JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

HPLC
TLC
Capillary Electrophoresis
Supercritical Fluid Techniques
Membrane Technology
Field-Flow Fractionation
Preparative & Analytical Separations

VOLUME 20 NUMBER 2 1997

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

January 1997

Aims and Scope. The journal publishes an outstanding selection of critical, peer-reviewed papers dealing with analytical, preparative and process-scale liquid chromatography of all types and related technologies such as TLC; capillary electrophoresis; supercritical fluid extraction and chromatography; membrane separation technology; field-flow techniques; and others. As new separation technologies are introduced, they will also be included in the journal. On a regular basis, special topical issues are devoted to specific technologies and applications. Book reviews, software reviews and a calendar of meetings, symposia and expositions are also included.

Identification Statement. *Journal of Liquid Chromatography & Related Technologies* (ISSN: 1082-6076) is published semimonthly except monthly in May, August, October, and December for the institutional rate of $1,750.00 and the individual rate of $875.00 by Marcel Dekker, Inc., P.O. Box 5005, Monticello, NY 12701-5185. Periodicals postage paid at Monticello, NY. POSTMASTER: Send address changes to *Journal of Liquid Chromatography & Related Technologies*, P.O. Box 5005, Monticello, NY 12701-5185.

Volume	Issues	Institutional Rate	Individual Professionals' and Student Rate	Foreign Postage: Surface	Foreign Postage: Airmail to Europe	Foreign Postage: Airmail to Asia
20	20	$1,750.00	$875.00	$75.00	$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: JLCTFC 20(2) i-vi, 167-334 (1997)
ISSN: 1082-6076

Printed in the U.S.A.

Subscribe Today!

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

Order Form

Journal of Liquid Chromatography & Related Technologies

Please enter my subscription to Vol. 20, 20 Numbers, 1997 at the ☐ institutional rate of $1750.00; ☐ individual rate of $875.00 *Individual subscriptions must be prepaid in American currency by personal check or credit card. Please add $3.75 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 pro forma invoice.
____ Check enclosed made payable to Marcel Dekker, Inc.
____ Charge my: ☐ MasterCard ☐ Visa ☐ American Express
____ Please bill my company: P.O. No. _____

Card No._____ Exp. Date_____

Signature_____

Name_____

Address_____

City/State/Zip_____

Does your library subscribe to the ***Journal of Liquid Chromatography & Related Technologies***? 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 & Related Technologies*** and would like to recommend the journal for acquisition.

Signature_____ Date_____

Name_____ Department _____

Journal of Liquid Chromatography & Related Technologies
Volume 20, 20 Numbers, 1997: $1750.00
ISSN: 1082—6076 CODEN: JLCTFC

Sample copy and pro forma 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 & Related Technologies* or to recommend the journal to your library for acquisition.

NO POSTAGE
NECESSARY
IF MAILED
IN THE
UNITED STATES

BUSINESS REPLY MAIL
FIRST-CLASS MAIL PERMIT NO. 2863 NEW YORK NY
POSTAGE WILL BE PAID BY ADDRESSEE

PROMOTION DEPT
MARCEL DEKKER INC
270 MADISON AVE
NEW YORK NY 10157-1928

Journal of Liquid Chromatography & Related Technologies

Editor: **JACK CAZES**
Coconut Creek, Florida

The *Journal of Liquid Chromatography & Related Technologies* now publishes an outstanding selection of critical, peer-reviewed papers dealing with analytical, preparative, and process-scale liquid chromatography of all types and related technologies such as TLC; capillary electrophoresis; supercritical fluid extraction and chromatography; membrane separation technology; field-flow techniques; and others. As new separation technologies are introduced, they will also be included in the journal.

On a regular basis, special topical issues will be devoted to specific technologies and applications. Book reviews, software reviews, and schedules of meetings, symposiums, and expositions are also included.

JOURNAL OF LIQUID CHROMATOGRAPHY
& RELATED TECHNOLOGIES

Editor:
DR. JACK CAZES

Editorial Manager:
ELEANOR CAZES

JLC & RT
P.O. Box 970210
Coconut Creek, Florida 33097

Associate Editor:

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

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*
D. CORRADINI, *Istituto di Cromatografia del CNR, Rome, Italy*
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*

(continued)

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

Editorial Board *(continued)*

W.L. HINZE, *Wake Forest University, Winston-Salem, North Carolina*
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*
I.N. PAPADOYANNIS, *Aristotelian University of Thessaloniki, Thessaloniki, Greece*
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*
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, *Budelco, B.V., Budel, The Netherlands*
R. WEINBERGER, *CE Technologies, Chappaqua, New York*

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

Indexing and Abstracting Services. Articles published in *Journal of Liquid Chromatography & Related Technologies* 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 © 1997 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 17005, 1001 JA Amsterdam, The Netherlands, Tel: +31-20-626-2629, Fax: +31-20-626-1437.

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.

J. LIQ. CHROM. & REL. TECHNOL., 20(2), 167-182 (1997)

MICELLAR ELECTROKINETIC CHROMATOGRAPHY USING MIXED SODIUM DODECYL SULFATE AND SODIUM CHOLATE

Haleem J. Issaq,* Paul Liang C. Horng,
George M. Janini, Gary M. Muschik

SAIC Frederick
NCI-Frederick Cancer Research and Development Center
P.O. Box B
Frederick, Maryland 21702-1201

ABSTRACT

The migration behavior of fifteen dansylated amino acids, two estrogens, and two polycyclic aromatic hydrocarbons (PAHs) was studied by micellar electrokinetic chromatography (MEKC) with mixed micelles. The buffer system consisted of 10 mM borate at pH 9.0, containing 50 mM total surfactant concentration. Five different ratios of sodium dodecyl sulfate (SDS) and sodium cholate (SC) surfactants were used, namely: 100:0, 75:25, 50:50, 25:75, and 0:100 (SDS:SC). The numbers refer to molar ratio percent. The migration time window was 50% larger in 50:50 (SDS:SC) compared to 100% SC, and 15% larger than that of 100% SDS.

The migration times of PAHs and estrogens exhibited a pattern similar to that of Sudan III in all the SDS/SC mixed micellar systems, due to their hydrophobic property. Although most amino acids are negatively charged at pH 9, their migration patterns were different depending on their R-group.

Copyright © 1997 by Marcel Dekker, Inc.

The migration times of amino acids with negatively charged R-groups increased continuously with increasing percent of SC in the SDS/SC mixed micellar systems, while the migration time of amino acids with positively charged R-group continuously decreased with increasing percent of SC in the SDS/SC mixed micellar system. For other groups of amino acids, the migration times in SDS/SC mixed micellar systems exhibited variation without specific pattern.

In general, the use of SDS and SC mixed micelles extended the migration time window to some extent, but it was not sufficient to get satisfactory resolution for the amino acids tested in this study.

INTRODUCTION

Capillary Electrophoresis (CE) is a highly efficient micro separation technique which can resolve charged compounds under the influence of a gradient electric field.[1] Micellar electrokinetic chromatography (MEKC) is a variation of CE whereby a micelle is added to the running buffer to effect the separation of uncharged compounds. Today, MEKC is used for the separation of both ionic and neutral molecules. The separation mechanism in MEKC is based on the differential partitioning of a molecule between the micellar pseudo-stationary phase and the aqueous phase.[2] There are many surfactants that can be used in MEKC. They can be grouped into four classes based on the charge characteristics of the head group, namely: anionic, cationic, neutral and zweitterionic. Normally, in MEKC one surfactant is added to the buffer to effect the separation. Modifiers, such as organic solvents,[3,4] cyclodextrins,[5] urea,[6,7] are added to the micellar buffer to enhance the separation. The addition of an additive to a micellar solution may: (a) affect the distribution of the solutes between the aqueous and the micellar phases; (b) shift the micellization equilibrium; (c) affect the additive-surfactant interactions in the aqueous and micellar phases; and (d) affect the aggregation number, shape and degree of ionization. This becomes especially prominent when two different micelles are used in the same buffer solution where solubilization of the solute in the micellar system may be affected. For example, it was observed that cholesterol was more soluble in mixed micelle compared to micelles of single bile salt.[8] The use of mixed micelle[9,10] resulted in significant extension of the elution window, and an improvement in resolution. Also, it influenced the retention of the solutes. Rasmussen et al.[11] were able to resolve benzene from benzaldehyde by using mixed Brij 35/SDS micelles. Little and Foley[12] were able to optimize their separation by changing the concentration of the

micelle. Ahuja et al.[10] studied the effect of changing the concentration of Brij 35 while keeping the concentration of SDS constant on the retention and resolution of n-alkylphenone homologues, using coated and uncoated fused silica capillaries. In both types of columns a plot of molar concentration of Brij 35 versus the reciprocal of electroosmotic and electrophoretic velocities resulted in a linear relationship which increased with increasing the molar concentration of Brij 35 while SDS molar concentration remained constant at 0.02 M. Bumgarner and Khaledi[9] used a mixed micellar system made of bile salt surfactants at different mole fractions as well as total micelle concentrations in order to optimize the resolution of a mixture of corticosteroids which were not resolved when SDS alone was used. Optimum separation was achieved using a ternary surfactant system made of 50 mM each of dehydrocholate, taurocholate and SDS. They also reported that the addition of SDS to a mixture of bile salt micelles resulted in a significant extension of the elution window.

The use of bile salt surfactants in combination with SDS is interesting because of the type and properties of the micelles that each group forms. SDS forms a spherical micelle, while bile salts forms helical micelles. The SDS micelle consists of a hydrophobic inner core and a charged external shell, while the bile salt micelle is the reverse, the hydrophobic region faces the outside while the hydrophilic region faces inward. Therefore, a mixture of a bile salt and a SDS will form a micellar system which possesses micelles with both hydrophobic and hydrophilic outer surfaces facing not only the aqueous buffer but the solutes in the buffer solution, which gives a micellar system that can be used to resolve mixtures of hydrophilic and hydrophobic compounds. This is an advantage not available using only a single surfactant.

The objective of this research is to study the effect of a binary mixed micelle system on the migration times of a group compounds having different physical/chemical properties. In this study, the binary mixed micelles SDS and sodium cholate (SC) were selected. Also three different groups of compounds of different chemical and physical properties, namely, two polycyclic aromatic hydrocarbons (fluoranthene and naphthalene), two estrogens (estrone and estriol), and dansylated amino acids were selected. The amino acids were divided into six groups: (a) polar but uncharged R group (serine and glutamine); (b) negatively charged R group (aspartic, glutamic, and cysteic acid); (c)positively charged R group (lysine and arginine); (d) hydrophobic with an aliphatic R group (alanine, isoleucine, and leucine); (e) hydrophobic with an aromatic R group (phenyalanine, tryptophan and tyrosine); and amino acids with special R group (glycine and proline).

EXPERIMENTAL

Chemicals

Dansylated amino acids and sodium cholate were purchased from Sigma Chemical Company (St. Louis, MO), estriol and estrone were purchased from Steraloids, Inc. (Wilton, NH), and fluoranthene and naphthalene were obtained from Aldrich Chemical Company (Milwaukee, WI). Sodium dodecyl sulfate (SDS) was from Fluka (Ronkonkoma, NY). All other chemicals for preparation of the electrolyte buffer were purchased from Fisher Scientific (Fair Lawn, NJ). Deionized water was generated from a Nanopure Water system manufactured by Barnstead/Themolyne (Dubuque, IA). All electrolyte solutions were filtered through a 0.45 μm nylon acrodisc filter purchased from Gelman Science (Ann Arbor, MI) and degassed.

Apparatus and Procedures

A Beckman CZE Model P/ACE 5510 equipped with diode array detector, an automatic injector, a fluid-cooled column cartridge and a System Gold data station were used in this study. All runs were performed at 25° C, using a 57 cm x 50 μm (50 cm to detector) fused silica capillary from Polymicro Technologies, Inc. (Phoenix, AZ). Each new capillary was washed with 0.1 N NaOH and deionized water then equilibrated with the buffer for 20 min. Capillary electrophoresis was operated at 18 kV. The resulting current ranged from 52-75 μA. The buffers with different concentration of sodium cholate (SC) and sodium dodecylsulfate (SDS) were prepared fresh in deionized water. Solutes were dissolved in the buffer system to be used, was specified in the figure legends. Injections were made using the pressure mode for 5 s at 0.5 psi. Dansylated amino acids, naphthalene and fluoranthene were detected at 200 nm while estrone and estriol were detected at 280 nm.

The running buffer throughout the experiment was prepared as borate buffer 10 mM, pH 9.0. SDS and SC were mixed in five ratios, namely: 100:0, 75:25, 50:50, 25:75, and 0:100 (SDS/SC). The numbers refer to molar ratio percent. In all, the total surfactant concentration was 50 mM, with total concentration of 50 mM. Solutes were dissolved in the buffer solution for injection. Sudan III was dissolved in methanol, then mixed with buffer solution containing the desired SDS/SC mixed ratios, which were then injected to determine the migration time of aqueous buffer solution (t_o) and micelles (t_{mc}). The capillary was rinsed with running electrolyte for 3.5 min between runs.

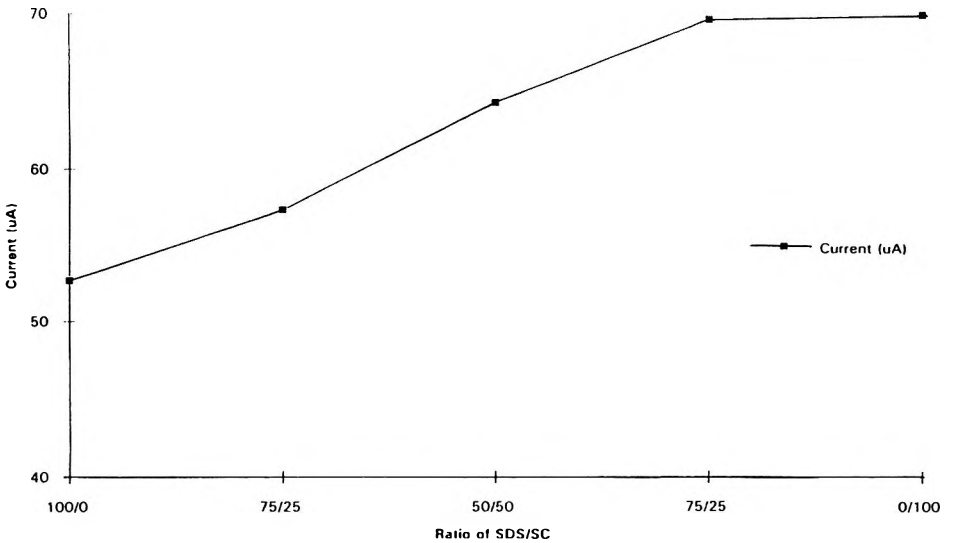


Figure 1. Current versus ratio of SDS/SC in mixed micelles. Instrument: Beckman Model P/ACE System 5510; Column: Bare fused-silica; Column dimensions: L_{total}=57cm; $L_{detector}$=50cm; i.d.=50um; Buffer: 10 mM borate, plus 50 mM surfactant in single or mixed micelles; pH=9.0; applied voltage: 18 kV.

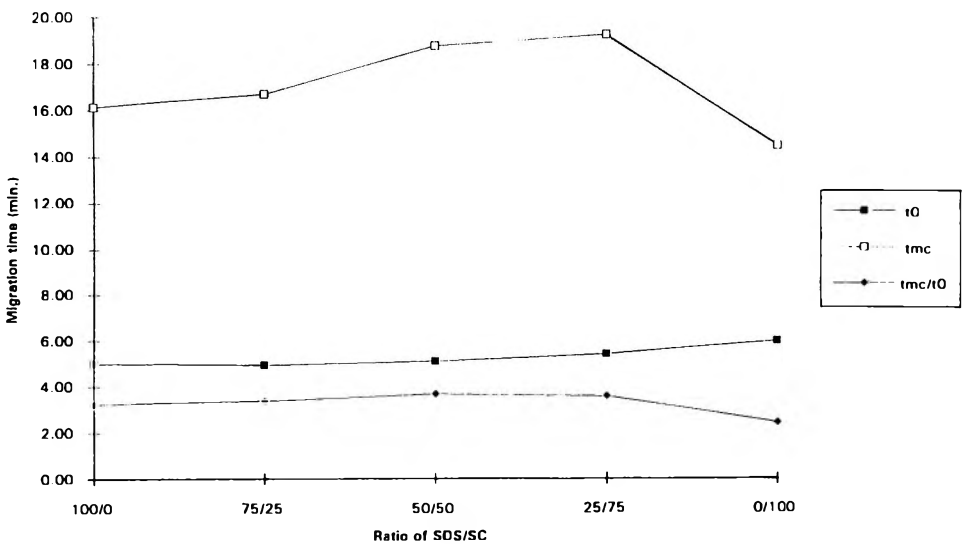


Figure 2. Effect of ratio of SDS/SC on the migration times of methanol (t_o) and Sudan III (t_{mc}) in MEKC. Experimental conditions as in Figure 1.

When different surfactant ratio systems were employed, the capillary was rinsed in sequence with water, 0.1 M NaOH, water, and running electrolyte 2 min. each. Samples were run in triplicates. Data of migration time were the average of three replications.

RESULTS AND DISCUSSION

Effect of Mixed Micelles on Current

The effect of mixing SDS and SC surfactants on current is shown in Figure 1. Using a borate buffer (10 mM, pH 9.0), at a voltage of 18 kV, the current flow was in the range of 52 - 75 μA. The figure shows that in 50 mM SDS, the current generated was 52 μA. The current proportionally increased as the percent of SC in SDS/SC mixed micellar system increased, then leveled off at 25:75% of SDS/SC.

Effect of Mixed Micelle on Migration Time Window

Sudan III is usually used in MEKC as an indicator to determine the migration time of the micelles, due to its hydrophobicity and high binding coefficient with the non-polar core of the micelles. Only one peak was detected as Sudan III in all five SDS/SC mixed micellar systems, which indicates that SDS/SC mixed micelles exhibited only one type of complex micelle, and not multiple micelles. This phenomenon was observed by others.[8-10] In 50 mM SDS buffer, the migration time of Sudan III (t_{mc}) was 16.13 min. The migration time increased from 16.13 to 19.28 min. as the percentage of SC in the buffer increased from 0-75%. However, the migration time dropped to 14.52 min. in 100% SC (Figure 2).

Bacci et al.[13] used an ionic hydrocarbon/non-ionic hydrocarbon mixtures of SDS/β-dodecyl maltoside (DM) to elucidate the theory of micellization of binary surfactant systems. The phenomenon is very similar to the SDS/SC mixed micellar system in the present study. In his model, in the presence of 0.2 M NaCl, when the mole fraction of DM increased from 0 to 75% of total surfactant, the micelle aggregation numbers also increased from about 110 in 100:0% SDS/DM to about 225 in 25:75% SDS/DM. When the mole fraction of DM changed to 100%, the aggregation numbers of micelles sharply decreased to about 100. The authors further indicated that when the mole fraction of DM in monomer changed from 0 to about 25%, the critical micelle concentration (CMC) of SDS in the SDS/DM mixed micellar system abruptly

decreased from about 6.4 to about 2 mM. A further increase in the mole fraction of DM to about 100%, the CMC of SDS surfactant was maintained at a flat level. Furthermore, as the mole fraction of DM in monomer increased from 0 to 25%, the mole fraction of DM in micelles was also sharply increased to >50% in SDS/DM mixed micellar system. When the mole fraction of DM in monomer continually increased to 100%, the mole fraction of DM in SDS/DM micelles system continually increased in sigmoid curve up to 100%.

If the theory is applicable to the SDS/SC mixed micelles, then the increase in migration time of micelles in SDS/SC mixed micellar system was due to the increase of aggregation numbers of micelles. This corresponds to the increase in size and total anionic charges on the surface of micelles, so the electrophoretic migration of micelles increased, and the migration time of micelle to the detector window also increased. The pure SC micelle, which tends to have lower aggregation number with smaller number of negative charges on the micelles, will results in slower electrophoretic migration, however, the dominant electroosmotic flow (EOF) makes the SC micelles to migrate faster than SDS because they are smaller.

Methanol, which is not retained in the micelle, is used for the determination of the migration time of the aqueous phase. It was observed that the SDS/SC mixed micellar systems, the migration time of methanol (t_o) slightly increased as the percentage of SC in SDS/SC mixed micellar systems increased.

It was suggested by Terabe[14] that t_o/t_{mc} value can be used for measurement of the migration time window of compounds in MEKC. For convenience, the t_o/t_{mc} value was transformed as t_{mc}/t_o value in later publications. The t_{mc}/t_o of pure SC and SDS micelles are 2.43 and 3.23, respectively. When the percent of SC in SDS/SC mixed micellar systems increased, the migration time window (t_{mc}/t_o) was proportionally increased and reached a peak value of 3.69, at 50:50% of SDS/SC. A further increase in percent of SC to 25:75, slightly decreased the migration time window (t_{mc}/t_o) to 3.57. The percent of SDS:SC at 50:50 produced an optimum migration time window, which is 50% higher than SC alone, and 15% higher than SDS alone.

Migration of Polycyclic Aromatic Hydrocarbons (PAHs) in SDS/SC Mixed Micelles

The pattern of migration times of fluoranthene in SDS/SC mixed micellar systems was very similar to the migration time of the tracer (Sudan III) run in SDS/SC MEKC. Because of its hydrophobic property, fluoranthene spent about

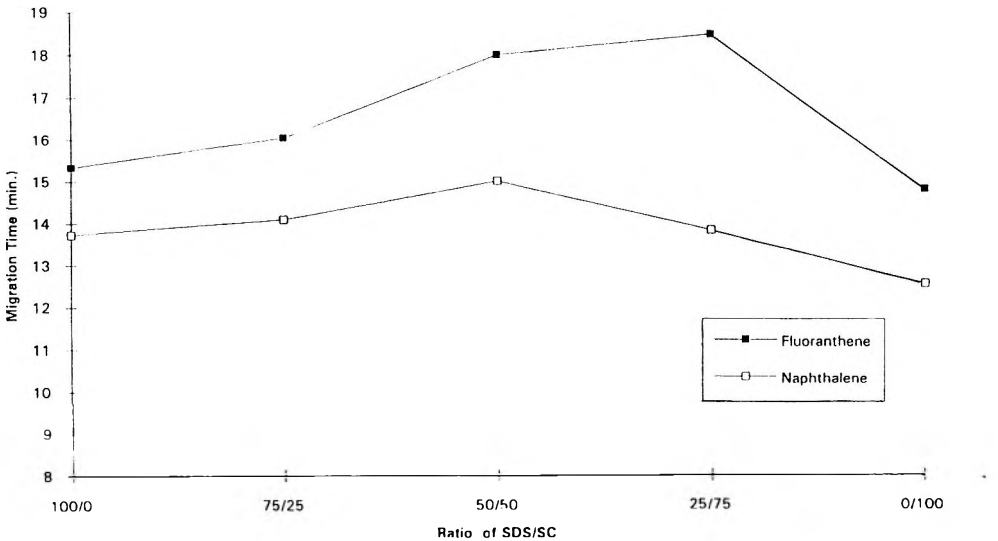


Figure 3. Effect of ratio of SDS/SC on the migration times of fuoranthene and naphthalene in MEKC. Experimental conditions as in Figure 1.

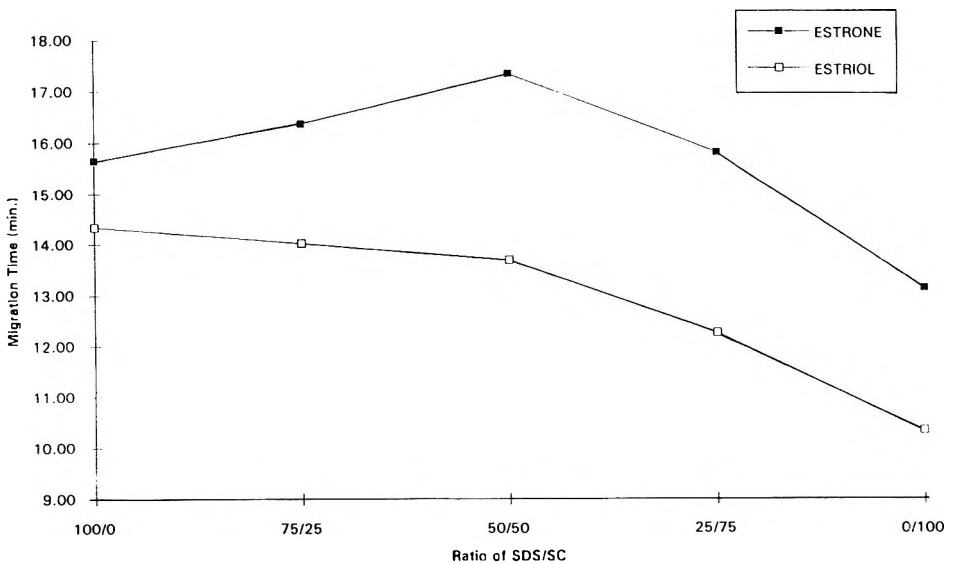


Figure 4. Effect of ratio of SDS/SC on the migration times of estrone and estriol in MEKC. Experimental conditions as in Figure 1.

95% of migration time in association with the micelles during the process of separation (Figure 3). This can be attributed to the high binding coefficient of this compound to the micellar entity.[15,16] Same phenomenon was observed for the naphthalene, which has two benzene rings, its binding coefficient to the SDS micelles was much smaller than fluoranthene, therefore, it eluted from the MEKC at a shorter time.

Migration of Estrogens in SDS/SC Mixed Micelles

The pattern of migration times of estrone in SDS/SC mixed micellar systems was similar to that of fluoranthene. Estrone spent about 82-97% of migration time associated with the micelles in the process of separation. Estriol is structurally similar to estrone, but more polar; this makes the binding coefficient of estriol in the SDS/SC mixed micellar systems less than that of estrone, which resulted in shorter migration times (Figure 4). Chan et al.,[17] in a study of separation of estrogens by MEKC, found that the migration time of estrone was longer than estriol under the same experimental condition, using a buffer system containing 50 mM SDS at pH 9.2.

Migration of Dansylated Amino Acids Containing Negatively Charged R Groups in SDS/SC Mixed Micelles

Dansylated aspartic acid, glutamic acid, and cysteic acid (CYA), at pH 9.0 are negatively charged.[18] In the pure SDS buffer solution, the negatively charged heads of SDS faced outside of the aqueous phase and the non-polar tails extended inside of the micellar spheres. As the negatively charged amino acids come in contact with the negatively charged surfaces of SDS, the electrostatic repulsion forces cause these amino acids to decrease their binding to the micelle aggregates. The result is that the amino acids eluted from the CE in a short time.

As the percentage of SC in the buffer increased and that SDS decreased to 75, 50, 25, and 0%, the electrostatic repulsion forces of SDS were proportionally decreased, but the binding forces of the hydrogen bonding, hydrophobic Van der Waals forces, and electrostatic attraction forces[19] increased, so the migration times of these acids increased proportionally (Figure 5). However, at 75% and 100% SC, the migration times of aspartic acid and glutamic acid increased abruptly. In all five SDS/SC mixed micellar systems, glutamic acid migrated faster than aspartic acid.

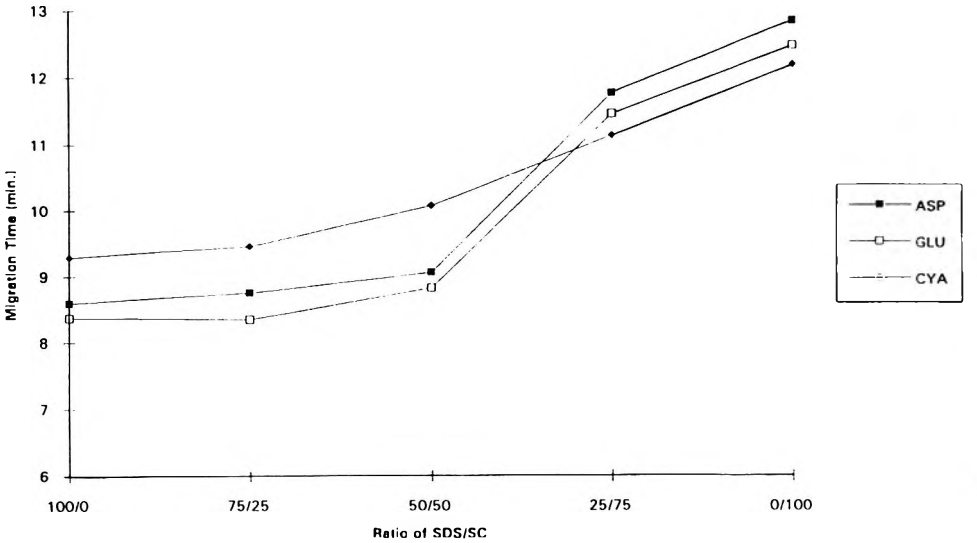


Figure 5. Effect of ratio of SDS/SC on the migration times of dansylated asparatic acid, glutamic acid, and cysteic acid (CYA) in MEKC. Experimental conditions as in Figure 1.

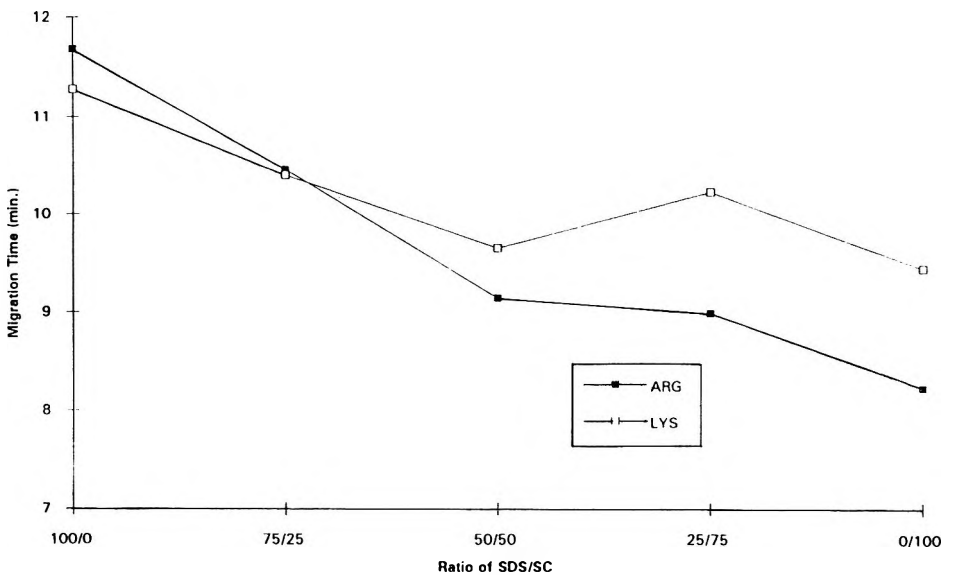


Figure 6. Effect of ratio of SDS/SC on the migration times of dansylated arginine and lysine in MEKC. Experimental conditions as in Figure 1.

The pattern of migration time of cysteic acid in SDS/SC five mixed micellar systems was similar to aspartic acid and glutamic acid. The migration time of cysteic acid proportionally increased with the increase in percentage of SC in SDS/SC mixed micellar system.

Migration of Dansylated Amino Acids with Positively Charged R-Group in SDS/SC Mixed Micelles

Dansylated arginine and lysine are basic amino acids. At pH 9.0, they are positively charged. In borate buffer solution containing pure SDS, the positively charged arginine and lysine are attracted to the negatively charged SDS aggregates, and are retained on the micelles surfaces, which resulted in longer migration time than the negatively charged amino acids. As the percent of SDS in SDS/SC mix micellar systems decreased to 75, 50, 25, and to 0%, the total negative charges of the mixed micelles also decreased, and the electrostatic binding forces to arginine and lysine were also correspondingly decreased, that resulted in a decrease of the migration time (Figure 6).

The migration time of arginine in pure SDS micelles was larger than that of lysine, but at SDS/SC (75:25%), their migration times became very close. As the percent of SDS in SDS/SC mix micellar systems decreased to 50, 25, and 0%, the migration time of arginine became smaller than that of lysine. This change in elution order was probably due to the change of binding coefficients of the solutes to the pseudo-stationary phase.

Migration of Dansylated Amino Acids with Aromatic R-Groups in SDS/SC Mixed Micelles

The pattern of migration time of di-dansylated tyrosine was similar to that of Sudan III in SDS/SC five mixed micellar systems, because didansylated tyrosine is relatively non-polar. When it is dissolved in the micelle, it would take long time to elute from the MEKC. This phenomenon was reported by Chrambach and Hjelmeland[20] in a study dealing with the CE separation of 20 dansylated amino acids in a micellar buffer made of 100 mM borate, pH 9.0, containing 50 mM SDS. The migration times of dansylated phenylalanine and tryptophan in all the SDS/SC mixed micellar systems were very close to each other (Figure 7). The use of SDS and SC mixed micellar system without the addition of modifiers were not sufficient to achieve good resolution and selectivity of phenylalanine and tryptophan under the present experimental conditions. The migration times of phenylalanine in SDS/SC five mixed micellar systems were slightly shorter than those of tryptophan (Figure 7).

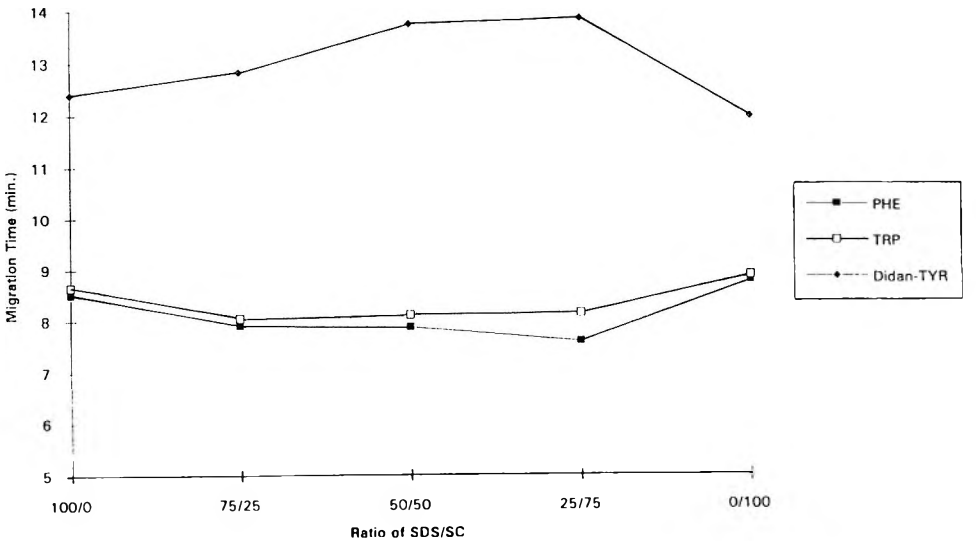


Figure 7. Effect of ratio of SDS/SC on the migration times of dansylated phenylalanine, tryptophan, and di-dansylated tyrosine in MEKC. Experimental conditions as in Figure 1.

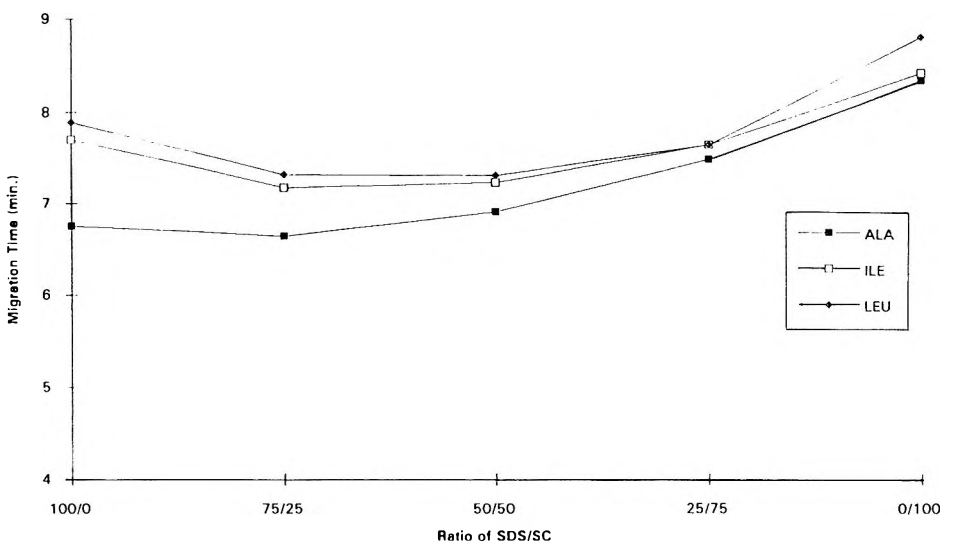


Figure 8. Effect of ratio of SDS/SC on the migration times of dansylated alanine, leucine, and isoleucine in MEKC. Experimental conditions as in Figure 1.

Migration of Damsylated Amino Acids with Aliphatic R-Group in SDS/SC Mixed Micelles

Dansylated alanine, leucine and isoleucine exhibited similar migration times in the five mixed micellar systems with slight variation at 100% SC (Figure 8).

Migration of Dansylated Amino Acids with Polar but Uncharged R-Group in SDS/SC Mixed Micelles

Dansylated glutamine and serine in SDS 100% micellar system migrated close to each other, but in the other four ratios of SDS/SC mixed micellar systems, the two compounds were separated (Figure 9). The migration times of glutamine were smaller than those of serine in SDS/SC five mixed micellar system.

Migration of Dansylated Glycine and Proline in SDS/SC Mixed Micelles

Dansylated glycine and proline were resolved in 100% SDS, 100% SC, and at 25:75% SDS/SC. However, the migration times of these two compounds in 75:25 and 50:50% were so close that there was no resolution between them (Figure 10).

Overall, the results indicated that the use of SDS and SC in five mixed micellar system were not sufficient in extending the migration time window to allow a satisfactory separation of a mixture of all amino acids used in this study. No optimization of such a separation was attempted, because this was not the objective of this research.

CONCLUSION

In this SDS/SC buffer system MECK, the migration time window, (t_{mc}/t_o), at 100% SC was found to be 2.43. The addition of SDS to the SC at the SDS/SC ratio of 50:50, the migration time window (t_{mc}/t_o), increased to 3.69, which is 50% higher than using pure SC and 15% higher than using pure SDS. The increase in migration time window in SDS/SC mixed micellar systems, is due to an increase in aggregation numbers of the micelles. The results show that the physical properties of the micelles, as well as the test solutes; hydrophobic, polar, positively or negatively charged influenced the migration times.

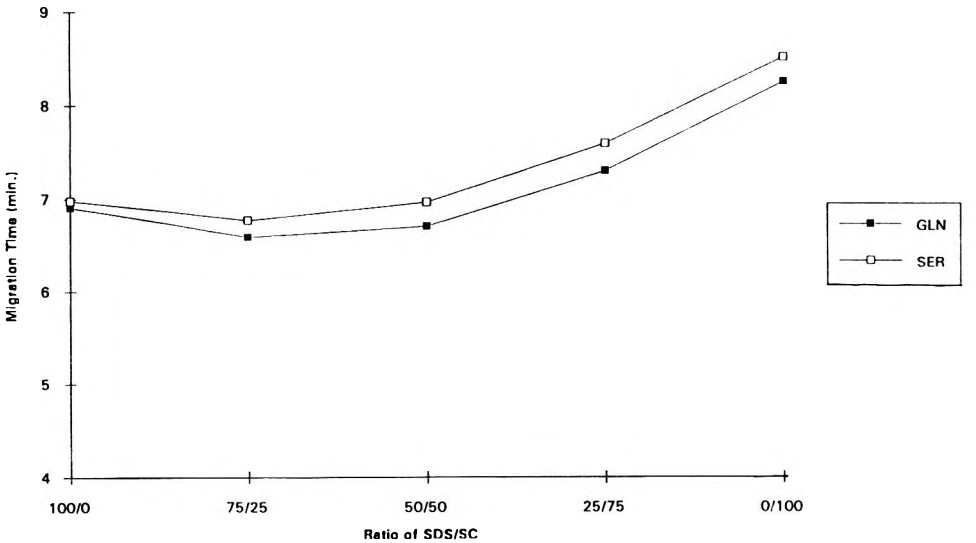


Figure 9. Effect of ratio of SDS/SC on the migration times of danslyated glutamine and serine in MEKC. Experimental conditions as in Figure 1.

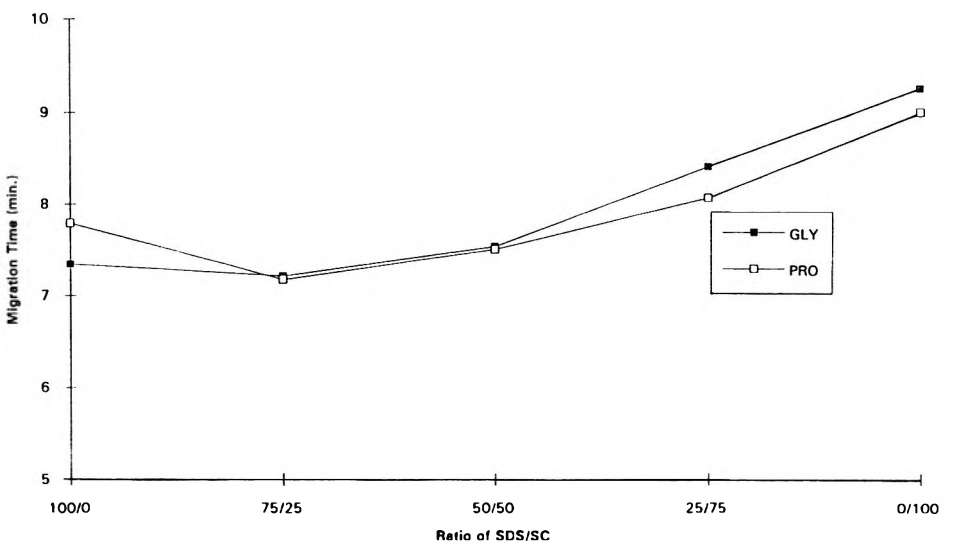


Figure 10. Effect of ratio of SDS/SC on the migration times of dansylated glycine and proline in MEKC. Experimental conditions as in Figure 1.

This can be explained by the ability of the solutes to interact with the micellar system or be repelled by it. Although most amino acids are negatively charged at pH 9, they showed different migration patterns in the five different mixed micelles due to the chemical/physical properties of their R-group. For example, the migration times of the positively charged amino acids decreased with an increase in SC concentration while the negatively charged amino acids eluted faster. Also, the hydrophobic PAHs, which are expected to bind to SDS eluted faster when the concentration of SC was increased to 100%.

ACKNOWLEDGMENT

By acceptance of this article, the publisher or recipient acknowledges the right of the U.S. Government to retain a nonexclusive, royalty-free license and to any copyright covering the article.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

REFERENCES

1. J. W. Jorgensen, K. D. Lukacs, Anal. Chem., **53**, 1298 (1981).

2. S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem., **56**, 111-113 (1984).

3. A. Balchunas, M. Sepaniak, Anal. Chem., **59**, 1466-1470 (1987).

4. J. Gorse, A. Balchunas, D. Swaileand, M. Sepaniak, J. High Res. Chromatogr., Chrom. Commun., **11**, 554-559 (1988).

5. S. Terabe, Y. Miyashita, O. Shibata, E. Barnhart, L. Alexander, D. Patterson, B. Karger, K. Hosoya, N. Tanaka, J. Chromatogr., **516**, 23-31 (1990).

6. H. Nishi, M. Matsuo, J. Liq. Chromatogr., **14**, 973-986 (1991).

7. S. Terabe, Y. Ishihama, H. Nishi, T. Fukuyama, K. Otsuka, J. Chromatogr., **545**, 359-368 (1991).

8. N. Nishikodo, "Solubilization in Mixed Micelles," in **Solubilization in Surfactant Aggregates**, S. D. Christian and J.F. Scamehorn, Editors, Surfactant Science Series, Vol. 55, Marcel Dekker, Inc., New York, N.Y., 1995, pp. 143-189.

9. J. G. Bumgarner, M. G. Khaledi, Electrophor., **15**, 1260-1266 (1994).

10. E. S. Ahuja, E. L. Little, K. R. Nielsen, J. P. Foley, Anal. Chem., **67**, 26-33 (1995).

11. H. T. Rasmussen, L. K. Goebel, H. M. McNair, J. Chromatogr., **517**, 549-555 (1990).

12. E. L. Little, J. P. Foley, J. Microcolumn Sepn., **4**, 145-154 (1992).

13. S. Bacci, C. Fagotti, V. Degiorgio, R. Pizza, Langmuir, 7, 824 (1991).

14. S. Terabe, K. Otsuka, T. Ando, Anal. Chem., **57**, 834-841 (1985).

15. D. W. Armstrong, Sepn. Purif. Methods, **14**, 212 (1985).

16. J. A. Zultewicz, S. Munoz, J. Phys. Chem., **90**, 5820 (1986).

17. K. C. Chan, G. M. Muschik, H. J. Issaq, P. K. Siiteri, J. Chromatogr., **690**, 149-154 (1995).

18. J. T. Smith, W. Nashabeh, Z. El Rassi, Anal. Chem., **66**, 1119-1133 (1994).

19. W. L. Hinze, "Organized Surfactants Assemblies in Separation Science," in **Ordered Media in Chemical Separations**," W. L. Hinze and D. W. Armstrong, Editors, ACS Symposium Series No. 342, ACS, Washington, D. C., 1987.

20. A. Chrambach, L. M. Hjelmeland, **Electrophoresis '83**, H. Hira, ed., de Grayter, Berlin, 1984, pp. 81-97.

Received August 1, 1996
Accepted August 18, 1996
Manuscript 4237

J. LIQ. CHROM. & REL. TECHNOL. 20(2). 183–201 (1997)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF SMALL AND LARGE MOLECULES WITH NONPOROUS SILICA-BASED STATIONARY PHASES

Jim Yu,† Ziad El Rassi*

Department of Chemistry
Oklahoma State University
Stillwater, OK 74078-3071

ABSTRACT

Nonporous monodispersed, microspherical silica particles, having mean particle diameters of 0.8 and 1.1 μm, were surface-modified with octadecyl or triphenyl functions as well as with lectin proteins for high performance liquid chromatography of small and large molecules. "Monomeric" or "polymeric" octadecyl-silica (C_{18}-silica) bonded phases were formed, depending on the silane compounds used. Monomeric C_{18}-silica stationary phases yielded high resolution in the separation of 2-pyridylamino derivatives of xyloglucan oligosaccharides and proteins. The effect of particle size of the support on resolution was also examined. For comparative study, the chromatographic behavior of large molecular weight proteins on a triphenyl-silica stationary phase was also conducted. Polymeric C_{18}-silica stationary phases yielded higher phase ratios and better support surface coverage which proved useful in the separation of small hydrophilic species such as derivatized chitooligosaccharides of lower degree of polymerization (d.p.).

Copyright © 1997 by Marcel Dekker, Inc.

The slopes of the linear dependence of logarithmic retention factor on volume percent of organic modifier were determined for the monomeric and polymeric C_{18}-silica stationary phases with low molecular weight aromatic compounds. Lectin affinity stationary phases with *lens culinaris* agglutinin and wheat germ agglutinin covalently bound to nonporous silica support were examined for the applications of nonporous stationary phases in lectin affinity chromatography of glycoproteins. In all cases, rapid separations in the time scales of seconds and minutes could be obtained because of the absence of mass transfer resistance in the nonporous stationary phase matrices.

INTRODUCTION

The development of rapid separation schemes by HPLC has been an important theme of research in recent years. This is because fast chromatographic separations ensure short analysis time, rapid development of analytical methods, high mass recovery with preserved bioactivity for biological species, and improved separation efficiency.

Mass transfer resistances and solute adsorption-desorption kinetics are the two main factors that influence band broadening in chromatography. To reduce the mass-transfer resistance arising from slow diffusion commonly encountered in traditional porous column packing materials, one approach is to use particles with flow-through macropores.[1] Another approach is to eliminate the pore structure by using nonporous packing. Nonporous silica packing of small particle diameter (0.5 - 2.0 μm) offers about the same specific surface area per unit column volume as a silica with 400 nm pore size, 5 to 10 μm particle diameter and a packing density of 0.5 g/mL.[2]

Columns packed with nonporous particles have been used for rapid separation of biomacromolecules, such as peptides, proteins, oligonucleotides and nucleic acid restriction fragments, by reversed phase,[3-5] ion exchange[6,7] and hydrophobic interaction chromatography.[8] Furthermore, the application of nonporous silica and zirconia packing materials for reversed phase chromatographic separation of low-molecular-weight compounds was recently investigated.[9-11]

This paper reports the applications of nonporous monodispersed, microspherical silica particles having mean particle diameter of 0.8 and 1.1 μm. These particles were modified with octadecyl or triphenyl functions to

yield nonpolar stationary phases, and with lectins (e.g., LCA and WGA) to produce lectin affinity stationary phases. The chromatographic properties of the various modifications on these nonporous supports were evaluated with small and large molecular weight compounds.

EXPERIMENTAL

Instrumentation

The liquid chromatograph was assembled from (i) an LDC Analytical (Riviera Beach, FL, U.S.A.) ConstaMetric 3500 solvent delivery system with a gradient programmer, which was used to control a ConstaMetric Model III solvent delivery pump; (ii) a sample injector Model 7125 from Rheodyne (Cotati, CA, U.S.A.); and (iii) a UV interference filter photometric detector Model UV-106 from Linear Instruments (Reno, NV, U.S.A.). Chromatograms were recorded with a computing integrator Model C-R6A equipped with a floppy disk drive and a cathode-ray tube (CRT) monitor from Shimadzu (Columbia, MD, U.S.A.).

Reagents and Materials

HPLC grade acetonitrile and methanol, reagent grade sodium phosphate mono- and di-basic, sodium hydroxide, sodium chloride, sucrose, glacial acetic acid, anhydrous ammonia, saturated ammonium hydroxide, trifluoroacetic acid (TFA), benzene, toluene, *p*-xylene, and naphthalene, and reagent grade as well as technical grade isopropanol, methanol, 200 proof denatured ethanol, and *N*, *N*-dimethylformamide (DMF) were from Fisher (Pittsburgh, PA, U.S.A.). Tetraethylorthosilicate (TEOS), trimethylchlorosilane, sodium cyanoborohydride and sodium periodate were purchased from Aldrich (Milwaukee, WI, U.S.A.). γ-Glycidoxypropyltrimethoxysilane, triphenylchlorosilane, octadecyldimethylchlorosilane and octadecyltrichlorosilane were obtained from Hüls America Inc. (Bristol, PA, U.S.A.).

Ribonuclease A from bovine pancreas, cytochrome c from horse heart, lysozyme from chicken egg, glucose oxidase from *Aspergillus niger*, ovalbumin, α_1-acid glycoprotein from bovine and human, *p*-nitrophenyl-*N*-acetyl-α-D- and β-D-glucosaminide, *p*-nitrophenyl-*N*-acetyl-β-D-*N*,*N*'-diacetylchitobioside were purchased from Sigma (St. Louis, MO, U.S.A.). *N*-acetyl-D-glucosamine, *N*-acetyl-D-chitobiose, *N*-acetyl-D-chitotriose, and *N*-

acetyl-D-chitotetraose were obtained from Seikagaku America, Inc. (Rockville, MD, U.S.A.) and were labeled with 2-aminopyridine using a well established procedure.[12] *Lens culinaris* agglutinin (LCA) and wheat germ agglutinin (WGA) were from Vector Laboratories, Inc. (Burlingame, CA, U.S.A.).

Silica Supports

Microspherical, monodispersed nonporous silica particles were prepared by seed-growth process of base-catalyzed hydrolysis of TEOS following the procedures described earlier.[10] Briefly, 500 mL of ethanol placed in a 1.0 L three-neck round bottom flask was first saturated with anhydrous ammonia by bubbling it through the solution. Thereafter, 127 mL of saturated ammonium hydroxide and 8.0 mL TEOS were added while stirring. After 10 h, addition of 6.0 mL of TEOS and 1.4 mL water were repeated at every 12 h interval for a total of six additions. Silica particles thus prepared have a nonporous texture, monodispersed spherical shape with a mean diameter of *ca.* 0.8 μm. Nonporous monodispersed silica particles in the size of 1.1 μm were prepared as the above procedure except that 16 mL TEOS and 1.4 mL water were added for a total of five additions.

Commercially available Nucleosil silica of 7 μm mean particle diameter and 300 Å mean pore diameter was obtained from Machery-Nagel (Düren, Germany), and used as supplied.

Stationary Phases

Octadecyl-silica stationary phases

Monomeric and polymeric C_{18}-silica stationary phases were synthesized according to the procedures described in an earlier study.[10] The silica microspheres were treated with octadecyldimethylchlorosilane to form "monomeric" stationary phase. Further treatment of some monomeric C_{18}-silica stationary phases with chlorotrimethylsilane was performed to end-cap the residual silanol groups that might remain unreacted.

Octadecyl-silica of the "polymeric" type was formed by reacting the silica with octadecyltrichlorosilane. Both modifications were carried out in toluene while refluxing at 120°C for 12 h. End-capping with chlorotrimethylsilane was carried out in toluene at 60°C for 12 h.

Triphenyl-silica stationary phase

Typically, 3.0 g of silica support were suspended in 30.0 mL of toluene, and heated to 120°C. Then 8.0 mL of triphenylchlorosilane were added to the solution. The reaction mixture was stirred for 12 h at 120°C. After the reaction, the silica thus treated was separated from the solution, and then washed successively with toluene and methanol, and let dry in the air.

Lectin stationary phases

Microspherical silica particles were first epoxy activated by reacting them with γ-glycidoxypropyltrimethoxysilane in 1.0 mM Na_2HPO_4, pH 6.0. The reaction mixture was stirred at 95°C for 4 h. Thereafter, the epoxy ring was opened with 0.01 M HCl at 90°C, and then converted to aldehyde by allowing the diol-silica phase to react with sodium periodate in glacial acetic acid-water (9:1, v/v) at room temperature. The aldehyde-activated silica microparticles were reacted with WGA or LCA. In both cases, 50 mM phosphate buffer, pH 6.0, containing sodium cyanoborohydride was used as the reaction medium.

Column Packing

All columns, 3.0 x 0.46 cm I.D. No. 316 stainless steel tubes (Alltech Associates Inc., Deerfield, IL, U.S.A.) were packed at 7000 *psi* using slurry packing technique with Shandon column packer instrument (Keystone Scientific, Bellefonte, PA. U.S.A.). Octadecyl-silica stationary phases were packed from an isopropanol slurry with isopropanol as packing solvent. Lectin stationary phases were packed from an aqueous sucrose-NaCl slurry containing 50% (w/v) sucrose, with 1.0 M NaCl as the packing solvent.

RESULTS AND DISCUSSION

Reversed-Phase Chromatography

Reversed-phase chromatographic properties

Benzene, toluene, *p*-xylene and naphthalene were employed as model solutes to evaluate the chromatographic properties of the nonporous C_{18}-silica of 0.8 μm average particle diameter using mobile phases composed of water-acetonitrile mixtures at various volume percent of acetonitrile. As shown in Figure 1A and 1B, plots of log k' *vs.* φ are linear and follow the expression usually found in RPC:[13]

$$\log k' = \log k_w - S\varphi \quad (1)$$

where S is the slope of the linear function, which is characteristic of the solute molecular weight for a given stationary phase, φ is the volume fraction of the organic solvent in the mobile phase, and k_w is the retention factor obtained with pure water. The value of S for a given solute has important consequences in the selection of optimum gradient elution conditions.[14] For porous stationary phases, and to a first approximation, S is given by[15]

$$S = 0.48M^{0.44} \quad (2)$$

where M is the molecular weight of the solute. The S values of the four aromatic solutes obtained on the monomeric and polymeric C_{18}-silica stationary phases are listed in Table 1. As can be seen in Table 1, the S values for the solutes obtained on both types of C_{18}-silica stationary phases increased with increasing molecular weight of the solute. Compared to the calculated S values using equation (2), which applies to porous packings, the S values obtained on the nonporous silica sorbent are in the same range, except for that of benzene. Conversely, the S values of large molecular weight proteins (MW in the range 12,000-162,000) were reported to be 10-fold smaller on nonporous than on porous support.[3] This may indicate that the rapid mass transfer of large molecules on the external surface of the packing affects the rate constants of adsorption and desorption in such a way that lower equilibrium constants and distribution coefficients will result.[3] For small molecules the difference in kinetics of the diffusion process will be much less significant, when comparing solute diffusion into and out of a pore system to solute diffusion on exclusively external surfaces. This may explain why the S values for small molecules obtained on nonporous ODS packings were similar to the calculated values.

Chromatographic behavior of 2-AP derivatized linear and branched oligosaccharides

In our previous studies,[10,11] it was found that polymeric C_{18}-silica stationary phases exhibit higher phase ratios and better support surface coverage than the monomeric ones. This was especially important in the separations of small molecules of closely related species, *e.g.* dansyl amino acids. Similarly, and because of its relatively high surface density in octadecyl function, the polymeric C_{18}-silica stationary phase was useful for the separation of hydrophilic species such as the 2-pyridylamino derivatives of chitooligosaccharides of low d.p. (see Figure 2). As can be seen in Figure 2, the four solutes could be separated in less than four minutes with gradient elution of up to 10% (v/v) acetonitrile in the mobile phase at a flow rate of 2.0 mL/min.

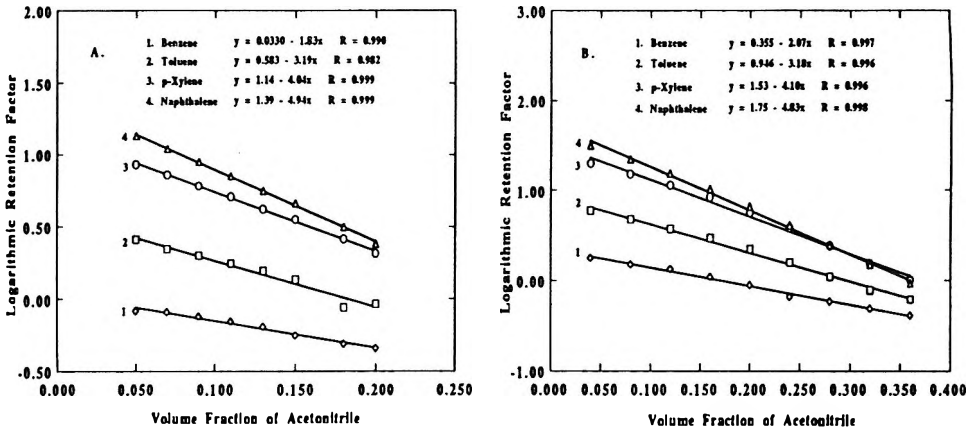


Figure 1. Plots of logarithmic retention factor versus the volume percent acetonitrile in mobile phase for end-capped monomeric octadecyl-silica stationary phase in A; and polymeric octadecyl-silica stationary phase in B. Columns, 30.0 x 4.6 mm; mobile phase, water at various volume percent acetonitrile; flow rate, 2.0 mL/min.

Table 1

Values of S Calculated using Equation (2) and Determined from the Linear Plots According to Equation (1)

		S-Value		
	M.W.	**Calculated**	**Monomeric C_{18}-silica**	**Polymeric C_{18}-silica**
Benzene	78.11	3.27	1.83	2.07
Toluene	92.14	3.51	3.19	3.18
p-Xylene	106.17	3.74	4.04	4.10
Naphthalene	128.17	4.06	4.94	4.83

Column. 30.00 x 4.6 mm. end-capped monomeric and polymeric octadecyl-silica stationary phases; mobile phase, water at various volume fraction of acetonitrile; flow rate, 2.0 mL/min.

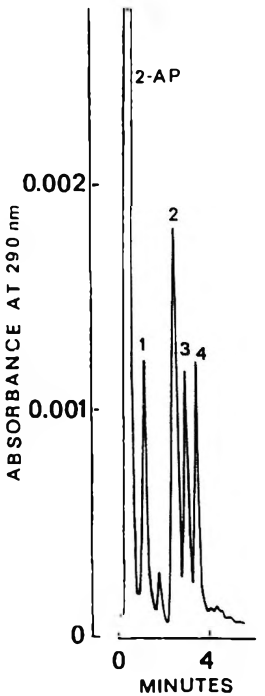


Figure 2 Chromatograms of 2-pyridylamino derivatives of chitooligosaccharides obtained on polymeric octadecyl-silica stationary phase. Column, 30.0 x 4.6 mm; linear gradient in 5.0 min from 0.0 to 10.0% (v/v) acetonitrile in 10.0 mM NaH_2PO_4, pH 3.0; flow rate, 2.0 mL/min; samples: 2-pyridylamino derivatives of 1, *N*-acetyl-D-glucosamine; 2, chitobiose; 3, chitotriose; 4, chitotetraose; detection, UV, l = 290 nm.

On the other hand, for branched oligosaccharides such as 2-pyridylamino derivatives of xyloglucan oligosaccharides, monomeric C_{18}-silica columns yielded better separation than polymeric C_{18}-silica stationary phases. Figure 3 illustrates the various maps of 2-pyridylamino derivatives of xyloglucan oligosaccharides (PA-XG) at different mobile phase flow rates and gradient duration, performed on a short column (30.0 x 4.6 mm) packed with 0.8 μm nonporous octadecyl-silica monomeric stationary phase. As can be seen in the chromatograms, upon increasing the flow rate from 1.0 mL/min to 3.0 mL/min and gradient steepness from 0.40 to 0.85% (v/v) acetonitrile/min, the analytical information about the content of the mixture remained almost unchanged, as far as the larger PA-XG fragments (*i.e.* retarded peaks) are concerned. In addition, the peaks were sharper at the mobile phase flow rate of 3.0 mL/min than at 1.0 mL/min. and, consequently, for the same signal the amount injected

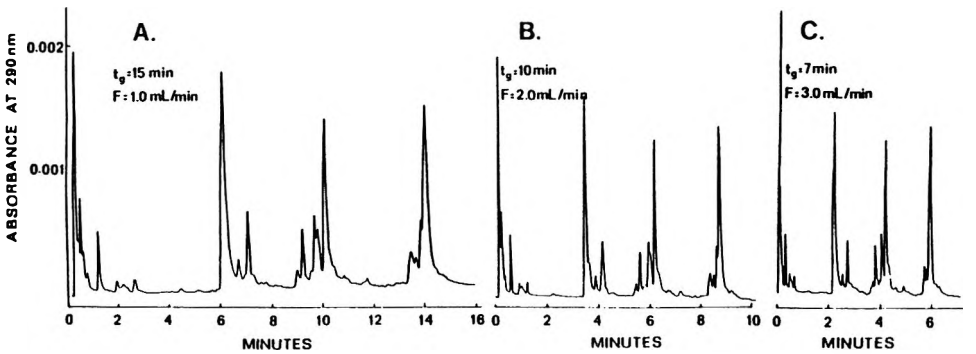


Figure 3. Chromatograms of 2-pyridylamino derivatives of xyloglucan oligosaccharides obtained on end-capped monomeric octadecyl-silica (d_p = 0.8 μm) stationary phase. Column, 30.0 x 4.6 mm; linear gradient from 0.0 to 6.0% (v/v) acetonitrile in 10.0 mM NaH_2PO_4, pH 3.0; the gradient duration (t_g) and flow rate (F) are indicated in each panel; detection, UV, l = 290 nm.

was 2 to 3 times lower at higher flow velocity. This clearly demonstrates that rapid reversed phase chromatography with appropriately designed nonporous octadecyl-silica stationary phase yields excellent separation for closely related oligosaccharides with high sensitivity and high separation efficiencies.

When the same oligosaccharide mixture was chromatographed on the same size silica gel (*i.e.*, 0.8 μm) but with "polymeric" octadecyl bonded phase, the amount of organic solvent needed to bring about the same time of analysis as with the "monomeric" octadecyl-silica packings is higher (*ca. 1.7*-fold, results not shown). This is expected as the phase ratio is increased. However, the resolution did not improve when going from monomeric to polymeric octadecyl bonded phase, and part of the analytical information about the mixture was lost, even when using the same gradient profile as with the monomeric octadecyl sorbent. This corroborates our previous findings[16] with porous octadecyl silica stationary phases in that higher phase ratio sorbents, which necessitates increased percent of organic solvent in the mobile phase, did not bring about good resolution for this mixture. This is probably due to organic solvent-induced conformational changes of the analytes. In higher organic solvent mobile phases, the difference in the molecular hydrophobic surface areas of the various sugar chains was diminished during the elution process of the oligomers from the stationary phase, and as a result lower selectivity was obtained.

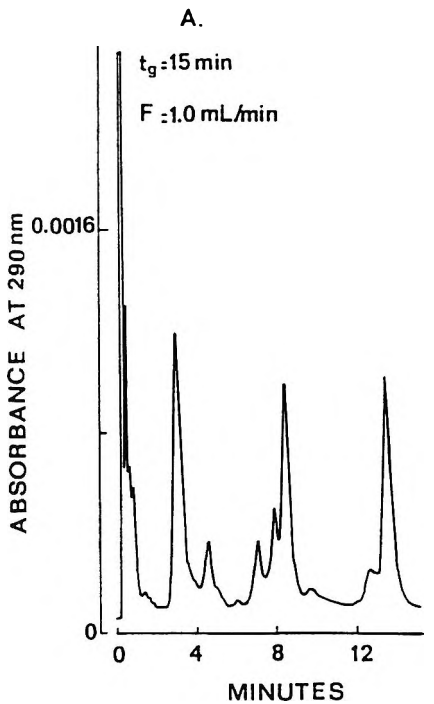


Figure 4. Chromatograms of 2-pyridylamino derivatives of xyloglucan oligosaccharides obtained on end-capped monomeric octadecyl-silica (d_p = 1.1 μm) stationary phase. Column, 30.0 x 4.6 mm; linear gradient from 0.0 to 6.0% (v/v) acetonitrile in 10.0 mM NaH_2PO_4, pH 3.0; the gradient duration (t_g) and flow rate (F) are indicated in the figure; detection, UV, l = 290 nm.

To determine the effect of particle size on the resolution of the oligomers, the PA-XG oligosaccharides were chromatographed on a 1.1 μm nonporous silica gel having monomeric octadecyl bonded phase. The results are shown in Figure 4. As expected, increasing the particle size by a factor of 1.4 resulted in decreased retentivity of the column as a result of the decreased specific surface area of the silica particles. The percent organic solvent in the mobile phase needed to bring about the same time of analysis as with 0.8 μm decreased by a factor of 1.5. Also, the resolution for the early eluting peaks decreased significantly, and part of the analytical information was lost. It seems that there is an optimum phase ratio that is best attained by decreasing the particle size of the support and using monomeric octadecyl bonded stationary phase for the rapid and high efficiency separation of these branched oligosaccharides.

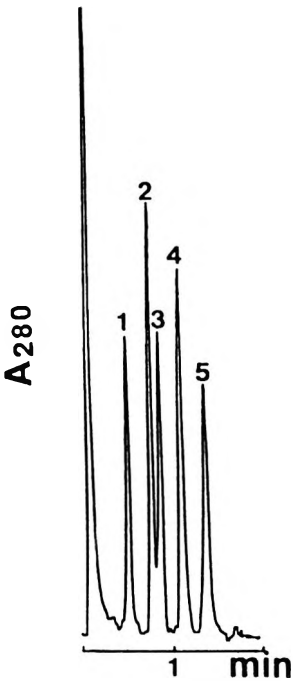


Figure 5. Chromatogram of proteins obtained on monomeric octadecyl-silica stationary phase without end-capping. Column, 30.0 x 4.6 mm; linear gradient in 2.5 min from 20.0 to 70.0% (v/v) acetonitrile in aqueous mobile phase with 0.05% (v/v) TFA; flow rate, 4.0 mL/min; samples: 1, ribonuclease A; 2, cytochrome c; 3, lysozyme; 4, albumin (bovine); 5, ovalbumin; detection, UV, l = 280 nm.

Chromatographic behavior of proteins

Although the low phase ratio of monomeric C_{18}-silica stationary phase on 1.1 μm silica support limits the resolution of hydrophilic species (see preceding section), this feature, on the other hand, can be regarded as an advantage in terms of bringing chromatographic retention to a practical range, especially in the chromatography of biomacromolecules, *e.g.*, proteins. Under these circumstances, biopolymers may be separated under milder elution conditions which would preserve their biological activity and allow their high mass recovery. Figure 5 is an example of the high speed separation of a group of five proteins on monomeric C_{18}-silica stationary phase without end-capping having a mean particle diameter of 1.1 μm.

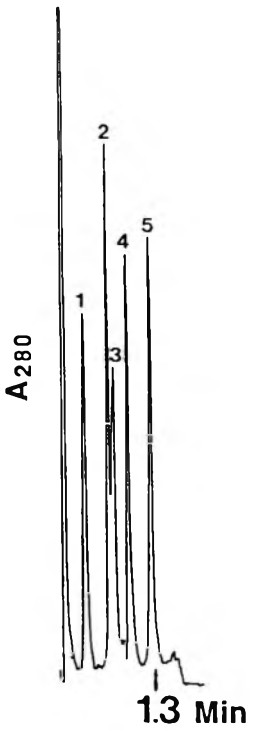


Figure 6. Chromatogram of proteins obtained on triphenyl-silica stationary phase. Column, 30.0 x 4 6 mm; linear gradient in 1.5 min from 20.0 to 70.0% (v/v) acetonitrile in aqueous mobile phase with 20.0 mM NaCl and 0.05% (v/v) TFA; flow rate, 4.0 mL/min; samples: 1. ribonuclease A; 2, cytochrome c; 3, lysozyme; 4, albumin (bovine); 5, ovalbumin; detection, UV, l = 280 nm.

The separation was accomplished in less than 1.5 min at mobile phase flow rate of 4.0 mL/min using a gradient elution from 20 to 70% (v/v) acetonitrile in the mobile phase in 2.5 min. Even though the phase ratio for this stationary phase was quite low, its application for the separation of macromolecules could still produce enough resolving power.

Silica microparticles (1.1 μm) were also modified with triphenyl functional groups. The same group of proteins chromatographed on the C_{18}-silica stationary phase was separated on the triphenyl-silica stationary phase, see Figure 6. The elution pattern was the same, except that 20.0 mM NaCl had to be added to the mobile phase since otherwise these proteins could not be eluted from the triphenyl stationary phase. This may indicate the presence of

unreacted surface silanols which act as cation exchange sites for polyionic species such as proteins. When the same hydro-organic mobile phase was used for both C_{18}-silica and triphenyl-silica stationary phases, slightly lower retention for all the proteins was observed on the triphenyl phase, possibly because of the lower hydrophobicity of the triphenyl functions when compared to the octadecyl functions. Better resolution between cytochrome c and lysozyme was obtained on the C_{18}-silica than on the triphenyl-silica stationary phase (compare Figures 5 and 6).

Lectin Affinity Chromatography of Glycoproteins and Small Glycoconjugates

Glycoproteins are heterogeneous species due to the variations in the oligosaccharide chains attached.[17] This heterogeneity is amplified when the glycoprotein solute is multiply glycosylated with variations in the number and location of the oligosaccharides as well as in the nature of the sugar chains. These microheterogeneities lead to multiple forms for a given glycoprotein, and they are referred to as glycoforms. These glycoforms have been shown to correlate with certain clinical syndromes.[18,19] Thus, the ability to separate and quantitatively measure the various glycoforms of a given glycoprotein has become increasingly important. In this regard, lectins which are sugar-binding proteins[20] are powerful tools for studying glycoprotein microheterogeneity.[21,22] In particular, lectin affinity chromatography can reveal the microheterogeneity of glycoproteins, imparted by their oligosaccharide chains. This is often manifested by the presence of three different peaks:

> (i) non-reactive population of the molecules, *i.e.*, molecules missing the oligosaccharide moieties that can be recognized by the lectin, which are eluted in the void volume of the column with the binding buffer;
>
> (ii) weakly reactive population of the molecules, *i.e.*, molecules containing weakly reactive oligosaccharide chains; and
>
> (iii) strongly reactive molecules which are specifically desorbed and eluted with the debinding buffer containing the appropriate haptenic sugar.[17]

Two lectins, LCA and WGA, were immobilized onto nonporous silica microparticles for rapid lectin affinity chromatography of glycoconjugates. Figure 7A and B show the rapid lectin affinity chromatography of glucose

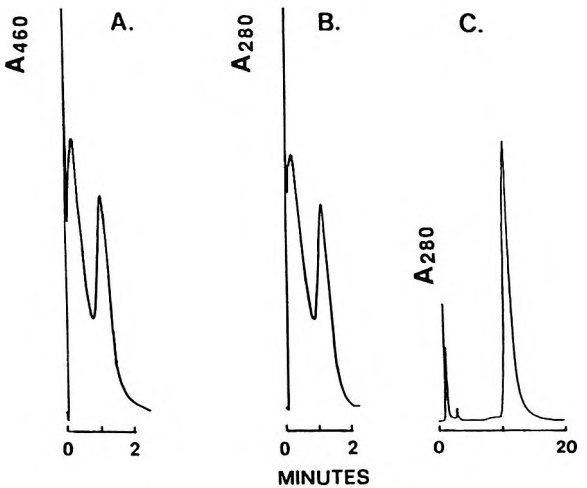


Figure 7. Chromatograms of glucose oxidase obtained on nonporous (0.8 μm) silica-bound LCA stationary phases in (A) and (B), and porous (7 mm, 300 Å) silica-bound LCA stationary phase in (C). (A) and (B): column, 30.0 x 4.6 mm; gradient from 0.0 to 25.0 mM methyl-α-D-mannopyranoside in 25.0 mM Na_2HPO_4, pH 6.0, and 200.0 mM NaCl, in 0.20 min, and isocratic elution for 2.0 min; flow rate, 3.0 mL/min; (C): column, 100.0 x 4.6 mm; linear gradient from 0.0 to 20.0 mM methyl-α-D-mannopyranoside in 20.0 mM $NaOOCCH_3$, pH 6.0, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 200.0 mM NaCl, in 1.0 min, and isocratic elution for 10.0 min; flow rate, 1.0 mL/min. Detection, UV, (A) l = 460 nm, (B) and (C) l = 280 nm.

oxidase performed on a short column (30.0 x 4.6 mm) packed with 0.8 μm nonporous LCA-silica. As can be seen in Fig. 7, the LCA column yielded three peaks for the protein. One peak eluted in the void volume of the column, and is believed to correspond to the non-reactive population of the glycoprotein molecules. A second peak corresponding to molecules missing the saccharide determinant, *i.e.*, weakly reactive components, was slightly retained, and eluted with the binding buffer. The third peak was specifically eluted with the debinding buffer containing the haptenic sugar, methyl-α-D-mannopyranoside, thus corresponding to the molecules containing the oligosaccharide chains with the sequences that are necessary for lectin recognition, *i.e.*, strongly reactive components. Note that glucose oxidase is a flavoprotein, the prosthetic group being flavin adenine dinucleotide which is well detected in the visible region at 460 nm (see Figure 7A); the chromatogram recorded at 280 nm corresponds to the specific UV absorbance of the peptide bonds of the protein. On the other hand, on porous silica (300 Å) with surface bound LCA, the weakly binding

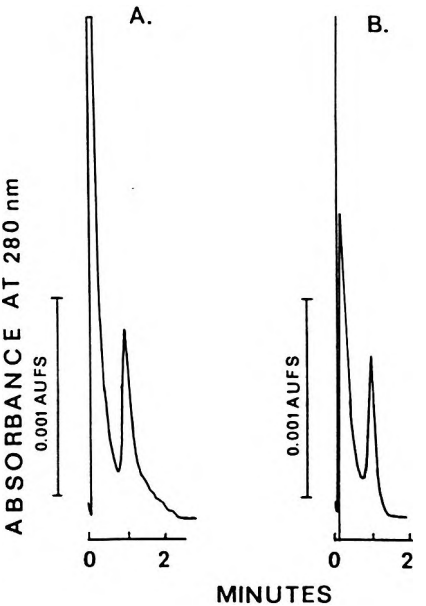


Figure 8. Chromatograms of α_1-acid glycoprotein from bovine in A and human in B obtained on nonporous silica-bound WGA stationary phase. Column, 30.0 x 4.6 mm; gradient from 0.0 to 25.0 mM *N*-acetylglucosamine in 25.0 mM Na_2HPO_4, pH 6.0, and 200.0 mM NaCl, in 0.20 min, and isocratic elution for 2.0 min; flow rate, 3.0 mL/min.

fraction of glucose oxidase (i.e., the second peak with the nonporous LCA column) seemed to elute with the lectin reactive fraction, and one single retained peak was recorded, see Figure 7C. This can be explained by the much higher phase ratio obtained on the porous support. The strong binding interactions exhibited by the highly populated lectin stationary phase rendered the two fractions of glucose oxidase to coelute at the same concentration of methyl-α-D-mannopyranoside in the eluent using gradient elution.

Figure 8 illustrates the rapid lectin affinity chromatography of bovine and human α_1-acid glycoproteins obtained on a column packed with 1.1 μm nonporous WGA-silica particles. The selectivity of the lectin column permitted the illustration of the microheterogeneity of the glycoproteins. α_1-Acid glycoprotein from bovine was separated into two fractions, see Figure 8A. One fraction eluted first, and is believed to contain the non-reactive and slightly reactive components of the glycoprotein. The second peak containing the strongly reactive components was highly retained and eluted with the haptenic

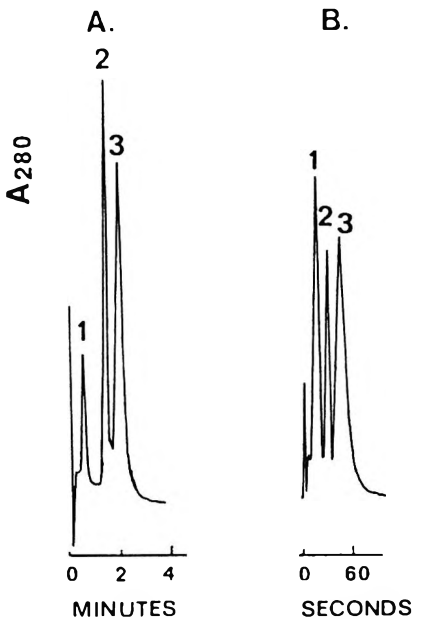


Figure 9. Chromatograms of *p*-nitrophenyl derivatives of saccharides obtained on nonporous silica-bound WGA stationary phase. Column, 30.0 x 4.6 mm; in A linear gradient from 0.0 to 50.0 mM *N*-acetylglucosamine in 100.0 mM Na_2HPO_4, pH 6.0, in 1.0 min, and isocratic elution for 2.0 min; in B linear gradient from 0.0 to 100.0 mM *N*-acetylglucosamine in 100.0 mM Na_2HPO_4, pH 6.0, in 0.50 min, and isocratic elution for 2.0 min; flow rate, 3.0 mL/min in both cases; samples, *p*-nitrophenyl derivatives of 1, *N*-acetyl-α-D-glucosaminide; 2, *N*-acetyl-β-D-glucosaminide; 3, b-D-*N*,*N*'-diacetylchitobioside; detection, UV, l = 280 nm.

sugar. α_1-Acid glycoprotein from human yielded three peaks (Figure 8B): one non-reactive, another slightly retained containing the weakly reactive components, and the third one was retarded containing the strongly reactive components.

Lectin affinity chromatography is characterized by its inherent slow sorption kinetics which often leads to bandspreading. With porous particles, axial dispersion at high flow velocities would be influenced by two independent processes, kinetic resistance and intraparticular diffusion resistance. With nonporous affinity stationary phases, however, since there is no solute mass transfer in and out of the pores, the major contribution to bandspreading comes only from the sorption kinetics. A more energetically uniform surface is

expected to result from the modification of support having totally exposed surface of nonporous texture than from the modification of porous sorbents. As a consequence, nonporous stationary phases may exhibit the sorption kinetics that are significantly faster than that of the porous stationary phases. The high efficiency of nonporous WGA-silica stationary phase was demonstrated with the rapid separation of *p*-nitrophenyl derivatives of mono- and disaccharides (see Figure 9). The high resolution was achieved even when steep gradient of strong eluent was used, and the separation was accomplished in less than 1min.

CONCLUSIONS

The chromatographic applications of nonporous monodispersed, microspherical silica particles were examined for rapid HPLC. Surface modification with octadecyl functions for the formation of reversed phases have shown that the polymeric C_{18}-silica stationary phases are suitable for the separation of derivatized homooligosaccharides of low d.p. (e.g., 2-pyridylamino-chitooligosaccharides). The monomeric C_{18}-silica, when the surface modification was properly carried out, can yield higher resolving power than the polymeric type toward derivatized branched heterooligosaccharides. Uniformly covered supports of low ligand density, such as monomeric C_{18}-silica without end-capping, are suitable for high-speed separation of biomacromolecules such as proteins. On the other hand, low ligand density silica bonded stationary phases having covalently bound triphenyl functions necessitated the addition of small amounts of sodium chloride to the mobile phase to bring about the elution and separation of proteins. The formations of lectin affinity stationary phases on the nonporous silica supports have proved to be useful for the rapid analysis of the microheterogeneity of glycoproteins.

REFERENCES

1. N. B. Afeyan, S. P. Fulton, F. E. Regnier, J. Chromatogr., **544**, 267-279 (1991).

2. K. K.Unger, G. Jilge, R. Janzen, H. Giesche, J. N. Kinkel, Chromatographia, **22**, 379-380 (1986).

3. K. K. Unger, G. Jigle, J. N. Kinkel, M. T. W. Hearn, J. Chromatogr., **359**, 61-72 (1986).

4. K. Khalghatgi, Cs. Horváth, J. Chromatogr., **443**, 343-354 (1988).

5. Y.-F. Maa, Cs. Horváth, J. Chromatogr., **445**, 71-86 (1988).

6. Y. Kato, T. Kitamura, A. Mitsui, T. Hashimoto, J. Chromatogr., **447**, 212-220 (1988).

7. Y. Kato, Y. Yamasaki, A. Onaka, T. Kitamura, T. Hashimoto, T. Murotsu, S. Fukushige, K. Matsubara, J. Chromatogr., **478**, 264-268 (1989).

8. R. Janzen, K. K. Unger, H. Giesche, J. N. Kinkel, M. T. W. Hearn, J. Chromatogr., **397**, 91-97, (1987).

9. H. Itoh, T. Kinoshita, N. Nimura, J. Liq. Chromatogr., **16**, 809-823 (1993).

10. J. Yu, Z. El Rassi, J. Liq. Chromatogr., **16**, 2931-2959 (1993).

11. J. Yu, Z. El Rassi, J. Chromatogr., **631**, 91-106 (1993).

12. W. Nashabeh, Z. El Rassi, J. Chromatogr., **536**, 31-42 (1991).

13. L. R. Snyder, "Gradient Elution," in **High-Performance Liquid Chromatography**, Cs. Horvath, ed., Vol. 1, Academic Press, New York, 1980, p. 207-316.

14. L. R. Snyder, J. L. Glajch, J. J. Kirkland, "Gradient Elution," in **Practical HPLC Method Development**, Wiley-Interscience, New York, 1988, pp. 153-178.

15. M. A. Stadalius, H. S. Gold, L. R. Snyder, J. Chromatogr., **296**, 31-59 (1985).

16. Z. El Rassi, D. Tedford, J. An. A. Mort, Carbohydr. Res., **215**, 25-38 (1991).

17. J. Montreuil, S. Bouquelet, H. Debray, B. Fournet, G. Spik, G. Strecker, "Glycoproteins," in **Carbohydrate Analysis**, M. F. Chaplin and J. F. Kennedy, eds., IRL Press, Oxford, 1986, pp. 143-204.

18. H. G. van Ejik, W. L. van Noort, G. de Jong, J. F. Koster, Clin. Chim. Acta, **165**, 141-145 (1987).

19. H. Stibler, O. Sydow, S. van der Berg, Pharmacol. Biochem. Behav., **13**, 47-51 (1980).

20. H. Debray, J. Montreuil, "Specificity of Lectins Toward Oligosaccharidic Sequences Belonging to *N*- and *O*-Glycosylglycoproteins," in **Affinity Electrophoresis, Principles and Application**, J. Breborowicz, A. Mackiewicz eds., CRC Press, Boca Raton, 1992, pp. 23-57.

21. H. Lis, N. Sharon, "Lectins: Their Chemistry and Application to Immunology," in **The Antigens**, M. Sela, ed., Vol. 4, Academic Press, New York, 1977, pp. 429-529.

22. I. Goldstein, C. Hayes, Adv. Carbohydr. Chem. Biochem., **35**, 127-340 (1978).

† Current affiliation: Laboratory of Biology of Addictive Diseases, The Rockefeller University, New York, NY 10021-6399, USA.

Received August 1, 1996
Accepted August 15, 1996
Manuscript 4238

J. LIQ CHROM & REL. TECHNOL., 20(2), 203–216 (1997)

DETERMINATION OF ASSOCIATION CONSTANTS IN CYCLODEXTRIN OR VANCOMYCIN-MODIFIED MICELLAR CAPILLARY ELECTROPHORESIS

Usha B. Nair, Kimber L. Rundlett, Daniel W. Armstrong*

Department of Chemistry
University of Missouri - Rolla
Rolla, MO 65401

ABSTRACT

The estimation of association constants of chiral analytes to chiral selectors is often useful in determining the nature and extent of interactions that exist between them. These determinations also provide insight into the mechanisms that are involved in the enantiorecognition of these analytes. Simple models have been developed previously to estimate association constants of analytes binding through a 1:1 stoichiometry with a ligand. These models have also been extrapolated to explain the binding behavior of analytes in systems containing two ligands. In the present study, the binding behavior of some chiral analytes was studied in micellar capillary electrophoresis (CE) systems modified with hydroxypropyl-β-cyclodextrin (HPBCD) or vancomycin as the chiral selectors. The pseudophase or three phase model was used as the basis for the determination of the absolute binding constants of the solutes to the selector and to the micellar phases.

Copyright © 1997 by Marcel Dekker, Inc.

However, these systems are complicated due to the interactions that exist not only between the analyte and the chiral selector/micellar phase but also between the ligands themselves. In the case of the hydroxypropyl-β-cyclodextrin/sodium dodecyl sulfate (SDS) system, the SDS monomers are included in the cyclodextrin cavity. Similarly, in the vancomycin/SDS system, vancomycin partitions itself into micelles. The limitations associated with the use of ideal mathematical models in these complex systems, are also discussed.

INTRODUCTION

Cyclodextrins are one of the more widely used selectors for chiral separations in CE. Although native cyclodextrins are used for specific applications,[1,2] there has been a constant search for better chiral selectors compatible to CE systems. Recently, several derivatized cyclodextrins have been introduced for use in CE.[3-5] These derivatized cyclodextrins are effective because they frequently have higher solubility, somewhat different enantioselectivities and many of them are charged. Another class of selectors that have proved to be very useful in chiral CE are the macrocyclic antibiotics.[6-8] The application range of commonly used chiral selectors was further expanded when they were used in conjunction with micellar media.[9-11] Enantiomeric separations based upon the selective partitioning of solutes between a chiral selector and a micellar pseudophase has been proven to have numerous applications in CE, particularly in the separation of neutral and hydrophobic compounds in aqueous run buffers.[12,13]

Several approaches have been utilized to measure the extent of binding of analytes to chiral selectors and/or micellar media and to help to understand some of the mechanistic features underlying enantioseparations. Many theoretical models (often derived from chromatographic systems) have been developed to determine the association or binding constants of solutes to commonly used chiral selectors or pseudophases.[3,10,14] Most of these studies have been done on simple systems involving a 1:1 association of a solute to a single ligand. Shibukawa et al.[15] studied the extent of binding of leucovorin and related compounds to native γ-cyclodextrin. Gahm and Stalcup[4] determined the extent of binding of dinitrobenzoylated amino acids to naphthylethylcarbamate-derivatized cyclodextrin, and Penn et al. measured the binding of tioconazole enantiomers to hydroxypropyl-β-cyclodextrin.[16] Similarly, the binding of solutes to micellar systems has been evaluated.[17] A variety of experimental, mathematical and graphical approaches have been used to determine these binding constants.[17] Equations have been developed

that allow the determination of equilibrium constants, with reasonable accuracy, for solutes binding in a 1:1 stoichiometry with a ligand. However, very few binding studies have been done for more complex systems such as those containing a polyvalent ligand[18] or systems where a solute is capable of binding to two different ligands.[10,19] In these cases, modified equations have been developed to determine the respective binding constants of the solute to each ligand.

In the present study, the binding constants of analytes to hydroxypropyl-β-cyclodextrin in sodium dodecyl sulfate (SDS)-modified run buffers were determined using the modified equation proposed for the vancomycin-SDS system.[10] Also, an attempt was made to recalculate some of the binding constants determined for solutes to the vancomycin-SDS system, using the simpler graphical plotting methods outlined in an earlier work,[17] instead of the more involved method adopted in a previous vancomycin-SDS study.[10] The difficulties and inconsistencies encountered in the accurate determination of binding constants in a multicomponent/multipseudophase system using these methods are discussed.

EXPERIMENTAL

The hydroxypropyl-β-cyclodextrin (average molar substitution = 0.6) used in this study was obtained from Astec (Whippany, NJ). Vancomycin hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO) and SDS was obtained from Bio-Rad (Richmond, CA).

The monosodium hydrogen phosphate used to prepare the run buffer was purchased from Aldrich (Milwaukee, WI). All the analytes evaluated were obtained from Aldrich (Milwaukee, WI) or from Sigma Chemical Co. (St. Louis, MO).

Capillary electrophoresis was performed using a 50 μm x 50 cm (57 cm to the detector) capillary at 15 kV, using a PACE 2100 CE unit (Beckman Instruments, CA). All analytes were detected using on-line UV detection at 254 nm. All experiments were carried out at 25° C. A 0.05 M phosphate buffer (pH = 7) was used as the run buffer.

The fused silica capillaries used in this study were purged for 10 minutes with a 0.1M KOH solution, followed by a water rinse for 5 minutes, at the beginning of each day.

Subsequent to each run, the capillary was rinsed with KOH, water and the run buffer for two minutes each. Methanol (Fisher Scientific , NJ) was used as the electroosmotic flow (eof) marker and quinine hydrochloride (Aldrich, WI) was used as the micelle marker. All buffers and analyte solutions were filtered using 0.45μm filters (Alltech, IL), before introduction into the capillary.

Statistical treatment of the data obtained was performed using the weighted linearized least square software.[20] A surface tensiometer (model 20, Fisher, St.Louis, MO) was used to perform surface tension measurements.

RESULTS

Simple pseudophase models can be used to describe the association of an analyte with various components in solution (e.g., chiral selectors, micelles, etc.).[10,17,21] Each distinct molecular or aggregational component in solution that participates in the separation (in an associative manner) can be treated as a distinct phase or more accurately, as a pseudophase. This includes the bulk solution (that can contain salts, buffer, etc., but not other pseudophase components) which is treated as a pseudophase. This terminology and approach was developed in the early days of micelles and cyclodextrin-based separations.[22-25] For example, when both micelles and cyclodextrins are present in the run buffer, the three pseudophases would be: a) the micelle b) the cyclodextrin, and c) the rest of the bulk solution. If two different cyclodextrins (one charged and one uncharged, for example) were used,[26] then each would constitute a distinct pseudophase.

In evaluating the binding of analytes to the selector and the micelle in the hydroxypropyl-β-cyclodextrin / SDS and vancomycin / SDS systems, the pseudophase model was used. Since it is expected that the mobility of the free analyte would be different from its mobility when bound to the selector or the micelle pseudophase, it is envisaged that the experimentally determined mobility of the solute (μ_i) is a weighted average of its mobilities in each of the three pseudophases.[2,10] Based on the three phase model, an equation was previously derived to determine the binding constants of a solute to the chiral selector and the micelle.[10] A general form of this equation is:

$$K_{mic} = \frac{1}{[M]_f}\left(\frac{\mu_f - \mu_i}{\mu_i - \mu_m}\right) + \frac{K_{sel}[S]_f}{[M]_f}\left(\frac{\mu_s - \mu_i}{\mu_i - \mu_m}\right) \qquad (1)$$

where K_{mic} and K_{sel} are, respectively, the equilibrium constants for binding of the solute to the micelle and of the solute bound to the selector. $[M]_f$ refers to the equilibrium concentration of the free micelle, $[S]_f$ is the equilibrium concentration of the free selector existing in solution . Also, μ_f represents the mobility of the free solute in the aqueous phase, μ_s is the mobility of the solute-selector complex and μ_m is the mobility of the solute-micelle complex. μ_i is the experimentally measured mobility of the solute at a given concentration of the selector and the micelle. This equation requires the independent determination of μ_s and K_{sel} in order to determine K_{mic}. The value of μ_s was estimated using the relationship $\mu \alpha f/M^{2/3}$ where μ is the mobility of the solute, f its fraction of charge and M is its molecular weight. By plotting mobility versus $f/M^{2/3}$ for standards of known charge and molecular weight, the mobility μ_s for the appropriate selector was determined.[10] This value of μ_s was then substituted into the equation, $K_{sel}[S]_f = (\mu_f-\mu_i)/(\mu_i-\mu_s)$ to determine K_{sel}.[10,16] The K_{sel} value, thus determined, was substituted into equation (1) to obtain K_{mic}. In order to circumvent the use of this procedure in this study, μ_s was determined by the graphical method outlined earlier.[17] The double reciprocal method was used, in which $1/(\mu_I-\mu_f)$ was plotted as a function of change in selector concentration. By measuring the slope and intercept of the linear plot obtained, μ_s and K_{sel} were calculated. The advantage of this method is that actual mobility of the solute-ligand complex, which is usually measured as the limiting mobility of the solute at saturating ligand concentrations, is not required to be determined for calculation of K_{sel} and hence a range of inconveniences and errors associated with its measurement can be avoided.[17] In an attempt to develop a method to simultaneously determine K_{sel} and K_{mic}, equation (1) was rearranged as follows :

$$\frac{K_{mic}}{K_{sel}} = \frac{1}{[M]_f K_{sel}}\left(\frac{\mu_f-\mu_i}{\mu_i-\mu_m}\right) + \frac{[S]_f}{[M]_f}\left(\frac{\mu_s-\mu_i}{\mu_i-\mu_m}\right) \qquad (2)$$

Equation (2) can be re-expressed as :

$$\frac{[S]_f}{[M]_f}\left(\frac{\mu_s-\mu_i}{\mu_i-\mu_m}\right) = \frac{K_{mic}}{K_{sel}} + \frac{1}{[M]_f K_{sel}}\left(\frac{\mu_i-\mu_f}{\mu_i-\mu_m}\right) \qquad (3)$$

By plotting $\frac{[S]_f}{[M]_f}\left(\frac{\mu_s - \mu_i}{\mu_i - \mu_m}\right)$ on the Y axis and $\frac{1}{[M]_f}\left(\frac{\mu_i - \mu_f}{\mu_i - \mu_m}\right)$ on the X axis for the linear equation (3), K_{sel} and K_{mic} can be conveniently and simultaneously determined using the relationships

$$K_{sel} = \frac{1}{\text{slope}} \text{ and } K_{mic} = \frac{\text{Intercept}}{\text{Slope}}.$$

In determining K_{mic} using equation (3), K_{sel} and μ_s were proposed to be determined simultaneously using the double reciprocal plot.

Determination of Binding Constants of Analytes in the Hydroxypropyl-β-Cyclodextrin System

The mobility of an analyte bound to hydroxypropyl-β-cyclodextrin (μ_{cd}) was determined by measuring the mobility of the analyte at various concentrations of hydroxypropyl-β-cyclodextrin [HPBCD] and plotting $1/(\mu_i - \mu_f)$ versus 1/[HPBCD].[17] Figure 1 shows the double reciprocal plots obtained for the first eluted enantiomer of some of the analytes studied. It must be noted that in the calculation of μ_i and μ_f the migration times were corrected in order to minimize effects of changes in the mobility of the solute due to changes in the viscosity of the solutions at various hydroxypropyl-β-cyclodextrin concentrations.[15,17] From the slopes and intercepts of these lines, K_{sel} was calculated as the ratio of intercept to slope and μ_{cd} was calculated from the intercept.[17]

The binding constants obtained for various analytes to hydroxypropyl-β-cyclodextrin using this method are shown in Table 1. After having determined μ_{cd}, the concentration of SDS in the run buffer was varied while maintaining a constant concentration of hydroxypropyl-β-cyclodextrin. The mobility of the analyte was determined at each of these concentrations. The mobilities thus measured were then substituted into equation (3) in order to directly and simultaneously determine K_{sel} and K_{mic} as mentioned above. The K_{sel} measured in this manner could be expected to be compared to K_{sel} previously calculated for the binding of the analyte to hydroxypropyl-β-cyclodextrin using the double reciprocal plot. However, it was found that this approach yielded negative values of K_{sel}.

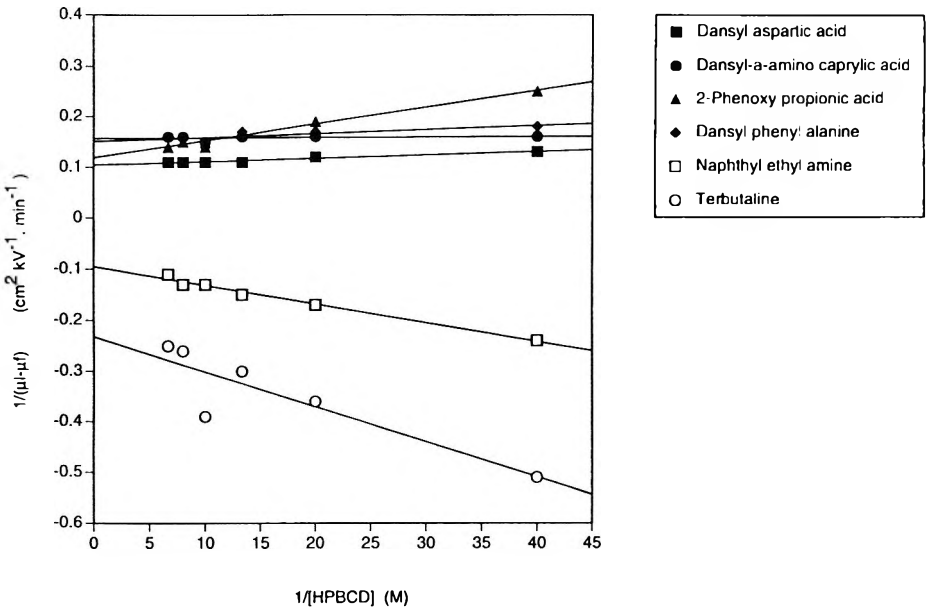


Figure 1. Double reciprocal plots obtained for solutes at various concentrations of hydroxypropyl-β-cyclodextrin.

Table 1

Binding Constants of Chiral Solutes to Hydroxypropyl-β-Cyclodextrin

Compound	$\mu_{cd}1$	$\mu_{cd}2$	$K_{cd}1$ (M^{-1})	$K_{cd}2$ (M^{-1})	$K_{mic}1$ (M^{-1})	$K_{mic}2$ (M^{-1})
Dansyl aspartic acid	-7.26	-7.29	76±10	68±8	5.3±0.6	5±0.6
Dansyl-α-amino caprylic acid	-2.79	-2.95	236±22	197±16	242±72	221±66
2-Phenoxypropionic acid	-5.12	-5.39	26±2	24±3	2.4±0.1	2.3±0.3
Dansyl phenylalanine	-3.52	-3.83	255±39	210±39	87±14	77±13
Naphthylethylamine	0.52	0.05	22±2	21±2	327±50	326±50
Terbutaline	3.94	3.50	46±12	42±16	196±38	193±39

These values of K_{sel}, which did not have any physical meaning, could be attributed to the fact that the rearranged equation (3) involves the use of a (μ_i - μ_f) term. In the SDS micellar systems, the mobility of the solute (μ_i) tended toward more negative values as compared to m_f of the solute in phosphate buffer. Therefore, (μ_i -μ_f) is always a negative quantity. Thus, the values plotted on the X-axis were always negative values while those plotted on the Y-axis were positive values. Such a plot yielded a line that always had a negative slope and usually a positive intercept, which when used in further calculation of K_{sel} yielded negative numbers. Thus, equation (3) could not be used for further determinations of K_{mic}. The method for determining K_{mic} in this multipseudophase system had to be restricted to the mathematical substitution of K_{sel} and μ_{cd} (obtained from the double reciprocal plots) into the original equation (1) rather than from the graphical estimation from equation (3). The K_{mic} values obtained in this manner are shown in Table 1.

It might be expected that the binding constants determined in the mixed micelle/cyclodextrin system should correlate well with the individual binding constants determined separately for the solutes binding 1:1 to hydroxypropyl-β-cyclodextrin or to SDS micelles. However, this may not be the case if interactions exist between hydroxypropyl-β-cyclodextrin and SDS. In order to ascertain if such an interaction existed between hydroxypropyl-β-cyclodextrin and SDS, surface tension measurements were carried out to determine the critical micelle concentration (cmc) of SDS at various concentrations of hydroxypropyl-β-cyclodextrin dissolved in 0.05 M phosphate buffer (pH 7). The cmc data obtained are compiled in Table 2. Clearly, the cmc increases almost linearly with the concentration of hydroxypropyl-β-cyclodextrin. This sort of phenomenon occurs when the solution concentration of free surfactant is reduced thereby increasing the apparent cmc.

It can be deduced from these data that there is considerable interaction (inclusion) of SDS monomers with the hydrophobic cavity of hydroxypropyl-β-cyclodextrin. Hence, in systems containing both micelles and cyclodextrins, the analyte would have to compete with monomeric SDS (which exists in equilibrium with micellar SDS) to form an inclusion complex with the hydroxypropyl-β-cyclodextrin. The competitive analyte/cyclodextrin binding constants in this more complex system could be very different from those measured in the absence of SDS. Obviously, competitive binding effects could affect the resolution and the separation mechanism of these analytes in capillary electrophoresis as well.

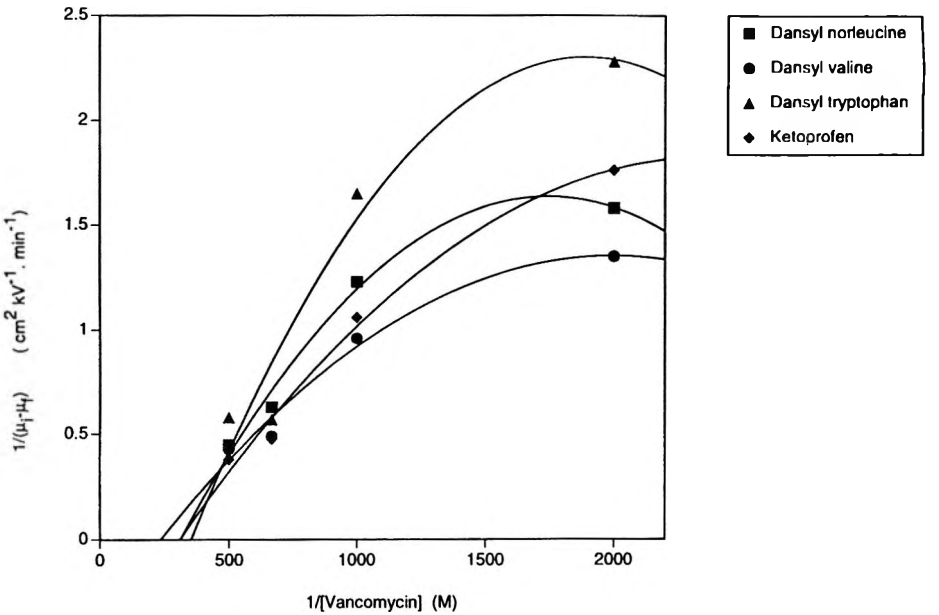


Figure 2. Double reciprocal plots obtained for solutes at various concentrations of vancomycin.

Table 2

Variation of the Critical Micelle Concentration of Sodium Dodecyl Sulfate with Hydroxypropyl-β-Cyclodextrin Concentration

Concentration of of HPBCD (mM)	CMC of SDS (mM)
3	3.8
5	5.8
10	10.6
25	23.2
50	45.9
75	59.8
100	86.2

Determination of Binding Constants in the Vancomycin System

As in the case of the hydroxypropyl-β-cyclodextrin/SDS system, the first step in the determination of binding constants of analytes to the vancomycin and micelle respectively, was to determine μ_S and K_{sel}. The graphical procedure adopted in the hydroxypropyl-β-cyclodextrin system was used to determine μ_{vanco} so as to estimate the extent of binding of solutes to the vancomycin selector. In order to do this, each solute was injected at various concentrations of vancomycin and their mobilities were determined from their migration times. Uncorrected migration times were used in the calculation of mobilities of the solutes in the vancomycin system.[10]

Figure 2 shows the double reciprocal plots obtained for the first eluted enantiomer of some of the solutes evaluated in the vancomycin system. The plot of $1/(\mu_i-\mu_f)$ versus 1/[vancomycin] made for these solutes was unlike the plots in the hydroxypropyl-β-cyclodextrin system. It is evident that the reciprocal of the mobility difference varied non-linearly with the reciprocal of the vancomycin concentration (Fig. 2). The values of μ_{vanco} and K_{vanco} cannot be determined from these plots (in the same manner as was done in the determination of μ_{cd} and K_{cd}) since the slope and intercept of a linear plot are required for determining these constants. It is envisaged that the non-linearity of these plots is a manifestation of the wall binding property of vancomycin.[6,10] It is evident that the simpler graphical procedure cannot be used in the direct determination of μ_{vanco} and K_{vanco} for further mathematical substitution into equation (1) to determine K_{mic}. Hence, the indirect estimation of μ_{vanco}, as was done in the previous study,[10] may be the only method suitable for the estimation of this parameter. For this reason, further recalculations of binding constants of the analyte to vancomycin and micelles in the vancomycin/SDS system were not repeated in this study.

DISCUSSION

The association of an analyte to two different ligands or pseudophase components can best be evaluated by equation (1) when there is little or no interaction between the ligands themselves. However, in the case of the hydroxypropyl-β-cyclodextrin/SDS system, the SDS monomer interacts with the cyclodextrin cavity. Since the interactions between hydroxypropyl-β-cyclodextrin and SDS do not significantly alter the binding behavior of the solute to each ligand, equation (1) can still be used to quantitate these interactions. However, it must be understood that the binding constant of an

analyte to cyclodextrin may be different in the presence of surfactant as compared to the identical measurement in the absence of the surfactant. The vancomycin/SDS system is more complex. In this case, the vancomycin substantially partitions into SDS micelles. There are several other factors that contribute to the difficulties in the accurate determination of absolute binding constants of solutes in vancomycin-modified micellar capillary electrophoresis. Firstly, the wall adsorption of vancomycin allows it to exist as a "fourth phase" besides the micelle pseudophase, the unadsorbed vancomycin phase and the bulk solution phase. Obviously, a three phase model cannot accurately account for such a "four phase" or pseudophase system.

Secondly, the partitioning of vancomycin into the micellar pseudophase may alter the physico-chemical nature of the mixed micelles. Also, at sufficiently high surfactant concentrations, the mixed micelles may be distinguished as a different phase from the neat SDS micelles. Further, in varying the concentration of SDS in the micellar system at constant vancomycin concentration, the nature and ratio of the micelle/mixed micelle may itself change.

The problem of wall binding of vancomycin may be overcome in the determination of binding constants of analytes to vancomycin, by studying the mobility of vancomycin in solutions containing various concentrations of the analyte.[27] The application of this method would, however, be restricted to measurements in systems where the selectors have large UV absorbance while the analytes have low UV absorbance and high solubility in the media being studied. For example, the absence of a UV chromophore on hydroxypropyl-β-cyclodextrin precludes the use of this method from being applied to that system. Another approach to minimize the effects of wall binding in the determination of absolute association constants would be to make these evaluations using coated capillaries.

CONCLUSIONS

In determining the binding constants of solutes to SDS micelles and the chiral selector phase, it became evident that the pseudophase model was effective in quantitating the binding of solutes to a simple system such as those containing hydroxypropyl-β-cyclodextrin and SDS. Its use was restricted in the complex vancomycin/SDS system wherein the existence of multiple equilibria made it impossible to accurately determine the absolute binding constants of solutes to the vancomycin and the micellar phases. However, since the major contribution to the total binding came from the partitioning of the solute to the vancomycin and the SDS phases, it was possible to determine the approximate

binding constants of solutes in these phases based on the three phase model. In making the determination of absolute binding constants, additional interactions from wall binding of vancomycin, its partitioning into SDS as well as changes in the nature of the micelle would have to be accounted for.

ACKNOWLEDGMENT

The authors would like to acknowledge the National Institutes of Health (grant NIH BMT 2R01 GM36292-05A2) for support to carry out this work.

REFERENCES

1. J. Snopek, H. Soini, M. Novotny, E. Smolkova-Keulemansova , I. Jelinek, J. Chromatogr., **559**, 215-222 (1991).

2. A. Guttman, A. Paulus, A.S. Cohen, N. Grinberg , B. L. Karger, J. Chromatogr., **448**, 41-53 (1988).

3. S. A. C. Wren, J. Chromatogr., **636** , 57-62 (1993).

4. K. H. Gahm , A. M. Stalcup, Anal. Chem., **67**, 19-25 (1995).

5. A. M. Stalcup , K. H. Gahm, Anal. Chem., **68**, 1360-1368 (1996).

6. D. W. Armstrong, K. L. Rundlett , J.-R. Chen, Chirality, **6**, 496-509 (1994).

7. K. L. Rundlett , M. P. Gasper, E. Y. Zhou, D. W. Armstrong, Chirality, **8**, 88-107 (1996).

8. R. Vespalec, H. Corstjens, H. A. H. Billiet, J. Frank, K. Ch. A. M. Luyben, Anal. Chem., **67**, 3223-3228 (1995).

9. S. Terabe, Y. Matsushita, Y. Ishihama , O. Shibata , J. Chromatogr., **636**, 47-55 (1993).

10. K. L. Rundlett , D. W. Armstrong, Anal. Chem., **67**, 2088-2095 (1995).

11. M. P. Gasper. A. Berthod. U. B. Nair , D. W. Armstrong. Anal. Chem., **68**, 2501-2514 (1996).

12. S. Terabe , K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem., **56**, 111-113 (1984).

13. D. W. Armstrong , K. L. Rundlett, J. Liq. Chromatogr., **18**, 3659-3674 (1995).

14. S. A. C. Wren, R. C. Rowe, J. Chromatogr., **603**, 235-241 (1992).

15. A. Shibukawa , D. K. Lloyd. I. W. Wainer, Chromatographia, **35**, 419-429 (1993).

16. S. G. Penn, D. M. Goodall, J. S. Loran, J. Chromatogr., **636**, 149-152 (1993).

17. K. L. Rundlett , D. W. Armstrong, J. Chromatogr., **721**, 173-186 (1996).

18. M. Mammen, F. A. Gomez , G. M. Whitesides, Anal. Chem., **67**, 3526-3535 (1995).

19. D. W. Armstrong , F. Nome. L. A. Spino , T. D. Golden. J. Am. Chem. Soc., **108**, 1418-1421 (1986).

20. G. L. Bertrand. **Weighted Linearized Least Square** software. Private communication .

21. D. W. Armstrong . Sepn. Purif. Methods. **14**, 213-304 (1985).

22. D. W. Armstrong , F. Nome, Anal. Chem., **67**, 1662-1666 (1981).

23. D. W. Armstrong , J. Fendler, Biochim. Biophys. Acta, **478**, 75-80 (1977).

24. D. W. Armstrong , G. Y. Stine, J. Am. Chem. Soc., **105**, 2962-2964 (1986).

25. D. W. Armstrong , Am. Laboratory, **13**, 14-20 (1981).

26. M. J. Sepaniak, C. L. Copper, K. W. Whitaker, V. C. Anigbogu, Anal. Chem., **67**, 2037-2041 (1995).

27. J. L. Carpenter, P. Camilleri, D. Dhanak, D. Goodall, J. Chem. Soc., Chem. Commun., **11**, 804-806 (1992).

Received June 7, 1996
Accepted June 19, 1996
Manuscript 4210

J. LIQ. CHROM. & REL. TECHNOL., 20(2), 217-226 (1997)

DETERMINATION BY HPLC OF THE UDP-GLUCURONYL TRANSFERASE ACTIVITY FOR 7-HYDROXYCOUMARIN IN RABBIT TISSUE SAMPLES

Ciaran F. Duffy, Declan P. Bogan,
Richard O'Kennedy*

School of Biological Sciences
Dublin City University
Glasnevin
Dublin 9, Ireland

ABSTRACT

HPLC was used to determine the uridine diphosphate (UDP) glucuronyl transferase activity in rabbit tissues. The crude tissue samples prepared were from the liver, kidney, bladder, large intestine, lung, spleen, heart and fat. A metabolic reaction mixture was prepared that included the rabbit tissue sample, 7-hydroxycoumarin and UDP-glucuronic acid (UDPGA). The method was used to determine the level of production of 7-hydroxycoumarin-glucuronide from 7-hydroxycoumarin. Seperation of the analyte was carried out under gradient elution by reverse phase chromatography on a C_{18} column, with UV detection at 320 nm. It was possible to determine 7-hydroxycoumarin-glucuronide (7-OHCG) produced over time as well as the decrease in 7-hydroxycoumarin concentration as the reaction progressed. The rate of the reaction was calculated from a plot of the concentration of 7–OHCG produced versus time for

Copyright © 1997 by Marcel Dekker, Inc.

each organ, and the rate calculated from the slope of the linear part of the curve. The liver showed the highest activity with a rate of production of 7-OHCG of 2.3 nmol per min per milligram of protein. The kidney showed an activity of 0.22 nmol of 7-OHCG produced per minute per milligram of protein, with the bladder and large intestine showing activities of 0.14 nmol and 7.8 pmol of 7-OHCG produced per minute per milligram of protein. The method used required minimal sample clean up and was reliable and accurate.

INTRODUCTION

Of all the organs in the body the liver has been established to be quantitatively and qualitatively the most important site for glucuronidation.[1] It has also been shown that glucuronidation occurs predominantly in the periportal reigon of the liver.[2,3] Other extra hepatic tissues also have the ability to glucuronidate compounds, although with a more restricted substrate specificity and capacity than hepatic tissue (Dutton 1980). In rat kidney UDPGT activity towards (-)-morphine and testosterone is absent, while transferase activities towards phenols and bilirubin are present.[4] In rats the gastro-intestinal tract exhibits glucuronidation capacity for phenols.[5] Other organs such as lungs,[6] bronchus,[7] and skin,[8] have also demonstrated activity to simple phenolic xenobiotics. Phenol UDPGT activity at very low levels has been shown in many rat tissues including spleen, thymus, brain and heart.[4]

Coumarin, 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and 4-hydroxycoumarin are all members of the benzo-pyranones. Coumarin and 7-hydroxycoumarin are found in nature and both have been used as trial drugs in cancer treatment.[10] 7-Hydroxycoumarin is metabolised by UDP-glucuronyl transferase to the glucuronide (Fig 1.). The glucuronide is principally excreted in the urine.[11]

The clinical role of 7-hydroxycoumarin-glucuronide is unclear. It can be detected in the plasma after coumarin administration.[12] Due to the active transport system proposed to exist for glucuronides,[4] it has been suggested that 7-hydroxycoumarin-glucuronide is transported into the cells where glucuronidases present within the cell reconvert the glucuronide to the 7-hydroxycoumarin. After it has exerted its pharmacological action it might then be reglucuronidated before excretion.[13] Analysis of 7-hydroxycoumarin-glucuronide and 7-hydroxycoumarin in metabolism studies, and in urine, plasma and serum samples almost always involves the deconjugation of 7-hydroxycoumarin-glucuronide to free 7-hydroxycoumarin, followed by

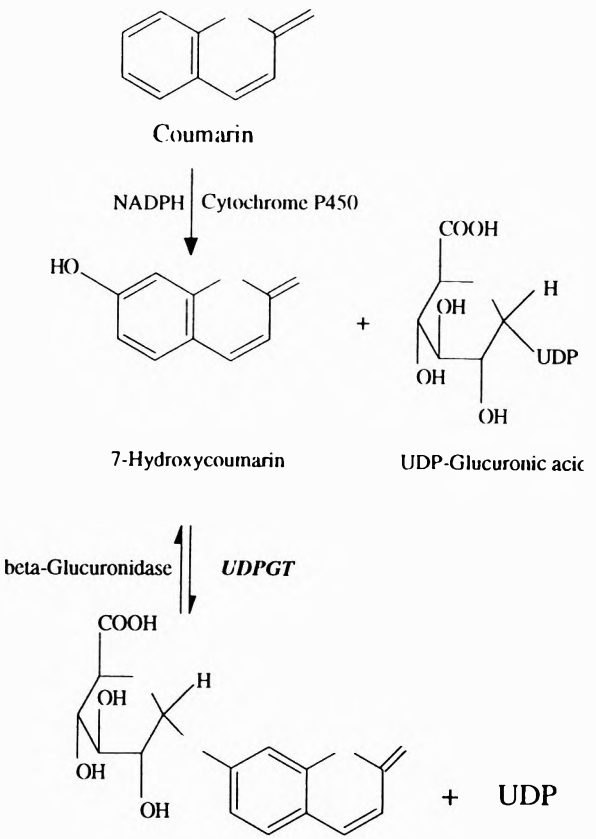


Figure 1. Reaction scheme for the in vivo metabolism of coumarin and 7-hydroxycoumarin.

extensive clean up procedures.[14,15] Killard *et al.*[16] reported on the direct determination of the glucuronide form of 7-hydroxycoumarin as well as the free 7-hydroxycoumarin without deconjugation with β-glucuronidase.

Crude tissue was prepared and used for studying the activity of UDP-glucuronyl transferase on 7-hydroxycoumarin in eight different rabbit tissues. The method applied here was adapted from Killard *et al.*[16] and applied to the separation and determination of 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and the internal standard, 4-hydroxycoumarin with minimal sample clean up.

EXPERIMENTAL

Chemicals

7-hydroxycoumarin and uridine 5'-diphosphate glucuronic acid (UDPGA) were purchased from Sigma (St. Louis, MO, USA). Trichloroacetic acid and acetic acid were purchased from BDH Chemicals Ltd., Poole, Dorset, England. 4-Hydroxy-coumarin was purchased from Aldrich Chemicals Ltd., Gillingham, England. 7-hydroxycoumarin-glucuronide was kindly donated by Schaper and Brummer, Salzgitter, Germany. HPLC grade methanol was purchased from Labscan, Dublin, Ireland. 1.1 mL screw cap vials were obtained from Labquip, Dublin, Ireland. Ultra pure water was used for serial dilutions and mobile phase.

HPLC

The HPLC apparatus consisted of a System Gold solvent module 126, detector module 166, and autosampler, module 507. All components were controlled by System Gold Software^{TM}, (Beckmen Instruments Ltd., California, USA). Separation was carried out on a Microbonda Pak C_{18} column (HPLC Technology, Cheshire, UK). with gradient elution of the compound of interest. The solvents employed were A, water-methanol-acetic acid (950:50:2, v/v) and B, 100% methanol. The eluent was monitored at 320 nm. Sample (20μl) was injected onto the column. The 1 mL min^{-1} gradient was as follows: 0-5 min 100% A → 50% A : 50% B ; 5-14 min 50% A : 50% B ; 14-15 min 50% A : 50% B → 100% A.

Preparation of Samples

Organs were obtained from a New Zealand White Rabbit and stored at -20°C until required. A sample of each organ was weighed and homogenised in 50mM Tris-HCL, pH 7.4. The protein concentration in each organ was determined by Bicinchoninic acid (BCA) assay (Pierce, Illinois, USA). A reaction mixture was set up and incubated at 37°C. Samples were taken every 15 mins from time 0 to 120 minutes. The reaction was terminated by addition of 200μl of reaction mixture to 40μl of 20% (w/v) trichloroacetic acid. This mixture was centrifuged prior to analysis.

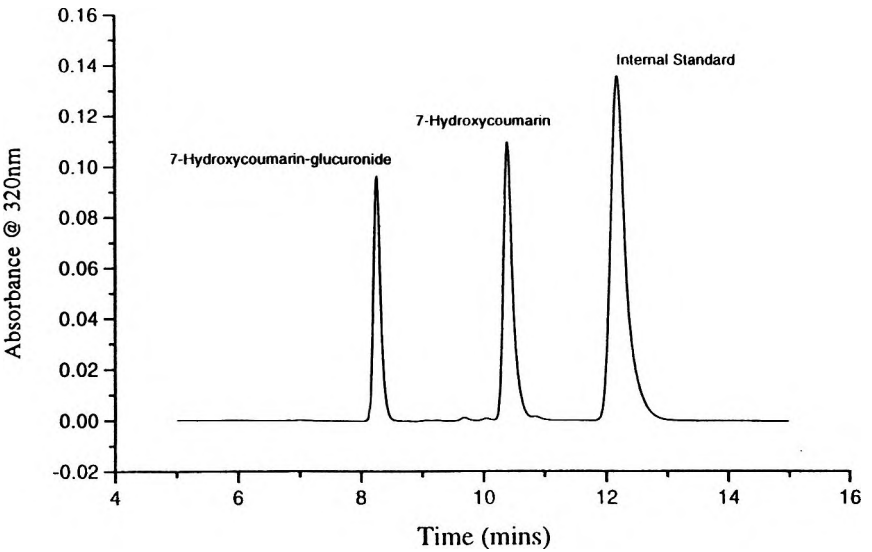


Figure 2. Chromatogram showing separation achieved for 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and the Internal Standard under experimental conditions.

Preparation of Standards

A series of standards of each analyte were prepared in ultra pure water. A 1 mg/mL stock solution of 7-hydroxycoumarin was prepared in 10% methanol / 90% water and the 1 mg/mL stock solution of 7-hydroxycoumarin-glucuronide was prepared in ultra pure water. The internal standard used was 4-hydroxycoumarin and was prepared in methanol. The standards used for determining concentration of analytes and also precision and accuracy of method were 0, 1, 5, 10, 20, 50, 80, 100 and 200 μg/mL.

RESULTS AND DISCUSSION

HPLC Separation

The purpose of this *in vitro* study was to gain more information on intra organ differences in the metabolism of 7-hydroxycoumarin to 7-hydroxycoumarin-glucuronide in rabbit. The focus of this study was the determination of the rate of production of 7-OHCG by each organ.

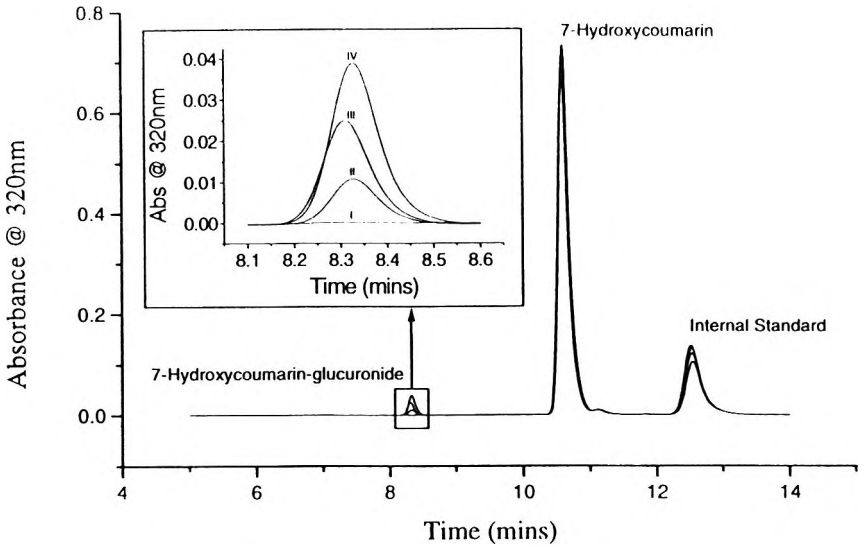


Figure 3. Overlay of four chromatograms of kidney reaction mixture showing analysis for 7-hydroxycoumarin-glucuronide at time 0 (I), time 30 (II), time 60 (III), and time 90min (IV). Reaction mixture analysed as outlined in experimental.

An HPLC method was adapted from that of Killard *et al.*[16] The parameters were changed by analysing a series of standards under different conditions until the optimum separation of analytes was achieved. Figure 2 shows the resolution obtained with the glucuronide eluting at 8.3, 7-hydroxycoumarin at 10.4, and internal standard (4-hydroxycoumarin) at 12.2 minutes, respectively.

The choice of solvents used for the separation were selected because they had been successfully used previously by Egan and O'Kennedy[14] and Sharifi *et al.*[17] The time between sample injection was 9 minutes as this was the time taken for the absorbance to return to zero after each analysis.

Glucuronidation of 7-hydroxycoumarin by UDPGT

There are very few methods that allow the direct determination of 7-hydroxycoumarin-glucuronide without deconjugation,[16,17] however, with method applied here the metabolite of 7-hydroxycoumarin was monitored over time and its increase could be seen clearly (Fig. 3). It was also possible to monitor the decrease in 7-hydroxycoumarin concentration (results not shown).

Table 1

Activities of Different Organs in Amount of 7-OHCG Producted in pmol per Minute per Milligram of Protein

Tissue	Concentration of 7-OHCG Produced per Min per Milligram of Protein (pmol)
Liver	2300
Kidney	220
Bladder	140
Large Intestine	7.8
Lung	0.0
Spleen	0.0
Heart	0.0
Fat	0.0

Table 2

Mean Concentrations of 7-hydroxycoumarin-glucuronide ± St. Dev.) Produced by Rabbit Kidney UDPGT and the % Relative Standard Deviation Over Time (n=3)

Time (min)	Mean 7-hydroxycoumarin glucuronide Concentration (μM)	% Relative Standard Deviation
0	0	0
15	2.4 ± 0.09	3.7
30	5.3 ± 0.2	4.0
45	10.3 ± 0.4	4.3
60	12.6 ± 0.6	4.9
75	17.3 ± 0.48	2.7
90	20.7 ± 1.53	7.4

The determination of 7-hydroxycoumarin or its metabolite allow a calculation of the metabolic rate of the reaction for the tissue in question. Eight organs; liver, kidney, large intestine, bladder, lung, spleen, heart and fat were selected for the study. UDPGT activity was not observed in all tissues. Table 1 shows the UDPGT activity in the various organs. The

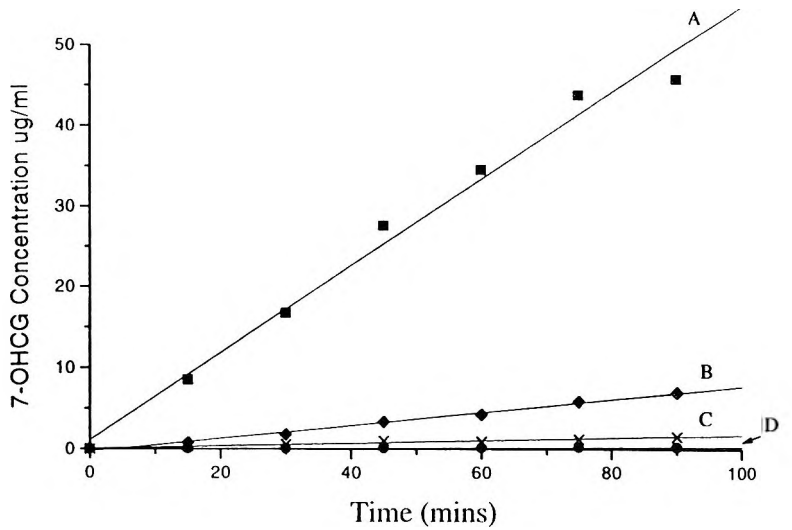


Figure 4. Graph showing rate of production of 7-hydroxycoumarin-glucuronide in four of the tissue samples, liver (A), kidney (B), bladder (C), and large intestine (D).

highest activity was observed in the liver, with the kidney and bladder showing less activity. Very low activity was observed in the large intestine. The spleen and lung showed trace concentrations of 7-hydroxycoumarin-glucuronide after 90 mins (results not shown), with the fat and heart tissue showing no detectable activity.

A plot of the mean 7-hydroxycoumarin-glucuronide concentration produced (n=3) versus time (Table 2) was used to calculate the activity of UDPGT in the tissue . The rate of reaction was calculated from the linear part of the curve (Fig. 4). It can be seen that the liver and kidney produced appreciable quantities of 7-hydroxycoumarin-glucuronide with relative activities of 2.3 nmol and 0.22 nmol of 7-hydroxycoumarin-glucuronide produced per minute per milligram of protein respectively.

The bladder showed an activity of 0.14 nmol of 7-OHCG produced per minute per milligram of protein, with a very low level of activity in the large intestine of 7.8 pmol of 7-OHCG produced per minute per milligram of protein.

CONCLUSION

UDPGT activity is present in many organs of the one species, here in the rabbit it has been demonstrated to be present in the liver, kidney and to a lesser extent bladder and large intestine. Trace amounts of the glucuronide were found in the spleen and lung, with no detectable activity in the heart and fat tissue. The method used is reliable and accurate and requires minimal sample clean up.

ACKNOWLEDGEMENTS

This research was supported in part by the Coumarin Research Fund, Forbairt, and the International Fund for Ireland. The authors would like to thank the Research and Postgraduate Committee, Dublin City University. We would also like to thank Brian Mehan and Tony Killard (Dublin City University).

REFERENCES

1. G. J. Dutton, **Glucuronidation of Drugs and Other Compounds**, CRC Press, (1991).

2. R. A. Branch, R Cothman, R. Johnson, J. Porter, P. V. Desmond, S. Schenker, J. Lab. Clin. Med., **102**, 805-812 (1983).

3. M. C. Mitchell, R. Hamilton, R.A. Branch, Xenobiotica, **19**, 389-400 (1989).

4. D J. Clarke.B. Burchell, Handb. Exp. Pharm., **112**, 3-43 (1994).

5. A. Koster, G. Schimmer, K. W. Bock, Biochem. Pharmacol., **35**, 3971-3975 (1986).

6. A. Aitio, Agents Actions, **6**, 531-533 (1976).

7. E. M. Gibby, G. M. Cohen, Biochem Pharmacol ., **33**, 739-743 (1984).

8. M. W. Coomes, A. Norsling, R. J. Pohl, D. Miller, J. R. Fonts, J. Pharmacol. Exp. Ther., **225**, 770-777 (1983).

9. A. Aitio, Int. J. Biochem., **5**, 325-330 (1974).

10. M. E. Marshall, J. L. Mohler, K. Edmonds, B. Williams, M. Ryles, L. Weiss, D. Urban, A. Buschen, M. Markiewicz, G. Cloud, J. Cancer Res. Clin. Oncol., **120** (suppl.) S39 (1994).

11. D. Bogan, G. Keating, H. Reinartz, M. R. Smyth, R. O'Kennedy, **Coumarins - Biology, Application and Mode of Action, John Wiley**, Chichester, England (1996).

12. S. Sharifi, E. Lotterer, H. C. Michaelis, J. Bircher, J. Ir. Coll. Phys. Surg., **22**, 29-32 (1993).

13. J. R. Casley-Smith, **In High Protein Odemas and the Benzo-pyranones**, CJ.B. Lippencott Company, 417-446 (1986).

14. D. Egan, R. O'Kennedy, J. Chromatogr. B, **582**, 137-143 (1993).

15. D. P. Bogan, B. Deasy, R. O'Kennedy, M. R. Smyth, U. Fuhr, J. Chromatogr. B, **663**, 371-378 (1995).

16. A. J. Killard, D. P. Bogan, R. O'Kennedy, J. Pharm. Biomed. Analysis, in press.

17. S. Sharifi, E. Lotterer, H. C. Michaelis, J. Bircher, J. Liq. Chromatogr., **16(6)**, 263 (1993).

Received May 1, 1996
Accepted May 20, 1996
Manuscript 4162

J. LIQ. CHROM. & REL. TECHNOL., 20(2), 227–243 (1997)

A NOVEL POLAR SEPARATION MEDIUM FOR THE SIZE EXCLUSION CHROMATOGRAPHY OF SMALL MOLECULES: UNIFORMLY SIZED, POROUS POLY(VINYLPHENOL-*CO*-DIVINYLBENZENE) BEADS

Kevin Lewandowski, Frantisek Svec, Jean M.J. Fréchet*

Department of Chemistry
Baker Laboratory
Cornell University
Ithaca, New York 14853-1301

ABSTRACT

A novel separation medium based on uniform size beads of 4-hydroxystyrene-divinylbenzene can be used for both the size exclusion chromatography of small molecules and their separation by reversed-phase chromatography. The new 5 μm material is prepared by the controlled swelling of monodispersed polystyrene particles, that serve both as porogen and shape template, with a polymerization mixture consisting of the monomers and dibutylphthalate, followed by a suspension polymerization. Removal of the acetoxy groups by hydrolysis with aqueous base leads to the final poly(4-vinylphenol-*co*-divinylbenzene) monodispersed beads. Polymerization conditions that favor the formation of very small pores were

Copyright © 1997 by Marcel Dekker, Inc.

developed to optimize the beads for the SEC separation of small molecules. The SEC calibration curve confirms that the optimized beads contain a large volume of pores suitable for the separation of solutes with a molecular weight of up to about 1000. The ability to separate alkylbenzenes according to their hydrodynamic sizes has been demonstrated using a column packed with this material. In addition, the phenol chemistry used in combination with a hydrophobic crosslinking monomer provides this separation medium with an unusual versatility that allows both reversed phase and normal phase chromatography to be run in the same column after a simple change of the mobile phase.

INTRODUCTION

The range of commercially available separation media and packed columns for HPLC, as well as the number of new chromatographic techniques, has grown rapidly in recent years. This development is stimulated by the need to achieve quickly and reliably the complete separation of an ever increasing variety of mixtures. The major share of the market for HPLC packings for both reversed phase and normal phase chromatography is accounted for by silica based materials, while polymeric media prevail for both the size-exclusion and the ion-exchange modes.[1] Although synthetic polymers with many different chemistries have been explored in order to obtain separation media with desired selectivity and efficiency, the vast majority of present day HPLC column packings are still based on only a few types of monomers, most frequently a combination of non-polar styrene and divinylbenzene.[2]

More polar packings have been prepared from monomers that contain charged groups or aliphatic hydroxyl groups. While the former are widely used for ion-exchange chromatography, the latter have found limited applications mainly in the aqueous size-exclusion chromatography of biopolymers. Crosslinked polymers with polar functionality derived from 2-hydroxyethyl methacrylate, 2,3-dihydroxyethyl methacrylate, vinyl alcohol, vinyl pyrrolidone and other hydroxylated monomers are examples of such materials.[1]

In contrast to these aliphatic monomers, vinylphenol is a good candidate for the preparation of styrenic-type polymers that contain hydroxyl groups. However, until recently, porous beads of vinylphenol remained inaccessible because this monomer is difficult to handle and does not polymerize well.[3] The situation changed dramatically when styrenic monomers containing protected phenolic groups became commercially available. We first demonstrated six

years ago that the standard suspension polymerization of 4-tert-butoxycarbonyloxystyrene[3] or its ring substituted derivatives with divinylbenzene, followed by removal of the hydroxyl protecting group, provides separation media that are suitable for the separation of amines by normal phase chromatography.[4,5] A very significant improvement of this earlier work was accomplished through a preparative method that afforded uniformly sized beads. In this method, small, monodisperse shape-template polymer particles are swollen with a mixture of 4-*tert* butoxycarbonyloxystyrene, divinylbenzene, and cyclohexanol (porogenic solvent), then subjected to suspension polymerization and thermal deprotection.[6] This uniformly sized phenolic separation medium, which has a broad pore size distribution with a maximum centered at about 50 nm, is very versatile in the separation of both small molecules and proteins using different chromatographic modes. Because the phenol group is a powerful hydrogen bond donor, the beads were also used as a very efficient tool in the measurement of relative hydrogen bond basicities of dilute compounds, which is a process that cannot be carried out using typical silica based phases due to silanophilic interactions.[7]

This report describes a novel approach to the preparation of uniformly sized porous poly(vinylphenol-*co*-divinylbenzene) beads that are uniquely suited for the hitherto elusive process of size-exclusion chromatography of small molecules, using commercially available acetoxystyrene.

EXPERIMENTAL

Materials

Styrene (99%, Aldrich) and divinylbenzene (91%, Dow Chemical) were extracted with 10% aqueous sodium hydroxide and water, dried over anhydrous magnesium sulfate, and distilled under vacuum. Azobisisobutyronitrile (AIBN) was obtained from Kodak, and 4-acetoxystyrene from Hoechst Celanese. Dibutylphthalate, benzoyl peroxide, 1-chlorodecane, and sodium dodecyl sulfate were purchased from Aldrich. All solvents were HPLC grade.

Preparation of Soluble Polystyrene Shape Templates

Monodisperse polystyrene shape templates obtained by emulsion polymerization[8] (1.0 μm, 22% solid in water, 0.3 mL) were swollen by adsorption of an emulsion of 1-chlorodecane (0.6 mL) in 0.25% aqueous

sodium dodecyl sulfate (SDS) solution (30 mL). After the droplets of the emulsified solvent had completely disappeared (about 24-30 h as verified by optical microscopy), a solution of benzoyl peroxide (0.13 g) in styrene (2.6 mL) emulsified in 0.25% aqueous SDS (30 mL) was added to the dispersion resulting from the previous step and the mixture was stirred slowly for 6 h. After transfer of the monomer to the shape templates was completed, sodium nitrite (0.01 g) and 5% solution of poly(vinyl alcohol) (PVA, MW 85,000-146,000, 88% hydrolyzed) was added to adjust the total concentration of PVA in the mixture to 1%, and the system was purged with nitrogen for 20 min. The polymerization was carried out in sealed 500 mL Erlenmeyer flasks placed in a shaker bath (Lab-Line) at 240 rotations/min and 70°C for 24 h. The resulting enlarged monodisperse particles of linear polystyrene porogen were used in the next step without further purification.

Preparation of Insoluble Porous Beads

A mixture containing p-acetoxystyrene (6 mL), divinylbenzene (4 mL), dibutylphthalate (3.8 mL), and AIBN (0.1 g) was emulsified by sonication in 0.25% aqueous SDS solution (30 mL), and added to the aqueous dispersion of polymer porogen particles. The mixture was stirred at room temperature until the emulsified liquid was completely transferred to the porogen particles. Sodium nitrite (0.009 g) and 5% aqueous PVA solution was added to adjust the total concentration of PVA to 1%, and the dispersion was purged with nitrogen for 20 min. The flask was sealed and the polymerization was carried out at 70°C and 240 rotations/min for 24 h. The resulting beads were decanted repeatedly with water and tetrahydrofuran till the supernatant liquid remained clear. The polymeric porogen was removed by extraction with toluene in a Soxhlet apparatus for 36 h, and the porous beads were dried under vacuum. The yield was 92%.

Hydrolysis of Porous Beads

A solution of potassium hydroxide (85%, 3.0 g) in water (15 mL) and methanol (15 mL) was added dropwise to poly(4-acetoxystyrene-*co*-divinylbenzene) porous beads (3.0 g) suspended in methanol (30 mL). After stirring at room temperature for 24 h, the suspension was diluted with water, and filtered through a 4-8 μm sintered glass filter. The beads were washed several times with water, followed by methanol, then dried under vacuum.

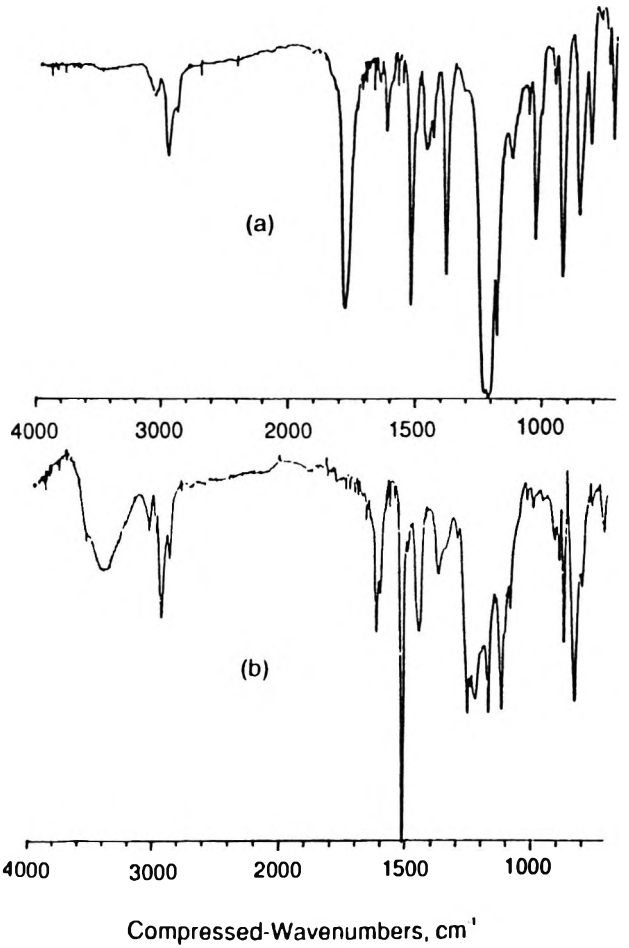


Figure 1. IR spectra of poly(acetoxystyrene-*co*-divinylbenzene) (a) and of the polymer after reaction with aqueous base (b).

Characterization of Porous Properties

The specific surface area of the beads was calculated from the BET isotherm of nitrogen. The pore size distribution in the dry state was determined by mercury porosimetry using an automated custom-made combined BET-sorptometer and mercury porosimeter from Porous Materials Inc., Ithaca, NY, USA.

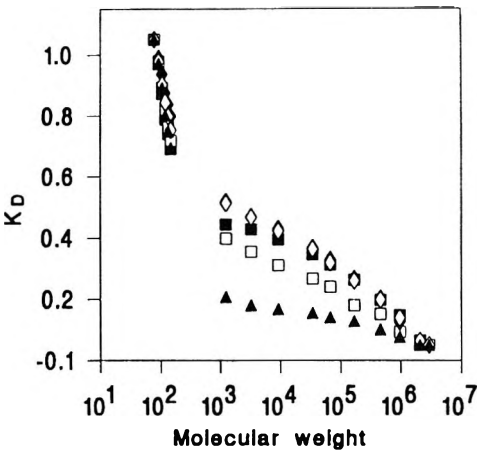


Figure 2. Size-exclusion calibration curve of poly(vinylphenol-*co*-divinylbenzene) beads with polystyrene standards and alkylbenzenes in tetrahydrofuran; co-porogen, 4-methyl-2-pentanol (■), decyl alcohol (□), 1-chlorodecane (♦), heptane (◊), dibutylphthalate (Δ). Conditions: column 150 x 4.6 mm i.d.; flow rate 1 mL/min.; UV detection at 254nm.

Chromatography

A Waters HPLC system consisting of two 510 HPLC pumps, a 717 plus autosampler, and a 486 UV detector, was used to carry out all the chromatography. The data was acquired and processed with Millenium 2010 software (Waters). Particles were packed from a tetrahydrofuran suspension into 150 x 4.6 mm i.d. and 300 x 7.8 mm i.d. stainless steel columns.

RESULTS AND DISCUSSION

In contrast to silica based porous packings, typical porous polymers are characterized by bimodal pore size distribution curves.[2,8-11] In addition to mesopores (2-50 nm) and macropores (over 50 nm), the polymers always contain some micropores with a diameter smaller than 2 nm.[10,11] Due to these micropores, the size-exclusion calibration curves exhibit a plateau for molecules with a molecular weight of less than about 500, instead of a sharp lower exclusion limit.

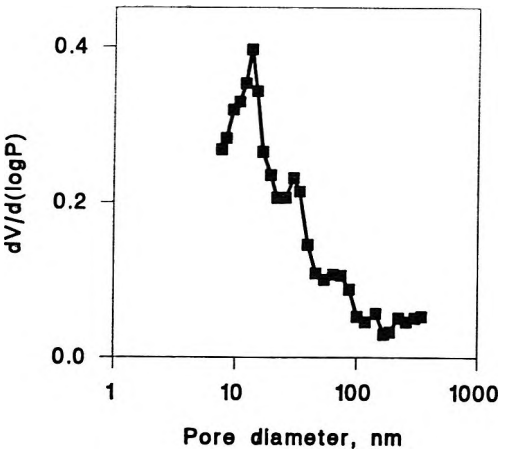


Figure 3. Differential pore size distribution curve of the poly(vinylphenol-*co*-divinylbenzene) beads measured by mercury porosimetry.

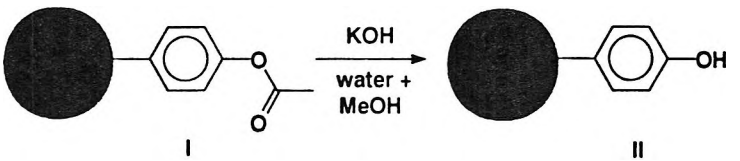
These pores do not play any role in the separation of large molecules;[8] however, it is believed that the micropores, which cannot be avoided, are the major difference between polymeric and silica based separation media in reversed phase chromatography of low molecular weight compounds, and affect both the column efficiency and the separation selectivity.[8,10]

Since it is impossible to prepare porous polymer beads without these micropores, we explored an unusual approach designed to enhance this formation particularly in a size range suitable for the size-exclusion chromatographic separation of small molecules.

Preparation of Beads

The method used for the preparation of uniform, porous poly(4-acetoxystyrene-*co*-divinylbenzene) beads I includes (i) the preparation of relatively large monodisperse polymer porogen beads from uniform latex shape-template particles, (ii) their controlled swelling with a polymerization mixture consisting of 4-acetoxystyrene, divinylbenzene and dibutylphthalate, and finally (iii) the suspension polymerization of the swollen polymeric porogen beads. This technique is well suited for monomers with a low water solubility, such as styrene and divinylbenzene, and also for 4-acetoxystyrene, which is relatively non-polar and hydrophobic. The extent of swelling, and the

ratio of the volumes of the various components of the polymerization mixture used in step (ii) to the volume of the polymer porogen beads, define the size of the final beads, and their porous characteristics.[12-15] Once the polymerization is complete, the porous poly(4-vinylphenol-*co*-divinylbenzene) beads II are obtained by base hydrolysis of the acetate groups (Scheme 1). The hydrolysis



reaction is readily monitored by IR spectroscopy because hydrolysis is accompanied by loss of the strong ester carbonyl band near 1765 cm^{-1}, and the appearance of a broad hydroxyl band centered at about 3400 cm^{-1} (Figure 1).

Physical Properties

Current separation media for HPLC are beads with a diameter in the range of 3-10 μm. The size uniformity of these beads contributes to both better efficiency and lower back pressure. Therefore, typical manufacturing processes involving beads prepared by suspension polymerization require a size fractionation step that is tedious, and a significant part of the batch ends in waste. Our method directly provides a high yield of monodisperse beads. The size of the beads used in this study was measured from scanning electron micrographs, and found to be 5 μm.

The use of a polymeric porogen allows fine tuning of the porous properties of monodisperse beads within a very broad range during the process of their preparation.[12-15] Because this study was aimed at beads optimized for the separation of small molecules, preparative conditions had to be adjusted in order to incorporate as many small pores as possible. The porogen system of linear polystyrene of molecular weight 64,000[15] and a co-porogen solvent, intentionally makes up only 31% by volume of the monomer/porogen mixture in order to keep the pores as small as possible. The use of various co-porogens results in beads that contain different percentages of pores, particularly in the size range above 50 nm (Table 1). The change in volume of large pores affects the distribution coefficients for alkylbenzenes and polystyrene standards (Figure 2). An increase in the volume of these pores results in decreased separation

Table 1

Effect of Co-Porogen on Total Pore Volume, V_p, Measured by Mercury Intrusion Porosimetry, of Poly(4-vinylphenol-co-divinylbenzene) Beads

Co-Porogen	V_p (mL/g)	Pore Volume in Range, %		
		<10nm	10-50nm	50-500nm
dibutylphthalate	0.27	15	59	26
4-methyl-2-pentanol	0.31	16	52	32
decyl alcohol	0.34	18	47	35
1-chlorodecane	0.38	11	53	37
heptane	0.32	13	53	34

selectivity of compounds with molecular weight less than 1000. The polymerization in the presence of dibutylphthalate provides beads with the best selectivity for the separation of small molecules. The pore size distribution of these beads, measured in the dry state by mercury porosimetry (Figure 3) shows that the beads have a total pore volume of 0.27 mL/g, which translates to a modest porosity of about 30% and correlates well to the total volume of porogens added to the polymerizing system. Although this level of porosity is low when compared to standard polymeric separation media, over 70% of the total pore volume is located in pores smaller than 80 nm in diameter. As a result, and in contrast to typical porous polymeric separation media, almost all of the pores have sizes that are nearly ideal for the separation of small molecules. In addition, because of their lower porosity, these beads have much better mechanical stability than standard polymeric media. The scanning electron micrographs in Figure 4 document the porous structure of the beads. The specific surface area of the beads was found to be 66 m^2/g, which is mainly due to the mesopores and is consistent with the pore volume.

Suitability in Interactive Chromatography

Because the polar surface chemistry of the poly(4-vinylphenol-*co*-divinylbenzene) beads is different from that of poly(styrene-*co*-divinylbenzene), it is useful to determine the capabilities of the packing in reversed phase and normal phase HPLC, since these are the most widely used modes of chromatography.

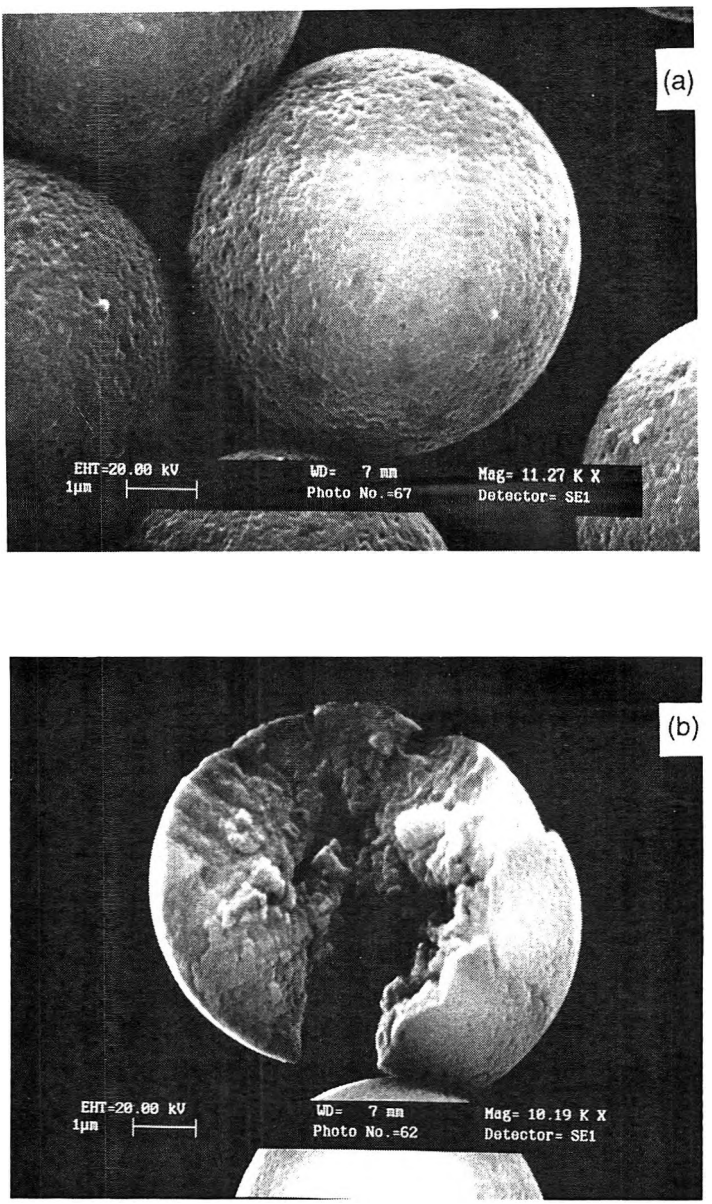


Figure 4. Scanning electron micrographs of the surface (a), and internal structure (b) of monosized poly(4-vinylphenol-*co*-divinylbenzene) beads.

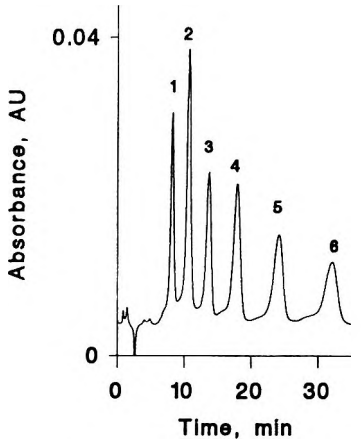


Figure 5. Separation of alkylbenzenes by reversed-phase chromatography. Conditions: 5 μm poly(vinylphenol-*co*-divinylbenzene) beads; column 150 x 4.6 mm i.d.; mobile phase: acetonitrile-water (1:1); flow rate 1 mL/min. Peaks: benzene (1), toluene (2), ethylbenzene (3), propylbenzene (4), butylbenzene (5), pentylbenzene (6).

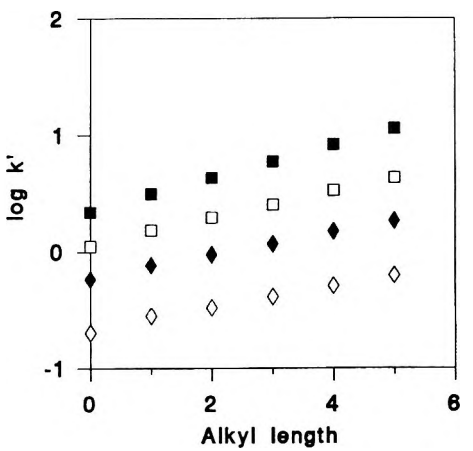


Figure 6. Variation of log *k'* with the number of carbon atoms in alkyl substituents for a homologous series of alkylbenzenes. Conditions: column 150 x 4.6 mm i.d.; flow rate 1 mL/min, mobile phase: acetonitrile/water 50/50 (■), 60/40 (□), 70/30 (♦), and 60/40 (◊).

The separation of a series of alkylbenzenes by reversed phase chromatography was investigated at different mobile phase compositions. With a mobile phase consisting of a 1:1 mixture of acetonitrile and water, the alkylbenzenes are baseline separated (Figure 5). Figure 6 shows that the standard linear relationship between the log k' and number of carbon atoms in alkylbenzenes in the series from benzene, which elutes first, to pentylbenzene is observed. As a result of the high polarity of the packing, separation is already achieved at a relatively low content of acetonitrile in the mobile phase. At a mobile phase composition of 1:1 acetonitrile and water, the line has a slope of 0.143, which is close to that found for styrene-divinylbenzene packings, but in a mobile phase containing 30% percent less acetonitrile.

A characteristic of these poly(vinylphenol-*co*-divinylbenzene) beads is that once packed in a column, they can withstand rapid changes of solvents without significant loss of separation ability. This allows their use in a variety of chromatographic modes. As expected, the presence of phenolic groups on the surface of the beads increases the polarity of the separation medium, making it useful also for separations in the normal phase mode.[5,6] Therefore, the uniformly sized phenolic beads prepared in this study were tested again in the normal phase separation of a mixture containing acidic (phenol), basic (N,N-dimethylaniline and aniline), non-polar (toluene), and electron acceptor compound (nitrobenzene), using hexane containing 5% ethyl acetate, 2% methanol, and 0.1% diethylamine as a mobile phase. All of the compounds are completely separated, and the overall selectivity is good, despite some tailing (Figure 7).

Size Exclusion Chromatography

Size-exclusion chromatography is a powerful technique used to separate molecules according to their effective size in solution. Molecules larger than the largest pore size of the column packing are excluded, and are first to be eluted. Molecules that can penetrate all the pores are entrapped, and are therefore the last to be eluted. The average residence time in the pores depends on the relative sizes of the molecules. While SEC of large molecules is a well established method for characterization of polymers, few chromatographic packings are available for the separation of small molecules using size-exclusion chromatography.[16]

In this work, the pore size distribution profile of the beads has been optimized for size-exclusion chromatography of small molecules. The volume of pores with a diameter in the range 10-300 nm, measured in the dry state by mercury porosimetry, represent about 0.3 mL/g of the polymer. Because the

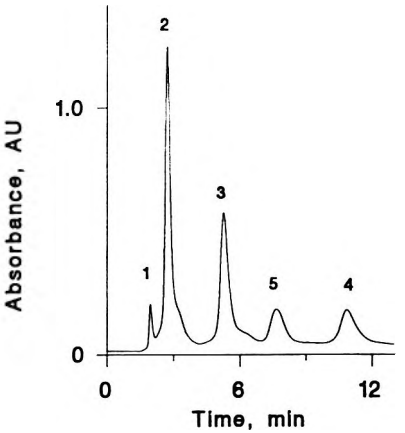


Figure 7. Separation of toluene (1), N,N-dimethylaniline (2), nitrobenzene (3), phenol (4), and aniline (5) by normal phase chromatography. Conditions: 5 μm poly(vinylphenol-*co*-divinylbenzene) beads; column 150 x 4.6 mm i.d.; flow rate 1 mL/min; mobile phase: hexane-ethyl acetate-methanol (93:5:2) with 0.1% diethylamine added.

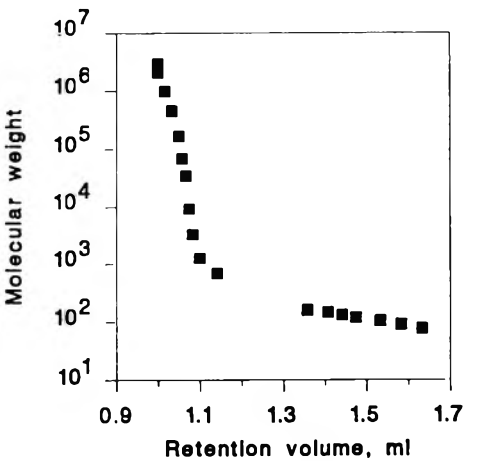


Figure 8. Size-exclusion calibration curve of poly(vinylphenol-*co*-divinylbenzene) beads with polystyrene standards and alkylbenzenes in tetrahydrofuran. Conditions: column 150 x 4.6 mm i.d.; flow rate 1 mL/min.; UV detection at 254nm.

150 x 4.6 mm i.d. column contains 1.7 g of the packing, the entire volume of pores in this column is 0.5 mL. This correlates well with the pore volume of 0.6 mL, found from the size exclusion calibration curve measured with a series of alkylbenzenes and polystyrene standards with molecular weights from 78 to 2,100,000 (Figure 8). However, about 80 % of this effective pore volume is only available for the separation of alkylbenzenes and styrene oligomer standards with molecular weight lower than 1000. The calibration curve is linear in the range of molecular weights 80-1000 and its slope is low. This translates into an excellent size-exclusion selectivity for low molecular weight solutes. The separation of larger molecules is poor as documented by the part of the calibration curve that becomes very steep at higher molecular weights. A low exclusion limit of the separation medium results from the low density of macropores, as indicated by the almost complete lack of separation of polystyrene standards above 1000 molecular weight.

A longer column (300 x 7.8 mm i.d.) packed with the beads was used in the separation of a mixture of benzene, ethylbenzene, amylbenzene, and two polystyrene standards. Figure 9 shows the good resolution achieved between the peaks of the low molecular weight species, which in the case of benzene and ethylbenzene, differ by only 28 g/mol. Column efficiency calculated for ethylbenzene is 32,600 plates/m at a flow rate of 1 mL/min.

A plot of plate height vs. linear flow velocity (Figure 10) obtained for the monodisperse 5 mm poly(4-vinylphenol-*co*-divinylbenzene) beads from a measurement with toluene in THF shows that the plate heights that can be achieved with these phenolic beads are comparable to those for poly(styrene-*co*-divinylbenzene) stationary phases.[3,16] The optimum linear velocity is obtained at about 0.8 mL/min. The back pressure of the column is a linear function of the flow rate and documents the pressure stability of the packing. Even at a flow rate of 3.5 mL/min, the back pressure was only 8 MPa.

CONCLUSION

The porous properties of poly(4-vinylphenol-*co*-divinylbenzene) beads prepared by the suspension polymerization of 4-acetoxystyrene and divinylbenzene within uniform particles of linear polystyrene, serving both as porogen and shape template, can be controlled. For example, to match the needs of a particular application, an optimized procedure provides monodisperse beads tailored for the separation of low molecular weight compounds. Although these chemically stable beads are a versatile packing material for both reversed phase and normal phase HPLC with great tolerance for solvent changes, their major field of application is in the size-exclusion

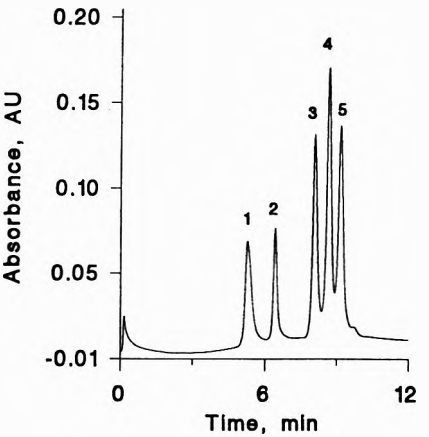


Figure 9. Separation of polystyrene standards with molecular weight 2 950 000 (1), 9200 (2), pentylbenzene (3), ethylbenzene (4), and benzene (5) by size-exclusion chromatography. Conditions: 5 μm poly(4-vinylphenol-*co*-divinylbenzene) beads; column 300 x 7.8 mm i.d.; flow rate 1 mL/min; solvent tetrahydrofuran.

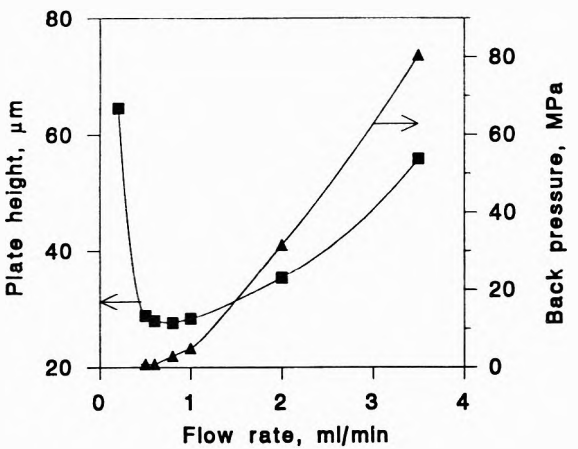


Figure 10. Effect of the linear flow velocity on efficiency and back pressure of column packed with 5 μm poly(4-vinylphenol-*co*-divinylbenzene) beads. Conditions: column 300 x 7.8 mm i.d.; mobile phase, tetrahydrofuran; analyte, benzene.

chromatography of small molecules with a molecular weight of less than 1000, which cannot be separated using reversed phase or normal phase chromatography. In this application, the separation of a homologous series of alkylbenzenes with very small differences in molecular weight is readily achieved.

ACKNOWLEDGEMENT

Support of this research by a grant of the National Institutes of Health (GM-44885) is gratefully acknowledged. This work made use of the MRL Central Facilities supported by the National Science Foundation under Award No. DMR-9121654. Thanks are also due to Hoechst Celanese for providing us with acetoxystyrene and Dow Chemical for a gift of high grade divinylbenzene.

REFERENCES

1. K. K. Unger, ed., **Packings and Stationary Phases in Chromatographic Techniques**, Marcel Dekker Inc., New York, 1990.

2. N. Tanaka, M. Araki, Adv. Chromatogr. **30**, 81 (1989).

3. J. M. J. Fréchet, E. Eichler, H. Ito, G. Wilson, Polymer, **24**, 995 (1983).

4. W. Rolls, F. Svec, J. M. J. Fréchet, Polymer, **31**, 165 (1990).

5. W. Rolls, J. M. J. Fréchet, J. Chromatogr., **504**, 97 (1990).

6. V. Smigol, F. Svec, J. M. J. Fréchet, J. Liquid Chromatogr., **17**, 259 (1994).

7. L.C. Tan, P. Carr, V. Smigol, J. M. J. Fréchet, Anal. Chem., **66**, 450 (1994).

8. N. Tanaka, K. Kimata, K. Hosoya, H. Miyanishi, T. Araki, J. Chromatogr., **656**, 265 (1993).

9. L. L. Lloyd, J. Chromatogr., **544**, 201 (1991).

10. N. Tanaka, K. Kimata, Y. Mikawa, K. Hosoya, T. Araki, J. Chromatogr., **535**, 13 (1990).

11. F. Nevejans, M. Verzele, J. Chromatogr., **342**, 325 (1987).

12. Q. C. Wang, F. Svec, J. M. J. Fréchet, Polym. Bull., **28**, 569 (1992).

13. Q. C. Wang, K. Hosoya, F. Svec, J. M. J. Fréchet, Anal. Chem., **64**, 1232 (1992).

14. Q. C. Wang, F. Svec, J. M. J. Fréchet, J. Polym. Sci., Polym. Chem., **32**, 2577 (1994).

15. M. Galia, F. Svec, J. M. J. Fréchet, J. Polym. Sci., Polym. Chem., **32**, 2169 (1994).

16. R. V. Vivilecchia, B. G. Lightbody, N. Z. Thimot, H. M. Quinn, J. Chromatogr. Sci., **15**, 424 (1977).

17. S. Coppi, A. Betti, C. Bighi, G. P. Cartoni, F. Coccioli, J. Chromatogr., **442**, 97 (1988).

Received March 4, 1996
Accepted June 18, 1996
Manuscript 4104

J. LIQ. CHROM. & REL. TECHNOL., 20(2), 245–256 (1997)

EXTRACTION AND QUANTIFICATION OF RECOMBINANT BOVINE SOMATOTROPIN FROM OLEAGINOUS VEHICLE

Jen P. Chang,* David A. Dickson, Thomas H. Ferguson,
Paul A. Record,† Snehlata S. Mascarenhas, Henry G. Richey

Lilly Research Laboratories
Eli Lilly and Co.
Greenfield, IN 46140, USA

† Lilly Research Laboratories
Indianapolis, IN 46285, USA

ABSTRACT

A non-denaturing assay for the determination of potency of a recombinant bovine somatotropin (somidobove) formulated in oleaginous vehicles was developed. In this paper, the optimal conditions for extraction of somidobove from oleaginous vehicle were investigated. Results demonstrated that somidobove monomer, oligomers, and soluble aggregates were quantitatively extracted by borate - ethylenediaminetetraacetate (EDTA) pH 9.5 buffer solution in a VanKcl dissolution system at 40°C for 3 hours. The extracted analytes, somidobove monomer, dimer, and soluble aggregates, were separated and determined by using a high performance size exclusion chromatographic system consisting of a TSK G3000SW column and a borate - EDTA buffer (pH 7.3) mobile phase. This method was validated for the effects of buffer type, extraction temperature, extraction time and

Copyright © 1997 by Marcel Dekker, Inc.

stirring speed. The average CV of intra- and inter-day precision obtained in this method were 2.3%. The recovery of somidobove monomer was 93.7% with a coefficient of variance (CV) of 3.71%. The CV for precision generated in three different labs ranged from 1.3 - 3.9%. This method was linearly correlated with the hypophysectomized rat body weight gain assay, regression coefficient = 0.953.

INTRODUCTION

Somidobove is a recombinant bovine somatotropin (rbST) composed of 199 amino acids with a molecular weight 22,818. The difference between somidobove and native bovine somidotropin is that at the NH_2-terminus end of the protein, somidobove contains 9 additional amino acids. Somidobove has been developed for administration to dairy cows to increase milk yield and the efficiency of milk production for years. Several sustained release delivery systems[1,2,3] have been developed for somatotropin to eliminate the need for daily injection. These delivery systems yield sustained release of bioactive somidobove for periods of 7 to 28 days, are relatively inexpensive and easy to manufacture as sterile preparations, stable, and easy to administer to the animal. In this study, a mixture composed of sesame oil, white wax, propyl gallate was used as an oleaginous vehicle for the delivery of somidobove.

Many physico-chemical interactions, such as hydrophobic interactions, pH, salts, and denaturants, can lead to protein aggregation.[4,5] Proteins can also be denatured in the presence of gas-liquid interface.[6,7] The formation of soluble and insoluble aggregates in somatotropin formulations effects the release and pharmacokinetics of the somatotropin and reduces the potency and quality of the product. Developing analytical assays for the quantification of somatotropin potency in formulated products has presented challenges not only in accuracy (recovery) and precision, but particularly, the separation of non-denatured somatotropin and aggregates. The hypophysectomized (hypox) rat body weight gain assay is available for the determination of bulk somatotropin potency but not for somatotropin formulated in oleaginous vehicles due to the interference of the oleaginous excipients. Several detergents such as sodium dodecyl sulfate, have been used to extract somatotropin from oleaginous vehicles but the protein was strongly denatured in the extraction process. Additionally, non-covalent bonded oligomers and aggregates present in samples would be decomposed to the denatured monomer. As a consequence, excessively high results were obtained in these methods. The key point in the development of a somatotropin potency assay for formulated drugs is that the method is required to have a non-denaturing extraction procedure with

quantitative recovery. In order to retain the bioactivity of somatotropin in the extraction process, therefore, mild extraction conditions including weak alkaline buffer solution without any denaturants and a suitable low temperature were used for the extraction of somatotropin from oleaginous vehicle.

Authors have reported a non-denaturing size exclusion chromatographic (SEC) assay for the determination of somidobove potency in bulk drugs.[8,9] This method can not only be used to determine the potency of the somidobove monomer, but also to separate and estimate the oligomers and soluble aggregates present. In addition, this method has been correlated to the hypox rat body weight gain assay and can be used for the determination of somidobove potency in routine analysis.

This paper describes a non-denaturing extraction procedure and a size exclusion chromatographic method for the determination of somidobove potency in oleaginous vehicles. The optimization of the extraction procedure including the incubation time, temperature, stirring speed, and buffer solution was investigated. This method was validated and was linearly correlated with the hypox rat body weight gain assay.

EXPERIMENTAL

Materials

Recombinant bovine somatotropin reference standard, bulk somidobove, and formulated somidobove in oleaginous vehicle (sesame oil, white wax, propyl gallate) were provided by Eli Lilly and Company. Water used in this study was obtained from a Millipore Milli-Q water purification system. All reagents and chemicals were of analytical-reagent grade and were used without further purification. Three buffer solutions, 20mM sodium borate - 1.44mM EDTA, 25mM sodium monohydrogen phosphate, and 50mM ammonium bicarbonate, adjusted pH to 9.5 with NaOH were used as extraction solutions.

Extraction

A Vankel Industries Vanderkamp 6000 six-spindle dissolution tester equipped with stir paddles and 1000 mL dissolution vessels was used for the extraction. The temperature of extraction solutions was controlled by a Vankel

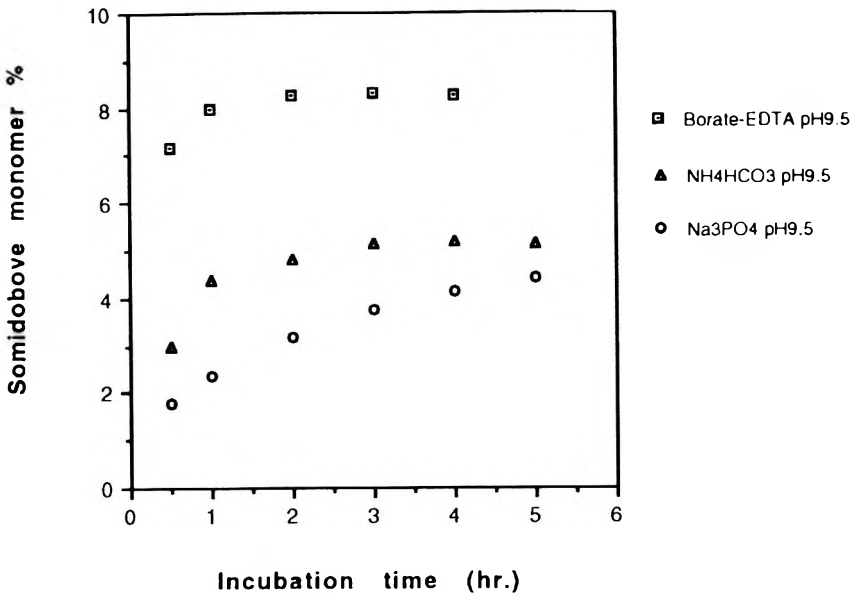


Figure 1. Extraction of somidobove from oleaginous vehicle by different buffer solutions. Extraction conditions: Formulated drug sample (Lot OA18); stirring speed 100 ± 4 rpm; incubation temperature was controlled at ± 0.2° C. Chromatography was performed on a TSK G3000SW column with a mobile phase of 20mM borate - 1.44mM EDTA buffer pH 7.3 at 0.5 mL/min flow rate. Elutes were detected at 280 nm.

C-2600 Heater/Circulator. Two to four grams of the formulated drug sample, depending upon the content of somidobove in the sample was accurately (±0.001g) weighed and transferred into the bottom of each dissolution vessel. 500 mL of the extraction buffer solution was added into each of the vessels. The vessels were placed into the dissolution apparatus with a temperature of 40 ± 0.5°C. Paddles were then lowered into the extraction solution until the top of each stir paddle was 5 mm below the surface of solution. The dissolution apparatus was set up to stir at 100 ±4 rpm. The covers were placed on each vessel and paddles were started in motion.

The incubation was maintained for 3 hours. After completion of the extraction, spindle motion was stopped. An aliquot of the extracted solution was removed by inserting a pipette into the solution placed within 1.5 - 2 inches from the vessel bottom.

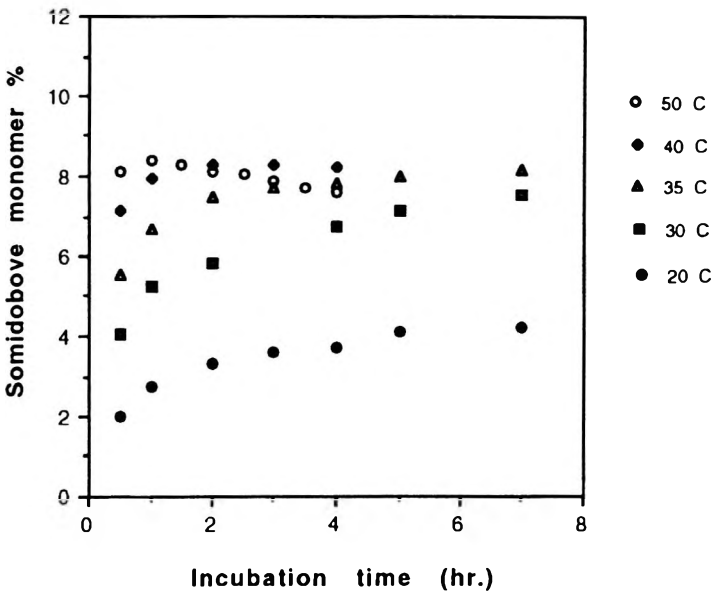


Figure 2. Effect of incubation time and temperature on the recovery of somidobove in extraction. Experimental conditions were the same as in Figure 1 except the extraction solution: 20 mM Na borate - 1.44 mM EDTA, pH 9.5.

Prior to sampling, a small amount of air was expelled from the pipette tip to remove any vehicle which may have been trapped in the pipette tip while inserting the pipette into the solution. The sample solution was filtered through a 0.45 μm Acrodisc filter and analyzed by HPLC.

Chromatography

HPSEC was performed on a HPLC system consisting of a Waters 625 LC system with a 991+ photodiode array detector and WISP 712 autosampler (Waters Chromatography, Milford, MA, USA). Varian 5000 Liquid Chromatograph (Varian Instruments, San Fernando, CA, USA) with a Spectroflow 757 absorbance detector (Kratos Analytical Inc., Ramsey, NY, USA) was used for the study. TSK G3000SW columns, 7.5x300mm, were operated at ambient temperature and with a 20 mL injection volume. The mobile phase was 20mM sodium borate - 1.44 mM ethylenediamine-tetraacetate di-sodium salt (EDTA) buffer solution adjusted to pH 7.3 with hydrochloric acid.

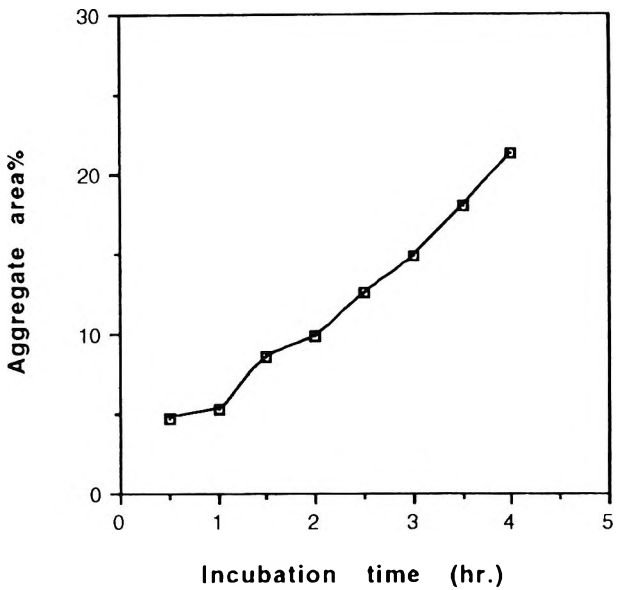


Figure 3. Effect of the incubation time on the formation of somidobove soluble aggregates at 50°C. Experimental conditions were the same as in Figure 2 except the incubation temperature at 50°C.

A flow rate of 0.5 mL/min was used for most of the studies in this work. Eluted components were detected at 280 nm. A linear regression plot of the reference standard in mg/mL versus peak area was used for the quantification of somidobove monomer and peak area normalization was employed for the estimation of the dimer and aggregates in formulated drug samples. A HP-1000 computer system (Hewlett - Packard, San Fernando, CA, USA) was used to collect, store and analyze the chromatographic data.

RESULTS AND DISCUSSION

Extraction

Somidobove maintains bioactivity in pH 9.5 borate-EDTA.[7,8] As a first choice, this buffer was used to extract somidobove from oleaginous vehicles. Several extraction procedures were investigated for the recovery of somidobove in the extraction process. When somidobove bulk drug was shaken with a

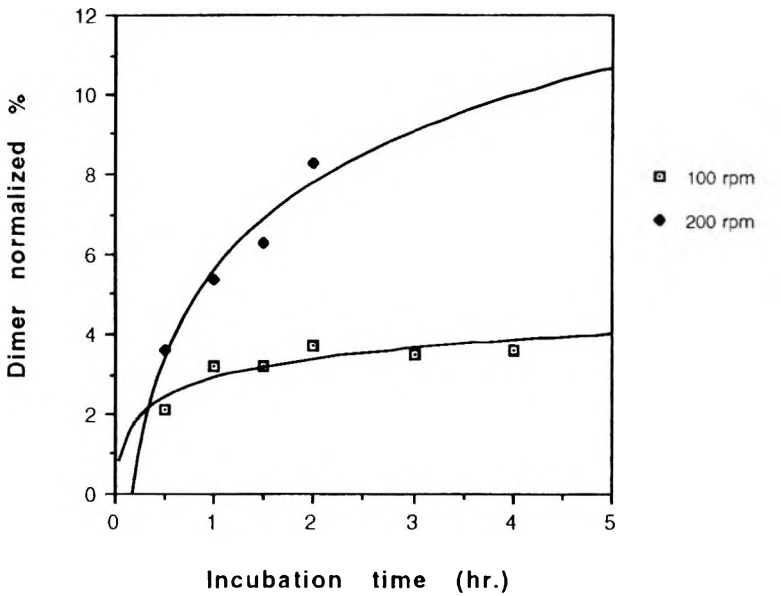


Figure 4. Influence of stirring speed in extraction on the formation of somidobove dimer. The experimental conditions were the same as in Figure 1.

borate - EDTA buffer solution heated at 40 - 50° C, the rate of protein denaturation increased resulting in the formation of both soluble and insoluble protein aggregates due to solid-liquid interfacial effect. Somidobove aggregates can also be formed in solution by strong stirring. This leads to poor sample recovery. In order to prevent the aggregation of protein in solution, mild conditions such as low incubation temperature, slow constant stirring, and weak alkaline bufer were employed in the extraction process.

Three different extraction solutions, 20mM borate - 1.4mM EDTA (pH 9.5), 25mM phosphate buffer (pH 9.5), and 50mM ammonium bicarbonate (pH 9.5) were evaluated for the extraction efficiency of somidobove from oleaginous vehicle. Figure 1 shows the influence of different buffer solutions on the recovery of somidobove monomer from oleaginous vehicle. Both phosphate and bicarbonate buffers showed poor extraction recovery for somidobove monomer. About 50-60% extraction recovery was found by using both buffers after 5 hours incubation. However, the borate-EDTA buffer provided excellent extraction recovery and stability. The somidobove extraction was quantitatively completed within 2 - 3 hours by using this buffer. The incubation temperature significantly affected the extraction recovery of somidobove from oleaginous

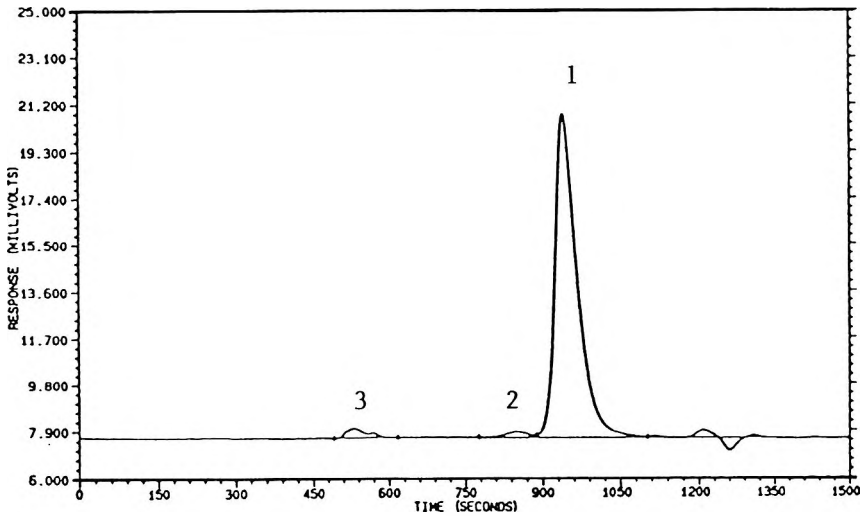


Figure 5. HPSEC chromatogram of somidobove extracted from the oleaginous vehicle lot 024. Peak 1: rbST monomer; Peak 2: Dimer; Peak 3: Soluble aggregates. Extraction and chromatographic conditions were the same as in Figure 1 except the incubation temperature was controlled at 40°C.

vehicles. It was found (Figure 2) that a low incubation temperature led to a lower recovery of somidobove even when the incubation time was prolonged to 7 hours in the extraction process. About 50% recovery was obtained in the extraction solution when incubated at 23°C and about 90% recovery was obtained at 30°C after 7 hours.

The formulated sample incubated at 35°C needed 6 hours to complete the extraction. Although a higher incubation temperature (50°C) reduced the incubation time, the somidobove potency was lost because more aggregates are formed in the extraction process (Figure 3). The results in this study revealed that the monomeric somidobove can be quantitatively extracted by using a borate-EDTA, pH 9.5, buffer solution at 40°C within 2-3 hours. Under these mild conditions, even if the incubation time was prolonged to 5 hours, the somidobove potency would be maintained.

Owing to the significant influence of strong shaking and stirring on the recovery in extraction, the optimal condition in stirring speed was investigated. A high stirring speed (200 rpm) caused a significant increase of somidobove

Table 1

Precision of Somidobove Monomer Determined in Formulated Drug Lots

Somidobove Monomer

Sample	Day 1		Day 2		Day 3		Mean	
Lot	%	CV%	%	CV%	%	CV%	%	CV%
024 (n=6)	8.37	1.56	8.40	1.18	8.65	1.94	8.47	1.75
4003A (n=6)	9.75	2.25	9.44	0.94	9.73	1.23	9.64	1.80
0A18 (n=6)	16.33	3.17	17.21	3.21	17.02	1.21	16.85	2.75

dimer (Figure 4). Overly slow stirring speed led to incomplete extraction and poor recovery. A stirring speed with 100 ± rpm revealed the optimal conditions for quantitative extraction of somidobove from oleanginous vehicle.

Quantification

Figure 5 shows a typical SEC chromatogram of somidobove extracted from oleaginous vehicle. Under the conditions described, the monomeric somidobove is baseline separated from dimer and soluble aggregates. The elution time of somidobove monomer is 15.6 min.

Linearity and Limit of Quantitation

The peak area response were linearly related to the concentration of somidobove in formulated vehicle in the range from 0.09 - 0.93 mg/mL. The regression equation was y (peak area response) = 4938.0 x (somidobove mg/mL) - 87.6. Correlation coefficient of the linearity was 0.999. The limit of quantitation (LOQ) in this method was 0.15mg/mL of somidobove monomer.

Precision and Reproducibility

Method precision for the determination of somidobove monomer potency in oleaginous vehicles was assessed through six weighings of three different formulated lots on three separate days. Table 1 summarizes the intra-day and

Table 2

Ruggedness of Precision and Recovery

Somidobove Monomer %

	Analyst 1	Analyst 2	Analyst 3	Inter-Lab
Lot 024				
Mean(n=3)	8.42	8.08	8.74	8.41
CV%	1.68	3.29	0.91	3.92
Lot 4003A				
Mean(n=3)	9.50	9.25	9.91	9.55
CV%	0.58	0.97	0.56	3.49
Lot 0A18				
Mean(n=3)	16.86		17.17	17.02
CV%	2.33		1.05	1.29
Lot 4004A				
Mean(n=3)	9.36	9.12	9.66	9.38
CV%	1.45	0.70	2.60	2.88

inter-day precision data. The coefficient of variance (CV) of intra-day precision in this study was 0.9 - 3.3% and CV of inter-day precision was 1.8-2.8%. System reproducibility was determined by injecting 5 times the same extracted solution of lot 0A18. The CV obtained in this experiment was 2.6%.

Accuracy (Recovery)

The method accuracy was assessed by fortifying two different amounts of somidobove bulk drug lot into a formulated drug lot 024 and analyzed by this method. The average recovery obtained was 93.7% with a CV of 3.71%.

Stability

The stability of somidobove in extraction solutions was evaluated by using two sets of identical sample solutions at room (22°C) and refrigerator (2-8°C). Results indicated that under the both temperature conditions, extracted monomeric somidobove in solution was stable for 2 days. The average CV was <1.6%.

Ruggedness

The ruggedness study was carried out by three analysts using 4 different formulated drug lots on different instruments at three different labs. The data of ruggedness were summarized in Table 2. The CV of precision generated at three different labs ranged from 1.3 - 3.9%.

Correlation with Hypox Rat Body Weight Gain Assay

The correlation between the potency data obtained by both of hypox rat body weight gain assay and this method was studied. Different amounts of somidobove formulated drug samples were extracted and assayed by this method. The extracted solutions with different concentrations of somidobove were then measured by hypox rat assay. The data obtained by both assays was linearly correlated. The regression coefficient obtained was 0.953.

ACKNOWLEDGMENTS

The authors thank Dr. Allison S. Kennington and Dr. Thomas K. Jeffers for reviewing this paper, and Ms. Terri Jackson for her technical assistance.

REFERENCES

1. T. H. Ferguson, R. K. McGuffey, D. L. Moore, R. E. Paxton, W. W. Thompson, J. F. Wagner, D. Dunwell, Proc. Int. Symp. Controlled Release Bioact. Mater., **15**, 55c-55d (1988).

2. S. M. Cady, W. D. Steber, R. Fishbein, Proc. Int. Symp. Controlled Release Bioact. Mater., **16**, 22-23 (1989).

3. K. N. Sivaramakrishnan, B. M. Rahn, B. M. Moore, and J. O'Neil. Proc. Int. Symp. Controlled Release Bioact. Mater., **16**, 14-15 (1989).

4. D. N. Brems, S. M. Plaisted, E. W. Kauffman, H. A. Havel, Biochemistry, **25**, 6539-6543 (1986).

5. H. A. Havel, E. W. Kauffman, S. M. Plaisted, D. N. Brems, Biochemistry **25**, 6533 (1986).

6. T. T. Donaldson, E. F. Boonstra, J. M. Hammond, J. Colloid Interface Sci., **74**, (1980) 441.

7. Henson, A.F. Mitchell, J. R.. Mussellwhite, P. R., ibid **32**, 162 (1970).

8. J. P. Chang, R. C. Tucker, B. F. Ghrist, M. R. Coleman, J. Chromatogr., **675**, 113-122 (1994).

9. J. P. Chang, T. H. Ferguson, P. A. Record, D. A. Dickson, D. E. Kiehl, A. S. Kennington, J. Chromatogr., In press.

Received April 3, 1996
Accepted May 13, 1996
Manuscript 4128

J. LIQ. CHROM. & REL. TECHNOL., 20(2), 257–272 (1997)

THE QUANTITATION OF A RESIDUAL QUATERNARY AMINE IN BULK DRUG AND PROCESS STREAMS USING CAPILLARY ELECTROPHORESIS

Bruce D. Johnson,* Nelu Grinberg, Gary Bicker, Dean Ellison
Analytical Research
Merck Research Laboratories
Merck & Co. Inc.
P. O. Box 2000
Rahway, NJ 07065

ABSTRACT

A capillary electrophoretic method for the determination of a residual alkyl quaternary amine, tetra-n-butylammonium ion (TBA^+), in bulk drug and process streams was developed. Since the analyte does not have a chromophore, detection was performed utilizing indirect photometric detection. The influence of the quinine, tetrahydrofuran and sodium acetate concentrations and pH_{app} in the background electrolyte solution upon the efficiency of the separation and effective electrophoretic mobility of both TBA^+ and electro-osmotic flow were studied. The ranges in which the various parameters were examined had considerable effects upon both the efficiency and the effective electrophoretic mobility of the electro-osmotic flow; however, the effective electrophoretic mobility of TBA^+ was not significantly affected. The optimized method was validated in terms of detector linearity, sensitivity, precision and accuracy.

Copyright © 1997 by Marcel Dekker, Inc.

INTRODUCTION

Capillary electrophoresis (CE) has been used to quantitate a broad range of organic and inorganic ions in a wide variety of matrices.[1-3] CE offers a uniquely different form of separation selectivity in comparison to conventional ion-analysis chromatographic methods. CE separates ions according to their mobility as they travel through a small diameter capillary filled with an electrolyte solution resulting in highly efficient separations and rapid analysis times. Additionally, CE offers the practical benefit of lower operating costs manifested by less solvent consumption and inexpensive column replacement.[1,2,4] Thus, CE is beginning to play an important role in pharmaceutical research towards the development and analysis of complex molecules.

Alkyl quaternary amines have proven to be difficult analytes to analyze for in drug substance matrices, due to the absence of a chromophore, which significantly limits detection by conventional UV detectors.[5] However, CE utilizing indirect photometric detection (IPD) is an appropriate option. In IPD, a UV-absorbing solute of the same charge as the separands (a co-ion) serves as an additive to the background electrolyte. This additive, known as a visualizing reagent (VR), elevates the baseline. When solute ions are present, they displace the VR and are measured as negative ions as they migrate past the detector window. IPD has proven to be a powerful technique due to its sensitivity without the need for derivatization.[1,2,6,7]

A literature survey revealed few reports of the separation of alkyl quaternary ammonium compounds including both CE[8-10] and IC.[11,12] This paper describes the quantitation of residual amounts of an alkyl quaternary amine, tetra-n-butylammonium ion (TBA^+), in both *indinavir* monohydrate intermediate and process streams. *Indinavir* monohydrate intermediate is the freebase form of the drug substance *Crixivan*, a potent inhibitor of the HIV protease.[13,14] The conversion step to form *indinavir* monohydrate intermediate from its precursor involves an alkylation reaction with 3-picolyl chloride in the presence of sodium iodide and tetra-n-butylammonium bromide in an aqueous/organic biphasic solution.[15,16] TBA^+, a commonly used phase transfer catalyst, facilitates rapid alkylation.

Upon completion of the alkylation reaction, the organic reaction mixture is extracted several times with water to remove TBA^+ along with other polar impurities. Alkyl quaternary amines are known to form micelles; thus, both the organic and aqueous cuts are analyzed for TBA^+.[17] Additionally, the isolated intermediate is analyzed for residual TBA^+ remaining from the extraction process.

The factors that contribute to the electrophoretic separation and quantitation of TBA^+ have been investigated. The effects of varying the concentration of THF, sodium acetate, quinine and the pH_{app} of the background electrolyte (BGE) upon the efficiency of the separation and the effective electrophoretic mobility of both TBA^+ and electro-osmotic flow are discussed. The method has been validated in terms of precision, accuracy, detector linearity and sensitivity. Additionally, the method was applied to the analysis of process streams and *inidinavir* monohydrate intermediate used in the manufacture of drug substance used for both clinical studies and commercial sale.

MATERIALS

Instrumentation

The CE experiments were carried out on a HP^{3D} CE (Hewlett-Packard, Germany) system. The HP^{3D} CE instrument was used with a 56 cm effective length fused-silica capillary (65 cm total length, 75μm I.D., Hewlett-Packard, Germany) and a diode-array detector (Hewlett-Packard, Germany). The wavelengths for the sample and reference signals were reversed to provide positive peaks for analysis. The capillary temperature was maintained at 30±0.1°C, a hydrodynamic sampling injection model was applied for 3 s at 50 mbar, and the applied voltage was 30 kV (current typically ~ 20 mA). The electrode polarity was in the 'standard' mode i.e. anode (+) at the inlet/injector and cathode (-) at the outlet/detector. Data collection, integration and efficiency calculations were accomplished by using the PE Nelson ACCESS CHROM system (Cupertino, CA). The spectra of the BGE at various pH_{app}'s was collected by making a 300-fold dilution of the BGE into 70:30 water:THF and measuring the spectra on a Shimadzu UV-2101PC UV-VIS spectrometer (Columbia, MD). The concentration of quinine was confirmed by potentiometric titrations using a Metrohm 716 DMS Titrino Dosimat and a Titrino Workcell titroprocessor (Brinkman, Westbury, NY).

Reagents

Indinavir monohydrate intermediate and reaction mixtures were obtained from the Chemical & Engineering department in Merck Research Laboratories, Rahway, NJ. The tetra-n-butylammonium bromide was purchased from Aldrich (Milwaukee, WI, USA), quinine, anhydrous obtained from either

Janssen Chimica (Belgium) or Aldrich, sodium acetate was obtained from Sigma (St. Louis, MO, USA), methanol and tetrahydrofuran (THF) were obtained from Fisher Scientific (Spring Field, NJ, USA) and concentrated acetic acid was obtained from EM Sciences (Gibbstown, NJ) . All commercial chemicals were used directly without any further purification. Water used in the study was purified with a HYDRO Picosystem (Hydro Service and Supplies, Inc., Research Triangle Park, NC, USA).

METHODS

Background Electrolyte (BGE) Solution

The BGE was prepared by dissolving sodium acetate in water. Separately, the quinine was dissolved with the THF and diluted to volume with the sodium acetate solution. The pH was adjusted with concentrated acetic acid and the solution was filtered through a 0.45 μm nylon membrane before use (the concentration of THF reported reflects the volume percent of the original amount of THF and water that were mixed).

Sample Solutions

Samples and standards were dissolved in methanol to provide sufficient solubility of the *indinavir* monohydrate intermediate and the reaction mixtures. The stock solution of tetra-n-butylammonium ion (TBA^+) was prepared by dissolving 360 mg of tetra-n-butylammonium bromide in 25 mL of water (approx. 4.5 mM TBA^+). Standard solutions were made from the stock solution by dilutions into methanol. Dry powder sample solutions were made by dissolving 700 mg of *indinavir* monohydrate intermediate into 10 mL of methanol. The organic layer reaction mixtures were prepared by dissolving approximately 40 g of material into 250 mL of methanol. The aqueous layer reaction mixtures were diluted with methanol. The methanol diluent was used as the neutral marker (NM).

Capillary Conditioning

New capillaries were conditioned with a 1*N* NaOH solution for 30 min., deionized H_2O for 10 min., 0.2 *N* NaOH solution for 30 min., deionized H_2O for 10 min., 75:25 (v/v) H_2O: THF for 10 min. and BGE for 15 minutes. Between each injection, the capillary was flushed with the BGE for 10 min.

The capillaries were flushed with 75:25 (v/v) H_2O: THF for 20 min subsequent to usage. Care was taken not to expose the capillary with BGE to aqueous solutions (i.e. water or NaOH solutions) due to the insolubility and precipitation of quinine.

Calculations

The electrophoretic mobility of the electro-osmotic flow (μ_{EOF}) and the net effective electrophoretic mobility of TBA^+ (μ_{TBA+}) were calculated using Equations 1 and 2, respectively,

$$\mu_{EOF} = \frac{l\ L}{V\ t_{EOF}} \quad (1)$$

$$\mu_{TBA-} = \left(\frac{1}{t} - \frac{1}{t_{EOF}}\right)\frac{l\ L}{V} \quad (2)$$

where l is the effective capillary length, L is the total capillary length, V is the applied voltage, t_{EOF} is the migration time of the NM (methanol), and t is the migration time of TBA^+.[18]

For calculation of the efficiency of the separation, the Foley-Dorsey equation was used.[19]

RESULTS AND DISCUSSION

Selection of Wavelength for Indirect Detection

The optimal wavelength for detection is determined by two factors. First, a wavelength where the maximum difference in the extinction coefficient between the analyte and the visualizing reagent (VR) is determined. Secondly, the absorbance of the VR at this wavelength should not be too high so as to saturate the detector.[2,6,7,20] Because TBA^+ does not possess a chromophore in the UV-visible wavelength ranges, the only wavelength consideration is the absorbance of the VR, quinine. To select the proper detection wavelength, spectra were obtained of the BGE as the pH was varied as follows: 3.5, 4.0,

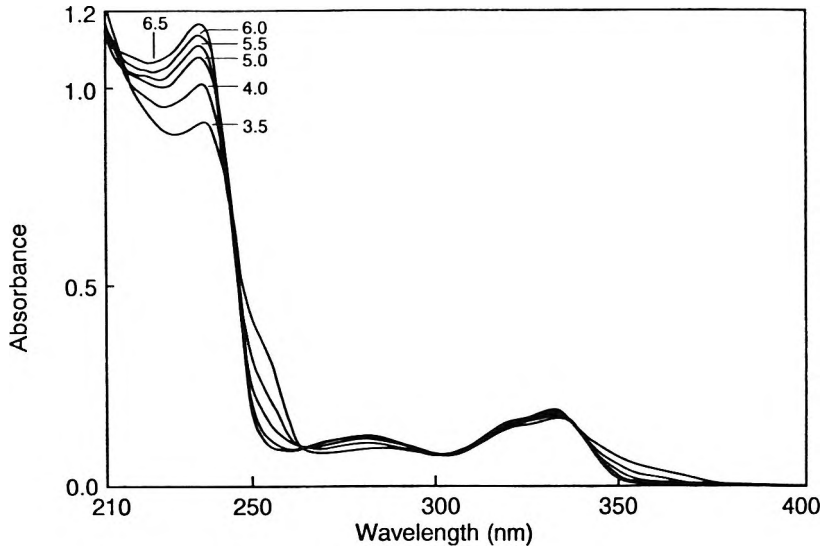


Figure 1. Effect of the pH_{app} on the absorbance spectra of quinine (absorbance decreases with decreasing pH_{app} at 235 nm). BGE: 7 mM sodium acetate, 12 mM quinine and 30 v/v % THF.

4.5, 5.0, 5.5 and 6.5 (Figure 1). Quinine exhibits absorbance maxima's at approximately 235, 285 and 335 nm's. The detector was saturated at the 235 nm maxima, however, an acceptable absorbance of approximately 0.5 AU was found for the 335 nm maxima. Additionally, the absorbance spectra of quinine is pH sensitive as it exhibits pKa's of approximately 5.1 and 9.7.[21] Therefore, the 335 nm maxima was selected as the optimal wavelength of detection because it does not saturate the detector and this wavelength is minimally sensitive to pH changes.

Influence of the pH_{app}

The pH_{app} of the BGE was varied between 3.5 and 6.5 in the same increments as described in the previous section. TBA^+ is a quaternary amine and, as expected, the μ_{TBA^+} changed minimally throughout the pH range studied. However, the μ_{EOF} increased over 180% when the pH_{app} was increased (Figure 2). The electro-osmotic flow (EOF) is generated by the silanol groups (SiO^-) present on the capillary wall and their ionization is significantly affected by pH_{app} changes.[1,18,22]

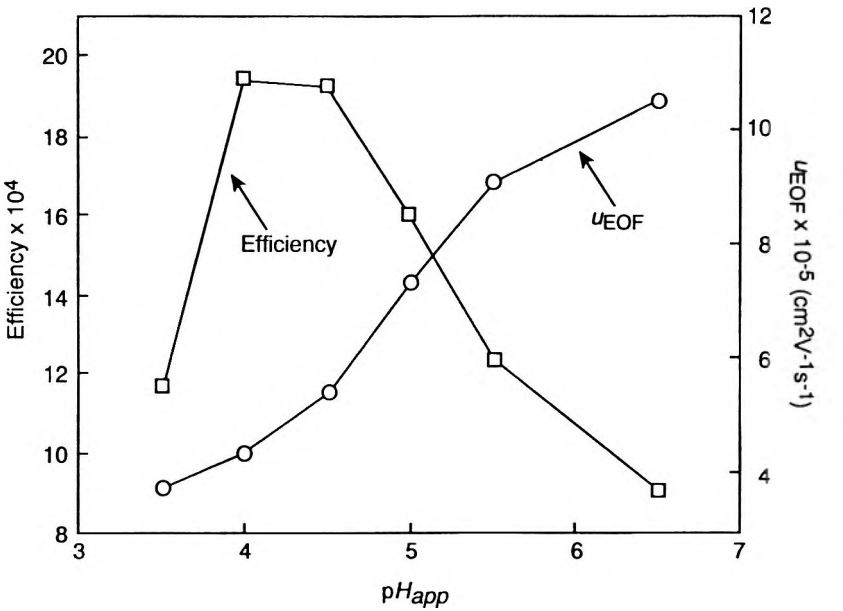


Figure 2. Effects of the pH_{app} on the effective electrophoretic mobility of the electroosmotic flow and efficiency of the separation. BGE: 7 mM sodium acetate, 12 mM quinine and 30 v/v % THF.

As the present study deals with the analysis of trace amounts of TBA^+, the method should be optimized such that the efficiency of the TBA^+ peak should be maximized to insure lower detection limits. From the data plotted in Figure 2, the maximum efficiency was obtained between the pH range of 4.0 to 4.5. The pH_{app} of 4.5 was selected because it is nearest to the pKa's of acetate (4.8) and quinine (5.1) thus, insuring the maximum buffering capacity. Therefore, in the following experiments the pH_{app} was maintained at this value.

Influence of the Concentration of Quinine

Quinine is an appropriate selection as a VR as it satisfies the general conditions required for successful capillary ion analysis of TBA^+.[1,2,23] Additionally, quinine has previously been utilized as a VR in CE.[10,24-26] The concentration of quinine in the BGE was varied between 5.3 and 26 mM as follows: 5.3, 11, 13, 21, and 26 mM. The μ_{TBA^+} varied minimally in the concentration range of quinine studied. However, the μ_{EOF} decreased more

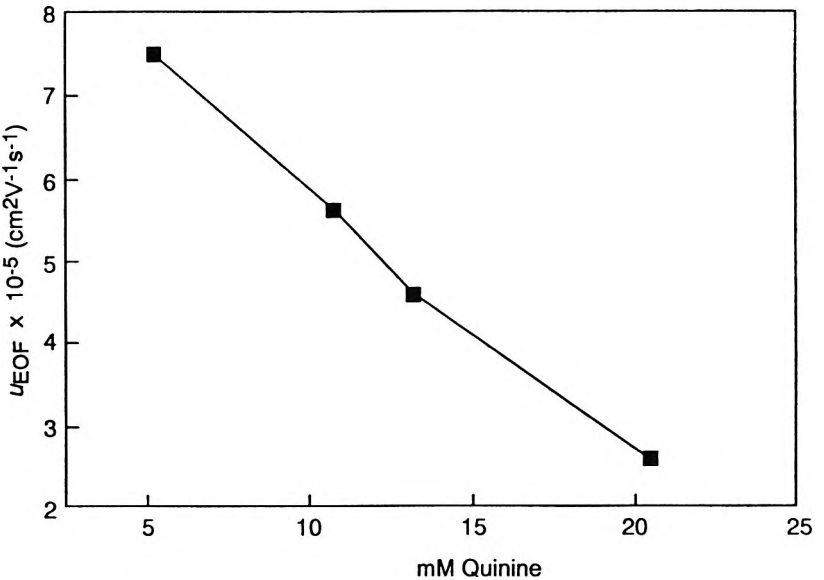


Figure 3. Effects of the concentration of quinine on the effective electrophoretic mobility of the electro-osmotic flow. BGE: 7 mM sodium acetate, pH_{app} 4.5 and 30 v/v % THF.

than 60% when the concentration of quinine was increased (Figure 3). This behavior is presumably due to the interaction of the positively charged quinine with the SiO^- groups on the capillary wall. As the concentration of quinine is increased, the zeta potential on the capillary wall is reduced thereby decreasing μ_{EOF}.

The increase of the concentration of quinine in the BGE has a positive effect on the efficiency of the TBA^+ peak (Figure 4). This efficiency increase is likely due to the minimization of the interactions of TBA^+ with the capillary wall. However, increasing the concentration of quinine in the BGE produced a decrease in area counts of TBA^+ (Figure 4). Therefore, an intermediate concentration of 12 mM quinine was maintained in the following experiments.

Influence of the Concentration of THF

THF was selected as an organic modifier as it provided solubility of quinine and is also known to inhibit the formation of micelles, a potential

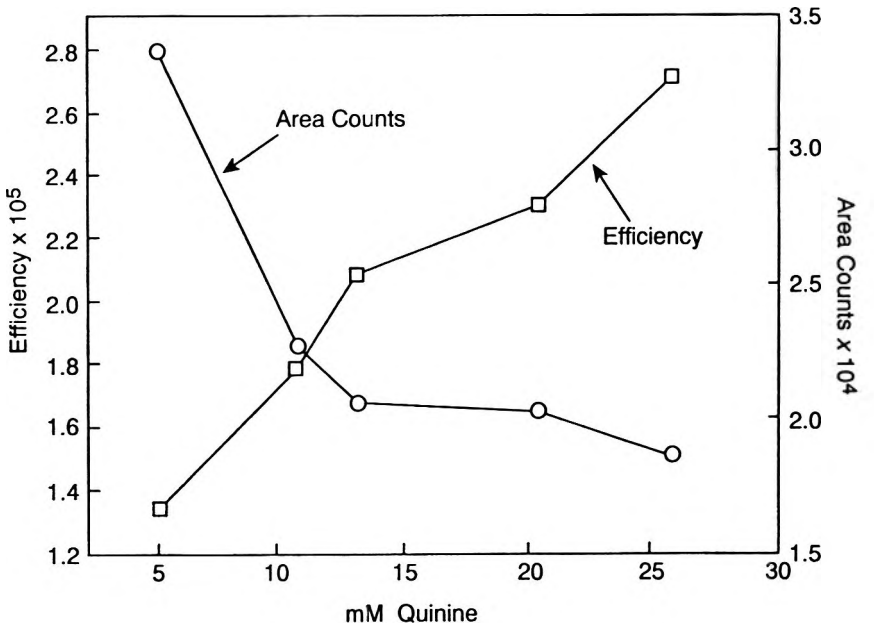


Figure 4. Effects of the concentration of quinine on the efficiency of the separation and the detector area counts. BGE: 7 mM sodium acetate, pH_{app} 4.5 and 30 v/v % THF.

problem towards the analysis of quaternary amines.[8,17,27] The volume percent of THF in the BGE was varied as follows: 20, 25, 30, 35, 40 and 50%. The μ_{TBA+} and the μ_{EOF} decreased by approximately 20 and 10%, respectively, as the concentration of THF was increased (Figure 5).

The reduction in μ_{TBA+} is attributed to the simultaneous decrease of current (approximately 30%) as the concentration of THF was increased. The decrease in μ_{EOF} is due to the interaction of THF with the silanol groups at the capillary wall resulting in a decreased zeta potential.[22,28-30]

The increase of the concentration of THF in the BGE generally decreased the efficiency of the TBA^+ peak (Figure 6). A maximum in efficiency was obtained at 25 v/v % THF, therefore, the v/v % THF was maintained at this value.

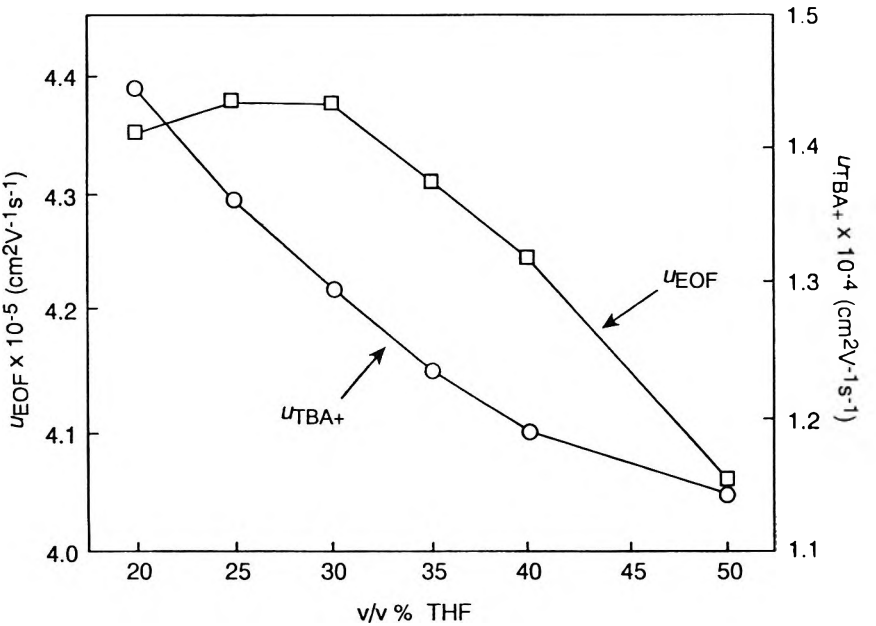


Figure 5. Effects of the v/v % THF on the effective electrophoretic mobility of the electro-osmotic flow and TBA^+. BGE: 7 mM sodium acetate, pH_{app} 4.5 and 12 mM quinine.

Influence of the Concentration of Sodium Acetate

Sodium acetate was utilized in the BGE to provide increased buffering capacity. The concentration of sodium acetate was varied between 3.0 and 30 mM as follows: 3.0, 7.0, 12, 15, 20, and 30 mM. The μ_{TBA^+} varied minimally for the range of sodium acetate concentration studied. However, the μ_{EOF} decreased 25% when the concentration of sodium acetate was increased (Figure 7). This behavior was due to shielding of the SiO^- groups on the capillary wall as the ionic strength of the BGE was increased resulting in a decreased zeta potential.[1,18,22]

The increase of the concentration of sodium acetate in the BGE has a negative effect on the efficiency of the TBA^+ peak (Figure 7). Therefore, in the following experiments the concentration of sodium acetate was maintained at 3.0 mM.

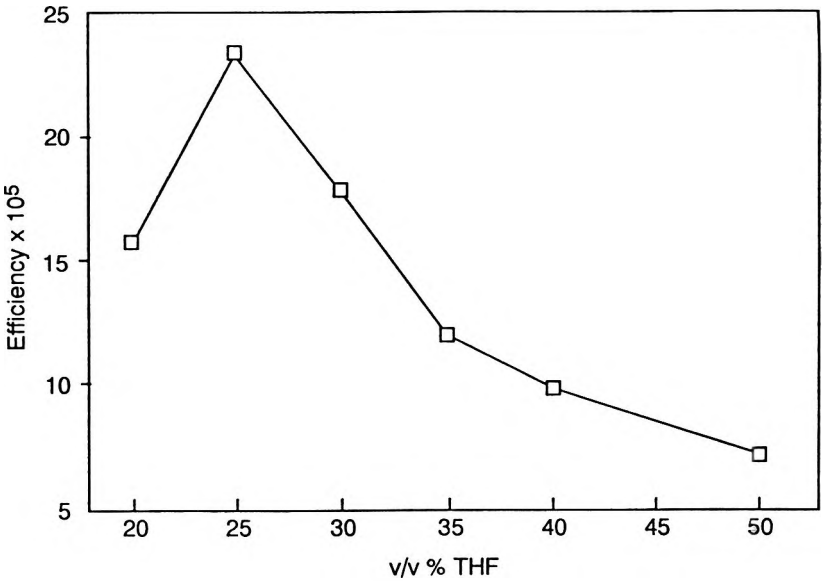


Figure 6. Effects of the v/v % THF on the efficiency of the separation. BGE: 7 mM sodium acetate, pH_{app} 4.5 and 12 mM quinine.

Influence of Methanol versus THF as the Organic Modifier

A direct comparison was made between methanol and THF as the organic modifier under the final BGE conditions. The μ_{TBA+} varied minimally, however, the μ_{EOF} was 20% slower with methanol. Methanol has a hydroxy group that can act both as an electron donor and acceptor while THF can only act as an electron donor. Thus, the decrease in μ_{EOF} is presumably due to the increased interactions of methanol with the capillary wall in comparison to THF. Additionally, the symmetry of the TBA^+ peak was reversed with methanol (fronting) in comparison to the THF (tailing). The asymmetry of a solute peak is a result of the difference between the electric field in the sample zone and the BGE.[1,2,18,31] The reversal of peak symmetry suggests that the conductivity of the BGE with methanol is lower (higher electric field) than the BGE with THF with respect to TBA^+. Because the efficiency and selectivity of the separation did not differ appreciably in the comparison between methanol and THF and because methanol does not inhibit the formation of micelles, THF was maintained as the organic modifer.[8]

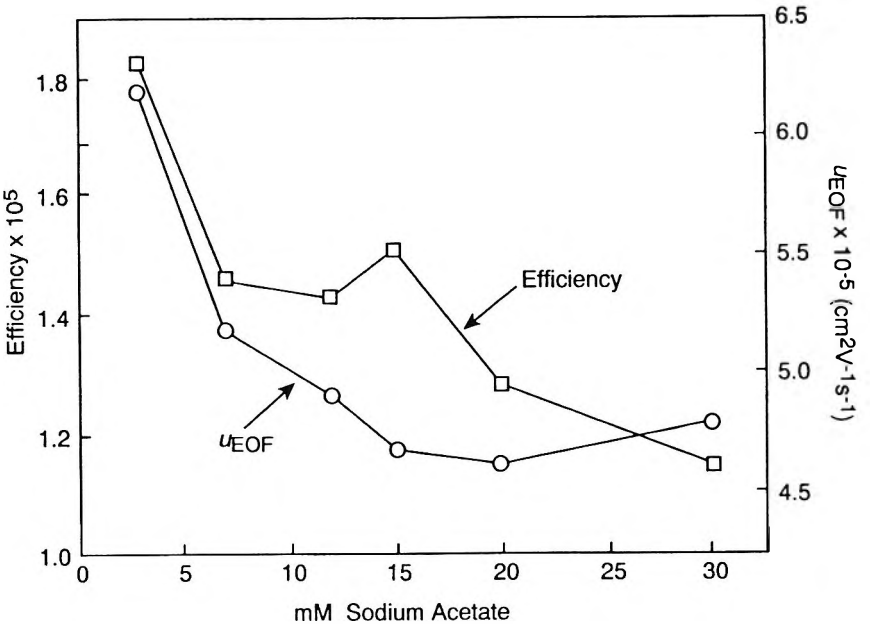


Figure 7. Effects of the concentration of sodium acetate on the effective electrophoretic mobility of the electro-osmotic flow and efficiency of the separation. BGE: 30 v/v % THF, pH_{app} 4.5 and 12 mM quinine.

The optimized composition of the BGE was 12 m*M* quinine and 3 m*M* sodium acetate in 75:25 (v/v) H_2O:THF at pH_{app} 4.5 and detection was performed at 335 nm. The ability to analyze real samples was the objective of this work and a typical electropherogram of an initial organic extraction layer (~0.1 mg/mL TBA^+) is shown in Figure 8. The method resolves several components in the process streams including potassium and sodium ions.

Validation Studies

The linearity of the detector response was evaluated using standard solutions of TBA^+ over the concentration range from 1.08 μg/mL to 1.08 mg/mL. This corresponds to approximately 15 ppm to 1.5 wt% TBA^+ in the *indinavir* monohydrate intermediate sample. Ten standard solutions were injected in triplicate and the detector response at 335 nm was found to be linear over the entire concentration range (R^2 = 0.9998). A signal to noise ratio (S/N) of three was measured for the lowest concentration tested during the linearity

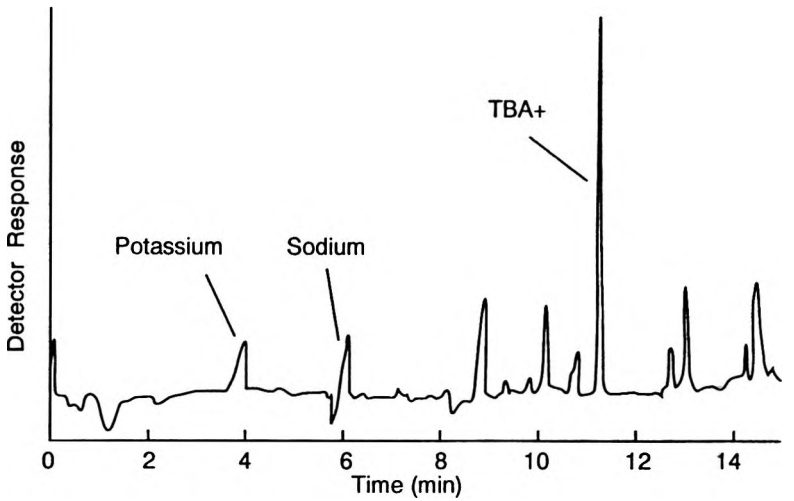


Figure 8. Typical electropherogram of an organic extraction layer. BGE: 25 v/v % THF, 3 mM sodium acetate, pH_{app} 4.5 and 12 mM quinine.

study, 1.08 μg/mL TBA^+. Therefore, the limit of detection and limit of quantitation (S/N=3xLOD) of the method was determined as 1.08 and 2.17 μg/mL, respectively. The linear range of the detector and sensitivity agrees well with the work of other laboratories quantitating structurally related alkyl quaternary ammonium compounds.[8-10]

The injection precision was evaluated by making three consecutive injections each of the standard solutions during the course of the linearity study. The %RSD for the area counts of TBA^+ ranged from 1.6% to 17.6% for the 1.08 mg/mL and 1.08 μg/mL standards, respectively. The average %RSD for the area counts and migration time of TBA^+ for each triplicate injection set of standards was 5.3 and 0.45%, respectively.

The accuracy of the assay was evaluated by the method of standard additions. Aliquots of TBA^+ stock solution (10.8 mg/mL) were spiked into a solution of *indinavir* monohydrate intermediate (71.7 mg/mL). The concentration of TBA^+ in the spiked samples ranged from 216 to 10.8 μg /mL. The amount of TBA^+ in the spiked samples was plotted against the amount of TBA^+ determined by linear regression of standards. A linear plot was obtained (R^2 = 0.998) with a slope close to 1.0 (0.98) and an intercept of zero.

Additionally, spiking analysis was performed at the 140 μg/mL level for both organic and aqueous reaction mixtures and recoveries between 96 and 102% were obtained.

CONCLUSIONS

The quantitation of TBA^+ in both drug substance and process streams was achieved by a CE method utilizing indirect detection. The studies described above demonstrated the effects of varying the pH_{app}, volume percent organic modifier, sodium acetate and quinine concentration in the BGE upon m_{TBA+}, μ_{EOF} and efficiency. The ranges in which the various parameters were examined had considerable effects upon both efficiency and m_{EOF}, however, μ_{TBA+} was not significantly affected. The method validation results demonstrated that the CE method is reproducible, accurate, precise and sensitive.

ACKNOWLEDGMENTS

The authors extend appreciation to Dr. Angelos Dovletoglou and Ms. Lili Zhou for the generous use of their CE system.

REFERENCES

1. J. P. Landers, ed., **Handbook of Capillary Electrophoresis**, CRC Press, Inc., Boca Raton, FL, 1993, Chapters 1 & 9.

2. D. Heiger, R. Weinberger, "Determination of Small Ions by Capillary Zone Electrophoresis with Indirect Photometric Detection," Application Note, Hewlett-Packard, Germany, Pub. No. 12-5963-1138E (1994).

3. P. Morin, C. Francois, M. Dreux, J. Liq. Chromatogr., 17, 3869-3888 (1994).

4. Environmental Notes, Waters Coporation, Milford, MA, **4(1)**, 1-9 (1995).

5. FDC Quality Control Reports: The Gold Sheet, Chevy Chase, MD, **27(11)**, 7 (1993).

6. H. Small, **Ion Chromatography**, Plenum Press, New York, NY, 1989, Chapter 8.

7. H. Small, T. E. Miller Jr., Anal. Chem, **54**, 462-469 (1982).

8. C. S. Weiss, J. S. Hazlett, M. H. Datta, M. H. Danzer, J. Chromatogr., **608**, 325-332 (1992).

9. S. A. Shamsi, N. D. Danielson, Anal. Chem., **67**, 4210-4216 (1995).

10. L. Gross, E. S. Yeung, Anal. Chem., **62**, 427-431 (1990).

11. T. A. Walker, T. V. Ho, J. Chromatogr. Sci., **28**, 254-257 (1990).

12. T. A. Walker, J. Liq. Chromatogr., **11**, 1513-1530 (1988).

13. B. D. Dorsey, R. B. Levin, S. L. McDaniel, J. P. Vacca, J. P. Guare, P. L. Darke, J. Zugay, E. A. Emini, W. A. Schlief, J. C. Quintero, J. Lin, I.-W. Chen, M. K. Holloway, P. M. Fitzgerald, M. G. Axel, D. Ostovic, P. S. Anderson, J. R. Huff, J. Medicinal Chem., **37**, 3443-3450 (1994).

14. J. P. Vacca, B. D. Dorsey, W. A. Schlief, R. B. Levin, S. L. McDaniel, P. L. Darke, J. Zugay, J. C. Quintero, O. M. Blahy, E. Roth, V. V. Sardana, A. J. Schlabach, P. I. Graham, J. H. Condra, L. Gotlib, M. K. Holloway, J. Lin, J.-W. Chen, K. Vastag, D. Ostovic, P. S. Anderson, E. A. Emini, J. R. Huff., Proc. Natl. Acad. Sci. USA, **91**, 4096-4100 (1994).

15. D. Askin, K. Eng, K. Rossen, R. Purick, K. Wells, R. Volante, P. Reider, Tetrahedron Lett., **35**, 673-676 (1994).

16. R. Calabria, S. Hoerrner, Merck Research Laboratories, personal communication, May 17, 1994.

17. Other structurally related alkyl quaternary amines similar to TBA^+ exhibit cmc's around 1 mM as per: P. Mukerjee, K. J. Mysels, **Critical Micelle Concentrations of Aqueous Surfactants NSRDS NBS 36**, National Bureau of Standards, Washington, D.C.

18. D. N. Heiger, **High Performance Capillary Electrophoresis - An Introduction**, Hewlett Packard, Germany, Pub. No. 12-5091-6199E, 2nd ed., 1992.

19. J. P. Foley, J. G. Dorsey, Anal. Chem., **55**, 730-737 (1983).

20. R. Thompson, N. Grinberg, H. Perpall, G. Bicker, P. Tway, J. Liq. Chromatogr., **17**, 2511-2531 (1994).

21. S. Budavari, ed., **The Merck Index,** Merck & Co. Inc., Rahway, NJ, 11th ed., 1989, p. 8080.

22. M. Jimidar, T. P. Hamoir, A. Foriers, D. L. Massart, J. Chromatogr., **636**, 179-186 (1993).

23. P. E. Andersson, W. D. Pfeefer, L. G. Blomberg, J. Chromatogr., A, **699**, 323-330 (1995).

24. R. Zhang, C. L. Cooper, Y. Ma, Anal. Chem., **65**, 704-706 (1993).

25. Y. Ma, R. Zhang, C. L. Cooper, J. Chromatogr., **608**, 93-96 (1992).

26. G. Zhou, Q. Yu, Y. Ma, J. Xue, Y. Zhang, B. Lin, J. Chromatogr., **717**, 345-349 (1995).

27. Y. Liu, K.-F. J. Chan, Electrophoresis, **12**, 402-408 (1991).

28. C. Schwer, E. Kenndler, Anal. Chem., **63**, 1801-1807 (1991).

29. W. Buchberger, P. R. Haddad, J. Chromatogr., **608**, 59-64 (1992).

30. S. M. Masselter, A. Zemann, Anal. Chem., **67**, 1047-1053 (1995).

31. F. E. P. Mikkers, F. M. Everaerts, Th. P. E. M. Verheggen, J. Chromatrogr., **169**, 1-10 (1979).

Received August 5, 1996
Accepted August 27, 1996
Manuscript 4229

J. LIQ CHROM. & REL. TECHNOL., 20(2), 273–282 (1997)

SEPARATION OF OXYTETRACYCLINE AND ITS RELATED SUBSTANCES BY CAPILLARY ELECTROPHORESIS

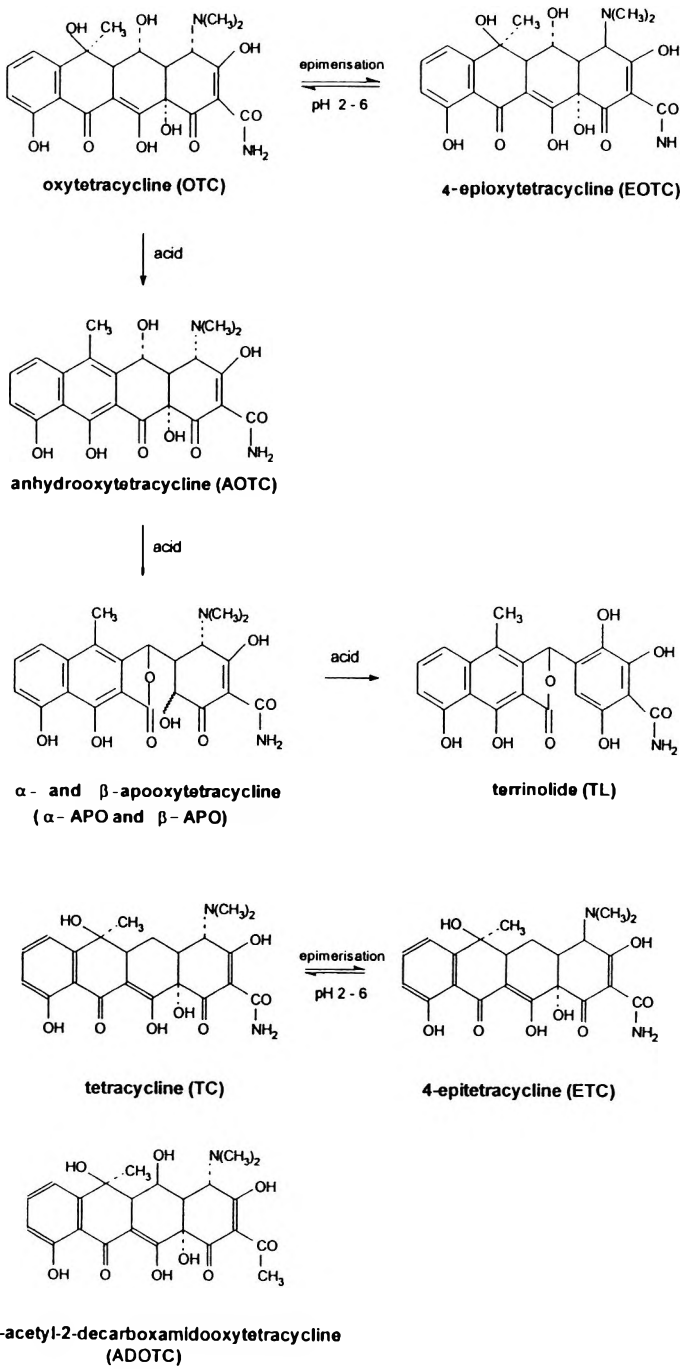
Y. M. Li, A. Van Schepdael, E. Roets, J. Hoogmartens

Laboratorium voor Farmaceutische Chemie
en Analyse van Geneesmiddelen
Faculteit Farmaceutische Wetenschappen
K. U. Leuven, Van Evenstraat 4
B-3000 Leuven, Belgium

ABSTRACT

A method is described for separation of oxytetracycline and its related substances by capillary electrophoresis. Oxytetracycline is a broad spectrum antibiotic obtained by biosynthesis. Main related substances are 4-epioxytetracycline, α-apooxytetracycline, β-apooxytetracycline, anhydrooxytetracycline, 2-acetyl-2-decarboxamidooxytetracycline, tetracycline, 4-epitetracycline and terrinolide. Method development was undertaken with a mixture consisting of oxytetracycline and its related substances mentioned. Using an uncoated fused silica capillary, the influence of buffer pH and its concentration was systematically investigated. Non-ionic surfactant Triton X-100 was added to the running buffer for improving separation selectivity. Quantitative data are reported and compared with those of liquid chromatography and capillary zone electrophoresis.

Copyright © 1997 by Marcel Dekker, Inc.


epimerisation
pH 2 - 6
oxytetracycline (OTC)
4-epioxytetracycline (EOTC)
acid
anhydrooxytetracycline (AOTC)
acid
α- and β-apooxytetracycline
(α-APO and β-APO)
acid
terrinolide (TL)
epimerisation
pH 2 - 6
tetracycline (TC)
4-epitetracycline (ETC)
2-acetyl-2-decarboxamidooxytetracycline
(ADOTC)

INTRODUCTION

Oxytetracycline (OTC) is a broad spectrum antibiotic obtained by biosynthesis. 2-Acetyl-2-decarboxamidooxytetracycline (ADOTC) and tetracycline (TC) are fermentation impurities of OTC. OTC can epimerise at position C-4. resulting in the formation of 4-epioxytetracycline (EOTC). The presence of a hydroxyl group at C-6 enables acid degradation, mainly occurring in OTC.HCl samples and forming anhydrooxytetracycline (AOTC). AOTC is quite unstable and rearranges in acid to two isomers, α-apooxytetracycline (α-APO) and β-apooxytetracycline (3-APO).[1] These can in turn react to form terrinolide (TL).[1] 4-Epitetracycline (ETC) can be present due to epimerisation of TC at position C-4.

The structures of OTC and its related substances are shown in Figure 1. Except for TC, the potential impurities are therapeutically inactive. Separation of OTC and its related substances is mainly performed by liquid chromatography (LC).[2,3]

However, capillary electrophoresis (CE) for analysis of OTC has also been reported in recent years. The separation of OTC from TC and chlortetracycline (CTC) with MECC[4] and the separation of OTC from six other tetracyclines[5] were described. A capillary zone electrophoresis (CZE) method to resolve OTC from its fermentation and degradation impurities was also investigated.[6] but an important impurity, ADOTC could not be separated from OTC in this system.

The method presented here is related to previous CE work concerning the analysis of tetracycline (TC),[7] doxycycline (DOX),[8] demeclocycline (DMCTC)[9] and minocycline (MC).[10]

In this paper, non-ionic surfactant Triton X-100 is employed to improve separation selectivity due to the interaction with the negatively charged analytes. This detergent moves at the speed of electroosmotic flow and has to be distinguished from the pseudostationary phase in electrokinetic chromatography which has its own electrophoretic mobility. Triton X-100 would also not allow the separation of neutral compounds.[11] The method enables the complete separation of OTC from its related substances AOTC, α-APO, β-APO, EOTC, TC, ETC, ADOTC and TL. The analysis time is shorter than with LC. Quantitative results are also compared with those of LC and CZE.

Figure 1 (left). Chemical structures of oxytetracycline and its related substances.

MATERIALS

Capillary electrophoresis was performed on Spectraphoresis 500 equipment (Thermo Separation Products, Fremont, CA, U.S.A.), coupled to a 3396 series II integrator (Hewlett Packard, Avondale, PA, U.S.A.). Tetracyclines were detected by UV absorption at 254 nm. Injection was done hydrodynamically for 4 seconds. pH measurements were performed on a Consort pH-meter (Turnhout, Belgium) using calibration buffers constituted according to the European Pharmacopoeia.[12] When necessary, the pH of running buffers was adjusted using 0.1 M HCl before making up to volume. Throughout the study, all samples were dissolved in running buffer to obtain better peak symmetry. To obtain repeatable migration times, it is advisable to wash the capillary each day consecutively with 0.1 M NaOH solution, 0.1 M phosphoric acid solution and 20 mM EDTA solution.

All reagents were of analytical grade (Merck, Darmstadt, Germany or Acros Chimica, Geel, Belgium). Oxytetracycline and its related substances were obtained from Acros Chimica. TL and AOTC were prepared by an existing method.[1] Small amounts of ADOTC were also prepared and isolated by a thin layer chromatographic method.[13] Fused silica capillary was from Polymicro Technologies (Phoenix, AZ. U.S.A.). Throughout the study, Milli-Q^{50} water was used (Millipore, Milford, MA, U.S.A.). All the solutions were filtered through 0.2μm nylon filters (Alltech, Laarne, Belgium).

RESULTS AND DISCUSSION

Method development was performed with an artifical mixture containing OTC, AOTC, α-APO, β-APO, EOTC, TC, ETC and TL. ADOTC was not available in sufficient quantities to include it in the mixture and therefore was only used in the final stage. Sodium carbonate was used as background electrolyte and 1 mM EDTA was added in all cases to prevent interaction of the tetracycline structure with metals through complexation. The following parameters were optimized consecutively: buffer pH, buffer concentration and Triton X-100 concentration. Influences of voltage and capillary temperature were also examined.

The pH is a very important parameter for improving selectivity in CE and small differences can cause the separation of closely related substances.[14] Therefore buffer pH was first optimized. This was performed with a sodium carbonate (20 mM) -EDTA (1 mM) buffer including 0.5 % (v/v) Triton X-100, at a voltage of 10 kV and a temperature of 10 °C. The variation of pH was

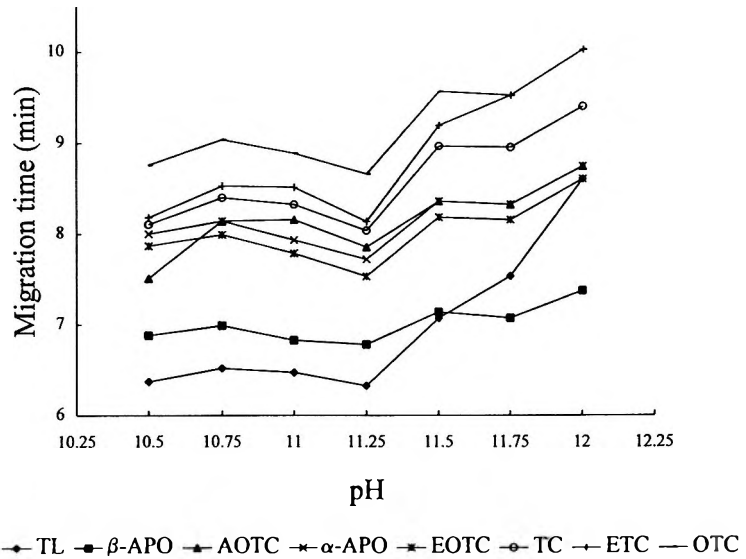


Figure 2. Influence of buffer pH on the separation of OTC and its related substances. Capillary: uncoated fused silica, L=44cm, l=38cm, ID=50μm; background electrolyte=sodium carbonate (20 mM)-EDTA (1mM) including 0.5 % (v/v) Triton X-100; temperature=10°C; voltage=10 kV.

restricted to the alkaline range to avoid sample adsorption on the capillary and epimerization of OTC. It was varied between 10.5 and 12.0 with steps of 0.25 pH unit. Since isoelectric points of OTC and its related substances are below the buffer pH, they are negatively charged and the electrophoretic mobility is opposite to the electroosmotic mobility. The changes of migration order and migration time are dependent on the sum of electrophoretic mobility of each solute and electroosmotic mobility. At one particular pH, the increase in the electrophoretic mobility of the negative ions might be dominant and result in an increase of migration time, and even migration order might change. However, at another pH, the increase of electroosmotic mobility can become dominant and result in a decrease of migration time which is unfavourable for resolution. Results are shown in Figure 2.

It was found that only at pH 11 and 11.25, separation of all eight substances could be obtained. A pH of 11 was retained because it gave a better resolution for the most critical separation TC/ETC. The order of migration

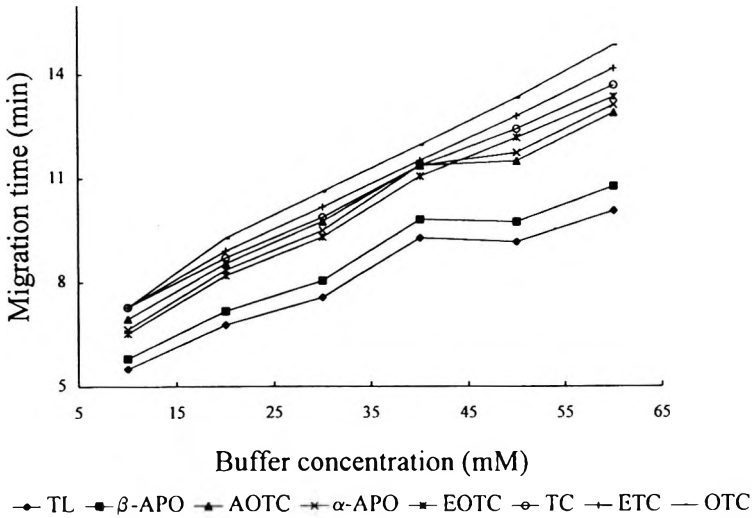


Figure 3. Influence of buffer concentration on the separation of OTC and its related substances. Capillary: uncoated fused silica, L=44cm, l=38cm, ID=50μm; background electrolyte=sodium carbonate (x mM)-EDTA (1mM) including 0.5 % (v/v) Triton X-100; pH=11.0; temperature=10°C; voltage=10 kV.

was: TL, β-APO, EOTC, α-APO, AOTC, TC, ETC and OTC. It should be pointed out that the buffer pH affected the order of migration in this system. At about pH 10.5, AOTC changed position around EOTC and α-APO. Above pH 11.5, the order of TL and β-APO also switched.

The next step was the optimization of buffer concentration. It has a main influence on electroosmotic flow and current produced in the capillary.[14] It was varied from 10 to 60 mM with steps of 10 mM, keeping other conditions constant (pH 11.0, 0.5 % Triton X-100, 10 kV, 10°C). Results are shown in Figure 3. All eight substances can be separated at 20 mM, 30 mM, 50mM and 60 mM. At 40 mM, their migration order changed. The explanation of the results is similar with the influence of the buffer pH. Under normal conditions, as the buffer concentration is increased, the electrophoretic and electroosmotic mobilities are both decreased. In this system, the decrease in the electroosmotic mobility with buffer concentration increase is dominant and results in an increase of migration time. 50 mM was finally chosen because it gave the best overall resolution.

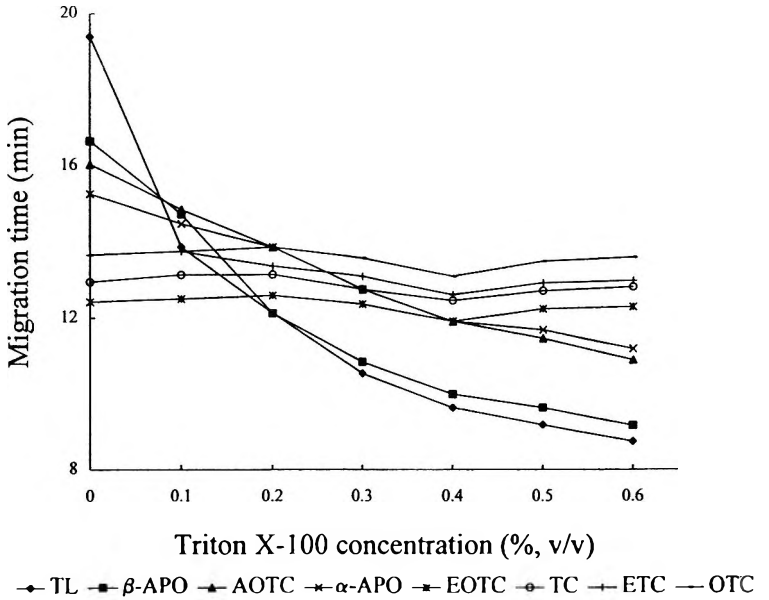


Figure 4. Influence of Triton X-100 concentration on the separation of OTC and its related substances. Capillary: uncoated fused silica, L=44cm, l=38cm, ID=50μm; background electrolyte=sodium carbonate (50 mM)-EDTA (1mM) including x % (v/v) Triton X-100; pH=11.0; temperature=10°C; voltage=10 kV.

The following step involved the optimization of Triton X-100 concentration in running buffer. It was varied from 0 to 0.6 % (v/v) with steps of 0.1 % (v/v). Only from 0.5 % (v/v) on, separation of all eight substances could be obtained. This concentration was finally retained because resolution between TC/ ETC decreased at 0.6 % (v/v). Results are shown in Figure 4 and can be explained by an interaction of the analytes with the alkyl chain of Triton X-100, possibly through hydrophobic interactions.

More hydrophobic compounds interact more strongly with the micelle and electrophoretic mobility decreases with increasing Triton X-100 concentration. So, migration time of the more hydrophobic compounds is shorter. This explanation can be confirmed by results of reversed phase LC[2,3] where elution order was almost opposite. In RPLC, the more hydrophobic compounds are retained longer. The selectivity of the system was markedly influenced by the Triton X-100 concentration, as migration order shows substantial changes.

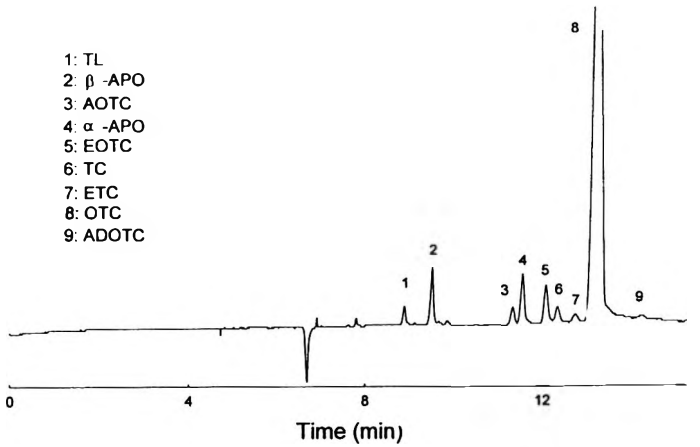


Figure 5. Electropherogram of oxytetracycline and its related substances. Capillary: uncoated fused silica, L=44cm, l=38cm, ID=50μm; background electrolyte=sodium carbonate (50 mM)-EDTA (1mM) including 0.5 % (v/v) Triton X-100; pH=11.0; temperature=10°C, voltage=10 kV.

It is shown from these results that in this system Triton X-100 concentration affected separation more than pH and buffer concentration. On the other hand, AOTC, TL and β-APO were affected more than other substances and it is difficult to explain this in detail from their structures.

In this stage, ADOTC was added to the sample. It was found that ADOTC can be separated from OTC with a resolution of 2.7. The order of migration under final optimal conditions is TL, β-APO, AOTC, α-APO, EOTC, TC, ETC, OTC and ADOTC. It is totally different from the CZE method,[6] where peak order was EOTC, TC, ETC, OTC, α-APO, AOTC, β-APO and TL.

Influence of capillary temperature and applied voltage on resolution was also investigated. It was found that separation became slightly worse with temperature and voltage increase, specifically for the critical pair TC/ETC. 10°C and 10kV were chosen as optimal conditions.

Figure 5 shows a typical electropherogram. Running buffer (excluding Triton X-100) was chosen as sample solvent, because it produced a better peak symmetry than 0.01 M HCl. A comparison with the performance of LC[2,3] shows that this method not only is better but also takes less time than LC. The latter analysis took over 30 min compared to 20 min for this method including

the washing procedure. Furthermore, the LC method needs a gradient elution. The repeatability was checked with the system shown in Figure 5 and using a spiked sample. The relative standard deviations (R.S.D.) (n=5) on corrected peak area (i.e. peak area divided by migration time) are given below, between brackets.

The sample used contained approximately 81.3 % w/w OTC (1.5 %), 5.0 % w/w of β-APO (5.7 %), , 4.4 % w/w of α-APO (3.4 %), 3.2 % w/w of EOTC (6.5 %), 1.4 % w/w of TC (13.5 %), 0.8 % w/w of ETC (8.2 %), 0.3 % w/w of ADOTC (22.7 %), 1.1 % w/w of TL and 2.5 % w/w of AOTC. The R.S.D.s of TL and AOTC are not given, because these compounds are too unstable in solution. Similar R.S.D.'s could be obtained with CZE (1.0 %, n=8).[6] As is generally the case, the R.S.D. for OTC is higher than with LC, where an R.S.D. value of 0.3 % was obtained for OTC.[2] The limit of detection (S/N = 3) was 0.05 % and the limit of quantification was 0.1 % for OTC (n=7, R.S.D.=17 %) with respect to the peak obtained with a sample solution containing 1.0 mg/mL OTC. The loadability of the system was also investigated. The fronting of the OTC peak increased with the loading. For the usually injected amount of approximately 7 ng (1 mg/mL, 4 sec), the peak symmetry factor was 0.7 and the separation of ETC and OTC was not affected until a solution containing 1.5 mg/mL of OTC was injected. The following calibration line was obtained for OTC: Y= 1396+140107 X, with Y= corrected peak area, X= concentration of the analysed solution in mg/mL, r= 0.9994, $S_{y,x}$ (standard error of y-estimate)=2702. investigated range =0.25-1.75 mg/mL, 7 points (n=2).

As a conclusion it can be stated that complete separation of OTC and its related substances was achieved by CE using a non-ionic surfactant. It offers the advantages of speed over LC and slightly better selectivity, but LC performs better in quantitative analysis.

ACKNOWLEDGMENT

The authors thank the Belgian National Fund for Scientific Research for financial support.

REFERENCES

1. F. A. Hochstein, C. R. Stephens, L. H. Conover, P. P. Regna, R. Pasternack, P. N. Gordon, F. J. Pilgrim, K. J. Brunings, R. B. Woodward, J. Am. Chem. Soc., 75, 5455-5475 (1953).

2. N. H. Khan, E. Roets, J. Hoogmartens, H. Vanderhaeghe, J. Chromatogr., **405**, 229-145 (1987).

3. J. Hoogmartens, W. Naidong, N. H. Khan, A. Malley, U. Hearty, R. Melamed, J. P. Gousset, P. Creed, C. Woollam, J. H. McB. Miller, J. Fuchs, H. Vanderhaeghe, Pharmeuropa, **2**, 77-85 (1990).

4. S. Croubels, W. Baeyens, C. Dewaele, C. Van Peteghem, J. Chromatogr. A, **673**, 267-274 (1994).

5. M. F. Tavares, V. L. McGuffin, J. Chromatogr. A, **686**, 129-142 (1994).

6. A. Van Schepdael, I. Van den Bergh, E. Roets, J. Hoogmartens, J. Chromatogr. A, **730**, 305-311 (1996)

7. A. Van Schepdael, J. Saevels, X. Lepoudre, R. Kibaya, Ni Zhi Gang, E. Roets, J. Hoogmartens, J. High Resolut. Chromatogr., **18**, 695-698 (1995).

8. A. Van Schepdael, R. Kibaya, E. Roets, J. Hoogmartens, Chromatographia, **41**, 367-370 (1995).

9. Y. M. Li, A. Van Schepdael, E. Roets, J. Hoogmartens, J. Chromatogr. A, in press.

10. Y. M. Li, A. Van Schepdael, E. Roets, J. Hoogmartens, J. Pharm. Biomed. Anal., in press.

11. M. Matsubara, S. Terabe, Chromatographia, **34**, 493-496 (1992).

12. European Pharmacopoeia, 2nd Edn, V.6.3.1. Maisonneuve, Sainte-Ruffine, France, 1980.

13. W. Naidong, S. Hua, E. Roets, J. Hoogmartens, J. Planar Chromatogr., 7, 297-300 (1994).

14. S. F. Y. Li. **Capillary Electrophoresis, Principles, Practice and Applications** (Journal of Chromatography Library, Volume 52), Elsevier Science Publishers, Amsterdam, 1992, pp 201-226.

Received April 14, 1996
Accepted April 30, 1996
Manuscript 4145

J. LIQ. CHROM. & REL. TECHNOL., 20(2), 283–295 (1997)

DETERMINATION OF FLUVOXAMINE AND PAROXETINE IN HUMAN SERUM WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ULTRAVIOLET DETECTION

M. Bagli, M. L. Rao, T. Sobanski, G. Laux

Psychiatrische Klinik und Poliklink der
Rheinischen Friedrich-Wilhelms-Universität
Sigmund-Freud-Str. 25
53105 Bonn, Germany

ABSTRACT

We optimized an isocratic reversed phase high performance liquid chromatography with ultraviolet detection to quantify, simultaneously, the selective serotonin reuptake inhibitors (SSRIs) fluvoxamine and paroxetine in human serum. Fluvoxamine was used as the internal standard for the determination of paroxetine, and paroxetine served as internal standard for the fluvoxamine assay. The method involved a pre-column technique for the on-line liquid-solid extraction with direct injection of serum samples and for their pre-concentration. Automation was achieved by column switching. The chromatographic separation was performed on an Ultrasep ES 100 CN-column with acetonitrile/methanol/phosphate buffer (58/19/23, v/v/v) as mobile phase. A linear relationship (r^2 >)

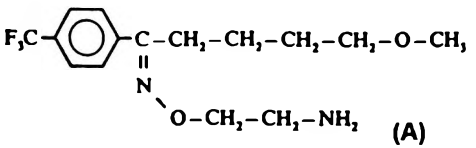
Copyright © 1997 by Marcel Dekker, Inc.

0.99) was noted between the concentrations of fluvoxamine or paroxetine and the detector signal. The lower limits of detection in human serum of fluvoxamine and paroxetine were 5 and 2 μg/L, respectively. The accuracy of the quality control samples deviated by ± 8% with a within-day and between-day precision of less than 12.1%, and 10.5% for fluvoxamine and paroxetine, respectively. The method is presently being applied in our clinic for the routine therapeutic drug monitoring of both SSRIs.

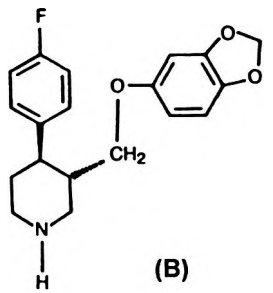
INTRODUCTION

Fluvoxamine (5-methoxy-1-[4-(trifluoromethyl)phenyl]-1 pentanone (*E*)-O-(2-aminoethyl)oxamine; Fig. 1A) and paroxetine ((-) *trans*-4-(p-fluorophenyl)-3-[[3,4-(methylenedioxyl)-phenoxy]methyl]piperidine; Fig. 1B) are selective serotonin reuptake inhibitors (SSRIs) and are used as antidepressants. The chemical structure of fluvoxamine and paroxetine differs from that of classical tricyclic antidepressants, a feature which goes hand in hand with the SSRI's pharmacological profile. Foglia et al.[1] reported that a good response to fluvoxamine is obtained at serum concentrations between 160 to 220 μg/L. However, other investigators did not observe any relationship between serum concentration and clinical efficacy for fluvoxamine[2-4] or paroxetine.[5-6] There are few investigations on the relationship between the serum concentration of the SSRIs and clinical efficacy or the occurrence of adverse events; the number of patients is too narrow to draw definitive conclusions. For paroxetine this could be due to the lack of reliable analytical methods for therapeutic drug monitoring of this drug.

Fluvoxamine was determined by gas chromatography[7,8] or by high performance liquid chromatography with fluorescence[9-11] or ultraviolet detection.[12-15] Paroxetine concentrations in human serum were determined by gas chromatography with nitrogen-specific detection[16] or high performance liquid chromatography (HPLC) with fluorescence detection.[17] These methods are time-consuming due to lengthy sample preparation procedures and derivatization for the fluorescence detection. In 1980 De Jong[18] described a method for the analysis of fluvoxamine using on-line pre-columns that allowed direct sample injection. Recently, Härter et al.[19] presented an automated method for the on-line determination of fluvoxamine using column-switching with HPLC and ultraviolet detection. In the present paper, we describe an HPLC method for direct sample injection to quantify fluvoxamine and paroxetine in human serum.



(A)



(B)

Figure 1. Chemical structures of (A) fluvoxamine and (B) paroxetine.

EXPERIMENTAL

Chemicals and Instrumentation

Fluvoxamine maleate (FGB 87B16A) and paroxetine hydrochloride (BRL 29060) reference standards were provided by Duphar Pharma (Hannover, Germany) and Beecham Pharmaceuticals (Sussex, England), respectively. Acetonitrile, water, methanol (J.T. Baker B.V., Deventer, Holland), and n-heptane (Rathburn, Walkerburn, Scotland) were all of HPLC-grade. All other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany).

The HPLC system was equipped with two HPLC pumps, a solvent degasser unit SDU 2003 (Bischoff, Leonberg, Germany), and a Waters 717 Autosampler equipped with a cooling module (Millipore-Waters, Eschborn, Germany). The ultraviolet detector SPD-10 A (Shimadzu, Duisburg, Germany) was set at 215 nm. The detector signals were analyzed by Hyperdata Chromsoft computer software with a model 1605 serial chromatography signal interface (Bischoff). The six-port switching valve, Rheodyne model 7010,

operated on an electronic actuator model 732 (both by Bischoff). The cartridge clean-up column (25 x 4 mm ID) filled with Lichrospher RP-18 ADS (25 µm particle size, Merck) was placed between pump B and the switching valve. The cartridge guard column (10 x 4 mm ID) and the analytical column (250 x 4.6 mm ID) contained Ultrasep ES 100 CN (4 µm particle size, Bischoff).

Standard Preparation and Sample Acquisition

Blood from healthy donors was collected into 500 mL bottles (without additives) and stored for 3 h at 20°C; serum was obtained by centrifugation at 2000 g for 15 min. The supernatant (serum) were combined and used immediately for the preparation of standards. The calibration range was 20-1600 µg/L for fluvoxamine and 10-1000 µg/L for paroxetine. Quality control samples contained 120 or 600 µg fluvoxamine/L serum or 40 or 300 µg paroxetine/L serum. The serum standards were prepared by adding the appropriate amount of fluvoxamine or paroxetine stock solutions in water to human serum. The quality control samples were run with each series of analysis. Patients' blood samples were drawn into 10 mL Venoject (Terumo, Leuven, Belgium) blood collecting tubes sealed with brown stoppers and without additives. After 30 min the serum was obtained by centrifugation of the patients' blood samples at 1800 g for 10 min at 4°C. Blood samples from patients on monotherapy with drugs which were used as a co-medication with the SSRIs were collected and prepared accordingly. Fluvoxamine was used as the internal standard for paroxetine and paroxetine as the internal standard for the fluvoxamine determination; 50 µL aqueous standard solution (6 µg/L fluvoxamine or 12 µg/L paroxetine) was added to 950 µL of the patients' serum sample; a second aliquot of 1 mL of the serum was stored without this additive to test putative interferences. The aqueous stock solutions, as well as serum standards, quality controls, and serum samples of patients were kept at -20°C.

On-Line Liquid-Solid Extraction and Chromatography

The mobile phase (solvent A) for the analytical chromatography consisted of 58:19:23 (v/v/v) of acetonitrile:methanol:0.05 M phosphate buffer (pH 6.8). Water:methanol 95:5 (v/v) was used as the mobile phase for the sample clean-up solvent (solvent B). Solvents A and B were filtered through a 0.45 µm Nylon filter (Millipore, Eschborn, Germany). The chromatography was conducted at two different modes, mode A for sample application and mode B for sample analysis (Fig. 2). The chromatographic system was set at mode A (sample application) and 100 µl of the serum sample was injected onto the

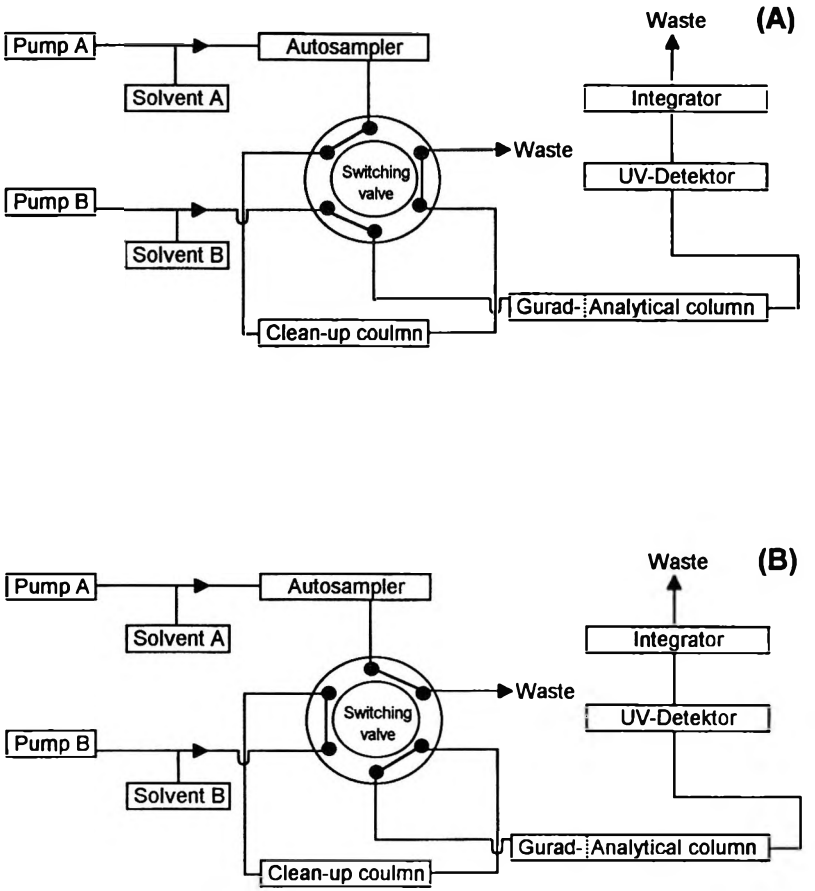


Figure 2. Instrumental arrangement with a six-port switching valve for the direct sample injection for high-performance liquid chromatography with integrated on-line liquid-solid analysis of fluvoxamine and paroxetine by column switching: mode (A) for sample injection and mode B for sample analysis (according to Härter et al. 1992).

clean-up column. The clean-up column was flushed with solvent B for 10 min at a flow rate of 1.5 mL/min. The drugs absorbed onto the clean-up column were then washed onto the analytical column and separated chromatographically with solvent A at a flow rate of 1.5 mL/min by switching the six-port valve to mode B (sample analysis). After 2.5 min the clean-up column was disconnected from the analytical column by back-positioning to

mode A (sample application) and the clean-up column was re-equilibrated with solvent B for 12.5 min for the next sample application. The chromatographic separation was achieved within 15 min.

Liquid-Liquid Extraction

Alternatively, to the on-line liquid-solid extraction, we used the liquid-liquid extraction procedure for weekly routine drug monitoring. This was carried out as follows: 2.0 mL water and 2.0 mL 2 M sodium hydroxide were added to the respective 0.5 mL serum standard, quality controls, or patient sample in 15 mL screw-capped borosilicate glass tubes, and the mixture was vortexed for 10 s. After the addition of 5.0 mL water-saturated n-heptane/isoamylalcohol (99:1) the mixture was gently shaken for 20 min; it was then centrifuged for 10 min at 2800 g and 4°C and the upper layer was aspirated. This procedure was repeated and the two extracts combined. The solvent was evaporated under vacuum to dryness, the residue dissolved in 500 µL of solvent A, and 100 µL of the latter mixture was analyzed following the same procedure for the on-line analysis of serum samples as described above.

Calculation, Recovery, and Interferences

The calibration was performed by linear regression of the peak height ratios of fluvoxamine to the internal standard (paroxetine) and of the peak height ratios of paroxetine to the internal standard (fluvoxamine) versus the respective standard concentration. The best fit was obtained with a weighing factor of $1/\text{concentration}^2$.

The recoveries of the extraction procedures were calculated by comparing the slope of the regression of peak heights obtained by direct injection of the aqueous standards onto the analytical column to those after liquid-solid or liquid-liquid extraction of the serum standards.

Interferences from endogenous serum constituents were accounted for by analysing serum from healthy medication-free subjects. During method validation, serum samples of patients treated with fluvoxamine or paroxetine and with antidepressants, benzodiazepines or neuroleptics were determined twice, namely with and without internal standard. Interferences were then checked by comparing the location of the peaks from co-medicated drugs with that of the respective internal standard (fluvoxamine or paroxetine). Serum samples of patients on monotherapy with other drugs were analysed following

the same protocol to tested putative interferences from the mother compound and that of the metabolites. The retention time of the mother compounds were determined with serum standards of the antidepressants, benzodiazepines or neuroleptics; the retention times of the metabolites with serum samples of patients on monotherapy with that drug.

RESULTS

The average recoveries of on-line liquid-solid and liquid-liquid extraction over the entire calibration range were 93% and 89% for fluvoxamine and 88% and 83% for paroxetine, respectively. The retention times of the mother compound or metabolites of drugs commonly used as add-on therapy and considered as possible interferences are given in Table 1. Occasionally, the analysis of serum sample from patients on monotherapy showed more than one peak in the chromatogram. These peaks corresponded to the spectrum of peaks of an administered drug and its metabolites.

The specificity of the assay was also monitored by checking the chromatograms of drug-free human serum for interfering peaks from endogenous components (Fig. 3). As Table 1 and Fig. 3 imply, there were no interferences from co-medicated drugs or endogenous serum components at the respective retention times of fluvoxamine and paroxetine. Typical chromatograms of serum samples from patients receiving 200 mg fluvoxamine per day (C) and 30 mg paroxetine per day (D) are shown in Fig. 3.

With an injection volume of 100 μL at a signal-to-noise ratio of 3, the detection limits were 5 and 2 μg/L for fluvoxamine and paroxetine, respectively. Sensitivity was improved by a factor of two, when the injection volume was increased to 300 μL. The calibration curves were linear for fluvoxamine (range: 20 to 1600 μg/L; r^2=0.9965 ± 0.0049) and paroxetine (range: 10 to 1000 μg/L; r^2=0.9960 ± 0.0026). The within-day precision determined with concentrations of 120 μg and 600 μg of fluvoxamine/L serum and 40 and 300 μg paroxetine/L serum (each n=10) was 7.5% and 2.1% for fluvoxamine and 7.6% and 3.0% for paroxetine.

The between-day coefficients of variation for the entire concentration range and the quality control samples were within 12.1% and 10.5% for fluvoxamine and paroxetine, respectively (Table 2). The accuracy of the standards and quality control samples deviated within ± 8% at the various concentrations of both SSRIs.

Table 1

Retention Times of Drugs (Mother Compound and Metabolites) Tested for Possible Interferences in the Determination of Serum Fluvoxamine and Paroxetine Concentrations by HPLC

Drug	Retention Time (Min)	
	Mother Compound[a]	Metabolites[b]
Alprazolam	n.o.*	n.o.*
Amitriptyline	8.3	11.7
Carbamazepine	n.o.*	n.o.*
Chlorproxthixene	4.7	8.4; 9.5
Clozapine	5.7	n.o.*
Diazepam	n.o.*	n.o.*
Doxepin	7.1	12.9
Flupentixol	n.o*	n.o*
Fluvoxamine	6.4	
Haloperidol	n.o*	n.o*
Imipramine	8.6	11.8
Levomepromazine	5.9	11.9
Lorazepam	n.o*	n.o*
Maprotiline	13.9	8.2
Melperone	9.1	n.o*
Mianserin	n.o*	9.2
Paroxetine	10.1	
Perazine	n.o*	n.o*
Promethazine	n.o*	n.o*
Thioridazine	8.8	7.7; 8.2; 8.6; 12.3

* n.o., not observed in the chromatogram

[a] The retention time of the mother compound was determined from serum standards.

[b] The retention time of the metabolites was determined from serum samples of patients on monotherapy with this drug.

Using the on-line liquid-solid extraction the sample extraction as well chromatographic analysis was accomplished within 25 min; approximately 100 samples could be determined within two days. The cost of one serum sample analysis, calculated according to Müller et al.,[20] with on-line liquid-solid or liquid-liquid extraction amounted to 31 or 41 US $, respectively.

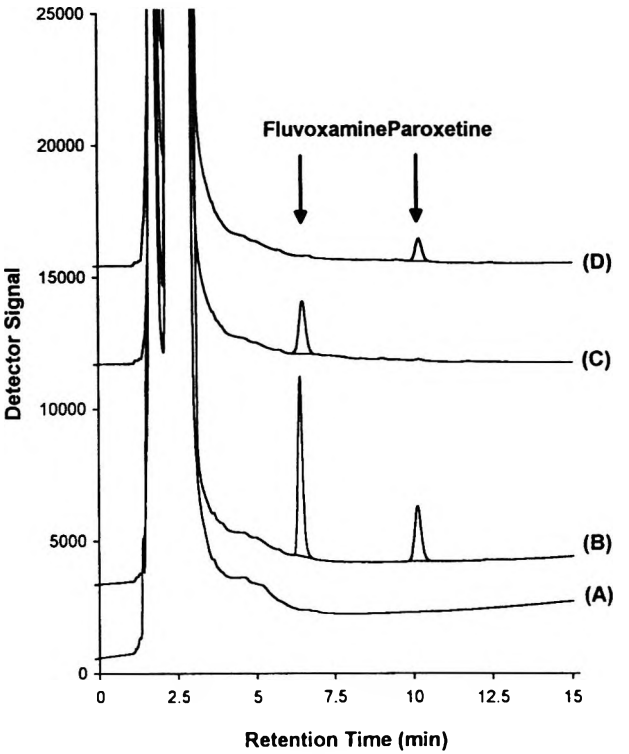


Figure 3. Chromatograms of (A) drug-free human serum, (B) serum standard containing 400 µg/L of fluvoxamine and 100 µg/L paroxetine, sera from patients receiving orally (C) 200 mg fluvoxamine or (D) 30 mg paroxetine per day. The retention times of fluvoxamine and paroxetine are indicated by the arrows and are 6.4 and 10.1 min, respectively.

DISCUSSION

In the present paper, we describe a sensitive, selective and accurate assay for the analysis of fluvoxamine and paroxetine in human serum. This is the first report on the analysis of paroxetine serum concentrations by HPLC and ultraviolet detection. The method involves on-line liquid-solid extraction by column switching for the direct injection of the serum samples, which permits sample preparation and chromatographic analysis within 25 min. A problem encountered during the analysis of serum samples by direct injection was the coating of the clean-up as well as of the analytical columns, attributable to

Table 2

Accuracy and Precision of the HPLC Determined Concentrations of Fluvoxamine and Paroxetine in Serum

Fluvoxamine			Paroxetine		
Concentration (μg/L)	Accuracy* %	CV** %	Concentration (μg/L)	Accuracy* %	CV** %
Calibration Standards					
20	1.2	5.8	10	3.9	8.3
40	0.1	7.7	20	2.6	8.3
100	2.6	5.0	40	2.0	6.0
200	0.8	10.5	100	4.6	8.3
400	6.6	3.5	400	2.8	6.0
1000	6.5	8.4	600	4.8	9.6
1600	3.5	8.6	1000	2.6	9.7
Quality Control Samples					
120	6.0	12.1	40	8.3	10.2
600	4.8	6.9	300	3.9	8.6

* The amount added was taken to be 100%.

** Inter-assay coefficient of variation.

impurities in the serum after clean-up. To maintain sensitivity it was necessary to replace the clean-up and the analytical column after approximately 250 runs. Although the frequency of changing the columns was reduced by using the new Lichrospher ADS clean-up column, recently introduced by Merck, liquid-liquid extraction of the serum samples with n-heptane/isoamylalcohol prior to analysis further improved the column life by a factor of up to three. Both extraction procedures were combined for the routine therapeutic drug monitoring of the SSRIs without changing the chromatographic equipment and conditions, owing to the internal standard technique and the fact that the recovery was similar after on-line liquid-solid and liquid-liquid extraction. This combines the advantages of both extraction procedures: direct serum sample injection and rapid sample analysis with the on-line liquid-solid extraction and cost reduction with the liquid-liquid extraction.

The determination of the SSRIs in serum from patients on co-medication posed no problems since there were no interferences. Testing the interferences of co-administered drugs by analysing the serum samples of patients on monotherapy is more reliable than analysing spiked serum samples, since not all metabolites are purchasable, and patients' serum samples certainly contain the mother compound plus the full spectrum of metabolites.

The lower calibration level of 20 µg/L fluvoxamine and 10 µg/L paroxetine proved to be sufficient. However, sensitivity is easily improved whenever necessary (for anticipated concentrations that are below the lower level of the calibration range) by increasing the injection volume; this requires only multiple injections on the clean-up column prior to the analytical chromatography.

We currently apply this method for the therapeutic drug monitoring of fluvoxamine and paroxetine to investigate the relationship between dose and serum concentration and between the drug's serum concentration and its therapeutic efficacy, as well as drug interactions during comedications.

ACKNOWLEDGEMENT

The provision of reference standards of fluvoxamine and paroxetine by Duphar Pharma (Hannover, Germany) and Beecham Pharmaceuticals (Sussex, England) is gratefully acknowledged. We thank Ms. S. Schmitz for her technical assistance and Prof. P. Hanfland, Institut für Experimentelle Haematologie und Transfusionsmedizin der Universität Bonn, for supplying drug-free serum from healthy subjects.

REFERENCES

1. J. P. Foglia, J. M. Perel, R. S. Nathan, B. G. Pollock, Clin. Chem., **36**,1043 (1990).

2. S. Bouquet, S. Vandel, G. Bertschy, B. Bonin, B. Vandel, D. Sechter, P. Bizouard., Clin. Neuropharmacol., **15 (1. Suppl.)**, 82-83A (1992).

3. S. Kasper, M. Dötsch, H. Kick, A. Vieira, H.-J. Möller, Eur. Neuropharmacol., **3**, 13-21 (1993).

4. J. E. M. De Wilde, D. P. Doogan, J. Affect. Disord., **4**, 249-259 (1982).

5. T. C. G. Tasker, C. M. Kaye, B. D. Zussman, C. G. G. Link, Acta Psychiatr. Scand., **80 (Suppl. 350)**, 152-155 (1989).

6. H. Kuhs, H. P. Schalke, L. H. Rolf, G. A. E. Rudolf, Acta Psychiatr. Scand. **85**, 364-369 (1992).

7. H. E. Hurst, D. R. Jones, C. H. Jarboe, H. De Bree, Clin. Chem., **27**, 1210-1212 (1981).

8. S. Dawling, N. Ward, E. G. Essex, B. Widdop, Ann. Clin. Biochem., **27**, 473-477 (1990).

9. J. Pommery, M. Lhermitte, Biomed. Chromatogr., **3**, 177-179 (1989).

10. R. H. Pullen, A. A. Fatmi, J. Chromatogr., **574**, 101-107 (1992).

11. C. Schweitzer, H. S. Spahn, E. Mutschler, J. Chromatogr., **382**, 405-411 (1986).

12. J. P. Foglia, L. A. Birder, J. M. Perel, J. Chromatogr., **495**, 295-302 (1989).

13. V. van der Mersch-Mougeot, B. Diquet, J. Chromatogr., **567**, 441-449 (1991).

14. S. H. Wong, H. R. Kranzler, S. DellaFera, R. Fernandes, Biomed. Chromatogr., **8**, 278-282 (1994).

15. A. Belmadani, I. Combourrieu, M. Bonini, E. E. Creppy, Hum. Exper. Ther., **14**, 34-37 (1995).

16. E. N. Petersen, E. Bechgaard, R. J. Sortwell, L. Wetterberg, Eur. J. Pharmacol., **52**, 115-119 (1978).

17. M. A. Brett, H.-D. Dierdorf, B. D. Zussman, P. E. Coates, J. Chromatogr., **419**, 438-444 (1987).

18. G. J. De Jong, J. Chromatogr., **183**, 203-211 (1980).

19. S. Härter, H. Wetzel, C. Hiemke, Clin. Chem., **38**, 2062-2066 (1992).

20. J. Müller, R. Hertrampf, B. Müller-Oerlinghausen, Pharmacopsychiat., **18**, 48-49 (1985).

Received April 1, 1996
Accepted April 22, 1996
Manuscript 4130

J. LIQ. CHROM. & REL. TECHNOL., 20(2), 297–310 (1997)

QUANTITATIVE ANALYSIS OF DEBRANCHED AMYLOPECTIN BY HPAEC-PAD WITH A POSTCOLUMN ENZYME REACTOR

K. S. Wong, J. Jane*

Department of Food Science and Human Nutrition
Iowa State University
Ames, IA 50011

ABSTRACT

The chain-length distribution of debranched amylopectin was analyzed by using high performance anion-exchange chromatography with an amyloglucosidase (AMG) reactor and a pulsed amperometric detector (HPAEC-ENZ-PAD). The effects of reactor length, reactor temperature, and additional α-amylase reactor were studied. Results showed that a 2 mm i.d. x 23 mm AMG reactor at 25°C was sufficient to convert HPAEC-separated amylodextrins to glucose; the PAD response for the hydrolyzed amylodextrins of different chain-length was consistent, and quantitative results were achieved. With this enzymatic treatment, amylodextrin with DP up to 77 was detected for a debranched tapioca amylopectin sample. The chain-length distribution profile of debranched tapioca amylopectin showed three fractions with peak DPs 48 ± 1 (F1), 19 (F2a), and 12 (F2b).

Copyright © 1997 by Marcel Dekker, Inc.

INTRODUCTION

Branch chain-length distribution of amylopectin is one of the most important parameters in understanding the relationship between the chemical structure and the functional properties of starch. Many techniques have been applied for the study of branch chain-length distribution; a common one is gel-permeation chromatography (GPC). The total carbohydrate and reducing value of the collected fractions from GPC were determined by a phenol-sulfuric acid method[1] and a modified Park-Johnson procedure,[2] respectively. The whole protocol of the technique is time consuming, and both the phenol-sulfuric acid and the Park-Johnson methods involve hazardous reagents.

High performance size-exclusion chromatography with a refractive index detector (HPSEC-RI)[3-5] can also be used to determine the chain-length distribution of debranched amylopectin in a much shorter time than GPC. When a laser light scattering detector is used as a second detector with HPSEC and a RI detector (HPSEC-LS-RI),[6-9] the technique provides molecular weight distribution information without the need of column calibration.[10,11] One major weakness of the HPSEC technique is the limited resolution of the size-exclusion column.

The development of high performance anion-exchange chromatography with a pulsed amperometric detector (HPAEC-PAD) provides a solution for this problem. The high separation power of the anion-exchange column allows amylodextrins to separate at individual homologous chain lengths, and the detector is selective and highly sensitive. By using the HPAEC-PAD, separation and detection of debranched amylopectin of normal maize starch up to DP 66 has been reported;[12] however, the detector response decreases as the chain-length of amylodextrin increases. Thus, the HPAEC-PAD chromatographic profile of debranched amylopectin does not provide a quantitative result.

To solve this problem, we decided to use an enzyme reactor to convert all the amylodextrins to glucose to achieve a consistent detection response. Amyloglucosidase (AMG) is a good choice for the purpose because it produces glucose exclusively, except for some residues, such as maltose and maltotriose, which are somewhat resistant to the enzyme hydrolysis. Larew and Johnson[13] have successfully demonstrated that the use of an AMG reactor resulted in quantitative conversion of HPAEC-separated maltooligosaccharides (G2 to G7) to glucose, which is then detected by PAD. In our studies, we used the HPAEC-PAD coupled with the AMG reactor to analyze debranched amylopectin. Because the debranched amylopectin sample contains amylodextrins with DP from 6 to over 70, the main challenges of this study

were to quantitatively convert all amylodextrins into glucose and maintain the chromatographic separation of each amylodextrin fraction. Effects of reactor length, reactor temperature, and additional α-amylase on the quantitative analysis of amylodextrin were investigated. Chain-length distributions of tapioca amylopectin obtained from the HPAEC-ENZ-PAD, HPSEC-RI, and GPC were compared.

EXPERIMENTAL

Chemicals

Amyloglucosidase (EC 3.2.1.3, from Rhizopus mold), α-amylase (EC 3.2.1.1, heat stable, from Bacillus licheniformis), soluble starch, normal maize starch, glucose, maltose, and glucose diagnostic kits (115-A) were purchased from Sigma Chemical Co. (St. Louis, MO). Acetic acid, citric acid, sodium acetate, sodium citrate, sodium hydroxide (5N), and sodium nitrate were purchased from Fisher Scientific (Fair Lawn, NJ). Nucleosil 300-10 and 1000-10 silica gels were purchased from Alltech (Deerfield, IL). Isoamylase (EC 3.2.1.68, crystal, from Pseudomonas amyloderamosa) and maltopentaose were purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). Maltotriose, maltotetraose, maltohexaose, and maltoheptaose were purchased from Aldrich Chemical Co. (Milwukee, WI). Sodium azide was purchased from Eastman Organic Chemicals (Rochester, NY). Tapioca amylopectin was a gift from Dr. J.-F. Chen. Water (18 MΩ cm) used in all preparations was obtained from the Milli-Q Reagent Water System (Millipore, Bedford, MA).

Apparatus

Anion-exchange chromatography of debranched amylopectin employed a Dionex HPAEC-PAD system described previously.[12] An AI-450 software/interface (Model 1, Full-Control System, Dionex, Sunnyvale, CA) was used for instrument control and data processing.

Other apparatus used in the postcolumn enzyme reaction and column efficiency studies included: a metering pump (AA-72-S Precision Metering Pump, Eldex Laboratories, Napa, CA), a pneumatic pump (Postcolumn Pneumatic Controller, Dionex) equipped with a mixing tee and reaction coil, an isocratic pump (HP series 1050 HPLC pump, Hewlett Packard, Wilmington,

DE) equipped with an injection valve (Model 7125, Rheodyne, Cotati, CA) and a 20 μL sample loop, and a Visco Mixer (10 μL, The Lee Company, Westbrook, CT). A heating circulating bath (Lauda MS-3, Brinkmann, Westbury, NY) was used for column temperature control.

Sample Preparation

Isolation of amylopectin from normal maize starch has been described previously.[12] Amylopectin was debranched by isoamylase according to the procedures described by Jane et al.[14]

Preparation of Enzyme Reactors

Immobilizations of AMG and α-amylase were performed according to the procedures described by Emnéus and Gorton.[15,16] AMG was immobilized onto a Nucleosil 300-10 silica gel, and α-amylase was immobilized onto a Nucleosil 1000-10 silica gel. The coupling yields of the enzymes to the supporting silica gels were determined by measuring the enzyme contents in the supernatant before and after immobilization.[15] The enzyme activities (the amount of glucose released per min. by 10 mg of enzyme) before and after immobilization were determined by the following procedures: to a beaker containing 150 mL of 1% (W/V) soluble starch solution, 3 mL of citrate buffer (1 M, pH 4) was added. The solution was mixed by magnetic stirring. After a volume of immobilized gel with a known amount of enzyme or an enzyme solution with an equivalent amount of enzyme as that in the immobilized gel was added to the solution, an aliquot of the solution was transferred to a test tube every one to ten minutes. The enzyme activity was stopped by heating the transferred solution in a boiling water bath, and the solution was subjected for glucose analysis by using a glucose diagnostic kit. The enzyme reactors were prepared by vacuum slurry packing of the enzyme immobilized support into a 2 mm i.d. x 23 mm column unless otherwise noted. The efficiency of the enzyme reactor was evaluated according to Larew and Johnson[13] with some modifications. A debranched amylopectin sample was injected into a HP 1050 isocratic pump equipped with a 20 μL sample loop, and was carried by a stream of acetate buffer (0.05 M, pH 4.5) at a flow rate of 0.2 mL/min into an AMG reactor. The eluate from the reactor was then merged with a gradient from the Dionex gradient pump before entering the anion-exchange columns for separation. The gradient composed of eluent A (100 mM NaOH) and eluent B (100 mM NaOH and 500 mM NaOAc) with the following gradient profile: 0-2 min, 80% A and 20% B; 2-60 min, linear gradient to 100% B. The flow rate of the gradient was 0.5 mL/min at all time.

High Performance Anion-Exchange Chromatography with Postcolumn Enzyme Reactor and Pulsed Amperometric Detector (HPAEC-ENZ-PAD)

The HPAEC-ENZ-PAD setup was based on that described by Larew and Johnson[13] with modifications. All enzyme reactors were maintained at 25°C unless otherwise noted. Studies on pushing agents[12] show that sodium nitrate provides a better resolution than sodium acetate for amylodextrins analysis, therefore, sodium nitrate was chosen as the pushing agent in this study. The separation of a sample employed anion-exchange columns with a gradient composed of eluent A (100 mM NaOH) and eluent C (100 mM NaOH, 300 mM $NaNO_3$) with a flow rate of 0.5 mL/min.

The separation gradient for the system using one AMG reactor was as follows: 0-5 min, 94% A and 6% C; 5-10 min, linear gradient to 10% C; 10-150 min, linear gradient to 30% C; 150-200 min, linear gradient to 40% C; 200-220 min, linear gradient to 45% C. Separation gradient for the system using two AMG reactors was as follows: 0-5 min, 94% A and 6% C; 5-10 min, linear gradient to 10% C; 10-30 min, linear gradient to 13% C; 30-150 min, linear gradient to 30% C; 150-200 min, linear gradient to 40% C. The pH of the eluate from the anion-exchange column was adjusted to 4.5 by mixing with a citrate buffer (pH 4.1, 0.5 M, 0.25 mL/min).

In the study of the effect of additional α-amylase reactor, separation gradient was as follows: 0-5 min, 92% A and 8% C; 5-10 min, linear gradient to 12% C; 10-30 min, linear gradient to 15% C; 30-200 min, linear gradient to 40% C, and the eluate from the anion-exchange column was adjusted to pH 5 by a citrate buffer (pH 4.5, 0.5 M, 0.25 mL/min). The eluate from the enzyme reactor was adjusted to pH 12 by mixing with a 0.75 M sodium hydroxide solution. The total flow rate through the detector cell was 1.05 mL/min.

High-Performance Size-Exclusion Chromatography with Refractive Index Detector (HPSEC-RI)

Size-exclusion chromatography of debranched amylopectin employed an HP 1050 isocratic pump equipped with an injector with a 20 μL sample loop and a refractive index detector (HP 1047A, Hewlett Packard). The separation of the debranched amylopectin sample utilized a series of TSK-GEL PWXL size-exclusion columns (Tosohaas, Montgomeryville, PA) including a guard column and G4000 and G3000 separation columns. The mobile phase was deionized water at a flow rate of 0.5 mL/min. All columns were maintained at 60°C with a circulated water bath.

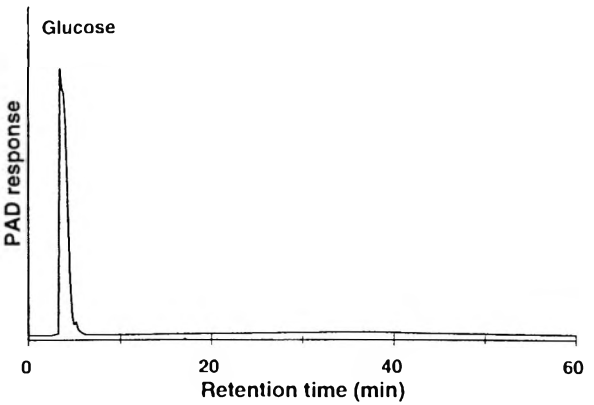


Figure 1. HPAEC chromatogram of debranched normal maize amylopectin (1.5 mg/mL) that passed through a 2 mm i.d. x 23 mm AMG reactor before the anion-exchange columns. Chromatographic conditions are described in 'Experimental'.

Gel-Permeation Chromatography (GPC)

Debranched amylopectin was analyzed by a Bio-Gel P-6 column (1.5 cm i.d. x 80 cm, Bio-Rad Laboratories, Richmond, CA)[14] followed by an anthrone sulfuric acid method for total carbohydrate analysis of each collected fraction.[17] The average branch chain-length was determined by analyzing the total carbohydrate (phenol-sulfuric acid method) and the reducing value (modified Park-Johnson method) of the three combined fractions at the peak.[14]

RESULTS AND DISCUSSIONS

Enzyme Reactors

The coupling yields of immobilizing α-amylase and AMG to one gram of the corresponding supporting silica gel were 16 mg and 243 mg, respectively. The activities of AMG before and after immobilization were 7.38±0.91 mg/min and 4.45±0.34 mg/min, respectively, and was 3.02±0.01 mg/min after the immobilized AMG reactor was kept at 4°C for eight months. Therefore, the enzyme activity was maintained at ca. 60% after immobilization, and the immobilized enzyme reactor remained ca. 68% of the reactor activity after a period of eight months. AMG is an exoenzyme that catalyzes the hydrolysis of

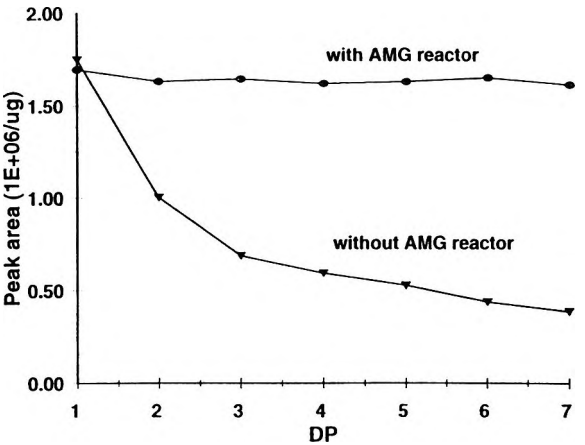


Figure 2. Sensitivity of PAD for unit mass of oligosaccharides with and without passing through an AMG reactor.

amylodextrin to glucose. The turnover rate of AMG increases as the molecular weight of oligosaccharides increases.[18] A study showed that the molar conversion of maltooligosaccharides to glucose by an immobilized AMG reactor reached 87-93% for G2 and G3 but up to 96% for G4-G7.[13] Because chain-lengths of amylopectin branches are at least 6 glucose units long,[8] it is likely that an AMG reactor can be sufficient in converting debranched amylopectin chains to glucose. The efficiency of the AMG reactor was evaluated by the injection of a 1.5 mg/mL debranched normal maize amylopectin. The result showed that glucose was the predominant product with a minor shoulder peak (peak area was less than 2% of the glucose peak) of maltose (Figure 1). The result confirmed that amylodextrins were effectively converted to glucose by the AMG reactor at 25°C.

Oligosaccharides and Debranched Tapioca Amylopectin Analyzed by HPAEC-ENZ-PAD

The HPAEC-ENZ-PAD system was first examined by injecting a standard oligosaccharide mixture (G1 to G7). All G1 to G7 had baseline separations and were quantitatively detected (Figure 2) after the in-line enzyme reactor

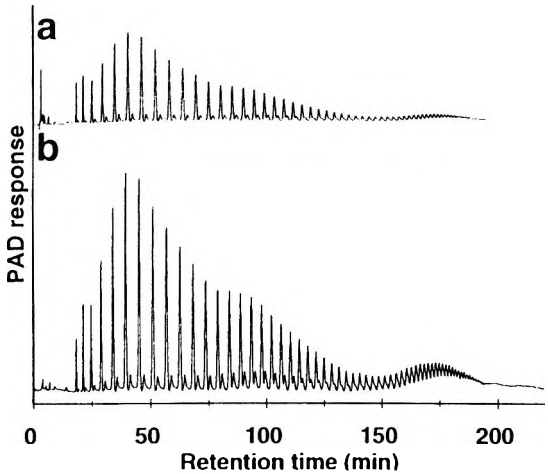


Figure 3. HPAEC chromatograms of 0.5 mg/mL debranched tapioca amylopectin (a) without and (b) with passing through a postcolumn AMG reactor. Chromatographic conditions are described in 'Experimental'.

treatment even though the enzymatic reaction time was less than 6 sec. The result was in good agreement with that reported by Larew and Johnson[13] in which enzyme reaction took place at 50°C. Figure 3 shows the analysis of debranched tapioca amylopectin by using the HPAEC-PAD with and without the AMG reactor. As expected, the PAD response for each amylodextrin was greatly enhanced by using the AMG reactor. On the basis of the AMG efficiency result shown in Figure 1 and that the concentration of each chromatographic fraction in this analysis was about 2% of that in the reactor efficiency study, we believe the amylodextrins were more than 98% converted to glucose.

Further studies on the effects of reactor length and reactor temperature showed that an increase in AMG reactor length from 23 mm (one AMG reactor) to 46 mm (two AMG reactors) or reactor temperature from 25°C to 50°C (optimum temperature for AMG) did not significantly change the chromatographic profile. The resulting normalized peak area plots of debranched tapioca amylopectin (Figure 4) showed that the detection of amylodextrin was to DP 77 when one AMG reactor was used but only to DP 68 when two AMG reactors were used. The decrease in detection sensitivity for

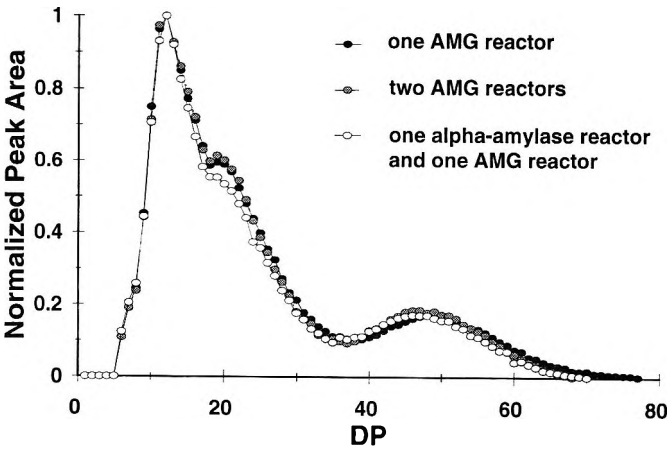


Figure 4. Normalized peak area plots for debranched tapioca amylopectin (0.5 mg/mL) analyzed by HPAEC-ENZ-PAD system with one AMG reactor, two AMG reactors, and a combination of an α-amylase reactor and an AMG reactor. Chromatographic conditions are described in 'Experimental'.

the latter was attributed to the diffusion effect introduced by the additional reactor. AMG has an optimum temperature at 50°C; however, it has been reported that a higher operating temperature shortens the lifetime of the AMG reactor.[19] The operation of the AMG reactor at 25°C instead of 50°C should prolong the life span of the enzyme reactor.

α-Amylase (B. licheniformis), an endo-enzyme that cleaves α-1,4-linkages within the starch molecule, can be used to increase the number of non-reducing ends of starch, thus, accelerating the AMG hydrolysis of starch. To investigate the effect of α-amylase on the hydrolysis of amylodextrin by an AMG reactor, an α-amylase reactor was connected in-line before the AMG reactor. α-Amylase and AMG have an optimum pH at 6 - 6.9[20] and pH 4.5,[21] respectively. To minimize the dilution and dispersion effects, we tried to select an appropriate working pH range for both enzyme reactors. In batch hydrolysis studies of soluble starch by a mixture of α-amylase and AMG at various pHs, the results showed that the enzyme mixture at pH 5 had the highest initial hydrolysis rate compared with that of the AMG alone at pH 4.5 and the enzyme mixture at pH 5.5. Therefore, pH 5 was selected for both the α-amylase and AMG reactors. The results showed that the efficiency of the conversion of

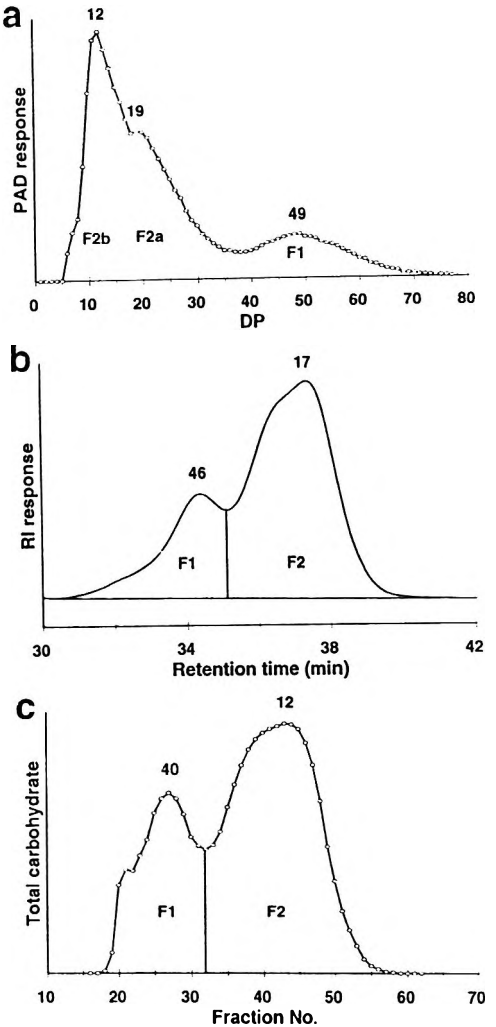


Figure 5. Chromatographic profiles of debranched tapioca amylopectin obtained by using (a) HPAEC-ENZ-PAD with one AMG reactor, (b) HPSEC-RI, and (c) GPC-total carbohydrate. Peak numbers indicate the degree of polymerization.

amylodextrin to glucose decreased when the combination of the α-amylase reactor and the AMG reactor was used (Figure 4). This difference may be attributed to the α-amylase hydrolyzing amylodextrin to short-chain oligosaccharides, in which maltose and maltotriose are more resistant to AMG hydrolysis.[13,22]

Table 1

Characterization of Branch Chain-Length Distribution of Tapioca Amylopectin by Using Different Chromatographic Techniques

Analytical Methods	Peak DP(% Peak Area) F1	F2a	F2	F2b
HPAEC-ENZ-PAD[a]	48 ± 1[b] (21)	19[b](32)		12[b] (47)
HPSEC-RI	46 ± 1[c] (17)		16.0 ± 0.5[c] (83)	
GPC	41 ± 3[b] (32)		12.5 ± 0.5[b] (68)	

[a] F1: DP 36-77, F2a: DP 19-35, F2b: DP 6-18.
[b] Three repetitions.
[c] Two repetitions.

Comparison of HPAEC-ENZ-PAD with HPSEC-RI and GPC for Debranched Amylopectin Analysis

After the in-line enzyme reactor treatment, the area (not the height) of each chromatographic peak corresponded to the total glucose concentration of the amylodextrin. Therefore, a plot of peak area against each DP represented a quantitative profile. Because there are no pure oligosaccharides with DP > 14 available as references, it is not possible to quantitatively calibrate each homologous peak. To further examine the effectiveness of HPAEC-ENZ-PAD for quantitative analysis of debranched amylopectins, we compared the HPAEC-ENZ-PAD profile of a debranched tapioca amylopectin with those from HPSEC-RI and GPC-total carbohydrate. Figure 5 shows the chromatographic profiles of debranched tapioca amylopectin obtained from these three techniques, and the chromatographic results are summarized in Table 1.

Because size-exclusion chromatography and anion-exchange chromatography have different separation mechanisms and different size-exclusion columns have different molecular size cut-offs, the profiles from the techniques varied. Figure 5a shows the HPAEC-ENZ-PAD chromatographic profile of debranched tapioca amylopectin in which the branch chain-length can be divided into three fractions with peak DPs of 48 ± 1 (F1), 19 (F2a), and 12 (F2b). HPAEC separates the amylodextrin mixture into individual homologous chain lengths; therefore, the DP of the peak can be conveniently determined by counting homologous peaks calibrated by using standard

references of G1 to G7. Figures 5b and 5c are the HPSEC-RI and GPC-total carbohydrate chromatographic profiles, respectively. Both HPSEC and GPC profiles of debranched tapioca amylopectin showed separated fractions F1 and F2. The peak DPs of the branch chain-length were 46 ± 1 (F1) and 16.0 ± 0.5 (F2) for HPSEC (calibrated by using pullulan standards) and were 40 ± 3 (F1) and 12.3 ± 0.5 (F2) for GPC (obtained by chemical analyses of the three fractions collected at the corresponding peak). Because HPSEC and GPC give broad and unresolved peaks, the DP at peak represents average DP of each arbitrary fraction. The close agreement in percentage areas of F1 and F2 between HPAEC-ENZ-PAD and HPSEC-RI (Table 1) provided further evidence in the quantitative analysis of the amylodextrin by using the HPAEC-ENZ-PAD. The source of discrepancy between the result of GPC-total carbohydrate and results of the other two is not clear.

CONCLUSION

The results of these studies indicated that the use of the AMG reactor alone at 25°C was sufficient to hydrolyze the HPAEC-separated amylodextrin. The use of extended reactor length did not improve the efficiency of the enzyme reactor but decreased the detection sensitivity of amylodextrin. HPAEC-ENZ-PAD provided excellent separation and quantitative detection for amylodextrin, and thus, the chain-length distribution was accurately determined by the HPAEC chromatogram and the result was reproducible.

ACKNOWLEDGEMENTS

The financial support from Grain Processing Corporation is greatly appreciated. The authors also thank Dr. D. C. Johnson for valuable information on enzyme reactors.

Journal Paper No. J-16584 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 3258, and supported by Hatch Act and State of Iowa funds.

REFERENCES

1. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, F. Smith, Anal. Chem., **28**. 350-356 (1956).

2. S. Hizukuri, Y. Takeda, M. Yasuda, Carbohydr. Res., **94**, 205-213 (1981).

3. A. G. W. Bradbury, A. B. Bello, Cereal Chem., **70**, 543-547 (1993).

4. S. Kobayashi, S. J. Schwartz, D. R. Lineback, J. Chromatogr., **319**, 205-214 (1985).

5. M. H. Ong, K. Jumel, P. F. Tokarczuk, J. M. V. Blanshard, S. E. Harding, Carbohydr. Res., **260**, 99-117 (1994).

6. T. Takagi, S. Hizukuri, J. Biochem. (Tokyo), **95**, 1459-1467 (1984).

7. S. Hizukuri, Carbohydr. Res., **141**, 295-306 (1985).

8. S. Hizukuri, Y. Maehara, Carbohydr. Res., **206**, 145-159 (1990).

9. C. Takeda, Y. Takeda, S. Hizukuri, Carbohydr. Res., **246**, 273-281 (1993).

10. W. Kaye, A. J. Havlik, Appli. Opt, **12**, 541-550 (1973).

11. A. C. Quano, W. Kaye, J. Polym. Sci, Polym. Chem. Ed., **12**, 1151-1162 (1974).

12. K. S. Wong, J. Jane. J. Liq. Chromatogr., **18**, 63-80 (1995).

13. L. A. Larew, D. C. Johnson, Anal. Chem., **60**, 1867-1872 (1988).

14. J. Jane, J. Chen, Cereal Chem., **69**, 60-65 (1992).

15. J. Emnéus, L. Gorton, Anal. Chem., **276**, 303-318 (1993).

16. J. Emnéus, L. Gorton, Anal. Chem., **276**, 319-328 (1993).

17. B. O. Juliano, Cereal Sci. Today, **16**, 334-360 (1976).

18. M. Abdullah, I. D. Fleming, P. M. Taylor, W. J. Whelan, Biochem. J., **89**, 35P-36P (1963).

19. D. D. Lee, Y. Y. Lee, P. J. Reilly, E. V. Collins, G. T. Tsao, Biotechnol. Bioeng., **18**, 253-267 (1976).

20. S. K. Hayashida, Y. Teramoto, T. Inoue, Appl. Environ. Microbiol., **54**, 1516-1522 (1988).

21. J. M. S. Cabral, J. F. Kennedy, J. M. Novais, Enzyme Microb. Technol., **4**, 343-348 (1982).

22. P. J. Reilly, in **Starch Conversion Technology**, G. M. A. Van Beynum, J. A. Roels, eds., Mercel Dekker, Inc., New York, 1985, p.114.

Received April 10, 1996
Accepted April 24, 1996
Manuscript 4140

J. LIQ. CHROM. & REL. TECHNOL., 20(2), 311–325 (1997)

MORPHINE DETERMINATION BY HPLC WITH IMPROVED CHEMILUMINESCENCE DETECTION USING A CONVENTIONAL SILICA BASED COLUMN

Elizabeth Amiott, Anthony R. J. Andrews*

Department of Chemistry
Ohio University
Athens, OH 45701-2979

ABSTRACT

A new high performance liquid chromatography-chemiluminescence analysis system for morphine is described. This system has an on-column limit of detection of 20 pg for morphine, allowing morphine determination down to 1 ng/mL, with a 20 µL injection size, without any concentration during sample preparation. Simultaneous determination of monoacetylmorphine to 15 ng/mL (300 pg on-column) is also achieved. The internal standard for both compounds is nalorphine and total analysis time is under 5 minutes.

INTRODUCTION

Morphine determination is of interest both in forensic cases as an indicator of heroin usage[1] and in routine pharmacokinetic studies.[2] The commonly used detection methods for morphine following high performance

Copyright © 1997 by Marcel Dekker, Inc.

liquid chromatography (HPLC) are ultraviolet (UV)[3] and electrochemical (EC).[4] UV detection lacks the sensitivity required for pharmacokinetic and trace forensic analysis and EC detection frequently requires extensive sample preparation for trace analysis.

Chemiluminescence (CL) detection, utilising the chemiluminescent reaction between morphine and acidic potassium permanganate was proposed as an ideal solution to these problems.[5] CL detection offers the sensitivity required, with a limit of detection (LOD) of 0.7 pg with flow injection analysis (FIA) and should offer enhanced selectivity as the CL reaction is non-universal. However when coupled with HPLC[6] the resulting on-column detection limits, 5 ng, fell short of those required for trace analysis. Attempts to improve upon this using electrogenerated CL met with mixed results.[7]

This disparity between the FIA LOD and the HPLC LOD was due to the problems of coupling the highly acidic conditions required for the CL reaction with the conditions required for HPLC analysis. In addition to a high LOD the previous HPLC-CL method used a specially synthesised compound as internal standard (IS) and was unable to determine monoacetylmorphine, a necessary requirement in forensic analyses.

By the use of an acidic permanganate solution the requirements for a highly acidic mobile phase are reduced and new HPLC conditions can be utilised. This paper presents a new HPLC method for morphine and monoacetylmorphine for coupling with CL. The on-column LOD has been reduced by almost three orders of magnitude over previous work, a commercially available compound is used as the IS and the simultaneous determination of monoacetylmorphine is now feasible, with a total analysis time of under 5 minutes.

EXPERIMENTAL

Equipment

The chromatographic system consisted of dual model LC-10AS solvent delivery modules (Shimadzu) and a Rheodyne 7725I injection valve with a 5 or 20 µL sample loop size. The UV detector was a SPD-10AV UV-Vis (Shimadzu), the CL detector a Shodex CL-2 (JM Science). The oxidant was mixed post-column at a stainless steel T-piece pumped through Teflon tubing (0.3 mm id x 1.58 mm od, Supelco) with a Minipuls 3 peristaltic pump (Gilson) using black/black silicone tubing (Elkay). Chromatograms were

recorded using EZChrom Chromatography software (version 6.5, Scientific Software) running on a 486DX-2 computer (Gateway 2000). HPLC columns used were Supelcosil ABZ+ (Supelco), Hypersil ODS (Alltech) and Inertsil ODS-2 and ODS-3 (Metachem).

Chemicals

Methanol (Aldrich) and tetrahydrofuran (Mallinckdrodt) were HPLC grade, water was obtained from a purification system (Millipore). Potassium permanganate (EM Science), polyphosphoric acid (Aldrich), sodium dihydrogen phosphate, disodium dihydrogen pyrophosphate, sodium hexametaphosphate (Fluka), and orthophosphoric acid (Fisher) were all reagent grade. All glassware for opiate solutions was treated with Surfsil siliconizing fluid (Pierce) prior to use. Buffer pH values were adjusted with orthophosphoric acid.

Aqueous morphine and monoacetylmorphine solutions were prepared from 1 mg/mL and 100 μg/mL methanol standards (Radian) respectively or from morphine sulphate (Sigma). Aqueous nalorphine solutions were prepared from a 1 mg/mL methanol standard (Alltech).

RESULTS

HPLC Column

Starting chromatographic conditions were based on the results of previous studies.[8] An original mobile phase of 90:10 sodium dihydrogen phosphate (0.01 M, pH = 3.8):methanol was used to compare 3 chromatographic columns for morphine peak shape and elution times. Results showed that the Supelcosil ABZ+ and Inertsil ODS-2 columns gave symmetrical peaks under these conditions and these columns were used in further studies.

Column Diameter Reduction

Reducing column diameter should result in narrower taller peaks and hence a decreased LOD. A comparison was made between three Supelcosil ABZ+ columns with ids of 4.6 mm, 2.1 mm and 1.0 mm using the mobile phase as described previously pumped at 1.0, 0.8 and 0.2 mL/min

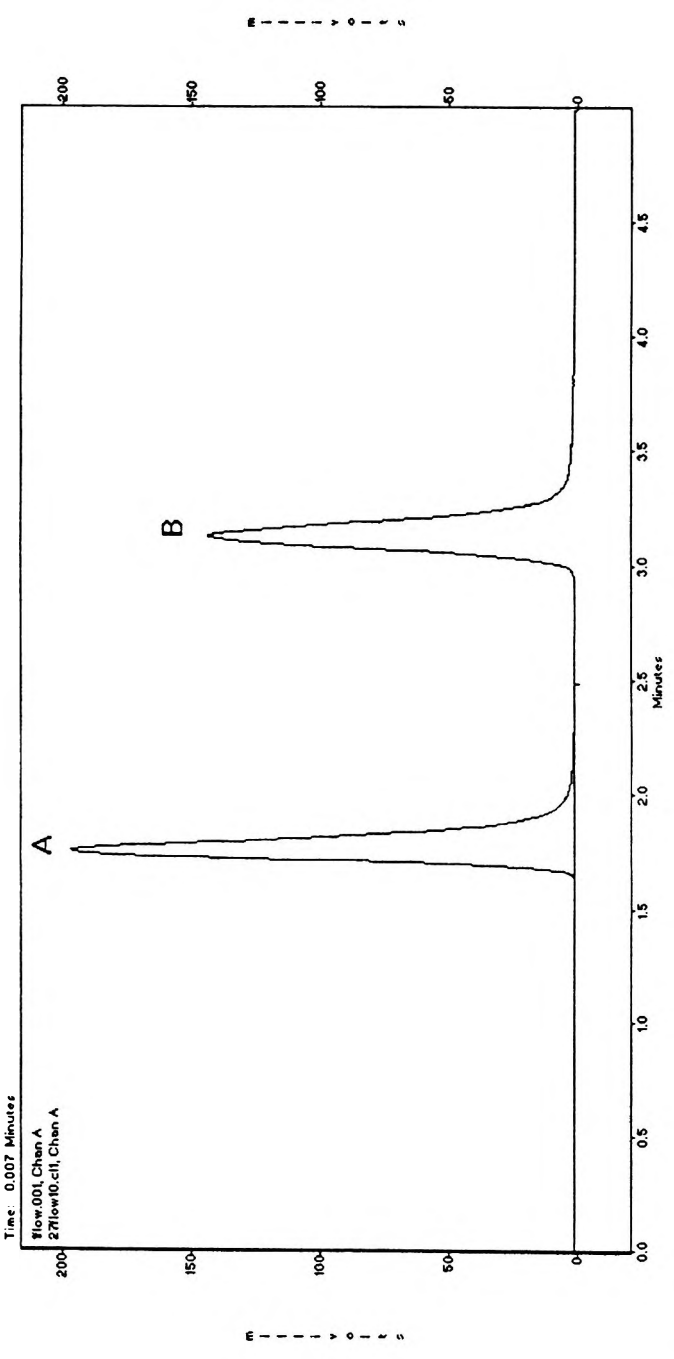


Figure 1. Comparison of two Inertsil ODS-2 columns with morphine. Both 15 cm length, column A = 2.1 mm id. and column B = 4.6 mm id. Mobile phase 90:10 buffer:MeOH.

Table 1

Effect of Three Phosphate Compounds as the Buffer on the CL Signal*

Buffer	% Relative Peak Area
disodium dihydrogen pyrophospate	75.4
sodium hexametaphosphate	100.0
sodum dihydrogen phosphate	64.9

* Results obtained using a 90:10 buffer:methanol mobile phase at 0.8 mL/min on a 2.0 mm Supelcosil column with standard oxidant conditions. Buffer (0.01 M) was pH adjusted to 3.8, 20 μL injections of 5×10^{-6} M morphine.

respectively and CL detection. It was found that reducing the column diameter from 4.6 mm to 2.1 mm increased peak height by 29.7 percent, but a further reduction to 1.0 mm caused a peak height decrease of 78.5 percent from the signal seen with a 4.6 mm id column. Figure 1 shows the increase seen in reducing column diameter from 4.6 mm to 2.1 mm. The decrease upon reduction to 1 mm is due to the fact that with this smaller bore column the dead volume between the T-piece and the detector cell becomes important. The lower column flow rate means that most CL emission occurs before the detector cell. A flow design as proposed by Danielson *et al.*[9] would eliminate this problem, however such a design is not commercially available and so is not feasible for most routine analysis laboratories. Columns with an id of 2.0 mm were used in all further studies.

Mobile Phase Buffer

Phosphates have been found to enhance the CL signal seen from the morphine-permanganate reaction. Two other phosphates were investigated to see if any provided enhanced CL signal over the currently used sodium dihydrogen phosphate buffer. Table 1 shows the results of a comparison of the 3 phosphate compounds.

Sodium hexametaphosphate resulted in the largest CL signal. This correlates with recent work by Barnett *et al.*[10] who used hexametaphosphate as a replacement for polyphosphoric acid.

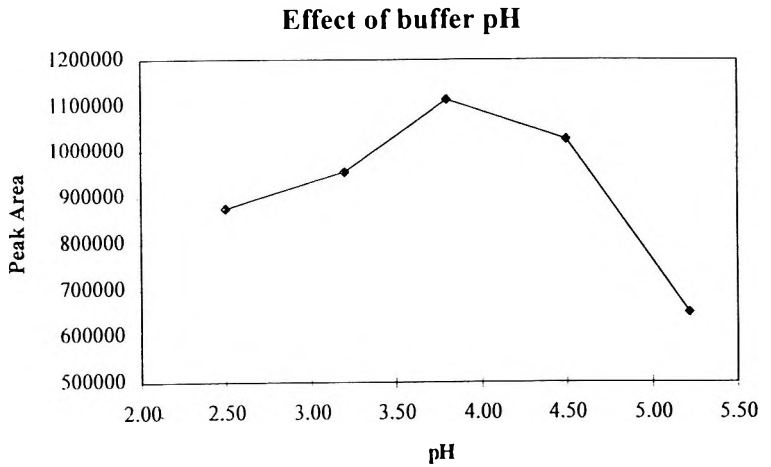


Figure 2. The effect of buffer pH upon CL signal seen.

Presumably the hexametaphosphate prolongs the emission so that more CL is generated in the detector cell. In this case the sodium hexametaphosphate is not acting as a pH buffer, but as a CL reaction enhancer.

Buffer pH

As pH is known to have a significant impact upon the CL signal, the pH of the buffer was varied over the range 5.2 to 2.5. Results are shown in Figure 2. The pH was not evaluated below 2.5 as a significant decrease in CL signal was already being seen. This is due to the fact that the pH affects the rate of the reaction that generates the CL. At pH values below 3.8 the CL reaction occurs prior to the sample entering the flow cell, at higher pH values other non-CL pathways begin to dominate and the emission is reduced. The use of this higher pH suits the analysis system well, as although the HPLC columns used are able to tolerate lower pH values than previous columns they are still limited to a pH of about 2 for extended use

Choice of IS

The requirements for the IS for this analysis system go beyond the standard need for chromatographic separation and commercial availability. The IS must also be a chemiluminescent compound otherwise it will not give

Table 2

Comparison of Capacity Factors on 3 Different Columns and 2 Mobile Phases*

	Mobile Phase					
	------ 90:9:1 ------			------ 94:5:1 ------		
Compound	**ABZ+**	**ODS-2**	**ODS-3**	**ABZ+**	**ODS-2**	**ODS-3**
morphine	0.92	0.34	0.60	1.74	1.26	1.05
nalorphine	1.82	1.30	2.21	4.55	5.08	3.66
MAM	4.26	2.95	4.48	10.89	13.01	8.42

* Mobile phase given as sodium hexametaphosphate (0.01 M, pH = 3.8):MeOH:THF @ 0.8 mL/min. except for ODS-2 run at 0.4 mL/min.

rise to a signal in the detector. Three commercially available compounds, nalorphine, naloxone, or hydromorphone are all chemiluminescent to some degree and have been used as the IS in other morphine determination methods.[4,11] Of these nalorphine is the most strongly chemiluminescent and has been used as an IS in other morphine analysis methods.[12] Preliminary studies showed that nalorphine was chromatographically resolved from both morphine and monoacetylmorphine and gave rise to a large CL signal. So nalorphine was used as the IS in all future work.

Column Type

As organic solvents such as methanol have been found to inhibit CL emission in this reaction, a mobile phase that contains a low an organic content as possible would be advantageous. To achieve a further reduction in organic content a Supelcosil ABZ+, Inertsil ODS-2 and a newly available Inertsil ODS-3 column were compared using two mobile phases. As tetrahydrofuran has been found to inhibit the CL reaction to a lesser extent than methanol experiments were conducted replacing the methanol with tetrahydrofuran. A comparison of the capacity factors for morphine, monoacetylmorphine and nalorphine with two mobile phases is shown in Table 2. One mobile phase replaces 1% of the methanol with an equal volume of tetrahydrofuran and the other a mobile phase replaces 5% of the methanol with 1% tetrahydrofuran and 4% buffer.

Whilst all columns baseline resolved all three compounds with both mobile phases, the ODS-3 column using the lower methanol content mobile phase offered the best combination of resolution and speed of elution. The elution time for monoacetylmorphine on the other two columns was considerably longer than that seen with the ODS-3 column, resulting in a significant amount of dead time in the chromatogram. It should be noted that the back pressure with the ODS-2 column was significantly higher than with the other columns and the maximum flow rate before going over the pump pressure limits (300 kgf/cm^2) was 0.4 mL/min.

Sample Injection Size

A comparison was made between the 5 and 20 µL sample loops. The morphine concentration was adjusted so that the on-column amount remained the same for both loop sizes. The 5 µL loop gave a 91.5 percent larger peak size. However the 20 µL loop was chosen as this allows four times as much sample to be injected onto the column and sample size is not expected to be a problem.

Oxidant Flow Rate and Concentration

The final parameters for optimisation were the oxidant flow rate and concentration. These need to be adjusted so that the CL detector receives the maximum amount light from the CL reaction. Results of these studies are shown in Figure 3. In each case the permanganate was prepared in a polyphosphoric acid solution ($1x10^{-4}$ M). The mobile phase was 94:5:1 buffer:methanol:tetrahydrofuran with a buffer concentration of 0.01 M, pH adjusted to 3.8 and the morphine concentration was 500 ng/mL.

Although a maximum signal was seen with a permanganate concentration of $1x10^{-4}$ M at a pump speed of 22 rpm, in order to conserve oxidant so that large volumes of solution need not be prepared and disposed of, a concentration of $6x10^{-4}$ M at a pump speed of 6 rpm was used to construct the calibration graph. This pump speed corresponds to a flow rate of 0.3 mL/min.

Calibration Graph

The final analysis conditions for morphine were an Inertsil ODS-3 column (15 cm x 2.1 mm id), mobile phase of 94:5:1 sodium hexametaphosphate (0.01 M, pH = 3.80):methanol:THF at a flow rate of 0.8

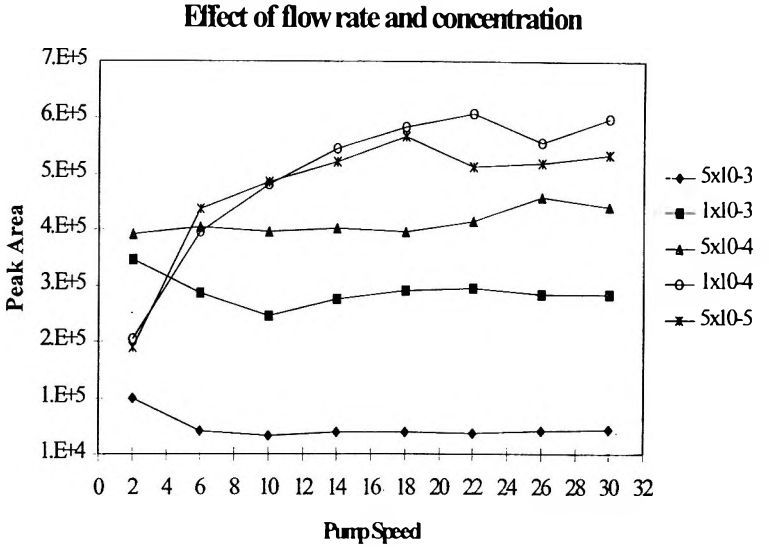


Figure 3. The effect of potassium permanganate flow rate and concentration upon CL signal seen. HPLC conditions given in the text.

mL/min. The oxidant to generate CL was potassium permanganate ($6x10^{-4}$ M) in polyphosphoric acid ($5x10^{-4}$ M) at 0.3 mL/min. Calibration graphs were obtained for both morphine and monoacetylmorphine over the concentration ranges 10 to 1000 ng/mL and 25 to 100 ng/mL respectively. Plotting peak height for morphine or monoacetylmorphine against nalorphine peak height gave a straight line relationship.

The morphine calibration graph is shown in Figure 4. Equation for the line is; $y = 0.00434x - 0.0441$ and $R^2 = 0.9996$. The calculated LOD (2 x noise) was 1 ng/mL (20 pg on-column) for morphine and 15 ng/mL for monoacetylmorphine. The linearity for monoacetylmorphine is expected to continue beyond that seen here. The concentration of starting standard prevented a larger calibration range being examined. More concentrated standards are now available and confirmatory work in this area will be undertaken shortly.

Figures 5 and 6 show chromatograms from the calibration graph runs with morphine at the 750 and 250 ng/mL levels respectively. Baseline resolution is clearly obtained with a total analysis time of under 5 minutes.

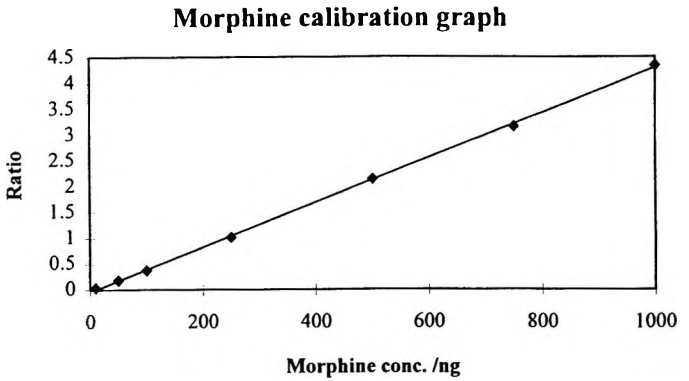


Figure 4. Calibration graph for morphine, range 10 to 1000 ng/mL for morphine, R^2 = 0.9996, equation is: y = 0.00434x - 0.0441.

Intra- and Inter-day Variability

To test the stability of the analysis system the intra- and inter-day variability was monitored. The intra-day ratio variability, given as relative standard deviation (rsd) of the ratio of morphine peak area to IS peak area was found to be 2.8% and 5.3% (n=3) and the interday ratio rsd over a week (n=5) was found to be 2.3 and 1.7% at the 500 and 50 ng/mL levels respectively.

DISCUSSION

The results seen upon reducing the column diameter demonstrate the importance of instrumental design when using CL detection. Utilising a smaller flow path from the T-piece to the detection cell should improve detection limits still further as it would then be possible to take advantage of the narrow peaks generated by 1.0 mm id columns. The importance of considering reaction kinetics and flow cell design was recently discussed by Nieman et al.[13]

The high pressure seen with the ODS-2 column and subsequent reduction upon going to the ODS-3 column is presumably due to the narrower particle size distribution of the ODS-3 column. A narrower particle size distribution means fewer numbers of small diameter particles which cause an increase in the back pressure.

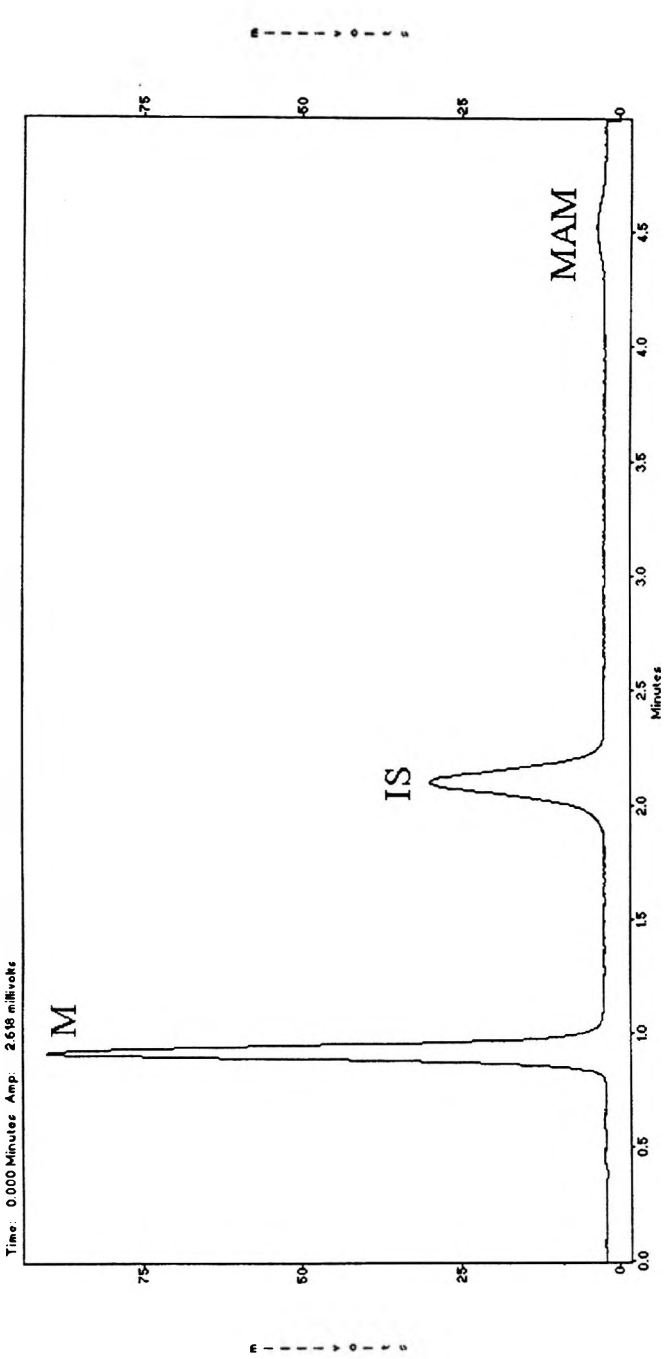


Figure 5. Chromatogram of calibration graph run, morphine (M, 750 ng/mL), nalorphine (IS, 500 ng/mL) and monoacetylmorphine (MAM, 75 ng/mL).

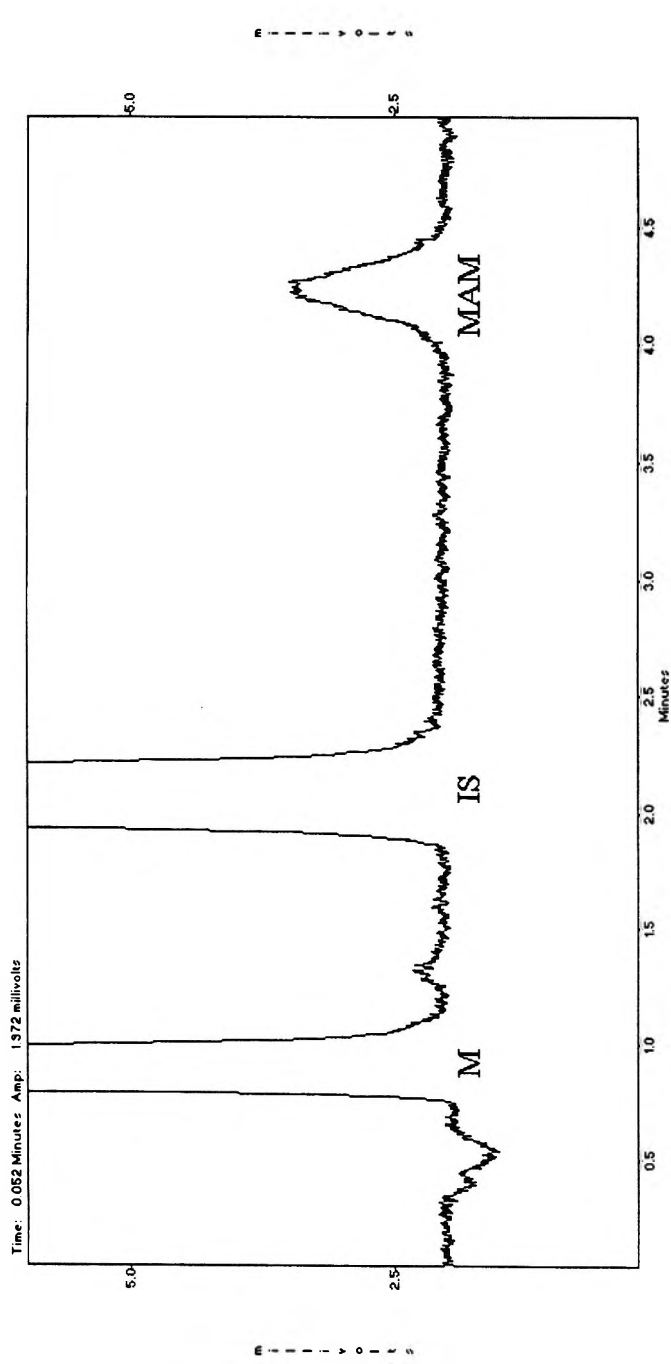


Figure 6. Chromatogram of calibration graph run, morphine (M, 250 ng/mL), nalorphine (IS, 500 ng/mL) and monoacetylmorphine (MAM, 25 ng/mL). The scale is adjusted to clearly show the MAM peak.

The decrease in capacity factor seen upon changing to the ODS-3 column also indicates the need to consider the surface coverage of the bonded phase and not just the column loading. The surface coverage can be calculated from the following equation:[14]

$$N = \frac{10^6 P_c}{\left[1200 n_c - P_c(m-1)\right] s} \qquad (1)$$

Where N is the surface coverage (in micromol/M^2), P_c the percent carbon loading, n_c the number of carbon atoms in the bonded phase,[18] m the molecular weight of the silica bonded phase (331) and s the surface area of the silica (M^2/g). Using P_c and s values of 18,5, 320 and 15, 450 for ODS-2 and ODS-3 respectively $N_{ODS\text{-}2} = 37.3$ and $N_{ODS\text{-}3} = 20.0$.

These values show that although the Pc values are changed only slightly, a 18.9% reduction, the surface coverage has a 46.4% reduction. This contributes to the large capacity factor reduction (35.3% for MAM) when THF is present in the mobile phase.

The retention mechanism is more complex than simple interactions with the bonded phase because when no THF is present in the mobile phase an increase in capacity factor is seen upon changing from ODS-2 to ODS-3 columns. This may be because interactions with the silica packing play a role in the retention and a decrease in surface coverage by the bonded phase would lead to an increase in the silica surface available for these interactions.

It appears that THF helps block these interactions in some manner, reducing retention time and hence capacity factor.

The low inter-day variability indicates the stability of the acidic permanganate solutions. Although some decrease in absolute values is seen over the course of the week the use of the IS accounts for these changes. This stability coupled with the use of a low flow rate allows solutions to be prepared on a weekly instead of daily basis.

This work has successfully coupled the use of an acidic permanganate solution and newly developed HPLC columns to obtain greater than two order of magnitude reduction in the on-column LOD for morphine whilst allowing for the determination of MAM in the same chromatographic run. The determination of MAM is critical for the method to be acceptable to

laboratories undertaking forensic analyses. The improved HPLC conditions also allow for the use of a more conventional IS when compared to the IS used in previous reports.

CONCLUSIONS

The final analysis conditions allow for the simultaneous determination of morphine and MAM at 1 and 15 ng/mL levels with 20 µL injections using CL detection. This is an on-column LOD reduction of over two orders of magnitude from previously published CL detection methods for morphine after HPLC separation. These improvements made to the HPLC-CL detection system make it a viable analysis method for morphine determination in forensic, pharmacokinetic and quality control situations. Studies on developing a simple extraction method for morphine and MAM from biological fluids to complement this determination method are in progress and will be reported on shortly.

REFERENCES

1. R. Wasels, F. Belleville, J. Chromatogr., A, **674**, 225-234 (1994).

2. A. I. Bouquillon, D. Freeman, D. E. Moulin, J. Chromatogr., **577**, 354-357 (1992).

3. B. L. Posey, S. N. Kimble, J. Anal. Toxicol., **8**, 68-74 (1984).

4. P. P. Rop, F. Grimaldi, J. Burle, M. N. De Saint Leger, A. Viala, J. Chromatogr., B: Biomed. Appl., **661**, 245-253 (1994).

5. R. W. Abbott, A. Townshend, R. Gill, Analyst, **111**, 635-640 (1986).

6. R. W. Abbott, A. Townshend, R. Gill, Analyst, **112**, 397-406 (1987).

7. G. M. Greenway, A. W. Knight, P. J. Knight, Analyst, **120**, 2549-2552 (1995).

8. A. R. J. Andrews, PhD Thesis, University of Hull, UK, 1990.

9. M. A. Targrove, N. D. Danielson, J. Chromatogr. Sci., **28**, 205-209 (1990).

10. N. W. Barnett, D. G. Rolfe, T. A. Bowser, T. Walter Paton, Anal. Chim. Acta, **282**, 551-557 (1993).

11. A. W. E. Wright, J. A. Watt, M. Kennedy, T. Cramond, M. T. Smith, Ther. Drug Monit., **16**, 200-208 (1994).

12. A. S. Low, R. B. Taylor, J. Chromatogr., B: Biomed. Appl., **663**, 225-233 (1995).

13. L. L. Schultz, J. S. Stoyanoff, T. A. Nieman, Anal. Chem., **68**, 349-354 (1996).

14. C. K. Poole, S. K. Poole, **Chromatography Today**, Elsevier, Amsterdam, 1991, p. 335.

Received April 8, 1996
Accepted June 18, 1996
Manuscript 4147

J. LIQ. CHROM. & REL. TECHNOL., 20(2), 327 (1997)

EDUCATION ANNOUNCEMENT

BASIC PRINCIPLES OF HPLC AND HPLC SYSTEM TROUBLESHOOTING

A Two-Day In-House Training Course

The course, which is offered for presentation at corporate laboratories, is aimed at chemists, engineers and technicians who use, or plan to use, high performance liquid chromatography in their work. The training covers HPLC fundamentals and method development, as well as systematic diagnosis and solution of HPLC hardware 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 and Optimization
- Ion-Pairing Principles
- Gradient Elution Techniques
- Calibration and Quantitation
- Logical HPLC System Troubleshooting

The instructor, Dr. Jack Cazes, is founder and Editor-in-Chief of the Journal of Liquid Chromatography & Related Technologies, Editor of Instrumentation Science & Technology, and Series Editor of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 35 years; he pioneered the development of modern HPLC technology. Dr. Cazes was Professor-in-Charge of the ACS Short Course and the ACS Audio Course on Gel Permeation Chromatography for many years.

Details may be obtained from Dr. Jack Cazes, P. O. Box 970210, Coconut Creek, FL 33097, USA. (954) 973-8516; E-Mail: jcazes@icanect.net.

J. LIQ. CHROM. & REL. TECHNOL., 20(2), 329–334 (1997)

LIQUID CHROMATOGRAPHY CALENDAR

1997

MARCH 16 - 21: PittCon '97, Atlanta, Georgia. Contact: PittCon '97, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

APRIL 13 - 17: 213th ACS National Meeting, San Francisco, California. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6059; FAX: (202) 872-6128.

APRIL 14 - 19: Genes and Gene 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.

MAY 5 - 9: 151st ACS Rubber Div Spring Technical Meeting, Anaheim, California. Contact: L. Blazeff, P. O. Box 499, Akron, OH 44309-0499, USA.

MAY 23 - 24: ACS Biological Chemistry Div Meeting, San Francisco, California. Contact: K. S. Johnson, Dept of Biochem & Molec Biol, Penn State Univ, 106 Althouse Lab, University Park, PA 16802, USA.

MAY 27 - 28: IInd Miniaturisation in Liquid Chromatography versus Capillary Electrophoresis Conference, University of Ghent, Ghent, Belgium. Contact: Prof. Dr. W. Baeyens, Faculty of Pharmaceutical Analysis, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium.

MAY 27 - 30: ACS 29th Central Regional Meeting, Midland, Michigan. Contact: S. A. Snow, Dow Corning Corp., CO42A1, Midland, MI 48686-0994.

MAY 28 - 30: 31st ACS Middle Atlantic Regional Meeting, Pace Univ, Pleasantville, NY. Contact: D. Rhani, Chem Dept, Pace University, 861 Bedford Rd, Pleasantville, NY 10570-2799, USA. Tel: (914) 773-3655.

MAY 28 - JUNE 1: 30th Great Lakes Regional ACS Meeting, Loyola University, Chicago Illinois. Contact: M. Kouba, 400G Randolph St, #3025, Chicago, IL 60601, USA. Email: reglmtgs@acs.org.

JUNE 22 - 25: 27th ACS Northeast Regional Meeting, Saratoga Springs, New York. Contact: T. Noce, Rust Envir & Infrastructure, 12 Metro Park Rd, Albany, NY 12205, USA. Tel: (518) 458-1313; FAX: (518) 458-2472.

JUNE 22 - 26: 35th National Organic Chemistry Symposium, Trinity Univ., San Antonio, Texas. Contact: J. H. Rigby, Chem Dept, Wayne State Univ., Detroit, MI 48202-3489, USA. Tel: (313) 577-3472.

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

SEPTEMBER 21 - 26: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Cleveland, Ohio. Contact: J. A. Brown, FACSS, 198 Thomas Johnson Dr, Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

OCTOBER 19 - 22: 49th ACS Southeast Regional Meeting, Roanoke, Virginia. Contact: J. Graybeal, Chem Dept, Virginia Tech, Blacksburg, VA 24061, USA. Tel: (703) 231-8222; Email: reglmtgs@acs.org.

OCTOBER 21 - 25: 33rd ACS Western Regional Meeting, Irvine, California. Contact: L. Stemler, 8340 Luxor St, Downey, CA 90241, USA. Tel: (310) 869-9838; Email: reglmtgs@acs.org.

OCTOBER 26 - 29: 8th Symposium on Handling of Environmental & Biological Samples in Chromatography and the 26th Scientific Meeting of the Group of Chromatography and Related Techniques of the Spanish Royal Society of Chemistry, Almeria, Spain. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland. FAX: 41-61-4820805.

OCTOBER 29 - NOVEMBER 1: 32nd ACS Midwest Regional Meeting, Lake of the Ozarks, Osage Beach, Missouri. Contact: C. Heitsch, Chem Dept, Univ of Missouri-Rolla, Rolla, MO 65401, USA. Tel: (314) 341-4536; FAX: (314) 341-6033; Email: reglmtgs@acs.org.

NOVEMBER 11 - 15: 5th Chemical Congress of North America, Cancun, Mexico. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6286; FAX: (202) 872-6128.

NOVEMBER 16 - 21: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey. Contact: S. Good, EAS, P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218.

1998

MARCH 1 - 6: PittCon '98, New Orleans, Louisiana. Contact: PittCon '98, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

JUNE 10 - 12: 53rd ACS Northwest Regional Meeting, Columbia Basin College, Pasco, Washington. Contact: K. Grant, Math/Science Div, Columbia Basin College, 2600 N 20th Ave, Pasco, WA 99301, USA. Email: reglmtgs@acs.org.

JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia. Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA. Tel: (804) 828-8483; FAX: (804) 828-7436.

AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6218; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 15th International Symposium on Medicinal Chemistry, Edinburgh, Scotland. Contact: M. Campbell, Bath University School of Chemistry, Claverton Down, Bath, BA2 7AY, UK. Tel: (44) 1225 826565; FAX: (44) 1225 826231; Email: chsmmc@bath.ac.uk.

SEPTEMBER 24 - 26: XIVth Conference on Analytical Chemistry, sponsored by the Romanian Society of Analytical Chemistry (S.C.A.R.), Piatra Neamt, Romania. Contact: Dr. G. L. Radu, S.C.A.R., Faculty of Chemistry, University of Bucharest, 13 Blvd. Reepublicii, 70346 Bucharest - III, Romania.

NOVEMBER 4 - 7: 50th ACS Southwest Regional Meeting, Resw Triangle Pk, North Carolina. Contact: B. Switzer, Chem Dept, N Carolina State University, Box 8204, Raleigh. NC 27695-8204, USA. Tel: (919) 775-0800, ext 944; Email: switzer@chemdept.chem.ncsu.edu.

1999

MARCH 7 - 12: PittCon '99, Orlando, Florida. Contact: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 22 - 26: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee. Contact: C. Feigerle, Chem Dept, University of Tennessee, Knoxville, TN 37996, USA. Tel: (615) 974-2129; Email: reglmtgs@acs.org.

2000

MARCH 5 - 10: PittCon 2000, Chicago, Illinois. Contact: PittCon 2000, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA.

MARCH 26 - 30: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 20 - 24: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2001

APRIL 1 - 5: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 19 - 23: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2002

APRIL 7 - 11: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 12: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

2003

MARCH 23 - 27: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2004

MARCH 28 - APRIL 1: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 22 - 26: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

2005

MARCH 13 - 17: 229th ACS National Meeting, San Diego, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 28 - SEPTEMBER 1: 230th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

The **Journal of Liquid Chromatography & Related Technologies** will publish, at no charge, announcements of interest to scientists in every issue of the journal. To be listed in the 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 & Related Technologies, P. O. Box 970210, Coconut Creek, FL 33097, USA.**

Improve your laboratory skills with the ***new edition*** *of the...*

Handbook of Thin-Layer Chromatography

Second Edition, Revised and Expanded

(Chromatographic Science Series/71)

Contains **timesaving** features such as an extensive glossary of **key** terms *and* a **useful** directory of manufacturers/suppliers of TLC instruments and products!

edited by
JOSEPH SHERMA and
BERNARD FRIED
Lafayette College, Easton, Pennsylvania

March, 1996
1128 pages, illustrated
$225.00

Written by over 40 internationally acclaimed authorities on thin-layer chromatography (TLC), this comprehensive ***Second Edition*** presents the **latest** techniques, instrumentation, and applications of overpressurized, rotational, and high-performance quantitative TLC.

Provides in-depth discussions of theory and optimization that facilitate a thorough understanding of experimental procedures!

Offering a **systematic** approach to TLC, the ***Handbook of Thin-Layer Chromatography, Second Edition*** covers

- introductory information
- sample preparation
- layers and mobile phases
- chromatographic techniques
- detection
- quantification
- applications to many sample types

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

Praise for the First Edition...

"...The wealth of practical detail can potentially save many hours of laboratory experimentation. The purchase price is easily covered by just one hour of saved labour."
—Chromatographia

"...overall this book should serve for many years as *the* source of information on TLC."
—Analytica Chimica Acta

"...one of the best practical books in the field."
—International Journal of Environmental and Analytical Chemistry

"...contains a wealth of expertise from the practitioner point of view and provides many practical tips on the application of thin-layer chromatography to anyone using this analytical technique."
—Journal of Labelled Compounds and Radiopharmaceuticals

"...extremely useful for those who wish to discover how a TLC separation has been performed."
—Talanta

The ***Second Edition*** contains **new**, practical information on

- the detection, identification, and documentation of chromatogram zones
- optical quantitation
- flame ionization detection
- automation and robotics
- nucleic acid derivatives

Furnishing more than **3150** up-to-date literature citations and over **800** drawings, photographs, tables, and equations, the ***Handbook of Thin-Layer Chromatography, Second Edition*** is an essential reference for analytical chemists, chromatographers, biochemists, biologists, laboratory technicians, medical technologists, biotechnologists, forensic and environmental scientists, veterinary toxicologists, pharmaceutical analysts, and graduate school students in these disciplines.

Contents

Principles and Practice of Thin-Layer Chromatography

Basic Techniques, Materials, and Apparatus. *Joseph Sherma*

Theory and Mechanism of Thin-Layer Chromatography. *Teresa Kowalska*

Optimization. *Qin-Sun Wang*

Sorbents and Precoated Layers in Thin-Layer Chromatography. *Heinz E. Hauk and Margot Mack*

Planar Chromatography (Instrumental Thin-Layer Chromatography). *Dieter E. Jaenchen*

Gradient Development in Thin-Layer Chromatography. *Wladyslaw Golkiewicz*

Overpressured Layer Chromatography. *Emil Mincsovics, Katalin Ferenczi-Fodor, and Ernő Tyihák*

Detection, Identification, and Documentation. *K.-A. Kovar and Gerda E. Morlock*

Thin-Layer Chromatography Coupled with Mass Spectrometry. *Kenneth L. Busch*

Basic Principles of Optical Quantitation in TLC. *Mirko Prosek and Marko Pukl*

Preparative Layer Chromatography. *Szabolcs Nyiredy*

Handbook of Thin-Layer Chromatography

Second Edition, Revised and Expanded

Contents *(continued)*

ISBN: 0—8247—9454—0

This book is printed on acid-free paper.

For Credit Card and Purchase Orders, and Customer Service
CALL TOLL-FREE 1-800-228-1160
Mon.-Fri. 8:30 a.m. to 5:45 p.m. (EST)
or FAX your order to 914-796-1772

Also in the ***Chromatographic Science Series...***

Thin-Layer Chromatography

Techniques and Applications

Third Edition, Revised and Expanded

BERNARD FRIED and **JOSEPH SHERMA** / 464 pages, illustrated / $185.00

"...an excellent reference book...gives an almost encyclopedic review of all aspects of TLC that should be appreciated by beginners and experts alike." —*Inform*

"...an honest effort has been made to revise and update the material in the earlier edition....succeeds admirably in providing an introductory text in TLC....extensively and well illustrated." —*Analyst*

"...offers an up-to-date survey of TLC techniques in the 1990s." —*Journal of Chromatography*

"...presents up-to-date developments in modern instrumental TLC, along with the conventional techniques of planar chromatography." —*Journal of Liquid Chromatography*

Contents

ISBN: 0—8247—9171—1

ORDER FORM *Mail today!*

Mail to **Promotion Dept., MARCEL DEKKER, INC.**
270 Madison Avenue, New York, N.Y. 10016

❑ Please send me _____ copy(ies) of the ***Handbook of Thin-Layer Chromatography, Second Edition*** edited by Joseph Sherma and Bernard Fried at $225.00 per volume.

❑ Please send me _____ copy(ies) of ***Thin-Layer Chromatography, Third Edition*** by Bernard Fried and Joseph Sherma at $185.00 per volume.

[illegible] Add $2.50 for postage and handling per volume. [illegible] Add $4.00 for postage and handling per volume.

I enclose payment in the amount of $ _____ by

❑ check ❑ money order ❑ Visa ❑ MasterCard ❑ Am Exp

Card No. _____ Exp. Date _____

Please bill my company P.O. No. _____

Signature _____
(must be signed for credit card payment)

Name _____

Address _____

City/State/Zip _____

N.Y. residents must add appropriate sales tax. Canadian customers add 7% GST. Prices are subject to change without notice.

Form No. 039611 Printed in U.S.A.

Achieve accurate and precise quantitative analysis with...

Generously illustrated with 160 richly detailed diagrams that clarify the construction of the detectors, facilitating understanding of their performance and function!

Chromatographic Detectors

Design, Function, and Operation

(Chromatographic Science Series/73)

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

July, 1996 / 536 pages, illustrated / $150.00

Written by a seasoned chromatographer with more than 40 years' experience in the field, this reference comprehensively covers the design, construction, and operation of gas chromatography (GC), liquid chromatography (LC), and thin-layer chromatography (TLC) detectors—*all in one convenient, up-to-date source.*

Emphasizing the essential use of common specifications to describe all detectors, allowing easy comparison of their attributes, this practical guide

- discusses the properties of chromatography detectors and the best way to measure their efficacy
- reviews factors that impair the column resolution *before* solutes reach the detector
- describes and explains the relatives merits of the more popular detectors, including the most recent commercially available models as well as lesser-known devices
- explores the exclusive number of TLC detectors available, including automatic scanning devices
- surveys those chromatograph/spectrometer tandem systems that have been satisfactorily developed and details their interfacing, function, and areas of application
- and more!

Chromatographic Detectors serves as an important on-the-job tool for chromatographers: analytical, physical, pharmaceutical, oil, petroleum, clinical, forensic, environmental, and synthetic chemists and biochemists; biotechnologists; and upper-level undergraduate, graduate, and continuing-education students in these disciplines.

Contents

ISBN: 0—8247—9779—5

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

Also in the Chromatographic Science Series...

Liquid Chromatography for the Analyst

RAYMOND P. W. SCOTT

344 pages, illustrated / $79.75

"...useful to students as an introduction to HPLC and also the experienced chromatographer who wishes to expand on their theoretical knowledge." —*Analyst*

Contents

An Introduction to Chromatography
Resolution, Retention, and Selectivity
Liquid Chromatography Phase Systems
The Liquid Chromatography Column
The Liquid Chromatograph
Liquid Chromatography Detectors
Sample Preparation
Qualitative and Quantitative Analysis
LC Applications

ISBN: 0—8247—9184—3

Techniques and Practice of Chromatography

RAYMOND P. W. SCOTT

416 pages, illustrated / $85.00

"The structure of this book by Raymond Scott, one of the pioneers in gas and efficient liquid chromatography, reflects his theoretical and practical experience....

"...recommend[ed]...for the first approach to gas, liquid and thin-layer chromatography because of a didactically instructive presentation of basic intermolecular forces and processes playing a substantial role in understanding the selectivity and resolution of separations, and a nice broad set of practical examples of applications."

—*Journal of Chromatography*

Contents

The Chromatographic Process
The Chromatographic Separation
The Mechanism of Retention
Peak Dispersion
Qualitative Analysis
Quantitative Analysis

Gas Chromatography
The Gas Chromatograph
Gas Chromatography Detectors
Gas Chromatography Columns
Gas Chromatography Applications

Liquid Chromatography
Liquid Chromatography Apparatus
Liquid Chromatography Detectors
Liquid Chromatography Columns
Liquid Chromatography Applications

Thin-Layer Chromatography
Thin-Layer Chromatography Apparatus
Thin-Layer Chromatography Techniques

ISBN: 0—8247—9460—5

Mail today! **Order Form**

Mail to: Promotion Dept., MARCEL DEKKER, INC.
270 Madison Avenue, New York, N. Y. 10016

❑ Please send me _____ copy(ies) of ***Chromatographic Detectors*** by Raymond P.W. Scott at $150.00 per volume.

❑ Please send me _____ copy(ies) of ***Liquid Chromatography for the Analyst*** by Raymond P.W. Scott at $79.75 per volume.

❑ Please send me _____ copy(ies) of ***Techniques and Practice of Chromatography*** by Raymond P.W. Scott at $85.00 per volume.

For USA & Canada: Please add $2.50 for postage and handling per volume on prepaid orders; add only $1.00 per volume. *For other countries:* Add $5.00 for postage and handling per volume.

I enclose payment in the amount of $ _____ by:

❑ check ❑ money order ❑ Visa ❑ MasterCard ❑ Am.Exp.

Card No. _____ Exp. Date _____

Please bill my company: P.O. No. _____

Signature _____
(must be signed for credit card payment)

Name _____

Address _____

City State Zip _____

Form No. 079611 Printed in U.S.A.

For Credit Card and Purchase Orders, *and* Customer Service
CALL TOLL-FREE 1-800-228-1160
Mon.-Fri., 8:30 a.m. to 5:45 p.m. (EST)
or FAX your order to 914-796-1772

Analyze bulk and formulated drug products with the ***new edition*** *of...*

Chromatographic Analysis of Pharmaceuticals

SECOND EDITION

(Chromatographic Science Series/74)

edited by **JOHN A. ADAMOVICS**
Cytogen Corporation, Princeton, New Jersey

October, 1996 / 544 pages, illustrated / $165.00

Praise for the first edition...

"There is no doubt that this book should have wide appeal to all those involved in pharmaceutical analysis. Over 1300 drugs and related substances are considered....**a veritable mine of information.**"—***Analyst***

"...an **excellent** reference for those who are involved in the chromatographic analysis of pharmaceutical compounds and their formulation."
—***Journal of Pharmaceutical Sciences***

"...**recommended** as a primary source of information for drug analysis in different drug forms."
—***Journal of Chromatography***

"...a **valuable** reference source for practicing chromatographers in the pharmaceutical industry."
—***Microchemical Journal***

"...provides a **comprehensive** overview of the chromatographic methods of analysis for pharmaceuticals."
—***Analytical Chemistry***

"...has a place as a **quick reference tool** and as a general introduction to chromatography in pharmaceutics."
—***LC-GC***

"...contains a **wealth of information** on the main chromatographic techniques and numerous examples on the application of these methods in the drug industry."
—***Journal of Labelled Compounds and Radiopharmaceuticals***

Updated and revised throughout, the ***Second Edition*** of ***Chromatographic Analysis of Pharmaceuticals*** explores the chromatographic methods used for the measurement of drugs, impurities, and excipients in pharmaceutical preparations—such as tablets, ointments, and injectables.

Contains a 148-page table listing the chromatographic data of over 1300 drugs and related substances—including sample matrix analyzed, sample handling procedures, column packings, mobile phase, mode of detection, and more!

Maintaining the features that made the first edition so popular, ***Chromatographic Analysis of Pharmaceuticals, Second Edition*** now offers

- *new chapters* on capillary electrophoresis and supercritical fluid chromatography
- *up-to-the-minute* information on proteinaceous pharmaceuticals
- *new coverage* of chromatographic methods from the Chinese Pharmacopoeia
- *updated data* from *US Pharmacopeia 23* and from the British and European Pharmacopoeias
- the *latest methods* developed by instrument and column manufacturers
- over 2100 key literature citations, including *recent references* from the chromatographic literature up to 1996

Written in a clear, easy-to-read style, ***Chromatographic Analysis of Pharmaceuticals, Second Edition*** serves as a timesaving resource for analytical, bioanalytical, pharmaceutical, organic, clinical, physical, quality control, and process chemists and biochemists; chromatographers; pharmaceutical scientists; and professional seminars and graduate-level courses in these disciplines.

Contents

ISBN: 0—8247—9776—0

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

Also of interest...

Analysis of Addictive and Misused Drugs

edited by **JOHN A. ADAMOVICS**

688 pages, Illustrated / $195.00

"...so rich in diverse and precious information, that it should be recommended to any analytical toxicologist...as a day-to-day guide to approach practical problems."
—Journal of Chromatography B: Biomedical Applications

"All toxicologists will find this a valuable and interesting addition to their library."
—Journal of Pharmacy and Pharmacology

"...a useful addition to the analytical literature concerned with testing for drug abuse and misuse."
—Talanta

For Credit Card and Purchase Orders, *and* Customer Service
CALL TOLL-FREE 1-800-228-1160
Mon.-Fri., 8:30 a.m. to 5:45 p.m. (EST)
or FAX your order to 914-796-1772

Mail today!

Contents

ISBN: 0-8247-9238-6

ORDER FORM

Send your order to your regular book supplier or directly to your nearest ***Marcel Dekker, Inc.*** *office:*

USA · Canada · South America
MARCEL DEKKER, INC., Promotion Dept.,
270 Madison Avenue, New York, N. Y. 10016 USA
Tel.: 212-696-9000 · Toll-Free 1-800-228-1160 · Fax: 1-914-796-1772

Europe · Africa · Middle East · Far East · Australia · India · China
MARCEL DEKKER AG
Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland
Tel.: +41-61-261-8482 · Fax: +41-61-261-8896

❑ Please send me _____ copy(ies) of ***Chromatographic Analysis of Pharmaceuticals, Second Edition*** (ISBN: 0-8247-9776-0) edited by John A. Adamovics at $165.00 per volume.

❑ Please send me _____ copy(ies) of ***Analysis of Addictive and Misused Drugs*** (ISBN: 0-8247-9238-6) edited by John A. Adamovics at $195.00 per volume.

For USA & Canada: Please add $2.50 for postage and handling per volume on prepaid orders; add only $1.00 per volume.
For other countries: Add $5.00 for postage and handling per volume.

I enclose payment in the amount of $ _____ by:

❑ check ❑ money order ❑ Visa ❑ MasterCard, EuroCard, Access ❑ Am.Exp.

Card No. _____ Exp. Date _____

Please bill my company: P.O. No. _____

❑ Please send me a pro forma invoice, including shipping and handling charges.

Signature _____
(must be signed for credit card payment)

Name _____

Address _____

City/State/Zip/Country _____

NY residents must add appropriate sales tax. Canadian customers add 7% GST. Prices are subject to change without notice.

Form No. 099620 **Printed in U.S.A.**

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

INSTRUCTIONS TO AUTHORS

The ***Journal of Liquid Chromatography & Related Technologies*** is published in the English language for the rapid communication of research results 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 & Related Technologies
P. O. Box 970210
Coconut Creek, FL 33097

Reprints
Due to the short production time for papers in this journal, it is essential to order reprints immediately upon receiving notification of acceptance of the manuscript. A reprint order form will be sent to the author with the letter of acceptance for the manuscript. Reprints are available in quantities of 100 and multiples thereof. Twenty (20) free reprints will be included with orders of 100 or more reprints.

Format of the Manuscript
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 addresses
Abstract
Text Discussion
References

2. **Title & Authors**: The entire title should be in bold-face capital letters and centered within the width of the printed area, located 2 inches (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 boldface, capitalized and centered, 2 lines below the addresses. This should be followed by a single-spaced, concise abstract. Allow 2 lines of space below the abstract before beginning the text of the manuscript.

4. **Text Discussion**: Whenever possible, the text discussion should be divided into major sections such as

INTRODUCTION
MATERIALS
METHODS
RESULTS
DISCUSSION
ACKNOWLEDGMENTS

These **major headings** should be separated from the text by two lines of space above and one line of space below. Each major heading should be typed boldface, in capital letters, centered.

Secondary headings, if any, should be placed flush with the left margin, 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 line of each paragraph** within the body of the text should be indented a half inch.

6. **Acknowledgments**, sources of research funds and address changes for authors should be listed in a separate section at the end of the manuscript, immediately preceding the references.

7. **References** should be numbered consecutively and placed in a separate section at the end of the manuscript. They should be typed single-spaced, with one line space between each reference.

Each reference should contain names of all authors (with initials of their first and middle names); do not use *et al.* for a list of authors. Abbreviations of journal titles will follow the American Chemical Society's Chemical Abstracts List of Periodicals. The word **REFERENCES**, in boldface type, should be capitalized and centered above the reference list.

Following are acceptable reference formats:

Journal:

1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18, 1979-1998 (1985).

Book:

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

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 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.**

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

Additional Typing Instructions

1. The manuscript must be prepared on **good quality white bond paper**, measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). International paper, size A4 is also acceptable. The typing area of the first page, including the title and authors, should be 6" (15.2 cm) wide 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 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 as part of the text, but in such a way as to separate them from the text by a 2-line space above and below the table. Tables should be inserted in 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** (followed by an Arabic number) should precede the table and should be centered above the table. The title of the table should have the first letters of all main words in capitals. Table titles should be typed single line spaced, across the full width of the table.

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

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

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

Manuscripts which require correction of English usage will be returned to the author for major revision.

J. LIQ. CHROM. & REL. TECHNOL., 20(2), (1997)

Contents Continued

JOURNAL OF LIQUID CHROMATOGRAPHY
& RELATED TECHNOLOGIES

Volume 20, Number 2, 1997

CONTENTS

(continued on inside back cover)

MARCEL DEKKER

MARCEL DEKKER, INC. NEW YORK • BASEL • HONG KONG

Contributions to this journal are published free of charge