V. Sanz-Nebot, Fresenius J. Anal. Chem., in press.

30. T. Mussini, A.K. Covington, P. Longhi, S. Rondinini, Pure Appl. Chem., 57: 865-876 (1985).

31. F.G.K. Bauke, R. Naumann, C. Alexander-Weber, Anal. Chem., 65: 3244-3251 (1993).

32. J. Barbosa, R. Bergés, V. Sanz-Nebot, J. Chromatogr., in press.

33. F. Jehl, C. Gallion, J. Debs, J.M. Brogard, H. Monteil, R. Minck, J. Chromatogr., 339: 347-357 (1985).

34. C.E. Fasching, L.R. Peterson, J. Liq. Chromatogr., 8(3): 555-562 (1985).

35. I. Nilsson-Ehle, J. Chromatogr., 416: 207-211 (1987).

36. C. Koechlin, F. Jehl, L. Linger, H. Monteil, J. Chromatogr., 491: 379-387 (1989).

37. C.F. Poole, S.A. Schuette, Contemporary Practice of Chromatography, Elseivier Science Publishers B.V., New York, 1984.

38. S. Rondinini, P.R. Mussini, T. Mussini, Pure and Appl. Chem., 59: 1549-1560 (1987).

39. R.G. Bates, Crit. Rev. Anal. Chem., 10: 247-277 (1981).

40. M.J. Kamlet, R.M. Doherty, J.L. Abboud, M.H. Abraham, R.W. Taft, J. Pharm. Sci., 75: 338-349 (1986).

41. J.H. Park, M.D. Jang, D.S. Kim, P.W. Carr, J. Chromatogr., 513: 107-116 (1990).

42. T.M. Krygowski, P.K. Wrona, U. Zielkowska, C. Reichardt, Tetrahedron, 41: 4519-4527 (1985).

43. W.J. Cheong, P.W. Carr, Anal. Chem., 60: 820-826 (1988).

44. Y. Marcus, Y. Migron, J. Phys. Chem., 95: 400-406 (1991).

45. M.J. Kamlet, J.L.M. Abboud, R.W. Taft, Prog. Phys. Org. Chem., 13: 485 (1981).

46. P.T. Ying, J.G. Dorsey, Talanta, 38: 237-243 (1991).

47. D. Barrón, J.A. Pascual, J. Segura, J. Barbosa, Chromatographia, in press.

48. D. Barrón, J.A. Pascual, J. Segura, J. Barbosa, Chromatographia, submitted for publication.

49. J. Barbosa, V. Sanz-Nebot, I. Toro, J. Chromatogr., submitted for publication.

50. D.J. Pietzyk, B.P. Kroeff, T.D. Rotsch, Anal. Chem., 50: 497-502 (1978).

51. C. Horváth, W. Melander, I. Molnár, Anal. Chem., 49: 142-154 (1977).

Received: April 16, 1995
Accepted: June 23, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(17), 3465-3471 (1995)

A DIRECT PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY PROCEDURE FOR THE ISOLATION OF MAJOR TRITERPENOIDS AND THEIR QUANTITATIVE DETERMINATION IN NEEM OIL

T. R. GOVINDACHARI*, G. SURESH, AND GEETHA GOPALAKRISHNAN
Centre for Agrochemical Research
SPIC Science Foundation
110, Mount Road, Madras 600 032, India

ABSTRACT

Isolation of the major triterpenoids from neem oil in pure form by a direct preparative High performance liquid chromatography procedure is presented. As neem oil is an important component of Neem based insecticidal formulations, a simple, isocratic analytical HPLC procedure for quantitation (relative abundance) of these major bioactive triterpenoids has been developed.

INTRODUCTION

Products from the neem tree, *Azadirachta indica* A.Juss. (Meliaceae) have been used in India from ancient times, both in medicine as well as for protection of plants from insect attack. The first crystalline compound nimbin was isolated by Siddiqui from neem oil[1] . But it was only after the isolation by Butterworth and Morgan[2] from neem kernels of azadirachtin, which was shown to be a potent antifeedant for the desert locust *Schistocerca gregaria* at a concentration of 40μg/l, that intense interest was aroused in the chemical investigation of *A. indica*. More than one hundred compounds have been isolated from all parts of the plant, many of them biologically active[3,4,5].

Copyright © 1995 by Marcel Dekker, Inc.

Azadirachtin A has been shown to possess antifeedant activity at concentrations of 10-100 ppm and also ecdysis inhibition activity at concentrations of 1-10 ppm in over two hundred species of insects[6]. Since azadirachtin and its congeners are non-toxic, a number of formulations have been developed for use in agriculture, some based on neem kernel extract and many others on neem oil. Formulations from neem oil contain less of the more polar compounds like azadirachtin A, compared to the kernel extract formulations.

Direct preparative HPLC has been used in this laboratory with great success for the isolation of Azadirachtins A,B,D,H, I and K from neem kernel extract[7,8,9]. During this study the less polar compounds in neem kernel extract such as salannin, nimbin, azadiradione, epoxy azadiradione and 6-deacetylnimbin have also been isolated and identified. In view of the wide use of formulations based on neem oil in India, it was of interest to isolate and identify principal triterpenoid compounds present in neem oil and also estimate their relative abundance. Major triterpenoids like salannin[10], nimbin[1,11], azadiradione, epoxyazadiradione[12], 6-deacetylnimbin[13] have been isolated at different times in different laboratories essentially by conventional column chromatography, using different adsorbents, eluents, and work-up procedures. In a very recent publication[14], HPLC analysis of neem oil using a semi-preparative reverse phase column has been carried out and a few major triterpenoids have been identified by HPLC-MS procedure. In this paper, we report the isolation of the major components of neem oil, in a single experiment by direct preparative HPLC as well as the estimation of the relative abundance of the major components.

MATERIALS AND METHODS

Preparative High Performance Liquid Chromatography was carried out using a Shimadzu LC8A HPLC system linked to CR4A data processor and the peaks detected at 215 nm. Two Shimpack reverse phase (C_{18}) preparative columns (25 cmx 50 mm i.d.) and (25 cm x 20 mm i.d.) were used for preparative runs and Shimpack reverse phase column (C_{18}) (25 cm x 4.6 mm) was used for analysis.

Neem oil, (1 lit) obtained by using a cold mechanical expeller was partitioned between n-hexane and 90% methanol and the methanol extract was concentrated to dryness *in vacuo* at 45° (62.8g). This was subjected to preparative HPLC for the isolation of the triterpenoids.

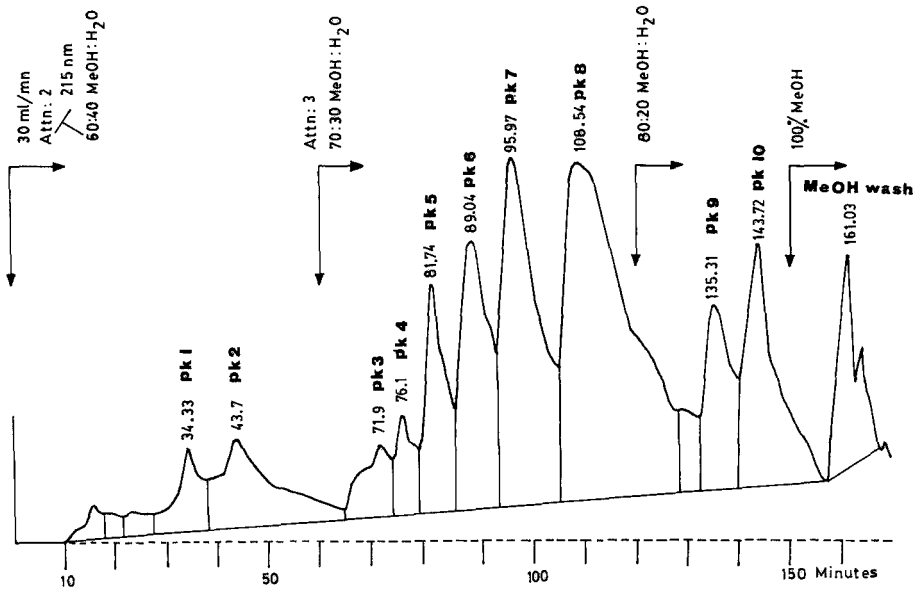


Fig.1 Preparative High performance liquid chromatogram of Neem oil triterpenoids

RESULTS AND DISCUSSION

For each preparative run 5 g of the above residue from methanol extract was dissolved in 20 ml of methanol, filtered through a Millipore filter (0.25μm) and then injected into the preparative column (25 cm x 50 mm i.d). The eluent flow rate was 30 ml/min throughout the run. During the first sixty minutes 60:40 MeOH: H_2O was used and the more polar compounds like azadirachtins were eluted. From 60 minutes the eluent was changed to 70:30 methanol:water and atleast six major peaks eluted out (Fig. 1).

Pk-1(rt 34.3 min.) and Pk-2 (rt 43.7 min.) were found to contain azadirachtins A, B, D, H and I in different proportions. Pk-3(rt 71.9 min.) and Pk-4 (rt 76.1 min.) gave only trace quantities of a material which showed the presence of a number of closely eluting compounds by analytical HPLC .Pk-5 (rt 81.7 min.) was found to be pure deacetyl nimbin (175 mg),while Pk-6 (rt 89 min.)(200 mg), Pk-7 (rt 95.9 min.)(448 mg) and Pk-8 (rt108.5 min.)(880mg), on evaporation and crystallisation in methanol:water, gave pure azadiradione, nimbin and salannin respectively.

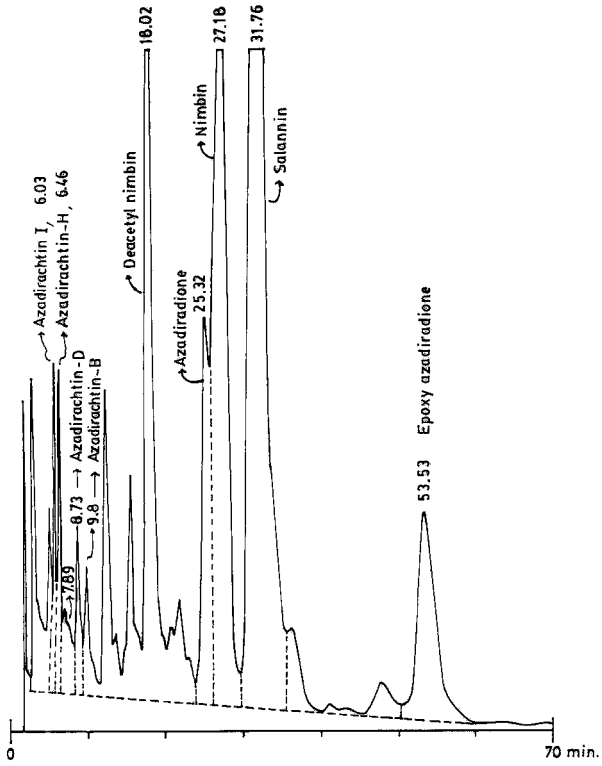


Fig.2 Analytical High performance liquid chromatogram of Neem oil triterpenoids

On changing the solvent to 80:20 methanol:water at 130 minutes, two major peaks were eluted with retention times 135.3 minutes and 143.7 minutes respectively. Both the peaks were collected and concentrated to dryness. The residue from the peak at 135.3 minutes was a complex mixture with a number of peaks in analytical HPLC and was not further investigated.

The peak eluting at 145 minutes (175 mg) was subsequently subjected to one more preparative HPLC using 25 cm x 20 mm i.d. column with 50:50 CH_3CN : H_2O as eluent and 15 ml/min flow rate. Peak eluting out at 45 min was collected and concentrated *in vacuo* to yield 110 mg of pure epoxy azadiradione.

Table 1. Quantitative estimation of major triterpenoids in neem seed oil

Compound	Retention time (min)	*Area/μg of pure compd.	mg. of compound/lit. of neem oil (in %)	
			R1	R2
Azadirachtin I	6.03	2117.40	1015.87 (0.109)	974.12 (0.097)
Azadirachtin H	6.46	1037.20	2521.40 (0.252)	2352.50 (0.235)
Azadirachtin A	7.89	2128.90	283.56 (0.028)	220.96 (0.022)
Azadirachtin D	8.73	1875.02	1141.85 (0.114)	1011.29 (0.101)
Azadirachtin B	9.8	1018.68	2107.2 (0.210)	1741.28 (0.174)
6-deacetyl nimbin	18.02	3658.76	4844.42 (0.48)	4971.46 (0.5)
Azadiradione	25.32	4694.86	2091.56 (0.21)	2102.76 (0.21)
Nimbin	27.18	4502.42	4956.9 (0.496)	4949.08 (0.496)
Salannin	31.76	4494.40	14010.6 (1.4)	14438.2 (1.44)
Epoxy azadiradione	53.53	7809.80	1313.4 (0.13)	1486.8 (0.144)

*based on average of 3 replicates and 4 concentrations

The identity and purity of the isolated triterpenoids were further confirmed by spectral data and comparison with standard samples by analytical HPLC[9].

For quantitation of the isolated triterpenoids, pure samples of Azadirachtins A, B,D,H, I, deacetylnimbin, azadiradione, nimbin, salannin, and epoxy azadiradione were accurately weighed (about 1 mg), dissolved in methanol and made up to 1 ml. Then three aliquotes of 20 μl, 15 μl, 10 μl and 5μl of each of the compounds were analysed on

a RP_{18} column (Shimpack ODS, 25 cm x 4.6 mm, detection at 215 nm, CH_3CN : H_2O 50:50 as the eluent at 1 ml/minute). Areas of peaks (valley to valley) of each concentration and compound were measured and average area per μg of each compound was calculated. The compounds eluted in the following order: Retention times are indicated in parantheses. Azadirachtin I (6.03 min.), Azadirachtin H (6.46 min.), Azadirachtin A (7.89 min.), Azadirachtin D (8.73 min.), Azadirachtin B (9.8), 6-deacetylnimbin (18.02 min.), Azadiradione (25.32 min.), nimbin (27.18 min.), salannin (31.76 min.) and epoxyazadiradione (53.53 min.). For quantitative analyses of these compounds in neem oil, a standard solution (32mg/10 ml) of the methanol extract residue (see above) was made and analysed with the same column and experimental conditions as for the pure compounds, injecting 10 μl of the solution. A representative HPLC chromatogram is given in Fig.2. Compounds are identified by retention times and area measurements (valley to valley) were used to calculate the amounts present.

Table 1 presents data on the relative abundance of the major triterpenoids in neem oil from two different sources.

REFERENCES

1. S.Siddiqui, Curr.Sci. 11: 278-279 (1942).

2. J.H.Butterworth, E.D.Morgan, J.Chem.Soc. Chem.Comm., 23-24 (1968)

3. C.Devkumar, Sukh Dev, "Chemistry" in Neem Research and Development, N.S.Randhawa, B.S.Parmar, eds., Society of Pesticide Science, India, 1993, pp. 63-96.

4. S.Siddiqui, B.S.Siddiqui, S.Faizi, T.Mahmood, J. Nat. Prod., 51: 30 - 4,(1988)

5. M.Jacobson, 1988 Focus on Phytochemical Pesticides, CRC Press Inc., Florida, 1989.

6. R.C.Saxena, "Insecticides from Neem" in Insecticides of Plant origin, J.T.Arnason, B.J.R.Philogene, P.Morand, eds., ACS Symp.Ser. 387, 1989, pp.110-135

7. T.R.Govindachari, G.Sandhya, S.P.Ganesh Raj, J.Chromatogr., 513:389-391 (1990)

8. T.R.Govindachari, G.Sandhya, S.P.Ganesh Raj, Chromatographia 31:303-305 (1991)

9. T.R.Govindachari, G.Sandhya, S.P.Ganesh Raj, Ind. J. Chem., 31B:295-298 (1992)

10. R.Henderson, R.McCrindle, A.Melera, K.H.Overton, Tetrahedron 24:1525-1528 (1968)

11. N.S.Narasimhan, Chem.Ber., 92: 769 -775 (1959)

12. D.Lavie, M.K.Jain, J.Chem.Soc.Chem.Comm., 278 (1967)

13. C.R.Narayanan, K.N.Iyer, Ind. J.Chem., 5:460 (1967)

14. J.D.Stark, J.F.Walter, J.Agric.Biol.Chem., 43: 507-512 (1995)

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

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(17), 3473-3494 (1995)

DETERMINATION OF COCAINE IN BIOLOGICAL MATRICES USING REVERSED PHASE HPLC: APPLICATION TO PLASMA AND BRAIN TISSUE

P. L. BONATE*, C. M. DAVIS,
P. B. SILVERMAN, AND A. SWANN
University of Texas Health Science Center at Houston
Mental Sciences Institute
Substance Abuse Research Center
1300 Moursund Avenue
Houston, Texas 77030

ABSTRACT

An HPLC assay using UV detection has been developed for the analysis of cocaine in biological matrices. Samples were precipitated with perchloric acid, extracted in 5% chloroform/pentane at pH 10, and back-extracted into 0.1 N hydrochloric acid. Samples were chromatographed using a Zorbax RX-C18®, 4.6 x 250 mm, 5 μm, column with the absorbance monitored at 235 nm. The method was applied to quantification of cocaine in plasma and brain tissue of Sprague-Dawley rats following intraperitoneal administration of 15 mg/kg cocaine. The method was characterized by its extreme ease of sample preparation and ruggedness over time. The average recovery for the method was marginal; 52 and 38% for cocaine and the internal standard, respectively, in plasma and 42 and 35% of the cocaine and the internal standard, respectively, in brain. However, due to the extreme cleanliness of the sample after preparation, the limit of detection of the method for cocaine was less than 5 ng/ml in plasma and less than 5 ng/g in brain. The limit of quantification was 25 ng/ml in plasma and 25 ng/g in brain.

Copyright © 1995 by Marcel Dekker, Inc.

The method was linear from 50-4000 ng/ml in both plasma and brain. Spiked quality control samples were assayed over a three month period. The inter-day coefficient of variation was 10.2% in plasma at 200 ng/ml (n=22), 11.6% at 200 ng/ml in brain (n=38) and 0.7% at 2500 ng/ml in both plasma and brain (n=23, n=43, respectively). The method would appear to be acceptable for the analysis of cocaine in both saliva and urine.

INTRODUCTION

Cocaine is an infamous drug of abuse. Cocaine acts as a psychostimulant in the central nervous system by inhibiting the reuptake of catecholamines into the presynaptic nerve terminal following release. It also acts as a local anesthetic in the peripheral nervous system by blocking fast opening sodium channels (1). Following repeated intermittent administration of cocaine to rats, progressive augmentation of locomotor activity and stereotyped behavior is observed. Following continuous administration of cocaine, progressive diminution of locomotor activity and stereotyped behavior is observed. The former process is termed sensitization, the latter is termed tolerance (2). One theory regarding the development of sensitization is that it may at least in part be due to increased cocaine brain levels following repeated administration (3). In order to test this hypothesis it is necessary to be able to quantify cocaine and, if possible, its metabolites in biological matrices, particularly plasma and brain tissue.

A variety of methods have been developed using HPLC to assay cocaine concentrations in biological matrices (4-11). However, none of these methods are ideal. Most are developed for a particular matrix, usually plasma (7,9,10), serum (5,6), or urine (4,9). Few are suitable for brain tissue (8, 11). Some require extensive sample preparation prior to actual injection on-column (4,9). Others sacrifice extraction efficiency for sample cleanliness (6,7,11). To

date, no validated method for cocaine analysis in multiple biological matrices, i.e., plasma, brain, urine, and saliva, has been developed.

Previous methods used to detect cocaine and its metabolites by reversed phase HPLC have been problematic both from a technical and theoretical viewpoint. One problem has been the use of lidocaine or other local anesthetics as an internal standard (5,6,8,9). Although lidocaine and bupivicaine (both of which have been used as internal standards in published HPLC methods) are local anesthetics, they are structurally unrelated to cocaine. Ideally, the internal standard would be similar both structurally as well as pharmacologically. Common commercial internal standards meeting the structural similarity requirement are propyl-benzoylecgonine and 3-tropanyl-3,5-dichlorobenzoate (MDL-72222). Unfortunately, these compounds are so lipophilic that long retention times are often seen when using an HPLC system consisting of a moderate percentage of organic in the mobile phase which makes them unsuitable when a rapid turn-around time is needed (Table 1). A seemingly ideal internal standard, structurally similar to cocaine, but less lipophilic than propyl-benzoylecgonine, is m-toluylecgonine perchlorate. This compound has been used in gas chromatography, but has yet to be applied to liquid chromatography (12). Unfortunately, it is not available commercially and must be synthesized by the user. A compromise internal standard is ethyl-benzoylecgonine, i.e., cocaethylene, which is both structurally and pharmacologically similar to cocaine (7). Although cocaethylene has a much shorter retention time than propyl-benzoylecgonine, it cannot be used clinically since cocaethylene is formed metabolically in vivo in subjects taking cocaine and alcohol in combination.

Another problem typically seen in the literature is related to chromatography of amines on octadecyl columns. Since cocaine and its metabolites are tertiary amines, using a reversed phase column, one would expect to see tailing of

TABLE 1
Retention Times and Capacity Factors for Various Drugs of Abuse and Psychotropic Drugs

NAME OF COMPOUND	Retention Time in min	Capacity factor, k'
procainamide	1.4	0.00
flupentixol	1.5	0.02
morphine	1.5	0.02
vancomycin	1.6	0.08
hydromorphone	1.7	0.19
codeine	1.7	0.20
quinine	1.8	0.26
quinidine	1.8	0.27
theophylline	1.9	0.30
hydrocodone	2.1	0.48
3,4-methylenedioxyamphetamine	2.2	0.50
3,4-methylenedioxymethamphetamine	2.4	0.68
caffeine	2.5	0.71
atropine	2.8	0.93
3,4 methylenedioxyethylamphetamine	2.9	1.03
lidocaine	3.0	1.12
chlorpheniramine	3.7	1.55
mepazine	4.2	1.96
methylphenidate	4.6	2.18
gepirone	4.8	2.36
clozapine	5.6	2.92
cocaine	6.5	3.40
procaine	6.9	3.83
meperidine	7.1	3.93
norcocaine	7.4	4.20
pseudococaine	7.9	4.51
chlordiazepoxide	8.3	4.81
phenylpropanolamine	8.5	4.97
salicylic acid	9.2	5.41
RTI-32	9.7	5.80
haloperidol	11.1	6.77
RTI-31	11.4	6.94
acetophenazine	11.4	6.94
pentazocine	11.5	7.01
buspirone	13.1	8.18
trazodone	14.5	9.16
benperidol	18.7	12.05
dextromethorphan	19.6	12.73
amoxapine	21.2	13.83
diphenhydramine	28.8	19.11

(Continued)

TABLE 1 (Continued)

NAME OF COMPOUND	Retention Time in min	Capacity factor, k'
oxazepam	63.5	43.39
methaquaalude	90.5	62.29
diazepam	106.0	73.13
butriptyline	nd	nd
proketazine	nd	nd
meprobamate	nd	nd
nortriptyline	nd	nd
amitryptiline	nd	nd
benztropine methanesulfonate	nd	nd
butaperazine	nd	nd
chlorpromazine	nd	nd
cyclopenzaprine	nd	nd
clomipraime	nd	nd
ethydorvynol	nd	nd
norfluoxetine	nd	nd
fluoxetine	nd	nd
fluphenazine	nd	nd
imipramine	nd	nd
methadone	nd	nd
methamphetamine	nd	nd
nicotine	nd	nd
PCP	nd	nd
phenothiazine	nd	nd
d-propoxyphene	nd	nd
pseudoephedrine	nd	nd
delta-9-tetrahydrocannabinol	nd	nd
ephedrine	nd	nd
propyl benzoylecgonine	28.8	19.14
pentobarbital	30.2	20.10
loxapine	31.1	20.75
flurazepam	33.4	22.36
mesoridazine	36.5	24.49
amphetamine	38.7	26.05
carbamazepine	44.2	29.88
3-tropanyl-3,5-dichlorobenzoate	52.1	35.45
secobarbital	54.6	37.15
acetaminophen	55.9	38.11
WIN 35428	56.2	38.27
hydergine (ergoloid mesylates)	56.3	38.39

Non-extracted samples (~1 μg on-column) were chromatographed for at least 90 min and monitored at 235 nm. Compounds listed as 'nd' were not detected under the conditions described.

peaks that are removed from the solvent front. Indeed, tailing and poor resolution are often seen in chromatograms using published methods (7,9,10). Some papers do not include chromatograms so it is difficult to evaluate these methods critically (8,11).

Our laboratory set out to devise a method for the analysis of cocaine in plasma and brain which was free of previous pitfalls in cocaine analysis. In particular, we wished to develop a method that could be applied to a variety of sample matrices, particularly brain tissue, and was rugged to slight modifications in terms of sample preparation or chromatographic conditions.

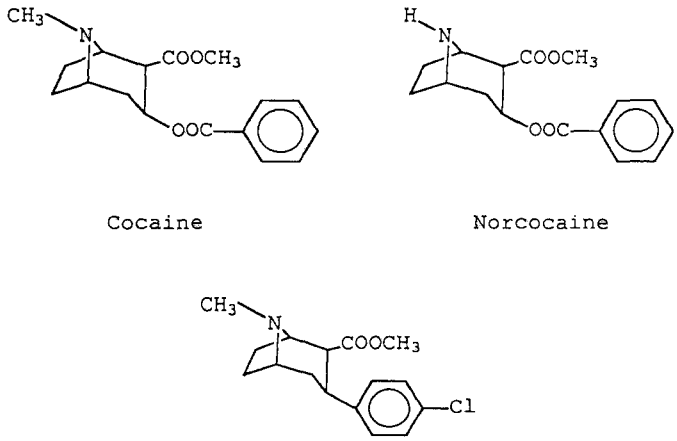
MATERIALS AND METHODS

Reagents and Standards

All standards were obtained from the National Institute on Drug Abuse (Research Technology Branch, Research Triangle Park, NC). The purity of these compounds were greater than 99% as determined by HPLC. All calculations were based on the free base concentrations. Solvents were purchased either from Fisher Scientific (Springfield, NJ) and were at least HPLC grade. All reagents were at least chemical grade. All glassware were silylated prior to use by vapor phase silylation (13). The internal standard, RTI-31 (Figure 1; IS), is available from Research Biochemical International (Natick, MA). Homogenization buffer consisted of 0.5% sodium fluoride in 100 mM sodium phosphate monobasic, pH 4.5. Plasma controls were a gift from UTAK Laboratories, Inc. (Canyon Country, CA).

Instrumentation and Data Analysis

All samples were analyzed using a Waters Assoc. (Milford, MA) 510 pump attached to a Waters Assoc. 710 WISP



Cocaine

Norcocaine

RTI-31
Internal Standard

FIGURE 1: Structure of cocaine, norcocaine, and RTI-31, the internal standard.

autosampler. A Brownlee RP-8 guard column, 15 x 3.2 mm, 7 μm, (Applied Biosystems, Foster City, CA) was attached in line between the injector and column. All samples were chromatographed on a Zorbax® RX-C18 column, 4.6 x 250 mm, 5 μm particle size (Mac-Mod, Chaads Ford, PA). Data were captured using a Waters 780 Data Module. Samples were detected at 235 nm on a Waters 210 variable wavelength ultraviolet detector set at 0.02 AUFS. Samples were centrifuged using a Sorvall RC-5B refrigerated superspeed centrifuge (Dupont Co., Newtown, CT) equipped with a SM24 rotor. All data were collected in peak height mode.

Calibration curves were calculated by linear regression analysis of the ratio of the cocaine peak height to internal standard peak height vs. theoretical cocaine concentration. All data were weighed by 1/concentration and unknown sample concentrations were estimated by inverse prediction. Since

the calibration curves were prepared as ng/ml concentrations, whole brain and striatal levels of cocaine, in ng/g, were determined using the following formula, assuming the density of brain to be 1.0 g/ml:

$$\text{Concentration, ng/g} = \frac{\left(\begin{array}{c}\text{Concentration From}\\ \text{Standard Curve, ng/ml}\end{array}\right)\left(\begin{array}{c}\text{Volume of}\\ \text{Standards, ml}\end{array}\right)\left(1 + \text{Dilution Factor}\right)}{\left(\begin{array}{c}\text{Volume of Tissue}\\ \text{Assayed, g}\end{array}\right)}$$

Sample Preparation

Unknown samples and controls were frozen at -80°C prior to analysis and were allowed to thaw at room temperature. After thawing, a 0.5 ml aliquot was removed for analysis. Plasma standards were prepared as follows: one-half milliliter (0.5 ml) blank plasma was mixed with 15 µl saturated sodium fluoride to inhibit the metabolism of cocaine to ecgonine methyl ester by pseudocholinesterase (14). The standards were then spiked with cocaine prepared in ethanol to give concentrations of 50 to 4000 ng/ml.

Whole brain or striata standards were prepared as follows: homogenized cow brain was diluted 1:8 or 1:4 (w/v), for striata or whole brain, respectively, with homogenization buffer and homogenized using a Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY). A 0.5 ml aliquot of homogenate was removed and spiked with cocaine prepared in ethanol to give concentrations of 50 to 4000 ng/ml.

From this point, standards, unknowns, and controls were treated identically. All samples were mixed with 75 µl 150 µg/ml internal standard and precipitated with 50 µl 50% perchloric acid. The samples were mixed vigorously for ~10 s, then allowed to sit at room temperature for 10 min. One (1.0) ml of distilled water was added to each tube. The samples were capped, mixed briefly, and centrifuged at ~2500 for ~30 min at ~10°C using an International Equipment Co.

(Needham Heights, MA) 7R centrifuge. After centrifugation, the supernatant was transferred to a clean 16x125 mm screw-top test tube and 750 µl saturated sodium carbonate added. The samples were briefly mixed and 7.5 ml 5% chloroform in pentane added. The samples were rocked gently for 10 min then centrifuged for ~2 min on a desk-top centrifuge. The samples were then placed in an acetone bath containing dry ice for ~2 min after which the organic layer was decanted into a 16x100 mm conical bottom screw-top test tube containing 250 µl 0.1 M hydrochloric acid. The samples were allowed to sit at room temperature until the aqueous layer was thawed. In some samples, decanting the supernatant onto the dilute acid briefly froze the dilute acid. After the samples had thawed, they were vigorously mixed for ~10 s, then centrifuged on a desk-top centrifuge for 1-2 min. After centrifugation, the samples were placed back in the dry ice bath for 3-5 min. The organic supernatant was discarded and the frozen aqueous layer allowed to thaw. After thawing, the samples were mixed and passed over a nitrogen stream for a few seconds to remove any lingering chloroform/pentane. Allowing the samples to sit at room temperature for ~1 h prior to injection achieved the same result. Seventy-five microliters (75 µl) of the samples was injected on-column. Batch processing of 40 samples could be accomplished within 3 h.

HPLC Conditions

The mobile phase consisted of acetonitrile mixed with 0.5% triethylamine in 100 mM potassium phosphate dibasic, adjusted to pH 2.7 with phosphoric acid (18/82%, v/v). Mobile phase was degassed using helium sparging prior introduction to the column. The flow rate was 2.0 ml/min with a back pressure of ~2700 psi. Under these conditions, cocaine and the internal standard had retention times of 6.5 and 11.4 min, respectively. Chromatography was done at room temperature.

Animal Studies

Female, Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis IN) were dosed intraperitoneally with 15 mg/kg cocaine. At 20 min after administration, the animals were decapitated, trunk blood collected and brain removed. To each ml of blood was added 10 µL saturated sodium fluoride and to the tube of blood was added 50 µl heparin sulfate (1000 U/ml). Striata were dissected out and pooled. Striata and the remaining brain tissue were diluted 1:8 or 1:4 (w/v), respectively, homogenized, and assayed for cocaine. Whole blood was centrifuged, the plasma removed and assayed for cocaine.

RESULTS AND DISCUSSION

Chromatography

Cocaine was adequately resolved from the internal standard (Figures 2 and 3). The only metabolite of cocaine which may interfere with the resolution of cocaine is norcocaine with a retention time of 7.4 min. However, in our studies, norcocaine was always less than 5% of the peak height of cocaine so this does not appear to be a problem. Figures 2 and 3 show chromatograms obtained from blank plasma and whole brain, respectively, spiked with cocaine, norcocaine, and internal standard. Although complete baseline resolution was not achieved between cocaine and norcocaine, the resolution was more than adequate to accurately quantify cocaines peak height. At higher doses, greater amounts of norcocaine may be formed, leading to decreased resolution of cocaine and norcocaine, which may require alterations in the mobile phase composition. Decreasing the percent organic in the mobile phase would achieve the goal of more complete resolution of norcocaine from cocaine, but at the expense of band broadening and

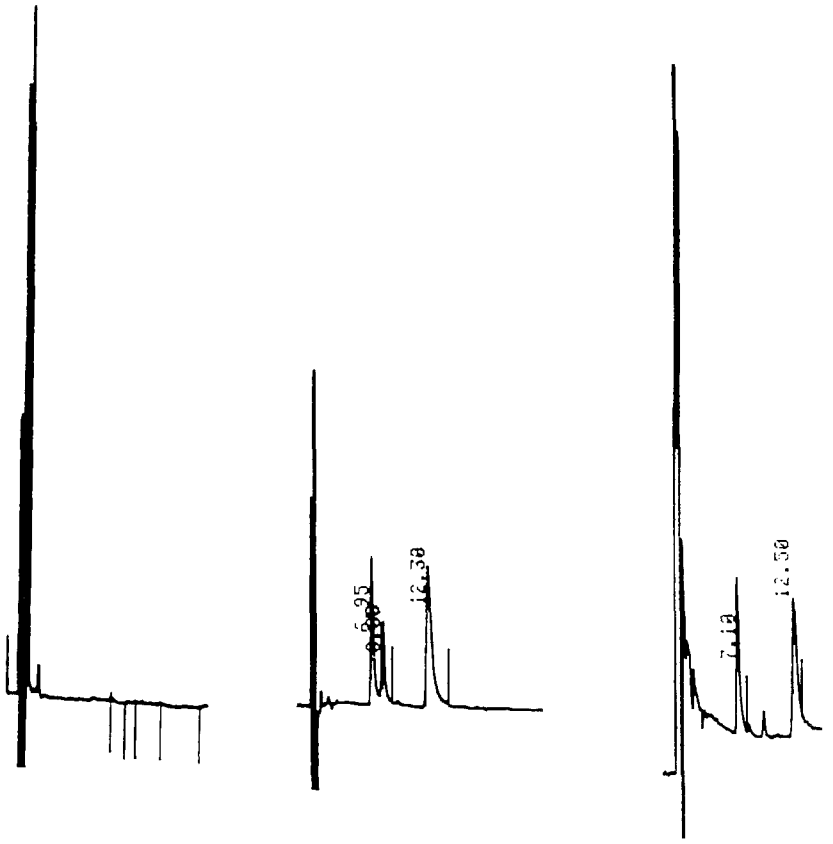


FIGURE 2: Chromatograms from blank plasma (left), plasma spiked with cocaine, norcocaine, and RTI-31 (middle), and plasma obtained from a rat dosed with 15 mg/kg cocaine ip (right).

higher LOD. Alternatively, use of a 3 μm rather than a 5 μm column, should increase resolution sufficiently to provide baseline resolution of cocaine and norcocaine and decrease the LOD, without affecting column pressure to any extent. The average recovery of norcocaine from brain and plasma was 32%.

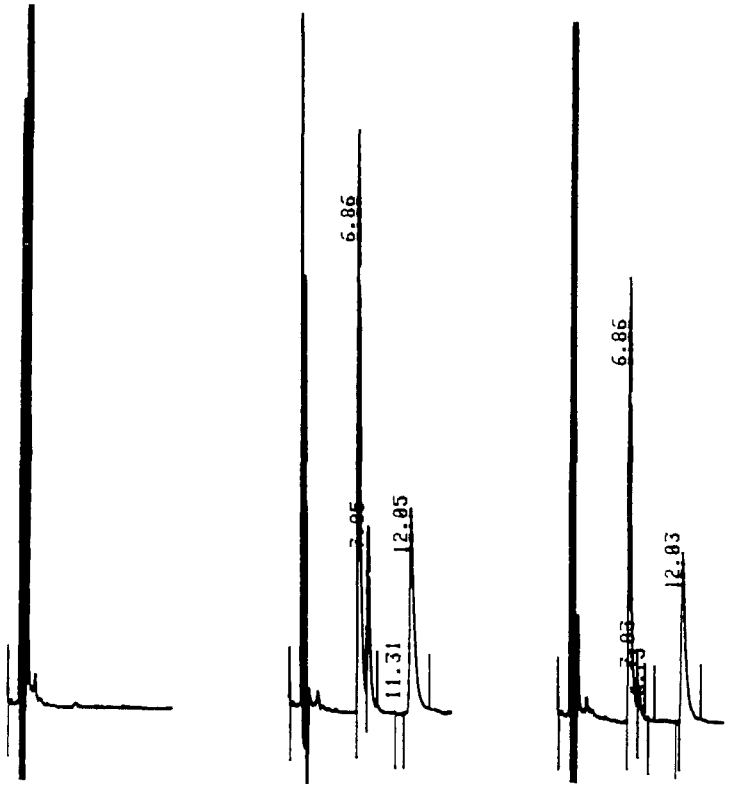


FIGURE 3: Chromatograms from blank whole brain (left), whole brain spiked with cocaine, norcocaine, and RTI-31 (middle), and whole brain obtained from a rat dosed with 15 mg/kg cocaine ip (left).

Figure 4 shows the effect of percent acetonitrile in the mobile phase on retention time of cocaine, norcocaine, and internal standard. As expected, the retention time of the compounds was log-linearly related to percent organic in the mobile phase. The final percent acetonitrile was chosen to maximize resolution of cocaine and norcocaine while minimizing the total run-time. Buffer pH has little effect

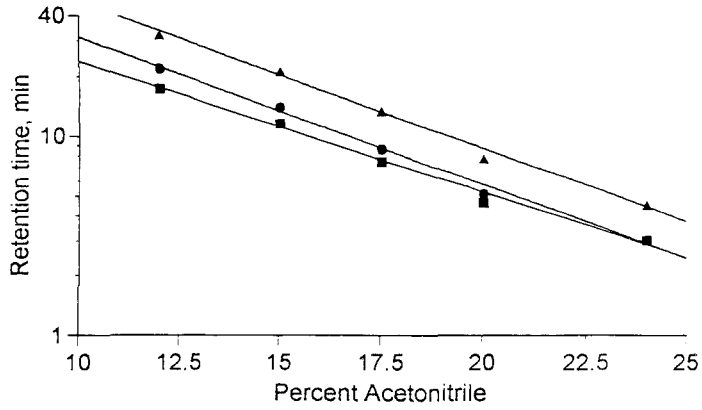


FIGURE 4: Effect of percent acetonitrile on retention time of cocaine (■), norcocaine (▲), and IS (●) when buffer pH was fixed at 2.7.

on retention time, except as the pH begins to exceed 4.0 (Figure 5). Here, pH 2.7 was chosen since it is less than one log unit from the pKa of phosphate ion.

One problem that was observed with the chromatography was the shift in retention time during the course of method development. This was probably a result of increased variability in room temperature as the building was shifted from heating to cooling. As such, our chromatography changed and coelution of cocaine and norcocaine became a problem. One way we tried to combat this problem was to add a column heater set to 37°C. To compensate for the expected decrease in retention time, the mobile phase proportions were changed to 15% acetonitrile to 85% buffer with a flow rate of 1.5 ml/min. This change produced similar retention times (cocaine, 7.9 min; norcocaine, 8.8 min; internal standard, 13.7 min), but band broadening significantly decreased peak resolution. As an alternative, and what was finally decided to be the best solution, we fit a cardboard box slightly larger than the column with foam. The column

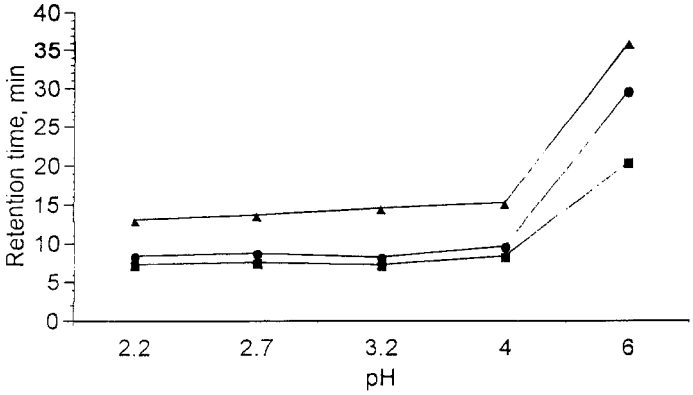


FIGURE 5: Effect of buffer pH on retention time of cocaine (■), norcocaine (▲), and IS (●) when percent acetonitrile was fixed at 18%.

was placed inside the box and sealed. This inexpensive insulation device proved more than satisfactory for any further temperature changes which occurred over time.

No endogenous interferences appear to be present in the assay (Figures 2 and 3). Of more than 70 drugs screened for possible interference, only pseudococaine and pentazocine have the possibility of interfering with the assay (Table 1). All the compounds with retention times in the range of 4.2 to 18.7 min were spiked into plasma to have a concentration of at least 10 μg/ml. In addition to the single drug of interest, each tube was spiked with cocaine, norcocaine, and the internal standard to have a concentration of at least 1 μg/ml. These samples were then processed as described above to determine if any of the drugs recovered to any significant extent and if they coelute with either cocaine, norcocaine, or the internal standard. Of the drugs tested, only methylphenidate, gepirone, clozapine, pseudococaine, chlordiazepoxide, and pentazocine were extracted to any significant extent. Of

these, only pseudococaine and pentazocine coeluted with one of the analytes.

Due to the cleanliness of the final samples, we utilized a "poor-man's" solvent recycling system to decrease the cost of solvent utilization. In effect, we fed our solvent waste line back into our mobile phase reservoir. Our solvent reservoir sat on a magnetic bar stirrer to maintain consistency in the mobile phase. Hundreds of injections were made before a change in resolution was noticed, although retention time began to drift after about 100 samples. Nevertheless, even as the retention time began to change, we were still able to use the method for sample analysis.

Two other biological matrices were examined from spiked samples: saliva and urine. The sample preparation was as used for plasma, described above. Both matrices have very clean chromatography with no endogenous interferences. Although not examined in vivo, saliva offers to be a non-invasive method which can be used to correlate behavioral effects to plasma concentration in humans (15).

Linearity and Limit of Detection

The method was used for routine analysis of brain and plasma samples over a three month period. Fitting daily calibration curves to a simple linear regression model resulted in coefficients of determination ranging from 0.9901 to 1.00 in plasma (n=8) and 0.9900 to 0.9996 in brain (n=14). Fitting each daily calibration curve to a quadratic polynomial resulted in a non-significantly quadratic term. Thus the method was linear from 50 to 4000 ng/ml. No attempt was made to determine the upper-most limit of linearity. The limit of detection (LOD) was calculated to be ~5 ng/ml in plasma and ~5 ng/g in brain. The limit of quantification calculated using a t-value of 10 was ~25 ng/ml in plasma and ~25 ng/g in brain (16). Should a lower

limit of quantification be required, the AUFS on the detector may be decreased to 0.01 with little alteration in baseline noise. The limit of quantification for norcocaine in plasma and brain was ~75 ng/ml and ~75 ng/g, respectively.

Recovery

There was good concordance between recovery from brain, plasma, and saliva. The average recovery from plasma was 52 and 38% for cocaine and the internal standard, respectively. Recovery from brain tissue was 42 and 35% of the cocaine and the internal standard, respectively. The average recovery from saliva was 46 and 40% for cocaine and the internal standard, respectively. Recovery data was not calculated for urine. Both precipitation and extraction from the centrifuged precipitant represent the steps at which the major loss in recovery occurred. Following precipitation, an ~15% loss in recovery was observed which was believed to be due to trapping of analytes in the precipitant. Base extraction from supernatant into 5% chloroform in pentane resulted in ~65% of the analytes being recovered. The final step, back extraction into dilute acid, was complete with ~100% of the analytes being recovered. Obviously, recovery is the major limitation for the method. However, low recovery must be balanced against sample cleanliness. Future studies may wish to examine the effect of other buffers with different pH values on percent recovery.

The solvent used for the extraction, 5% chloroform in pentane, is relatively non-polar in nature and as such should not be expected to extract endogenous, polar substances. However, some sample matrices, possibly forensic samples, may be so "dirty" that some endogenous substances may be extracted into the solvent and interfere with the assay. During the course of method development it was discovered that 35% ethyl acetate in pentane, a relative polar solvent, fails to extract the analytes to any

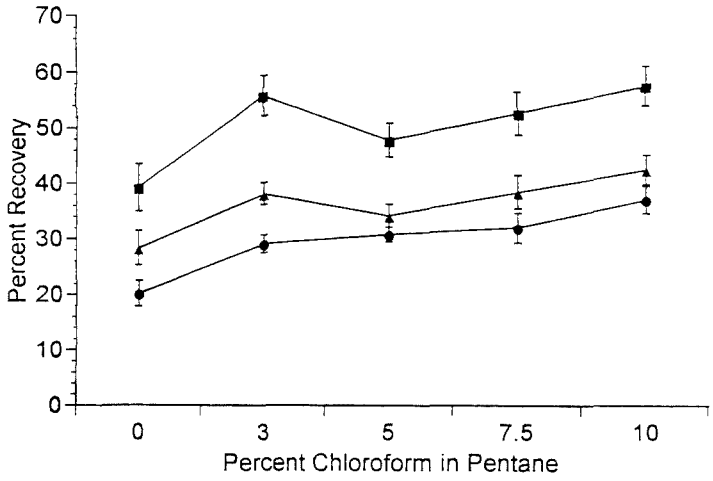


FIGURE 6: Effect of chloroform on percent recovery of cocaine (■), norcocaine (▲), and IS (●) from plasma following a 35% ethyl acetate in pentane wash.

significant extent. This allows polar substances to be extracted but leaves the majority of the analytes behind. Therefore, after precipitation by perchloric acid and transfer to a clean test prior to the addition of saturated sodium carbonate, a wash step using 35% ethyl acetate in pentane may be used as a further clean-up step. For the sample matrices examined herein, plasma, brain, saliva, and urine, this wash step was unnecessary. Figure 6 shows the effect of percent chloroform in pentane on recovery following ethyl acetate washing.

Stability and Precision

Samples were prepared and analyzed. Using a refrigerated autosampler set at 15°C, the samples were reanalyzed 72 h later and the peak heights compared. Using the Wilcoxon signed rank test for differences between days, no significant differences were found ($p > 0.05$).

Therefore, the analytes were stable in 0.1 M hydrochloric acid for a period of 72 h. This should be sufficient time to analyze a 48-position WISP sample carousel.

Blank plasma and cow brain samples were spiked with 200 or 2500 ng/ml (ng/g for brain) cocaine and stored at -80°C until analysis. These samples were treated and stored as if they were unknowns. Over a three-month period, these samples were analyzed in conjunction with experimental samples. The method exhibited adequate within and between-day precision. The average inter-day coefficient of variation (CV) was 10.2% in plasma at 200 ng/ml (n=22), 11.6% at 200 ng/ml in brain (n=38) and 0.7% at 2500 ng/ml in both plasma and brain (n=23, n=43, respectively). The average intra-day CV ranged from 4.7 to 12.7% in plasma at 200 ng/ml, 0.8 to 9.9% in plasma at 2500 ng/ml, 5.6 to 14.3% in plasma at 200 ng/g, and 2.4 to 14.5% in brain at 2500 ng/g. The mean relative error was 5.0% for both brain and plasma at 200 ng/ml and 3.0% for both brain and plasma at 2500 ng/ml.

A single lyophilized plasma control for cocaine with a target value of 500 $\pm$ 50 ng/ml was reconstituted in 5.0 ml water, mixed with 75 ul saturated sodium fluoride, and stored at -80°C until analysis. This control was assayed twice over a period of three weeks. The mean back-calculated concentration was 475 $\pm$ 17 ng/ml (n=6, mean $\pm$ std dev) after discarding the highest and lowest control with an inter-day CV of 3.6%. Without discarding the highest and lowest control, the mean back-calculated concentration was 488 $\pm$ 79 ng/ml with an inter-day CV of 16%.

Whole brain samples obtained from animals dosed with 15 mg/kg cocaine intraperitoneally were homogenized using the phosphate buffer/sodium fluoride buffer were assayed and frozen at -80°C. A random sample was reassayed six-eight months later. Using stochastic regression, the slope of the line relating the reassayed concentration to the original concentration was 0.85, indicating 15% of the cocaine in the

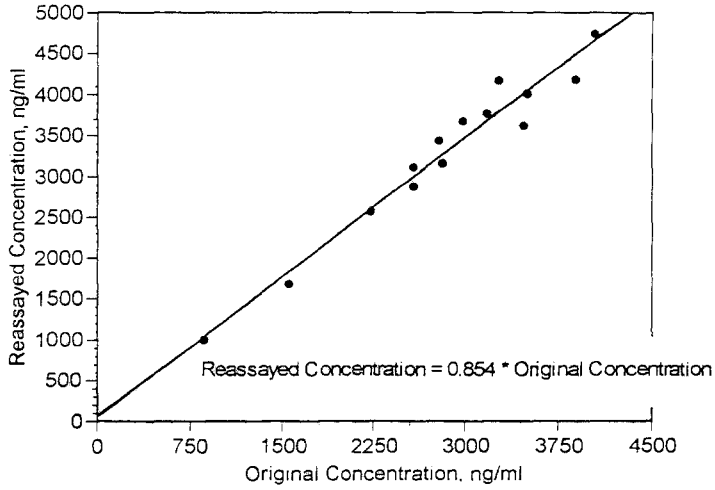


FIGURE 7: Plot of reassayed cocaine concentrations vs. their original concentration after being stored at -80°C for six-eight months. The line is the stochastic regression least-squares fit to the data.

original sample had degraded (Figure 7). This translates to ~2% loss per month.

Plasma and Brain Samples

Table 2 shows the concentrations for the samples and the brain to plasma ratios. Norcocaine was not detected in either plasma or brain. After correction for dose, the reported concentrations are agreement with other studies in animals (17).

CONCLUSIONS

An assay for the quantification of cocaine in both plasma and brain has been developed. The method development

Table 2
Striatum, Whole Brain, and Plasma Cocaine Concentrations in Sprague-Dawley Rats 20 Minutes After Intraperitoneal Administration of 15 mg/kg Cocaine

Animal	Whole Brain Concentration, ng/g	Striatum Concentration, ng/g	Plasma Concentration, ng/ml
1	8.84	9.61	2752
2	8.27	8.79	1582
3	11.54	10.12	1776
4	8.98	11.61	1406
5	11.24	11.66	2080
Mean	9.77	10.36	1919
Std Dev	1.50	1.26	528
CV, %	15.4	12.14	27.5

had three objectives. One was that it would be easy to perform and batch sample processing would be easily accomplished. The ideal method would require no special sample preparation simply because the matrix was not plasma. The second goal was that the chromatography would be similar across sample matrices without any modification of the mobile phase. Finally, the assay would be sensitive and specific for cocaine. The method achieves each goal. The biggest drawback is that the method suffers from marginal recovery, but this is compensated for by a low limit of detection.

We have strived to make this method as rugged as possible. At every point in the assay we have tried to make the method as stable as possible from slight modifications. Hence, during sample preparation no buffers are prepared to a specific pH. We have used saturated buffers and rely on their pKa to maintain the precise pH. Only the mobile phase is prepared to an exact pH and even this was found to be a non-critical factor. These factors all act to increase the stability of the method over time. In summary, a validated HPLC method for the analysis of cocaine has been presented

which is rapid, sensitive, specific, and rugged to slight changes in sample preparation.

REFERENCES

1.) Goodman and Gilman's The Pharmacological Basis of Therapeutics, 8th edition. A.G. Gilman, T.W. Rall, A.S. Nies, and P. Taylor: editors. Pergamon Press, New York, 1990.

2.) P. Jatlow: Cocaine: analysis, pharmacokinetics, and metabolic disposition. Yale J Biol Med 61, 105-113, 1988.

3.) P.W. Kalivas and J. Stewart: Dopamine transmission in the initiation and expression of drug- and stress-induced sensitization of motor activity. Brain Res Rev 223-244, 1991.

4.) P.J. Jatlow, C. van Dyke, P. Barash, R. Byck: Measurement of benzoylecgonine and cocaine in urine, separation of various cocaine metabolites using reversed phase high-performance liquid chromatography. J Chrom 152, 115-121, 1978.

5.) C.E. Lau: Determination of cocaethylene, cocaine and their metabolites in rat serum microsamples by high performance liquid chromatography and its application to pharmacokinetic studies in rodents. J Chrom 582, 167-172, 1992.

6.) C.E. Lau, F. Ma, and J.L. Falk: Simulataneous determination of cocaine and its metabolites with caffeine in rat serum microsamples by high performance liquid chromatography. J Chrom 532, 95-103, 1990.

7.) R.L. Miller and C.L. DeVane: Determination of cocaine, benzoylecgonine and ecgonine methyl ester in plasma by reversed-phase high-performance liquid chromatography. J Chrom 570, 412-418, 1991.

8.) C. Moore, S. Browne, I. Tebbett, and A. Negrusz: Determination of cocaine and its metabolites in brain tissue using high-flow solid-phase extraction columns and high performance liquid chromatography. Forensic Sci Int 53, 215-219, 1992.

9.) Sandberg J and Olsen GD: Microassay for the simultaneous determination of cocaine, norcocaine, benzoylecgonine and benzoylnorecgonine by high performance liquid chromatography. J Chrom, 525, 113-121, 1990.

10.) P. Jatlow and H. Nadim: Determination of cocaine concentrations in plasma by high-performance liquid chromatography. Clin Chem 36, 1436-1439, 1990.

11.) M. Benuck, A. Lajtha, and M.E.A. Reith: Pharmacokinetics of systemically administered cocaine and locomotor stimulation in mice. J Pharmacol Exp Ther 243, 144-149, 1987.

12.) P. Jacob, B.A. Elias-Baker, R.T. Jones, and N.I. Benowitz: Determination of benzoylecgonine in biological fluids by automated gas chromatography. J Chrom 417, 277-286, 1987.

13.) D.C. Fenimore, C.M. Davis, J.H. Whitford, C.A. Harrington: Vapor phase silylation of laboratory glassware. Anal Chem 48, 2289-2290, 1976.

14.) D.S. Isenschmid, B.S. Levine, and Y.H. Caplan: A comprehensive study of the stability of cocaine and its metabolites. J Anal Toxicol 13, 250-256, 1989.

15.) T. Inaba, D.J. Stewart, and W. Kalow. Metabolism of cocaine in man. Clin Pharmacol Ther 23, 547-52, 1978.

16.) J.C. Miller and J.N. Miller: Statistics for Analytical Chemistry. Ellis Horwood LTD., Chichester UK, 1984.

17.) P.K. Nayak, A.I. Misra, S.J. Mule: Physiological disposition and biotransformation of ^{3}H-cocaine in acutely and chronically treated rats. J Pharmacol Exp Ther 196, 556-569, 1976.

Received: April 22, 1995
Accepted: May 20, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(17), 3495-3505 (1995)

AUTOMATED DETERMINATION OF TRIMIPRAMINE AND N-DESMETHYL-TRIMIPRAMINE IN HUMAN PLASMA OR SERUM BY HPLC WITH ON-LINE SOLID PHASE EXTRACTION

SEBASTIAN HÄRTTER, BIRGIT HERMES, AND CHRISTOPH HIEMKE*
Department of Psychiatry
University of Mainz
Untere Zahlbacher Strasse 8
D-55131 Mainz, Germany

ABSTRACT

A fully automated method including column-switching and isocratic high performance liquid chromatography (HPLC) was developed for determination of the tricyclic antidepressant trimipramine (T) and its N-demethylated metabolite N-desmethyltrimipramine (DT).
The limit of quantification was below 10 ng/ml for T and DT. The assay revealed linearity between detector response and drug concentration in a therapeutically relevant range of 10 to 500 ng/ml. The mean intra- and interassay variabilities were 6.2 and 12.3 % , respectively, for T and 4.7 and 8.7 %, respectively, for DT. The method can be applied to therapeutic drug monitoring of patients under T therapy and may be useful for pharmacokinetic studies.

INTRODUCTION

Trimipramine (T) is a tricyclic antidepressant (TCA) which has been applied to the therapy of depression for over 20 years. Although, the chemical structure of T is closely related to other TCAs, like imipramine, it differs from the other TCAs in some pharmacological aspects [1]. The most striking difference is the induction of supersensitivity of the noradrenergic system after long term treatment with T [2]. This casts doubts on the

Copyright © 1995 by Marcel Dekker, Inc.

concept that receptor down-regulation by TCAs is relevant for the antidepressant effect [3, 4]. Moreover, preliminary clinical studies suggest that high doses of T have antipsychotic efficacy without inducing extrapyramidal side effects [5].

Supervision of T treatment by therapeutic drug monitoring (TDM) of blood concentrations is rare. Only few methods are so far available for the determination of T in body fluids. Methods reported in the literature use gas chromatography [6, 7] or high performance liquid chromatography (HPLC) [8-11]. They require laborious and time consuming sample pretreatment by either solvent-solvent extraction [6-10] or off-line solid phase extraction [11]. The assay described here enables simple and rapid quantification of T and DT by direct injection of plasma or serum samples on a column-switching system coupled to isocratic HPLC.

METHODS AND MATERIALS

Chemicals

Trimipramine methansulfonate and N-desmethyltrimipramine (free base) were kindly donated by Rhône-Poulenc (Vitry sur Seine, France). Clomipramine and its main demethylated and hydroxylated metabolites, maprotiline, and carbamazepine were gifts from Ciba Geigy AG (Basel, Switzerland). Fluvoxamine maleate was donated by Duphar (Weesp, Netherlands), paroxetine by SmithKline Beecham (Worthing, UK), haloperidol and risperidone by Janssen (Beerse, Belgium), chlorprothixene by Tropon (Köln, Germany), moclobemide and diazepam by Hoffmann -La Roche AG (Basel, Switzerland), lorazepam by Wyeth (Münster, Germany), and biperidene by Nordmark (Uetersen, Germany).
Acetonitrile and methanol were of LiChrosolve® quality and di-potassium hydrogenphosphate and orthophosphoric acid of analytical grade (Merck, Darmstadt, Germany). Water was deionized and filtered by a Milli-Q water processing system (Millipore, Eschborn, Germany).

Standards

Stock solutions were prepared by dissolving 10 mg T and 10.3 mg DT in 10 ml methanol, each. They were diluted with deionized water and mixed with drug free plasma or

serum to obtain calibration standards with concentrations of free bases ranging between 10 and 500 ng/ml (10, 50, 100, 300, and 500 ng/ml).

Quality control samples (QCs) were prepared by mixing the 1 mg/ml methanolic stock solutions with plasma or serum to acchieve concentrations of 20, 100, and 250 ng/ml T and DT, respectively.

All standards were stored at -20°C until use.

Plasma or serum samples

Plasma or serum samples were obtained from either healthy non-medicated volunteers to obtain drug-free plasma or from patients treated with T for at least 7 days. Patients' blood for the preparation of plasma or serum was collected in the morning immediately before the first daily dose.

Instrumentation

The HPLC system consisted of a CMA/200 autosampler (CMA/Microdialysis AB, Stockholm, Sweden), a six-port switching valve (Besta, Wilhelmsfeld, Germany) coupled to the autosampler and two HPLC pumps A and B, both from Bischoff, type 2200 (Leonberg, Germany). Pump A eluted the analytical mobile phase, pump B was used for loading plasma or serum sample onto the clean-up column and subsequent washing. Moreover, a variable wavelenght ultraviolet (UV) detector, type SPD-10A (Shimadzu, Kyoto, Japan) was included. Chromatograms were recorded and integrated by a Kontron chromatogram integration software PCIP (Kontron, Milan, Italy).

Analytical columns (250 x 4.6 mm) and pre-columns (20 x 4.6 mm I.D) were packed with Spherisorb CN (5 µm particle size) by MZ-Analysentechnik (Mainz, Germany). Clean-up columns (10 x 4.0 mm I.D.), packed with Hypersil CPS (10 µm particle size), were also supplied by MZ-Analysentechnik.

Chromatographic procedure

0-5 min: After centrifugation (3000 g for 5 min), 100 µl plasma or serum was injected automatically onto the clean-up column. Proteins and other interfering compounds were

washed to waste by deionized water containing 5 % (V/V) acetonitrile at a flow rate of 1.5 ml/min by pump B.

5-8 min: After the six port valve had been switched at 5 min, the analytes to be determined were eluted onto the analytical column (foreward flush) and separated by a mobile phase (pump A) consisting of 23 % (v/v) 8 mM phosphate buffer (adjusted to pH 6.2 with orthophosphoric acid) / 58 % acetonitrile / 19 % methanol (V/V) at a flow rate of 1.5 ml/min. The compounds were detected by UV-absorption at 214 nm.

8-16 min: Eight min after start of the analytical run the switching valve was reset, 16 min later, the next sample was processed.

The clean-up column was replaced after having processed about 80 plasma or serum samples.

Interferences

Drugs that are frequently used in combination with T were checked for interferences. The following drugs were included: tricyclic antidepressants and metabolites, maprotiline, paroxetine, fluvoxamine, and moclobemide. The neuroleptics haloperidol, perazine, chlorprothixene, and risperidone were included. Moreover, the tranquilizers diazepam, flunitrazepam, and lorazepam, and the anti-parkinsonian drug biperidene were tested.

Calculations

Peak heights versus nominal concentrations of the calibration samples were submitted to weighted ($1/y^2$) linear regression to calculate correlation coefficients, slopes and intercepts. Concentrations of drugs in patients' samples or QCs were calculated from the regression lines.

Precision and accuracy were determined from 5 replicate runs of QCs for assessment of intraassay variability and by analyses of QCs on different days (at least 10 for each concentration) for assessment of interassay variability.

Recovery was determined by comparing the peak heights of standard samples containing three different concentrations (20, 100, and 250 ng/ml; N=5 for each

concentration) by processing the samples without and with inclusion of column-switching and a clean-up precolumn.

RESULTS

Chromatographic separation and inferences

The chromatographic conditions described here provided baseline separation of the analytes within less than 20 min including sample purification on the clean-up column. No interferences were detectable either with plasma or serum constituents or with suggested trimipramine metabolites as shown in Fig.1. Both, T and DT were also well separated from most drugs that were tested for interference. The only interference observed was 8-hydroxy-N-desmethylclomipramine which was not separated completely from DT.

Recovery

The recovery rates of T were 79 ± 5, 78 ± 6, and 85 ± 9% for 20, 100, and 250 ng/ml, respectively (N = 5). The recovery rates for DT were 100 ± 6, 89 ± 7, and 99 ± 12& for 20, 100, and 250 ng/ml, respectively (N = 5).

Linearity and Limit of Quantification Limit

Linear regression analyses of calibration curves of serum specimen supplemented with T and DT and measured on 6 independent days revealed linearity between peak heights and concentrations with correlation coefficients always greater than 0.99 for both, T and DT. Concentrations as low as 10 ng/ml could be quantified with a mean CV< 20% (N = 6) for both analytes.

Precision and accuracy

Table 1 shows precision and accuracy data according to the determination of within-run variabilities and between-run variabilities which ranged from 3 to 15% and from 0.4 to 6.8%, respectively.

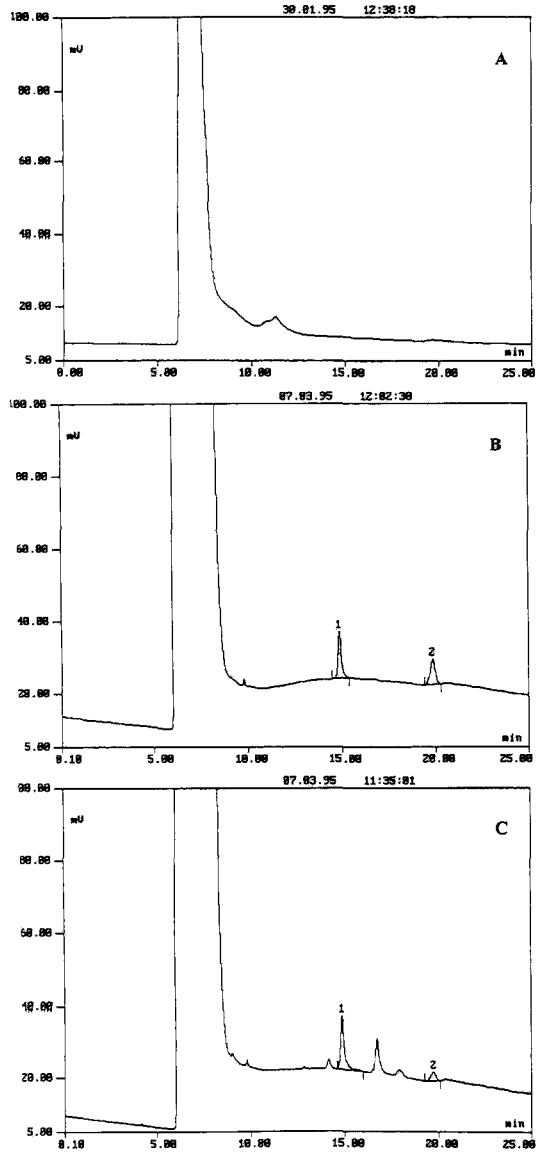


Figure 1. Representative chromatograms of serum samples analysed by the described column-switching HPLC system. (A) Blank serum obtained from a nonmedicated healthy volunteer; (B) blank serum supplemented with 100 ng/ml of each, trimipramine (1) and desmethyltrimipramin (2); (C) serum obtained from a patient treated for two weeks with daily oral doses of 100 mg trimiprmine. The calculated concentrations in sample C were 113 ng/ml for trimipramine and 37 ng/ml for desmethytrimipramine.

TABLE 1

Assay reproducibility and accuracy according to the analysis of quality control samples.

Intraassay precision and accuracy

Analyte	Nominal Conc. (ng/ml)	Precision (%)	Inaccuracy (%)	N
Trimipramine				
	20.00	10.8	-6.8	5
	100.0	6.1	+2.4	5
	250.0	4.9	-5.4	5
N-Desmethyltrimipramine				
	20.00	7.1	+3.6	5
	100.0	3.9	+4.1	5
	250.0	3.1	+2.7	5

Interassay precision and accuracy

Analyte	Nominal Conc. (ng/ml)	Precision (%)	Inaccuracy (%)	N
Trimipramine				
	20.00	15.1	+2.5	10
	100.0	11.2	+1.2	11
	250.0	10.6	+0.4	10
N-Desmethyltrimipramine				
	20.00	11.5	+2.3	11
	100.0	6.1	+0.7	10
	250.0	8.4	+1.5	10

TABLE 2

Resulting T and DT plasma or serum levels of patients treated with different doses of T.

Pat. No.	T-dose (mg/day)	Co-medication	T conc. (ng/ml)	DT conc. (ng/ml)
1	175	no/	87	<10
2	150	haloperidol.lorazepam	219	27
3	100	paroxetine, fluvoxamine, lorazepam	297	51
4	200	risperidone	90	7
5	100	paroxetine	38	14
6	100	no	113	37
7	150	perazine	293	319
8	100	fluvoxamine, clomipramine, carbamazepine	274	<10
9	150	no	343	517
10	200	moclobemide	128	38
11	25	clomipramine, haloperidol, lorazepam	42	<10
12	100	no	20	<10
13	150	no	63	27
14	200	risperidone, biperidene	82	12
15	100	no	123	347
16	50	moclobemide	148	46
17	125	diazepam, chlorprothixene	102	29
18	150	perazine, diazepam	250	195
19	50	moclobemide, maprotiline	265	76
20	100	no	153	289
21	75	paroxetine	80	265

Analysis of patients' plasma or serum

The analyses of plasma or serum samples from 21 patients who were treated for at least 7 days with different oral doses of T revealed highly variable blood levels without obvious correlations between blood level and the given dose (table 2). Seven of these patients received T monotherapy while all other patients had concomitant medications.

DISCUSSION

The method described were seemed advantageous over methods described by others [6-11]. The column-switching technique allowed fully automated processing of plasma or serum samples. Determinations could be accomplished within 20 min. The assay yielded sufficient precision, accuracy and sensitivity. It can therefore be applied to pharmacokinetic studies and also to therapeutic monitoring of patients under T therapy.

Analysis of patient samples indicated poor correlation between the given dose and the resulting blood concentrations. This was in agreement with findings reported for other tricyclic antidepressants [12, 13]. However, the poor correlation might also have been due to the co-medication with other drugs. Some comedications are suggested to interact with the metabolism of T [14]. Nevertheless, variabilities in the metabolic capacities for degradation of T must also be considered [15].

In conclusion, the described HPLC method established for the determination of T and its N-demethylated metabolite in plasma or serum is to our knowledge the first fully automated method that enables a reliable and sensitive determination of these two compounds without time-consuming sample pre-purification. The method enabled measurements of therapeutically relevant T and DT blood concentrations. It is suitable for pharmacokinetic studies and may also be helpful to investigate suggested drug interactions between T and other psychotropic drugs [14].

ACKNOWLEDGEMENTS

This research project was supported by the Stiftung Rheinland-Pfalz fuer Innovation (grant 836-386261/51). We thank Dr. W. Fischer, Rhone-Poulenc (Köln, Germany) and the pharmaceutical companies for supplying us with pure drug substances.

REFERENCES

1. Gross, X. Xin, and M. Gastpar, Neuropharmacol. 30: 1159-1166 (1991)

2. Hauser, H.-R. Olpe and R.S.G. Jones, Eur.J.Pharmacol. 111: 23-30 (1985)

3. Sulser, In Usdin et al (Eds.) Frontiers in biochemical and Pharmacological Research in Depression Raven Press, New York pp 249-261 (1984)

4. Duncan, I.A. Paul, K.R. Powell, J.B. Fassberg, W.E. Stumpf and G.R. Breese, J. Pharmacol. Exp. Ther. 248: 470-77 (1989)

5. Eikmeier, K. Muszynski, M. Berger and M. Gastpar, Pharmacopsychiatry 23: 212-214 (1990)

6. Abernethy, D.J. Greenblatt and R.I. Shader, Clin. Pharmacol. Ther. 35: 348-352 (1984)

7. Hatori, E. Takashima, T. Yamada and O. Suzuki, J. Chromatogr. 529: 189-193 (1990)

8. Visser, M. Oosterblos and P. Toll, J. Chromatr. 309: 81-93 (1984)

9. Eap, L. Koeb, E. Holsboer-Trachsler and P. Baumann, Ther. Drug Monit. 14: 380-385 (1992)

10. Gulaid, G.A. Jahn, C. Maslen and J. Dennis J. Chromatogr. 556: 228-233 (1991)

11. Mazhar and S.R. Binder, J Chromatogr. 497: 201-212 (1989)

12. Preskorn, S.H., J. Clin. Psychiatry 54 (Suppl.): 14-34 (1993).

13. Richelson, E., May Clin. Proc. 69: 1069-1081 (1994).

14 Seifritz, Holsboer-Trachsler, E., Hemmeter, U., Eap, C.P. and Baumann, P. Europ. Neuropsychopharmacol. 4: 15-20 (1994)

15. Bolaji, O.O., Coutts, R.T. and Baker, G.B., Res. Comm. Chem. Pathol. & Pharmacol. 82: 111-120 (1993).

Received: April 19, 1995
Accepted: May 9, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(17), 3507-3517 (1995)

SEPARATION AND DETERMINATION OF SOME CORTICOSTEROIDS COMBINED WITH BAMIPINE IN PHARMACEUTICAL FORMULATIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

J. E. KOUNTOURELLIS[1]*, C. K. MARKOPOULOU[1],
K. O. EBETE[1], AND J. A. STRATIS[2]
[1]*School of Pharmacy*
[2]*School of Chemistry*
Aristotelian University
54006 Thessaloniki, Greece

ABSTRACT

A simple and reliable h.p.l.c. method for the determination of bamipine, hydrocortisone, dexamethasone, betamethasone and beclomethasone has been developed. The procedure, which simultaneously resolves all five compounds, could be employed for the analysis of each component in different pharmaceutical formulations. Chromatographic separation was accomplished under isocratic conditions using a Bondapak 10 μm, C_{18} column 250x2.1 mm and a mobile phase of acetonitrile: water (48:52) and 0.65% acetic acid pumped at a rate of 1 ml/min. Column effluent was monitored with an U.V. detector at 251 nm. The behaviour (k' values) of the compounds were also investigated under different chromatographic conditions. The compounds were eluted in the range from 2.54 to 8.78 mins. Linearity, reproducibility and recovery (% of labelled amount) were satisfactory for all compounds. The method has been successfully applied to the analysis of ointment, cream, gel and lotion.

Copyright © 1995 by Marcel Dekker, Inc.

INTRODUCTION

Antihistamines are one of the most widely used group of drugs. They are formulated as single-components or combined with other drugs with different pharmacological actions. Bamipine is an antihistamine chemically defined as N-benzyl-N-(1-methyl-4-piperidyl) aniline. It is used topically to treat allergic dermatitis and pruritus caused by insect sting and similar conditions.

On the other hand, corticosteroids which are also applied topically are usually the most helpful in relieving both the inflammation and associated pruritus. Although potent topical corticosteroids may be effective when less potent corticosteroids are inadequate, the risk of adverse reactions is increased. In some cases, it is claimed that combination of a corticosteroid with an antihistamine requires considerably less amount of the former whereas the therapeutic result is the same as when corticosteroid is used as a single therapeutic compound.

There are only a few reports on the determination of hydrocortisone (1-6), dexamethasone (6-7), beclomethasone (9), betamethasone (6.8) and the antihistamine bamipine (10-12) in pharmaceutical formulations or in the bulk drug using HPLC. None of these methods is suitable for the determination of every single compound or their combination with bamipine: In the proposed HPLC method, each drug can be analysed as a single component or in mixtures with bamipine using the same chromatographic system. The method is simple, fast and is applied easily to the determination of the drugs in oitments, creams, gels and lotions.

EXPERIMENTAL

A. Apparatus

A Perkin Elmer Series 3B high performance liquid chromatography equipped with two reciprocating pumps controlled by a microcomputer, a Reodyne 7010 20 µl loop injector valve and a LC 75 UV spectrophotometric detector with a single-beam variable wavelength system was used. The spectrophotometer was operated at 0.04 Absorbance Units Full Scale (AUFS). The use of a higher sensitivity was unnecessary for these determinations. The

spectrophotometer was insensitive to flow noise and to changes in the refractive index of the solvents. The chromatographic peaks were recorded by employing a LKB 2210 Bromma potentiometetric recorder connected to the spectrophotometer, with an operating voltage of 10 mV and chart speed of 1 mm/min.

The analytical column was a Bomdapak C_{18} particle size 10 µm, 250 x 2.1 mm I.D. stainless steel. The column was equilibrated with mobile phase therefore when a stable line was achieved the standard and sample solutions were injected onto the column. A flow rate of 1 ml/min eluted the compounds in the range from 2.54 to 8.78 as illustrated in Table I. The wavelength was set at 251 nm.

B. Mobile phase

The mobile phase consisted of acetonitrile : water, 48:52 and 0.65% CH_3COOH (pH 3.18). It was degassed by vacuum filtration through a 0.2 µm Sartorius S 11 807 polytetrafluoroethylene membrane filter while the flask was in an ultrasonic bath.

C.Chemicals:

HPLC grade acetonitrile, water (acetonitrile Chromasolv^R, wasser G Chromosolv^R Riedel-de Haen) and acetic acid glacial (Merck).

The standards hydrocortisone-21-acetate, betamethasone-17-valerate, beclomethasone dipropionate and dexamethasone were purchased from Sigma Chemical Company. Bamipine lactate was kindly donated by Knoll (Ludwigshafen, W. Germany). The concentrations of the standard solutions are presented in Table II.

D.Sample Preparation

The main source of problems in sample preparations is related to the quantitative recovery of the active ingredients from single or multicomponent preparations.
In pharmaceutical formulations like creams, ointments, gels and lotions, sample preparation by extraction can be a serious problem because of sometimes poor recovery of compounds

TABLE I

High Performance Liquid Chromatographic characteristics of the drug separation

Compound	$t_{R(min)}$	k'*	R_s
Bamipine Lactate	6.83	2.42	-
Hydrocortisone-21-Acetate	2.54	0.29	5.66
Dexamethasone	3.67	0.83	3.45
Betamethasone-17-Valerate	5.17	1.58	1.80
Beclomethasone Dipropionate	8.78	3.39	1.44

t_0=2.00 min

to be analysed. Creams are viscous liquid or semisolid emulsions of oil-in-water type. Similarly ointments are generally more viscous based on either hydrocarbon or water-soluble excipient mixtures. Therefore most methods applied in the analysis of creams and ointments involve liquid-liquid extraction to separate the drug component from fatty-base components. Usually a dispersion of the sample in methanol is extracted with a solvent of very low polarity, such as cyclohexane. Alternatively from the polar constituents, an aqueous suspension of the sample is extracted with mostly chloroform.

Sample also are dissolved in a suitable solvent mixture and portions of the solution are analysed directly. The most convenient solvent with the ability of dissolving the active ingredients is ethanol. However, it is neccessary to combine it with other solvents to achieve complete dissolution of both the excipients and the active compounds, The use of a precolumn provides a good clean up of the multicomponent samples since the non-polar excipients are strongly retained on the pre-column relative to the drug substances.

After each day experiments, only the precolumn was flushed with methanol-tetrahydrofurane (75:25) at 1.0 ml/min flow for at least 30 minutes.

TABLE II

Concetration range and "linear regression and correlation data" of calibration curves for the compounds determined at 251 nm

Compound	Concentration µg/ml*	Peak Heights mm*	Intercept	Slope	r
Bamipine Lactate	4.65-16.26	32.0-115	0.89	7.07	0.9990
Hydrocortisone-21-Acetate	2.75-9.61	35.0-124	-0.91	12.91	0.9999
Dexamethasone	5.95-20.83	5.95-145	2.93	6.83	0.9998
Betamethasone-17-Valerate	6.76-23.65	6.76-112	0.21	4.74	0.9987
Beclomethasone Dipropionate	14.56-50.96	14.56-126	-1.79	2.51	0.9980

* Mean of four replicates

Ointments:

Ointments are monophasic, semi-solid often anhydrous greasy preparations sometimes consisting mainly of polyethylene glycol. Therefore 1 part of ethanol was combined with 3 parts of pentane to dissolve a suitable amount of ointment in a volumetric flask. The mixture was sonicated in a water bath for 20 minutes. An aliquot of this solution was then transferred into 100 ml flask and made to volume with ethanol. Because some particles were formed from the fatty excipients the solution was left for some time to precipitate. From the clear supernatant solution a portion was certifuged and filtered, then injections were made onto the column.

Creams:

Creams are semi-solid dermatological products consisting of two immiscible phases, one of which is dispersed in the other. Usually the continuous phase is the more polar one, that is, the emulsion is of the type o/w. Therefore a mixture of water: ethanol:tetrahydrofurane 50:25:25 was used for the dispersion of the excipients together with the active ingredients of the formulation. Water and ethanol were used to dissolve the external hydrophylic phase whereas tetrahydrofuran together with ethanol dissolves the internal phase and the active ingredients. Since tetrahydrofurane and water are immiscible, ethanol also helps to mix them fully.

Therefore a certain amount of cream was weighed in a volumetric flask, solvent mixture was added and stirred for 15 minutes at 40°C using magnetic stirrer. The whole mixture was made to volume and cooled in an ice bath where some fatty excipients were precipitated. From the clear supernatant solution, a certain amount was certifuged and filtered. Then appropriate dilutions were made by using certain amounts from the stock solution.

Lotions:

The same process of sample preparation was used for lotions as it is described for creams.

Gels:

The sample was dissolved in ethanol, ultrasonicated in water bath, then filtered and injected directly onto the chromatographic column.

RESULTS AND DISCUSSION

The optimum chromatographic conditions were investigated by varying the tiny amount of acetic acid added in the mobile phase. Figure 1(A) shows that by varying the amount of acetic acid present in the mobile phase, there was a dramatic change in the k' value of bamipine whereas there was no considerable, change in the k' values of the rest of the compounds. However the presence of acetic acid was essential for the elution of bamipine in reasonable time compared with the rest of the corticosteroids analysed. Similarly the amount of water present in the mobile phase was also studied. It was also observed that proportion of water in the mobile phase greater than 45% resulted in longer retention time which also reflected in greater k' values. These are illustrated in Figure 1(B).

The retention time of bamipine together with the rest of the corticosteroids were found to be reproducible under the present chromatographic conditions for a period over twenty days, which makes the method most suitable for screen testing. A typical HPLC chromatogram showing a simultaneous complete separation of the drugs used in different pharmaceutical formulations together in spiked placebos is illustrated in Figure 2.

The resolution factors R_s, were calculated between the chromatographic peak of bamipine and each separate peak of the rest of corticosteroids from the equation $R_s = 2(t_2 - t_1)/W_1 + W_2$, where t_1, t_2 are the retention times of the two peaks and W_1, W_2 are the peak widths at the base of the two respective peaks. The resolution R_s was more than 1.10, signifying complete separation between bamipine and each corticosteroid drug. The numerical values of the parameters R_s, t_R and k' are shown in Table I.

Calibration graphs were constructed of peak height versus concentration. The linear regression equations and correlation coefficients showed that the method is linear. The characteristics of the regression equations are presented in Table II.

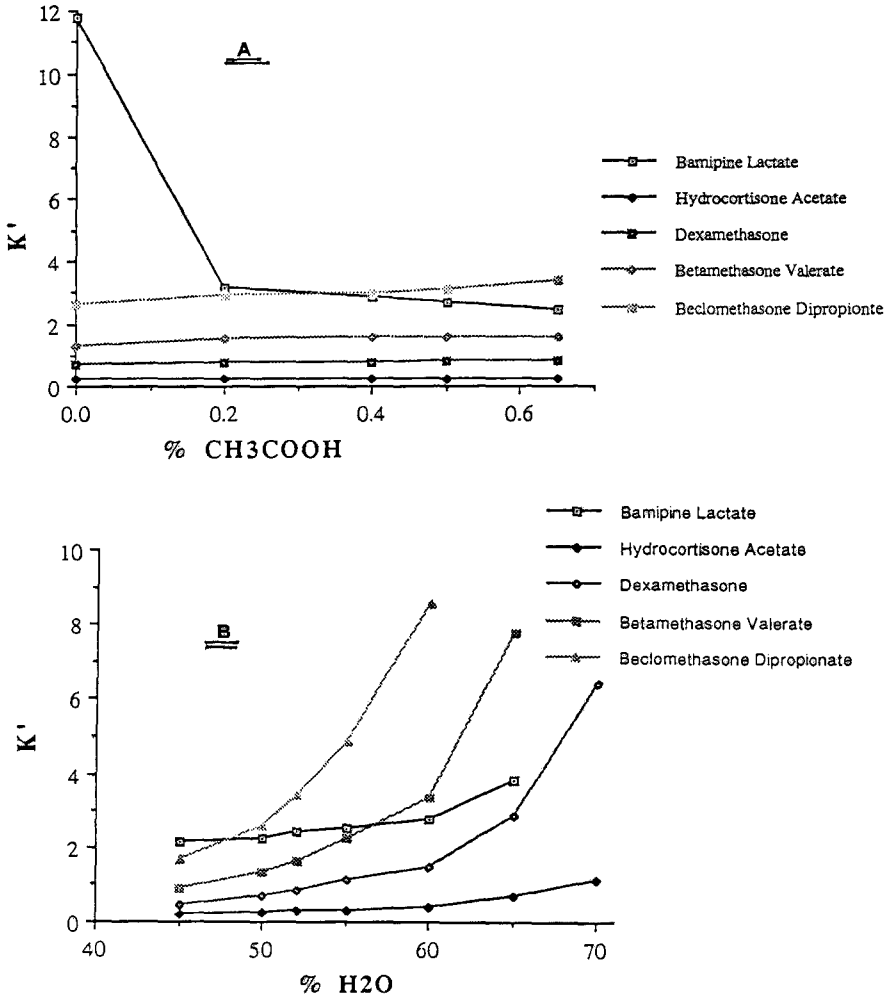


Figure 1. The relationship between (A) the percentage of acetic acid and (B) the water content in the mobile phase and the capacity ratio (k') of the compounds analysed by h.p.l.c.

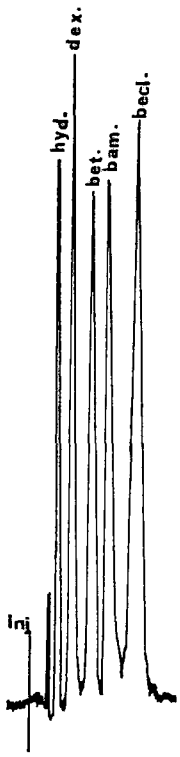


Figure 2. High Performance Liquid Chromatography of the simultaneous separation of hydrocortisons (hyd) 8.24 µg/ml, dexamethasone (dex) 17.86 µg/ml, betamethasone (bet) 20.27 µg/ml, bamipine (bam) 13.94 µg/ml and beclomethasone (becl) 43.68 µg/ml. The retention times are presented in Table I.

The results of the quantitations of bamipine, hydrocortisone, dexamethasone, betamethasone and beclomethasone in pharmaceutical formulations and in spiked placebos are shown in Table III. These are in agreement with the labelled amount. No noticeable interference from the excipients was observed in the chromatograms. The coefficient of variation was in the range 1.21 - 2.45.

TABLE III

Results of analysis of six drugs present in pharaceutical formulations

Pharmaceutical Formulation	Active Ingredients	Labelled Amount*	HPLC Resultes**	Coefficient of Variation % C.V	% Found
Cream	Bamipine Lactate	20.00	20.02	1.24	100.90
	Hydrocortisone-21-Acetate	2.50	2.41	2.08	96.34
Cream	Hydrocortisone-21-Acetate	10.00	9.84	2.36	98.44
Gel	Bamipine Lactate	20.00	20.87	1.48	104.35
Lotion	Dexamethasone	0.25	0.23	2.14	94.16
Ointment	Betamethasone-17-Valerate	10.00	10.35	2.45	103.45
Spiked Placebos	Bamipine Lactate	20.00	19.97	1.65	99.85
	Beclomethasone dipropionate	2.50	2.56	1.21	102.67

* mg/g

** Mean of four replicates

CONCLUSION

In conclusion, the results of the present study show that the proposed HPLC method is an efficient and reliable means of quantitating bamipine and some corticosteroids in pharmaceutical formulations. The active ingredients can be determined either as a single component or in combination with bamipine.

ACKNOWLEDGEMENT

The authors would like to thank Mr A.E. Tsolakopoulos for his technical assistance.

REFERENCES

1. Van de Vaart, F.J., Indemans, A.W.M., Hulshoff, A. and Lake, O.A., Chromatographia 16, 247-250, 1982.
2. Rego, A. and Neslon, N., J. Pharm. Sci., 71(11) 1219-1233, 1982.
3. Conley, D.L., Int. J. Pharm. 13(2) 205-217, 1983.
4. Phoon, K.W. and Stubley, C., J. Chromatogr. 246(2), 297-303, 1982.
5. Ezzedeen, F.W., Stohs, S.J. and Masoud, A.N., J. Pharm. Sci., 72(9), 1036-1039, 1983.
6. Pryce-Jones, R.H., Carr, B.E., Hossein, A.M., J. Pharmacol., 39(Suppl.) 47P, 1987.
7. NG,L.L., J. Assoc. Off. Anal. Chem., 70(5), 829-833, 1987.
8. Raju, N.R., Pramila, C. and Pai, A., Indian Drugs 26(8), 425-429, 1989.
9. Mulholland, M., Rudd, D.R., Anal. Proc., 22(5), 140-141, 1985.
10. Kountourellis, J.E., Markopoulou, C.K., Georgakopoulos, P.P., J. Chromatogr., 502, 189-192, 1990.
11. Kountourellis, J.E., Markopoulou, C.E., Georgakopoulos, P.P., Analytical Letters, 23(5), 883-891, 1990.
12. Kountourellis, J.E., Markopoulou, C.K., J. Liquid Chromatogr., 14(15), 2969-2977, 1991.

Received: April 20, 1995
Accepted: May 11, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(17), 3519-3527 (1995)

DETERMINATION OF CHLORAMPHENICOL RESIDUES IN MEAT SAMPLES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

I. CANIOU[1], E. NIKOLAIDES[1], H. TSOUKALI[3], J. A. STRATIS[2]*, AND G. A. ZACHARIADIS[2]

[1]Institute of Food Hygiene
Ministry of Agriculture
Thessaloniki, Greece
[2]Laboratory of Analytical Chemistry
Department of Chemistry
Aristotole University of Thessaloniki
Thessaloniki 54006, Greece
[3]Laboratory of Forensic Medicine and Toxicology
Faculty of Medicine
Aristotole University of Thessaloniki
Thessaloniki 54006, Greece

ABSTRACT

The results of a comparative study of chloramphenicol (CAP) determination in meat, by high performance liquid chromatography are presented. A spectrophotometrical detector at 278 nm was used for measurement of CAP absorbance and subsequent quantitative determination. Two different mobile phases, were tested. The first phase consisted of acetonitrile - water (30:70 v/v) and the second of acetonitrile - di-ammonium hydrogen phosphate 0.005M (25:75 v/v). The two procedures were compared by means of the retention time and the sensitivity achieved. Recoveries of CAP from meat samples ranged between 63-79 %. The detection limits for the two procedures were 14.1 $\mu g \cdot kg^{-1}$ and 18.0 $\mu g \cdot kg^{-1}$ respectively.

Copyright © 1995 by Marcel Dekker, Inc.

INTRODUCTION

Chloramphenicol (CAP D(-)threo-2,2-dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl) ethyl] acetamide, CAS No. 56-75-7) has a molecular weight of 323.1 and a chemical structure described in figure 1 (the molecular formula $C_{11}H_{12}C_{12}N_2O_5$).

It is a broad-spectrum antibiotic, with clinical applications similar to tetracyclines, sulfonamides, nitrofurans etc. It acts as an inhibitor of aminoacids incorporation in the peptides, because of its greater affinity to bound with microbe ribosomes (1).

During the period of 1950-1980 it was extensively used in animal disease treatment, specially in respiratory or intestinal infections, caused by various microbes such as *Salmonella typhosa, Hemophilus influenzae, Streptococcus pneumoniae, Staphylococcus epidermidis* and *Staphylococcus aureus*. Its use in meat-, milk- or egg-producing animals was banned in USA (1984) and in European Union (1986) (2,3) because of its toxicity to humans. However, it is still one of the therapeutic agents of last resort for some pathogens with resistance to other antimicrobial drugs (4).

Chloramphenicol may be very toxic to humans and should not be used for any purpose that might result in the presence of its residues in food for human consumption. Some of the undesirable effects it causes to humans are blood dyscrasias, erythropenia, thrombocytopenia, aplastic anaemia and in higher doses (>75 mg $\cdot$ kg^{-1} $\cdot$ day^{-1}) the so called "gray syndrome" (1, 5).

Residues of CAP in edible meat products must be lower than 10 $\mu g \cdot kg^{-1}$, according to EC directive 675/92 (6), but in the near future, almost zero tolerance levels must be in force, following the development of more sensitive analytical methods.

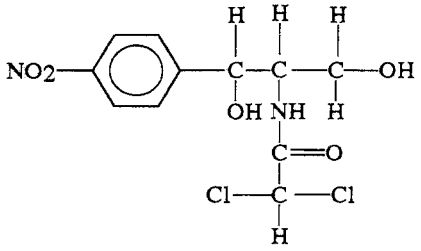


FIGURE 1. Chemical structure of chloramphenicol (CAP)

Because of its special clinical interest, various methods have been developed, based on almost all types of chromatography (GC, LC, TLC), with sufficient recoveries (7-10). The detection limits of these methods are strongly depended on the chromatographic system applied i.e. the mobile and stationary phase, detectors, flow characteristics, etc. The range of detection limits for GC and LC methods is 1-10 $\mu g \cdot kg^{-1}$ (11-15) while for TLC the level of conentrations is higher, 10-200 $\mu g \cdot kg^{-1}$ (16, 17). The sensitivity is significantly improved by the use of electron capture or fluorescence detectors.

In this work, high performance liquid chromatography was applied for the analysis of chloramphenicol in pig and ovine meat. A spectrophotometrical detector was used for measurement of CAP absorbance at λ_{max}=278 nm and subsequent quantitative determination. Two different mobile phases were tested. The first phase (A) consisted of acetonitrile - water (30:70 v/v) while the second (B) of acetonitrile - di-ammonium hydrogen phosphate 0.005M (25:75 v/v). The two procedures were compared by means of the retention time and the sensitivity achieved.

MATERIALS AND METHODS

Reagents and Solvents

Chloramphenicol (Sigma, C-0378) was used without further purification. CAP stock solution was prepared by dissolving 100 mg of CAP in 100 ml methanol and the calibration standard solutions were prepared by diluting the stock solution in double distilled water.

Meat samples (5g) were treated and extracted to ethyl acetate (Merck, p.a.) according to the sample preparation procedures published in the frame of BCR programme, by the Commission of the European Communities [18]. A Moulinette meat homogenizer, a vortex mixer and a table centrifuge (Heraeus) were also used for sample preparation and extraction procedures. Two sequential extractions were performed, and the combined extracts were evaporated to almost dryness in a rotary evaporator. The residues were reconstituted by a mixture of hexane-chloroform (50:50 v/v), and then by water. After stirring and centrifuging, the supernatant liquid was injected to the valve.

HPLC grade acetonitrile (Merck) and ammonium dihydrogen phosphate (Merck, p.a.) were used for the preparation of the eluents. Water was purified by demineralization (conductivity <1 µS/cm). These eluents were filtered through 0.22 millipore membrane filters (47 mm diameter) and degassed in ultrasonic bath prior to their use. The two mobile phases prepared as eluents were: (A) acetonitrile - water (30:70 v/v) and (B) acetonitrile - 0.005M di-ammonium hydrogen phosphate (25:75 v/v).

Apparatus and Conditions

The above phases were tested using the following analytical instumentation, respectively:

A) Liquid chromatograph GILSON model 303 (isocratic) combined with a GILSON UV-Vis detector at 278 nm (λ_{max} of CAP).

The analytical column was a Lichrospher RP-18, 250X4 mm, and 5 μm particle size. The eluent flowrate was 1 $ml \cdot min^{-1}$.

B) Liquid chromatograph JASCO 880-PU (isocratic), combined with a JASCO 870 UV-Vis detector at 278 nm.

The analytical column was also a Lichrospher RP-18, 250X4 mm, and 5 μm particle size. The eluent flowrate was 1 $ml \cdot min^{-1}$.

RESULTS AND DISCUSSION

Series of typical chromatograms of chloramphenicol, obtained during calibration study with the two mobile phases are given in figures 2 and 3.

CAP is eluated in significantly smaller retention times with mobile phase A (3.8 min) than with mobile phase B (10.5 min), because the latter contains ammonium dihydrogen phosphate and is more polar than the first. The rest of the peaks are due to elution of other substances with similar pK to CAP, but they don't interfere with its determination.

The sensitivities achieved with the above two procedures were compared by means of the calibration curves obtained for a concentration range of CAP 0.5-5 $mg \cdot kg^{-1}$. The calibration curves obtained for the two procedures are described by the intercepts and

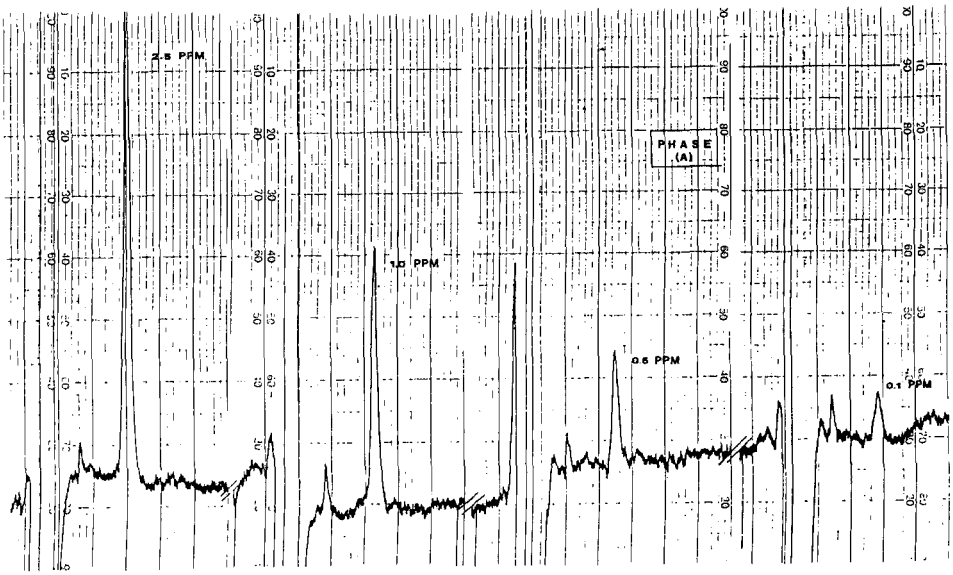


FIGURE 2. Series of typical chromatograms of chloramphenicol, obtained during calibration study with mobile phase (A) acetonitrile - water, (30:70 v/v). Attenuation 2mV, recorder chart speed 1 $cm \cdot min^{-1}$.

slopes, calculated by the regression analysis and given in Table 1. According to these results, better sensitivity (b=8.05) was gained by mobile phase A, than by method B (b=5.52).

This, together with the fact that the retention time for the elution with mobile phase A (acetonitrile-water) is significantly smaller, leads to the conclusion that this is the preferred procedure for a rapid and sensitive determination.

The reproducibilities of the two methods are comparable, and they are expressed by a relative standard deviation of 17%, for six succesive determinations of a standard solution at the 1.0 mg l^{-1} concentration level. The detection limits of the two methods

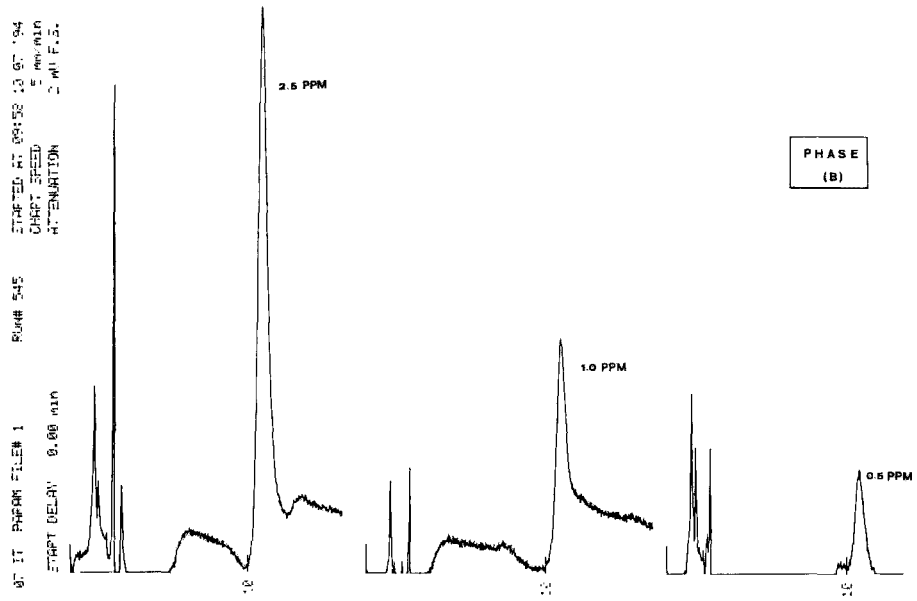


FIGURE 3. Series of typical chromatograms of chloramphenicol, obtained during calibration study with mobile phase (B) acetonitrile - di-ammonium hydrogen phosphate 0.005 M, (25:75 v/v).
Attenuation 2mV, recorder chart speed 0.5 $cm \cdot min^{-1}$.

TABLE 1.

Regression Analysis Data for CAP Determination with Two Different Mobile Phases.

Mobile phase	Slope b	Inter-cept a	Estimation error	Correlation coefficient r	Squared coefficient r^2
A	8.05	+0.05	0.71	0.98	0.96
B	5.52	-0.21	1.36	0.96	0.93

TABLE 2.

Recovery Study of Chloramphenicol from Fortified Meat Samples.

Sample	CAP added (ng)	CAP found (ng)	Recovery (%)
1	300	235	78.3
2	300	230	76.6
3	200	140	70.0
4	200	135	67.5
5	150	100	66.6
6	150	95	63.3

calculated as the mean of six determinations of control samples with negligible CAP concentration ± three times the standard deviation of the six results, are 14.1 $\mu g \cdot kg^{-1}$ and 18.0 $\mu g \cdot kg^{-1}$ respectively.

In Table 2, the results of the recovery study from fortified meat samples are listed. Recoveries between 63-79 were calculated using mobile phase A. The corresponding recoveries for method B are slightly higher than these and could be further improved by increasing the ratio of acetonitrile in the eluent mixture.

REFERENCES

1. H.M. Feder, Jr, C. Osier and E.G. Maderazo, Rev. Infect. Dis., **3:** 479-491 (1981)

2. Food and Drug Administration clamps down on chloramphenicol use in food animals, J. Am. Vet. Med. Assoc., 184: 1041 (1984)

3. Comite des Medicaments Veterinaires, Groupe de travail pour "Securite des residus", 2e reunion, Bruxelles, (1986)

4. A.G. Grilman, L.S. Goodman and A. Grilman, Goodman and Gilman: The Pharmacological Basis of Therapeutics, 6th Ed. MacMillan Publishing Co. Inc., New York, NY, (1980)

5. H.C. Meissner and A.L. Smith, Pediatrics, 64: 348-356 (1979)

6 . Commission of the European Communities, 675/92

7. E.H. Allen, J. Assoc. Off. Anal. Chem., 68: 990-999 (1985)

8. J.M. Degroodt, B. Wyhowski de Bukanski, J. De Groof, H. Beernaert and S. Spebrnik, J. Liq. Chrom., 15: 2355-2371 (1992)

9. V.M. Moretti, C. van de Water and N. Haagsma, J. Chrom., 583: 77-82 (1992)

10. C. van de Water, D.Tebbal and N. Haagsma, J. Chrom., 478: 205-215 (1989)

11. G. Samouris, Ph.D.Thesis, Aristotle University, Thessaloniki (1990).

12. R. Malisch, U. Sandmeyer and K. Kypke-Hutter, Lebensmittelchem. Gerichl. Chem., 38: 11-12 (1984)

13. H. Holtmannspotter, H.P. Thier, Deutsch. Lebensm. Rundsch., 78: 347-350 (1982)

14. M. Becheiraz, A. Haldemann and R. Etter, Mitt. Geb. Lebensmittelunters Hyg., 74: 147-155 (1983)

15. M. Petz, Z. Lebensm. Unters Forsch., 176: 289-293 (1983)

16. I. Karkocha, Chem. Abstr., 89: 127809p (1978)

17. I. Karkocha, Chem. Abstr., 91: 37562q (1979)

18. Commission of the European Communities, Agriculture, Veterinary Drug Residues, Residues in Food-Producing Animals and their products: Reference Materials and Methods, Edited by R.J. Heitzman, Brussels pp. 153-172 (1992)

Received: April 21, 1995
Accepted: May 19, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(17), 3529-3543 (1995)

HPLC DETERMINATION OF CHLORAMPHENICOL AND THIAMPHENICOL RESIDUES IN GAMEBIRD MEATS

A. M. DI PIETRA[1], V. PIAZZA[2], V. ANDRISANO[1], AND V. CAVRINI[1]
[1]*Department of Pharmaceutical Sciences*
University of Bologna
via Belmeloro 6
40126 Bologna, Italy
[2]*Institute of Avian Pathology*
University of Bologna
via Tolara di Sopra 30
40064 Ozzano Emilia, Italy

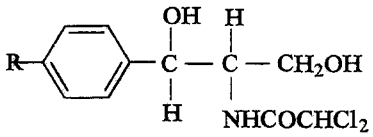
ABSTRACT

Liquid chromatographic methods for the determination of chloramphenicol (CAP) and thiamphenicol (TAP) residues in gamebird (pheasant, mallard and quail) meats were developed. The drugs were extracted from the homogenised tissues (muscle and liver) by water and the extracts were purified by subsequent partitions with Chem Elut CE 1020 cartridge - ethyl acetate and water - toluene. The recoveries were 67% for CAP and 72% for TAP. Chromatographic separations were performed on a Hypersil C-18 column and the peak identification and quantitation was made with a photodiode array detector. Using the first derivative of the chromatographic peak the selectivity of the analysis was improved. The elimination kinetics of CAP and TAP from the quail tissues were also evaluated.

Copyright © 1995 by Marcel Dekker, Inc.

INTRODUCTION

Chloramphenicol (CAP) and Thiamphenicol (TAP) are synthetic broad-spectrum antibiotics which are used by veterinary practice in the prevention and treatment of many bacterial infections (1, 2). The drugs are structurally correlated, the nitrogroup (R= $-NO_2$) in CAP being replaced with a methylsulphonyl group (R= $-SO_2CH_3$) in TAP.



CAP may display toxic side-effects to humans such as irreversible aplastic anaemia (3); these toxic effects have not been observed for TAP. For its toxicity CAP is banned for food producing animals in the United States; in the European Community (EC) the CAP use is prohibited for poultry and it is restricted for other animals. Consequently, appropriate control programs based on a proposed maximum residue level (MRL) of 10 μg CAP/Kg are recommended (4).

Among the various techniques, several liquid chromatographic (HPLC) methods have been proposed for the selective and sensitive determination of CAP residues in meat and fish (4-13, 15, 17, 18,) milk (4, 16, 17) and eggs (14, 15).

For the determination of TAP residues in edible tissues (8, 19, 21) and milk (23) few HPLC methods have been described.

The present study was undertaken to provide HPLC procedures suitable for the determination of CAP residues in gamebird meats whose importation in Italy has turned prominent in the last years. The animal "type" traded (gamebirds

shooted in game reserve but almost always obtained by intensive breeding) and the provenience from countries where the drug use is differently regulated, led us to investigate about a possible presence of CAP residues in the pheasant and mallard meat. The work also included the Japanese quail, a gamebird which is intensively reared in large quantity in hour country. To this end, a reversed-phase liquid-chromatographic (HPLC) method was developed as useful screening method for monitoring CAP and TAP residues in commercial gamebird meats.

Diode array UV-VIS detection was used for the peak purity evaluation and the drug identification. The HPLC method was also applied to achieve informations on the CAP and TAP elimination kinetics from the muscle tissues after the drug administration at therapeutic doses.

EXPERIMENTAL

Chemicals

Chloramphenicol (CAP) was obtained from Fluka (Switzerland); thiamphenicol (TAP) was supplied from Zambon S.p.A. (Italy). Solvents for chromatography were of HPLC grade from Mallickrodt (MO, USA); triethylamine (TEA) and all the other chemicals were from Farmitalia-C. Erba (Italy). Chem Elut CE 1020 cartridges from Analytichem International (USA) were used for liquid-liquid extraction.

Apparatus

Omni-mixer (Sorval Inc., Connecticut, USA), Sonorex super AK 102H (Bandelin, Germany) and Centrifuge 4225 ALC (Italy) were used.

The chromatographic system consisted of a 5020 Varian liquid chromatograph equipped with a Rheodyne 7125 model injector (100 µL sample

loop) and connected to a HP 1040A photodiode array detector (Hewlett-Packard, USA).

The chromatographic separations were performed on a 5 μm RP-18 Hypersil column (150x4.6 mm I.D.) using a mobile phase consisting of 0.05M TEA phosphate buffer (pH 3) - acetonitrile 79:21 (v/v) for CAP and 86:14 (v/v) for TAP at a flow rate of 1 mL /min.

UV detection at 278 and 224 nm was used for the routine analysis of CAP and TAP, respectively.

Sample preparation

Fresh or thawed meat was homogenised with Omni-mixer and an accurately weighed sample (about 10g of muscle and 5g of liver) was subjected to ultrasonication with 40 mL of water at ambient temperature for 8 min. The mixture was then centrifuged for 10 min and 20 mL of the supernatant were applied to a Chem Elut CE 1020 cartridge allowing to equilibrate for 15 min. The drug was then eluted with 50 mL of ethyl acetate and the eluate was evaporated to dryness with a rotavapor. The residue was mixed with 0.5 mL of water and extracted (2x1mL) with toluene (gentle vortex mixing and centrifugation). After careful removal of toluene, the aqueous phase was filtered through a 0.45 μm filter and injected (100 μL) into the LC system.

This general procedure was applied to the analysis of all the samples (Blank tissues, samples fortified with known quantities of CAP and TAP, and real samples).

Assay

Sample solutions and working standard solutions were sequentially subjected to the HPLC analysis according to an alternate order. The content of CAP and

TAP in each sample was calculated by comparison of the analyte peak height of the sample with that of standard solutions equivalent to initial concentrations of 20 μg/Kg for CAP and 80 μg/Kg for TAP.

Calibration graph

Chloramphenicol- Homogenised samples (quail muscle) were fortified with known quantities of CAP to give sample containing 10-75 μg/Kg of the drug. The described sample handling was followed and the peak heights were plotted against the corresponding added drug concentrations to obtain the calibration graphs.

Alternatively, when the first derivative of the chromatographic peak was used, the amplitude of the first satellite peak to the zero line was measured and plotted against the added drug concentration to obtain the calibration graph.

Thiamphenicol - The same procedures described for CAP were applied to homogenised samples (quail muscle) fortified with TAP at the level of 10 -100 μg/Kg.

Each sample solutions for the calibration graphs of CAP and TAP were also compared with the corresponding standard solution in order to evaluate the drug recovery at different concentration levels.

Elimination of CAP and TAP residues

Chloramphenicol- 18 quails were orally dosed with a 0.8 g/L CAP aqueous solution for three days for a mean total dose of 85 mg/Kg. From the fourth day, three quails were daily slaughtered and the breast tissues were subjected to the described extraction and HPLC methods for quantifying the CAP residues. Tissues from control quails and tissues fortified with CAP at 40 μg/Kg level were concurrently analysed.

Thiamphenicol- 14 quails were orally dosed with TAP for three day for a mean total dose of 108 mg/Kg. Then, two quails were slaughtered at different time intervals up to 16th day. The muscle tissues from treated and control quails were then concurrently analysed with tissues fortified with TAP at 80 μg/Kg level.

RESULTS AND DISCUSSION

Sample preparation

For the extraction of CAP or TAP from edible tissues ethyl acetate (7, 8, 19, 21-24), ethyl ether (11), water (4-6, 10, 27), diphasic dialysis (16) and matrix solid-phase dispersion (26) have been used. In this study, to obtain a rapid and practical sample clean-up suitable for a screening method, an aqueous extraction (4-6) was chosen with minor modifications. Briefly, the homogenised tissues was subjected to ultrasonication with water; an aliquot of the aqueous extract was applied to a Chem Elut CE 1020 cartridge and the drug eluition was carried out with ethyl acetate. After the solvent evaporation the residue was subjected to water-toluene partition to remove interfering co-extractants and to give a suitable aqueous sample solution. Ethyl acetate was used instead of dichloromethane (4-6) for the cartridge eluition and this resulted in improved overall recoveries (67%; RSD% = 5.1-8.2) for CAP and acceptable overall recoveries (72%; RSD% = 5.4-9.1) for TAP in the 10 - 75 μg/Kg range. The repeatability, as indicated by RSD%, was decreasing with the drug concentration. When aqueous standard solutions of CAP and TAP were applied to the Chem Elut column, the ethyl acetate elution gave mean recoveries of about 85%; this suggest that considerable analyte losses take place during the cartridge elution.

Chromatography

The described sample clean-up allowed adequate chromatographic separations to be performed under isocratic conditions.

Routine analyses were carried out on 5 μm Hypersil C-18 column using binary mixtures of TEA phosphate solution (pH 3.0) - acetonitrile 79:21 (v/v) for CAP and 86:14 (v/v) for TAP as the mobile phase.

Typical chromatograms obtained from the HPLC analysis of quail, pheasant and mallard tissues fortified at 75 μg/Kg level of CAP are reported in Figs 1, 2 and 3, respectively. In each case, by comparing the sample chromatogram with that from the corresponding control tissues the absence of matrix interferences can be ascertained. UV Diode Array Detection (DAD) was used to check the peak purity and to confirm the analyte peak identity (5, 7). In order to avoid false positive results, three dimensional plots (spectrochromatograms) were obtained, giving direct informations about the peak identity (retention and UV spectrum) and the background interferences.

A 3D plot for TAP is shown in Fig. 4; similar 3D plots for CAP were obtained according to previous reports (11). The described HPLC method with DAD detection enabled a reliable identification of CAP at ≥8 μg/kg level, with a detection limit (S/N = 3) of about 2 μg/Kg. This, should prevent false positives at the proposed tolerance level of 10 μg/Kg. On the other hand, positive samples should be confirmed by approved confirmatory methods, such as GC-Mass spectrometry (4).

Analysis of CAP and TAP residues

For the quantitation of the CAP and TAP residues in the muscle tissues a calibration graph for each drug was constructed. Quail muscle homogenates were

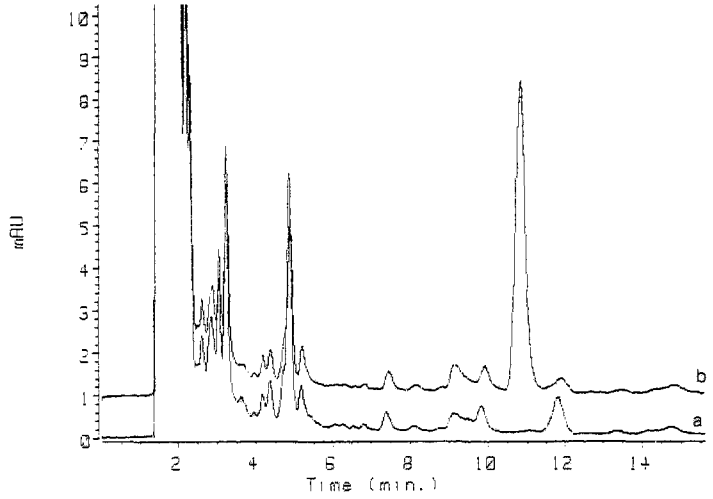


Fig. 1 - HPLC chromatograms obtained from quail muscle tissues: a) control tissue, b) tissue fortified with CAP at 75 µg/Kg level. Chromatographic conditions: 5 µm Hypersil C-18 column (150x4.6 mm I.D.) using a mobile phase of 0.05M TEA phosphate (pH 3.0) - acetonitrile 79:21 (v/v) at a flow - rate of 1 mL/min.

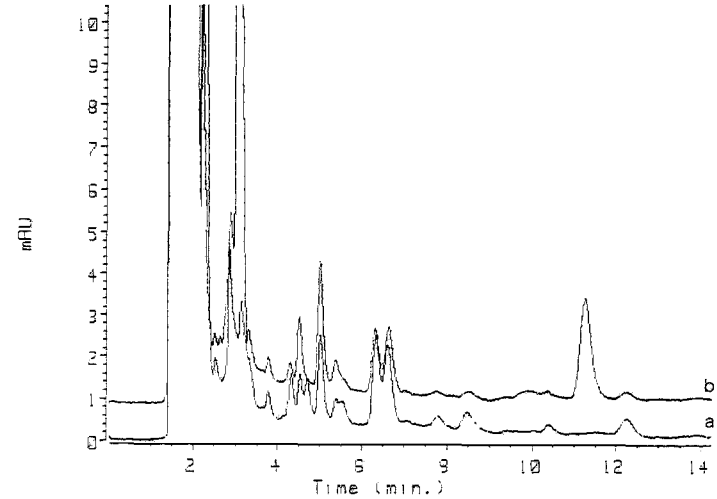


Fig. 2 - HPLC chromatograms obtained from pheasant muscle tissues: a) control tissue, b) tissue fortified with CAP at the level of 75 µg/Kg.
Chromatographic conditions as in Fig. 1.

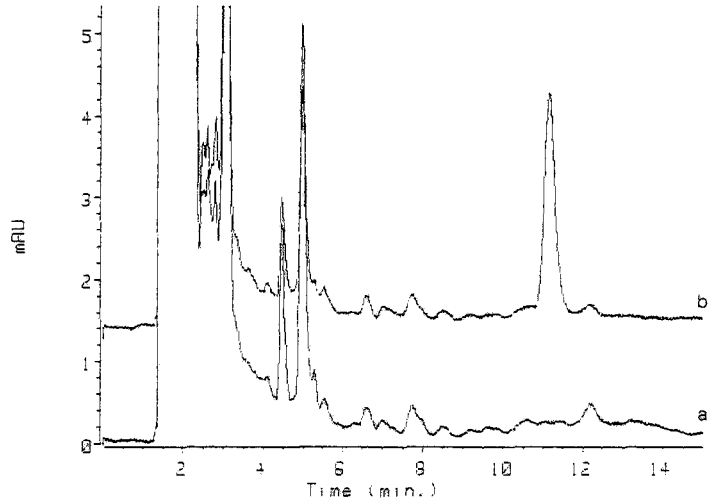


Fig. 3. - HPLC chromatograms obtained from mallard muscle tissues: a) control tissue, b) tissue fortified with CAP at the level of 75 µg/Kg.
Chromatographic conditions as in Fig. 1.

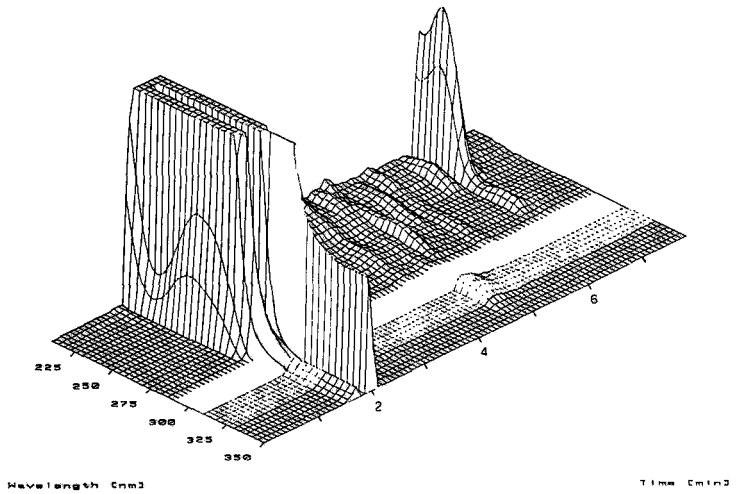


Fig. 4 - Three dimensional plot (spectrochromatogram) of a homogenised muscle tissue from a quail orally treated with TAP. Retention time of TAP: 6.62 min.

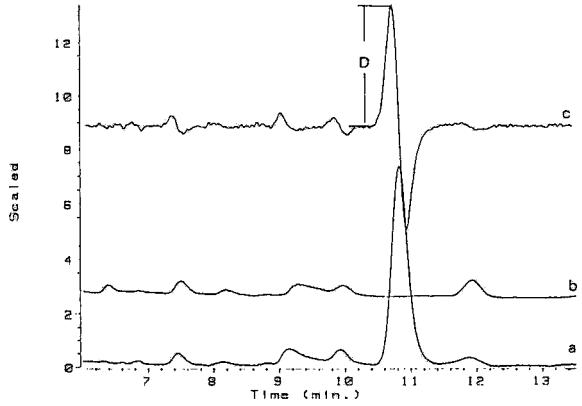


Fig. 5 - HPLC chromatogram obtained from quail muscle tissues: a) zero-order chromatogram of tissue fortified with CAP at 40 μg/Kg level, b) zero-order chromatogram from a control tissue, and c) first-order derivative of the chromatogram (a).
The amplitude D was used for the CAP quantitation.

fortified with known quantities of the drug over the 10-75 μg/Kg range; after the sample clean-up process and the HPLC analysis a linear relationship ($y=0.606x + 2.86$; $r=0.9987$; $n=5$) was obtained by plotting the CAP peak height (y) against the CAP concentration (x; μg/Kg). The obtained linear curve exhibits a significant y-intercept; in effect, the UV spectra (upslope, apex, downslope) showed the peak not to be homogeneous, owing to some interference at higher times. Thus, the first-order derivative spectrum of the chromatographic peak was recorder and the amplitude (D) of the first positive peak was measured (Fig. 5). By plotting this amplitude (y) against the drug concentration (x) an improved linear relationship ($y=0.310x + 0.205$; $r=0.9993$; $n=5$) with reduced y- intercept was obtained. Similar effect was observed when the analysis of TAP was carried out. The best linear relationship was observed using the first

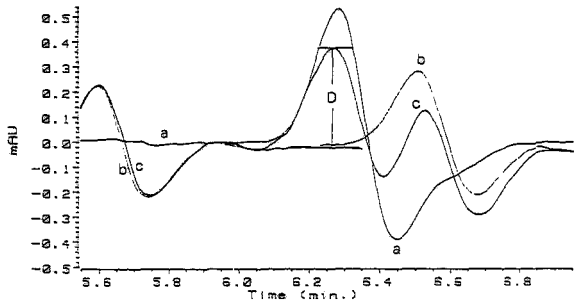


Fig. 6 - First-order derivative chromatograms obtained from: a) standard solution of TAP (100 ppb), b) control quail tissue, and c) tissue from quail orally treated with TAP. The amplitude D was measured for quantitative applications.

derivative of the TAP chromatogram (Fig. 6) and measuring the amplitude (D) of the first positive peak ($y=0.0056x - 0.0031$; $r=0.9996$; $n=5$). Thus, the application of the derivative approach to the analyte chromatographic peak allowed a more specific "analytical window" to be used in order to reduce the matrix background interferences.

The described HPLC method was applied to the determination of CAP residues in quails (52 muscle tissues), pheasants (33 muscle and 10 liver tissues) and mallard (30 muscle and 7 liver tissues) obtained from the market. No positive results were found; therefore, according to the method sensitivity, the presence of CAP at > 8 µg/Kg level was excluded for all the analysed samples. On account of this result, it was considered of interest to achieve informations on the elimination kinetic of CAP from the tissues after the drug assumption at therapeutical level. Thus quails were dosed with CAP and the resulting drug residues in the muscle tissues were monitored over a 6 days period. Analogous investigations were then performed on the elimination of TAP

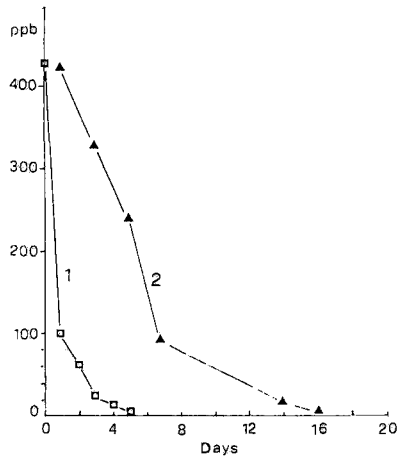


Fig. 7 - Curves concentration - time obtained for quail muscle tissues after CAP (1) and TAP (2) oral administration at therapeutical doses.

residues. The resulting curve concentration - time observed for each drug are reported in Fig. 7.

As shown, after five days the CAP residues were lower of the maximum residue level (10 μg/Kg). The observed rapid elimination of CAP from quail tissues was comparable to previous results obtained for chicken tissues (27).

Differently, TAP residues were found to be longer present in the quail tissues; after 14 days TAP residues at the level of 20 μg/Kg were still present. According to this slow elimination, the italian low states a withdrawn period of 21 days before the slaughter of food animals medicated with TAP. These data are consistent with the known different pharmacokinetics of CAP and TAP (2).

In summary, convenient HPLC screening methods have been developed for the determination of CAP and TAP residues in edible tissues of a variety of gamebird (pheasants, mallards and quails). The methods offer adequate

selectivity and sensitivity; using photodiode array detection the peak identity can be confirmed and the interferences from co-extractants in the quantitative assay can be eliminated by a derivative approach to the chromatographic peak evaluation.

Acknowledgements

The authors thank F. Ferrari for is valuable technical assistance. Financial support from MURST (Rome, Italy) is gratefully acknowledged.

REFERENCES

1. Martindale, The Extra Pharmacopoeia, The Pharmaceutical Press, London, 1989.

2. E. Gennazzani, A. Giotti, P. Mantegazza, G. Pepeu, P. Periti, Trattato di Farmacologia e Chemioterapia, USES Ed. Scientifiche, Firenze, 1983.

3. Huber, "Allergenicity of Antibacterial Drug Residues", in Drug Residues in animals, A.G. Rico Ed., Academic Press Inc., 1986.

4. Keukens, M.M.L. Aerts, W.A. Traag, J.F.M. Nouws, W.G. De Ruig, W.M.J. Beek , J.M.P. Den Hartog, J. AOAC Int., 75; 245-256 (1992).

5. Keukens, W.M.J. Beek, M.M.L. Aerts, J. Chromatogr., 235; 445-453 (1986).

6. Aerts, H.J. Keukens, G.A. Werdmuller, J. Assoc. Off. Anal. Chem., 72; 570-576 (1989).

7. Degroodt, B. Wyhowski De Bukanski, J. De Groof, H. Beernaert, S. Srebrnik, J. Liq. Chromatogr., 15; 2355-2371 (1992).

8. Nagata , M. Saeki, J. Liq Chromatogr., 15; 2045- 2056 (1992).

9. Yu, Fenxi Ceshi Tongbao ,10; 75-6 (1991); Chem. Abstr. 116,104591y.

10. Simonella, L. Torreti, C. Filipponi, J. High Resolut. Chromatogr., 12; 55-7 (1989).

11. Tjaden, D.S. Stegehnis, B.J.E.M. Reeuwijk, H. Lingeman, J. Van der Greef, Analyst (London), 113; 171- 4 (1988).

12. Pochard, G. Burger, M. Chevalier, E. Gleizer, J. Chromatogr., 409; 315-23 (1987).

13. Ramos, T. Reuvers, A. Aranda, J. Games, J. Liq. Chromatogr., 17; 385-401 (1984).

14. Samouris, B. Nathanael, H. Tsoukali-Papadopoulou, N. Papadimitriou, Vet. Hum. Toxicol., 35; 406-9 (1993).

15. Bellomonte, C. Filesi, A. Macri, M. Mosca, E. Sanzini, Ital. J. Food Sci., 5; 247-53 (1993).

16. Bayo, M.A. Moreno, J. Prieta, S. Diaz, G. Srarez, L. Dominguez, J. AOAC Int., 77; 854-6 (1994).

17. Blauchflower, A. Cannavan, R.J. McCracken, S.A. Hewitt, D.G. Kennedy, Residues Vet. Drugs Food, Proc. EuroResidue Conf., 2nd, 1, 196-200 (1993).

18. Gips, M. Bridzy, S. Barel, S. Soback, Residues Vet. Drugs Food, Proc. EuroResidue Conf., 2nd 1, 313-17 (1993).

19. Nagata , M. Saeki, J. Chromatogr., 565; 471-6 (1991).

20. Sato, Y. Mishima, T. Seki, A. Tsunoda, Kankyo Kagaku, 3; 97-100 (1993); Chem. Abstr. 119, 115630y.

21. Psomas, E.G. Iosifiday, J. Liq. Chromatogr., 16; 2653-2660 (1993).

22. Senders, P. Guillot, M. Dagorn, J.M. Delmas, J. Assoc. Off. Anal. Chem., 74; 483-486 (1991).

23. Yoshida, F. Kondo, J. Liq. Chromatogr., 17; 2625-2632 (1994).

24. Abjean, J. AOAC Int., 77; 1101-1104 (1994).

25. Epstein, C. Henry, K.P. Holland, J. Dreas, J. AOAC Int., 77; 570-576 (1994).

26. Barker, A.R. Long, J. Liq. Chromatogr., 15; 2071-2089 (1992).

27. A.Simonella, S.Carli, L.Torreti, C.Filipponi, C.Di Francesco, L.Ambrosi, Arch. Vet. Ital., 41; 23-33 (1990).

Received: April 22, 1995
Accepted: May 9, 1995

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(17), 3545-3547 (1995)

THE BOOK CORNER

HANDBOOK OF DERIVATIVES FOR CHROMATOGRPAHY, Second Edition, edited by K. Blau and J. M. Halket, John Wiley & Sons, Ltd., Chichester, England, 1993, xxii + 370 pp, £65.00; ISBN: 0-471-92699-X

The first edition of this book was published in 1977; it provided a comprehensive summary of chemistry of derivatization, ranging from classical esterification and acylation to other, more esoteric methods for derivatizing inorganic anions, which was primarily suitable for gas chromatography. However, with the rapid development in HPLC and other analytical modalities, the second edition includes several new chapters, such as colored and UV-absorpting derivatives for HPLC to increase selectivity and detectibility. Also, a new chapter addresses the derivatization of optically active compounds to maximize their potential for separation by liquid chromatography.

Chapter 12 deals with derivatization for fast atom bombardment mass spectrometry (FAB-MS) and to supercritical fluid chromatography (SFC). Thus, the new edition is currently needed to provide more comprehensive information and new advances in chemical derivatization in liquid chromatography.

The book consists of 16 chapters, and each one ends with a list of pertinent references. Among other topics discussed in detail are:

⇒ silylation and alkylation

⇒ derivative formation by ketone-base condensation

⇒ post chromatographic derivatives

This book provides information of great value for chromatographers, mass spectroscopists in particular and analytical chemists in general. It should be available in the laboratories and libraries of academic institutions, chemical and pharmaceutical industries and research centers.

HANDBOOK OF SIZE EXCLUSION CHROMATOGRAPHY, edited by Chi-san Wu. Chromatographic Science Series Volume 69, Marcel Dekker, Inc., New York, U.S.A., viii + 472 pp; $175.00; ISBN:0-8247-9288-2

Size exclusion chromatography (SEC) is a technique given to the liquid chromatographic separation of macromolecules by molecular size. This book discusses the details and particular use of SEC in characterizing the molecular distribution of important polymeric materials. The book is written by 25 internationally recognized contributors in the field. It consists of 17 chapters with over 1000 references and 175 tables and illustrations. The topics included in the book are:

- Introduction to Size Exclusion Chromatography
- Semirigid Polymer Gels for Size Exclusion Chromatography

  Modified Silica-Base Packing Materials for Size Exclusion Chromatography
- Molecular Weight-Sensitive Detectors for Size Exclusion Chromatography
- Determination of Molecular Weight Distributions of Copolymers by Size Exclusion Chromatography
- Size Exclusion Chromatography of Polyamides, Polyesters, and Fluoropolymers
- Size Exclusion Chromatography of Natural and Synthetic Rubber
- Size Exclusion Chromatography of Asphalts
- Size Exclusion Chromatography of Acrylamide Homopolymer and Copolymers

- Aqueous Size Exclusion Chromatography of Polyvinyl Alcohol
- Size Exclusion Chromatography of Polyvinyl Acetate
- Size Exclusion Chromatography of Vinyl Purrolidone Homopolymer and Copolymers
- Size Exclusion Chromatography of Cellulose and Cellulose Derivatives
- Size Exclusion Chromatography of Lignin Derivatives
- Size Exclusion Chromatography of Startch
- Size Exclusion Chromatography of Proteins
- Size Exclusion Chromatography of Nucleic Acids

The book is an important reference and excellent addition to the field of separation science. It is a practical tool for chromatographers, analytical chemists, biochemists and polymer chemist faced with use of SEC to determine the absolute molecular distribution of polymers among other applications. The book should be on the shelves of all those interested in SEC analyses.

These books were reviewed by:

Hassan Y. Aboul-Enein, Ph.D.,FRSC
Bioanalytical and Drug Development Laboratory
Biological and Medical Research Centre
King Faisal Specialist Hospital and Research Centre
P.O.Box 3354, Riyadh 11211, Saudi Arabia

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(17), 3549 (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(17), 3551-3556 (1995)

LIQUID CHROMATOGRAPHY CALENDAR

1995

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

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

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

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

NOVEMBER 2: Anachem Symposium, Dearborn, Michigan. Contact: Prof. C. Evans, University of Michigan, Chem Dept, 4807 Chemistry Bldg, Ann Arbor, MI 48109-1055, USA.

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

FEBRUARY 5 - 7: PrepTech'96, Sheraton Meadowlands Hotel, East Rutherford, New Jersey. Contact: Dr. R. Stevenson, 3338 Carlyle Terrace, Lafayette, CA 94549, USA.

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 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.

APRIL 17 - 19: VIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Université de Nice, Fracnce. Contact: Prof. W. R. G. Baeyens, University of Ghent Pharmaceutical Institute, Lab or Drug Quality Control, Harelbekestraat 72, Ghent, Belgium. Email: willy.baeyens@rug.ac.be.

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 Liquid Chromatography Calendar, 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.**

INSTRUCTIONS TO AUTHORS

The **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 complete original manuscript, and two (2) clear copies, with figures, must be submitted for peer review. After all required revisions have been completed, and the final manuscript has been accepted, the author will be asked to provide, if possible, a 3½" or 5¼" **PC-compatible** computer diskette containing the complete manuscript. **Microsoft Word, Word for Windows, WordPerfect, WordPerfect for Windows** and **ASCII** are preferred formats. Text (including tables), and figures (if in electronic format) should be saved in separate files on the diskette. Label the diskette with the corresponding **author's last name**, the **title of the manuscript** and the **file number** assigned to the manuscript.

Submission of a manuscript on diskette, in a suitable format, will significantly expedite its publication.

Manuscripts and computer diskettes should be mailed to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography
P. O. Box 2180
Cherry Hill, NJ 08034-0162

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 corresponding 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 only with orders of 100 or more reprints.

Format of the Manuscript

NOTE: Failure to adhere to the following guidelines will delay publication of a manuscript.

1. The preferred dimensions of the **printed area of a page** are
   6" (15.2 cm) width by 8.5" (21.6 cm) height
   Use Times Roman 12 point font, if possible.

The general organization of the manuscript should be:

Title
Author(s)' names and full address(es)
Abstract
Text Discussion
Acknowledgements
References

2. **Title & Authors:** The entire title should be in boldface capital letters and centered within the width of the printed area, located 2" (5.1 cm) from the top of the page. This should be followed by 2 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, H. R. Brown
Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

3. **Abstract:** The heading, **ABSTRACT**, should be typed capitalized and centered, 2 lines below the addresses. This should be followed by a single line space, then a concise abstract. Allow 2 lines of space below the abstract before beginning the text of the manuscript.

4. **Text Discussion:** Text should be typed with full (left & right) justification. 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 text by two lines of space above and one line of space below the headings. Each major heading should be typed centered in capital letters.

Secondary headings, if any, should be placed flush with the left margin, bold face, 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, with Arabic numerals, and placed at the end of the manuscript. They should be typed single-spaced. Each reference should contain names of all authors (with initials of their first and middle names placed **before** their last names); do not use *et al.* for a list of authors. Abbreviations of journal titles should 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).

2. N. H. Foda, "Quantitative Analysis of Fluvoxamine Maleate in Tablet Formulations by HPLC," J. Liquid Chrom., **18**, 1591-1601 (1995).

Book:

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

Chapter in a Book:

1. 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 the printed 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. **Hand-drawn characters are not acceptable.**

Additional Typing Instructions

1. The manuscript must be prepared on good quality US Letter **size** or international A4 size **white bond paper**. The **typing area of each page**, including the title and authors, should be 6" (15.2 cm) width by 8.5" (21.6 cm) height.

2. All **text** should be typed **single-spaced**.

3. It is essential to use **dark black** typewriter or printer ribbon, or a laser printer 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 within the body of the text, but in such a way as to separate them from the text by a two-line space above and below the table. **Tables should be inserted** into the text as close to the point of reference as possible. 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**, on a line by itself, (typed bold-face, capitalized and followed by an Arabic number) should precede the table and should be centered above the table. The title of the table, on the next line, should have the first letters of all main words in capitals. Table titles should be typed single line spaced, bold-face across the full width of the table.

5. **Figures** (drawings, graphs, etc.) should be professionally drawn in **black** India ink (or computer-generated) on separate sheets of **white** paper and should be placed at the end of the text. **Figures should not be inserted into the body of the text.** They should not be reduced to a small size. Preferred size for figures is from 5" x 7" (12.7 cm x 17.8 cm) to 8½" by 11" (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 number. All figures and lettering must be of a size that will remain legible after a 20-30% reduction from the original size. Figure numbers, name of senior author and an arrow indicating "top" should be written with a 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. The format for references should be as given above.

Contributors, particularly those unfamiliar with English usage, are encouraged to seek the assistance of colleagues who are well versed in English language usage prior to submission.

Obtain the ***best possible results*** *by understanding the essentials involved in the chromatographic process with...*

LIQUID CHROMATOGRAPHY FOR THE ANALYST

RAYMOND P. W. SCOTT
Georgetown University, Washington, D.C., and Birbeck College, University of London, United Kingdom

January, 1994
344 pages, illustrated
$75.00

This **practical guide** provides a clear presentation of the chromatographic process—demonstrating the functions of all associated instrumentation *and* the procedures necessary to obtain accurate qualitative and quantitative results.

Supplies a* host of applications *from a variety of sources to help identify the best equipment, the most appropriate columns, and the most suitable phase systems for specific samples!

Written by an international expert with over 45 years of industrial and academic experience, ***Liquid Chromatography for the Analyst***

- covers essential fundamental theory
- explains the chromatographic process using established physical chemical terminology
- illustrates chromatographic behavior with current practices
- gives **useful examples** to aid in applying principles to actual problems
- furnishes supporting experimental evidence for theoretical explanations
- and much more!

With its direct, jargon-free style that permits easy access to information, ***Liquid Chromatography for the Analyst*** is an invaluable resource for analytical chemists, laboratory technicians, and upper-level undergraduate, graduate, and continuing-education courses in analytical chemistry or separation science.

CONTENTS

ISBN: 0—8247—9184—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

Keep state-of-the-art theoretical and applied research developments at your fingertips with...

Advances in Chromatography

Volume 36

edited by

PHYLLIS R. BROWN
University of Rhode Island, Kingston

ELI GRUSHKA
Hebrew University of Jerusalem, Israel

October, 1995 / 464 pages, illustrated / $175.00

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 separations, this critically praised series separates the most important advances from an overabundance of supplementary materials.

Internationally acclaimed experts analyze the most current innovations in their areas of specialization!

Providing more than **850** bibliographic citations, allowing for further, in-depth study of recent trends in research, **Volume 36** examines timely subjects such as

- multilinear regression, canonical correlation, and factor and principal component methods of analysis in the evaluation of retention data matrices
- molecular recognition mechanisms in the liquid chromatographic separation of fullerenes
- the **latest techniques** in the use of capillary electrophoresis and mass spectrometry for sequencing antisense oligonucleotides
- analytical tools and separation methods for the analysis of drugs of abuse in biological fluids
- the applications of high-performance liquid chromatography coupled with nuclear magnetic resonance spectroscopy
- 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—9551—2

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

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(17), (1995)

Contents Continued

JOURNAL OF LIQUID CHROMATOGRAPHY

Volume 18, Number 17, 1995

CONTENTS

(continued on inside back cover)

MARCEL DEKKER, INC. New York, Basel, Hong Kong

Contributions to this journal are published free of charge