JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

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

VOLUME 20 NUMBER 13 1997

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

July 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	Airmail to Europe	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(13) i–iv, 1995–2154 (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

JOURNAL OF LIQUID CHROMATOGRAPHY
& RELATED TECHNOLOGIES

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

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.

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.

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(13), 1995–2007 (1997)

ENRICHMENT AND SEPARATION OF HOLMIUM AND ERBIUM BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

Kenichi Akiba,[1] Hiroyuki Hashimoto,[1]
Shigeto Nakamura,[1] Yoshihiro Saito[2]

[1]Institute for Advanced Materials Processing
Tohoku University
Katahira-2, Aoba-ku
Sendai 980-77, Japan

[2] Shimadzu Corporation
1, Nishinokyo-Kuwabaracho
Nakagyo-ku
Kyoto 604, Japan

ABSTRACT

Selective enrichment of rare earth elements and subsequent separation were performed by high-speed countercurrent chromatography (CCC) using a multilayer coiled column filled with a toluene solution of 2-ethylhexylphosphonic acid mono-2-ethylhexyl ester as a stationary phase. A large volume of aqueous solution containing adjacent rare earth elements, holmium and erbium, was passed through the column, and then the rare earth elements enriched in the stationary phase were chromatographically eluted by the mobile phase of an appropriate pH value.

1995

Mutual separation with sufficient resolution was accomplished, and approximately linear relationship existed between the area of elution peak and the quantity of rare earth element injected.

INTRODUCTION

A high-speed countercurrent chromatography (CCC) using a multilayer tubing coil is a novel technique for the separation of chemically similar materials.[1-4] This chromatographic method is attractive because the stationary and mobile phases can be selected on the basis of liquid-liquid extraction behavior. The CCC method was applied to the separation of several organic materials in the beginning, and in recent years it has seen development in the application of separations of inorganic materials.[5-9]

Rare earth elements are chemically similar and this makes it difficult to separate one another. Mutual separation of rare earth elements has been accomplished through CCC by employing acidic organophosphorus compounds as stationary phase materials.[10-12] A CCC stationary phase consisting of an excellent extracting reagent, can afford good separation of rare earth elements, without the addition of any other chemicals such as complexing agents for enhancing metal separation.

For the determination of trace elements, a preconcentration step is required from a large amount of sample. The CCC column can also serve as a continuous extractor for selective preconcentration of trace elements in the column by the passing through of a large volume of sample solution.[5,7,9] The enrichment of materials of interest and their subsequent separation can be accomplished by means of a single CCC column.

This successive procedure through the CCC column is desirable, because it has no extra enrichment step and is free from problems such as, contamination and loss of desired materials sometimes attributed to support materials in the column.

The present study deals with enrichment and separation of small amounts of the adjacent rare earth pair, holmium and erbium, through the CCC column, containing 2-ethylhexylphosphonic acid mono-2-ethylhexyl ester (EHPA) in toluene as a stationary phase.

EXPERIMENTAL

Materials

The extractant, 2-ethylhexylphosphonic acid mono-2-ethylhexyl ester (EHPA; PC-88A, Daihachi Chemical Ind. Co. Ltd.) was washed with a sodium hydroxide solution to remove acidic impurities and diluted with toluene.[13] Arsenazo III (Dojindo Lab.) was used as a post column reagent.

Apparatus

A prototype high-speed countercurrent chromatograph apparatus (HCC-1A; Shimadzu Corporation) holds two identical column holders. The column consists of three layers (about 300 helical turns) of polytetrafluoroethylene (PTFE) tubing with a 1.5 mm inner diameter and ca 150 m length, and a total capacity of coils is about 268 cm^3.[12] This apparatus was placed in an air-thermostated box kept at 35°C.

Procedure

The coiled column was first filled with a toluene solution containing 0.02 M $(EHPA)_2$ (M = mol dm^{-3}). An aqueous mobile phase was pre-equilibrated by contacting with toluene, and pumped "head to tail" into the column under operating conditions, i.e., a revolutional speed of 800 rpm, a flow rate of 5 cm^3 min^{-1} at 35°C. Two alternative methods of sample injection were employed as: a) Equilibrium injection - The column was fully equilibrated with the mobile phase before the sample injection; b) Nonequilibrium injection - A sample solution was directly injected into the column without equilibration of the two phases and then the aqueous mobile phase was passed through the column displacing a portion of organic stationary phase.

The sample solution, containing 10^{-7} to 10^{-3} M of holmium and erbium, was injected through a sample loop in the case of a small volume (1 cm^3) or through the feeder of the aqueous mobile phase in the case of a large volume, up to 2000 cm^3. The pH of sample solution was controlled with 10^{-2} M (H,Na)HCOO or 10^{-3} M $(H,Na)CH_3COO$.

Metal ions were eluted with the mobile phase of an appropriate pH buffered with 0.1 M $(H,Na)Cl_2CHCOO$. The eluted rare earth elements were monitored by spectrophotometric detection of the absorbance, at 650 nm of rare

earth complexes, by a post-column reaction with 0.014%(w/v) Arsenazo III in 50%(v/v) ethanol.[13] The flow cell was periodically washed by flowing ethanol to obtain a stable base line.

After the desired rare earth elements were eluted, the apparatus was stopped, and by introducing a pressurized nitrogen gas into the column, the column contents were emptied into a graduated cylinder to measure the volume of the stationary phase retained in the tubing coil.

RESULTS AND DISCUSSION

Liquid-Liquid Extraction of Rare Earth Elements

Extraction equilibrium of rare earth elements (RE) with 2-ethylhexylphosphonic acid mono-2-ethylhexyl ester (EHPA) has been studied previously.[12,13]

Overall extraction equilibrium with EHPA for the rare earth ions into the toluene solution of EHPA can be expressed by:

$$M^{3+}_{aq} + 3(HA)_{2,org} \Leftrightarrow M(HA_2)_{3,org} + 3H^{+}_{aq} \quad (1)$$

$$K_{ex} = \frac{\left[M(HA_2)_3\right]_{org}\left[H^-\right]^3_{aq}}{\left[M^{3+}\right]_{aq}\left[(HA)_2\right]^3_{org}} \quad (2)$$

where M denotes the rare earth element and $(HA)_2$, the dimer of EHPA.

The extraction constant (K_{ex}) and the separation factor (α_D), defined as the ratio of their K_{ex} values, were previously obtained in the toluene/ 0.1M(H,Na)Cl_2CHCOO system at 35°C; the log K_{ex} = -0.80 and -0.35 for holmium and erbium, respectively, and hence, their separation factor is 2.8.[12]

The extractability with EHPA in aromatic solvents such as toluene is lower than that in aliphatic solvents for rare earth elements.[14] For the chromatographic separation process, the low extractability in toluene is rather favorable, because a weakly acidic solution is available for a mobile phase.

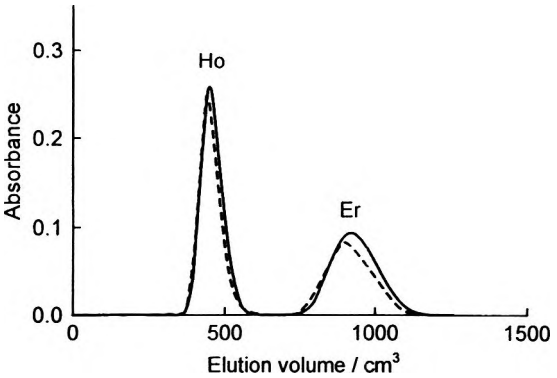


Figure 1. Comparison of equilibrium and nonequilibrium injections of the sample solution containing holmium and erbium. Revolutional speed: 800 rpm; flow rate: 5 cm^3 min^{-1}; 35°C. Stationary phase: 0.02 M $(EHPA)_2$ in toluene; mobile phase: 0.1 M $(H,Na)Cl_2CHCOO$, pH 2.05. Sample: 10^{-3} M RE, 1 cm^3; broken: equilibrium injection, solid: nonequilibrium injection.

Chromatographic Behavior by Equilibrium and Nonequilibrium Sample Injections

The retention volume (V_R) of a desired component is given by the mobile phase volume (V_m), the stationary phase volume (V_s) and the distribution ratio (D) as:

$$V_R = V_m + D\,V_s \qquad (3)$$

On the basis of the extraction data, chromatographic conditions of stationary and mobile phases were optimized for the separation of the adjacent elements.

In CCC separation, it is important to provide the stable retention of a suitable organic solution as the stationary phase in the coiled column. The volume of stationary phase retained in the column has been found to increase with increasing revolutional speed and with decreasing flow rate of mobile phase.[12] The revolution of 800 rpm and the flow rate of 5 cm^3 min^{-1} were adopted to retain an adequate volume of stationary phase.

Figure 1 illustrates typical chromatograms obtained for a mixture of holmium and erbium, by means of equilibrium and nonequilibrium injections of 1 cm^3 sample solution. The elution peaks after equilibrium injection are well

separated, and almost the same elution peaks with good resolution are also obtained by nonequilibrium injection. Thus, these two sample injection methods before and after phase equilibration, gave no significant effect on chromatograms and yielded sufficient separation of individual peaks.

Extraction Enrichment of Rare Earth Elements in Column

The enrichment of rare earth elements from different volumes of sample solution was examined by keeping the total quantity of metal to be constant. A small volume of sample solution can be readily injected, usually, through a sample loop; however, a large volume of sample solution should be directly injected through a inlet path for the mobile phase. Figure 2 presents CCC chromatograms obtained by nonequilibrium injection of different volume solutions containing a constant amount (10^{-6} mol) of each of holmium and erbium at pH > 3. Rare earth elements of low concentration are sufficiently enriched and well separated.

In the nonequilibrium injection method, a large volume of aqueous sample solution passes through the column, simultaneously, displacing the part of organic solution and forming the suitable stationary phase. There is a considerable saving in time and in the volume of buffer solution over the equilibrium injection method. The stationary phase can be available for repeated runs in the equilibrium injection method, however the volume of the stationary phase was found to decrease about 1.5 % by one recycled use. In the present experiments, the V_s values before and after chromatographic operations or repeated runs were confirmed to remain within the region of 125 - 133 cm^3 through four repeated runs.

Figure 3 shows the effect of the volume of sample solution on the chromatographic parameters. The V_R values for erbium and holmium remain unaltered, irrespective of the sample volume, ranging from 1 to 2000 cm^3, and the almost constant value of the separation factor (α_c); a relatively high resolution (R_S) was obtained.

After the chromatographic operation, each elution fraction was collected in order to evaluate the recovery of metals. The rare earth elements fractionated were extracted into 0.02 M $(EHPA)_2$ in toluene, and then stripped and concentrated in the nitric acid solution. The recovery determined by ICP-AES was found to decrease slightly from almost quantitative at 1 cm^3 to around 85% at 2000 cm^3 of sample solution.

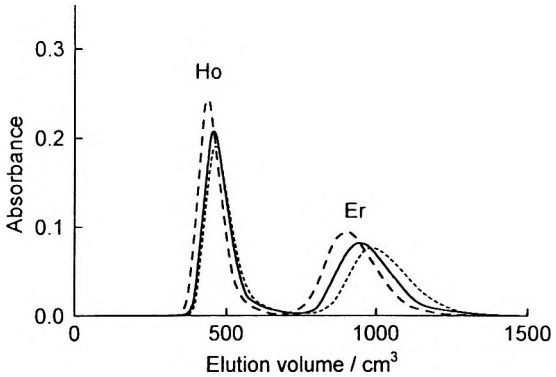


Figure 2. Chromatograms for a constant amount of holmium and erbium enriched from different volumes of sample solution. Mobile phase: pH 2.02-2.06. Nonequilibrium injection; broken: 5 x 10^{-6} M RE, 200 cm^3, pH 3.47; solid: 10^{-6} M RE, 1000 cm^3, pH 3.14; dotted: 5 x 10^{-7} M RE, 2000 cm^3, pH 3.46.

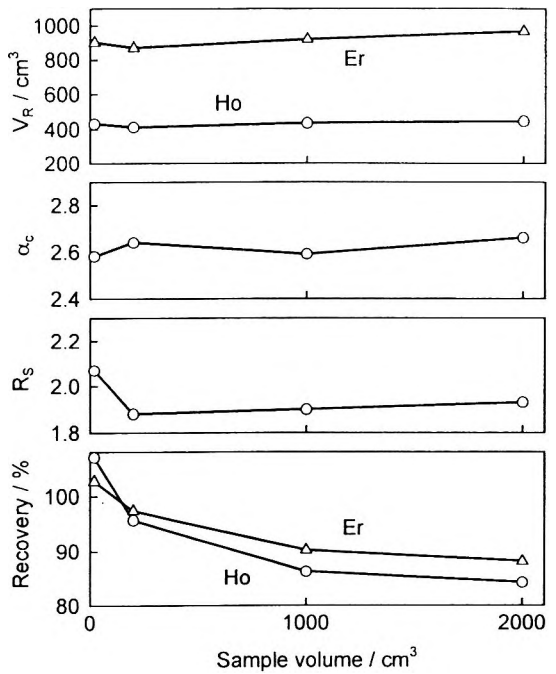


Figure 3. Effect of sample volume on the chromatographic parameters.

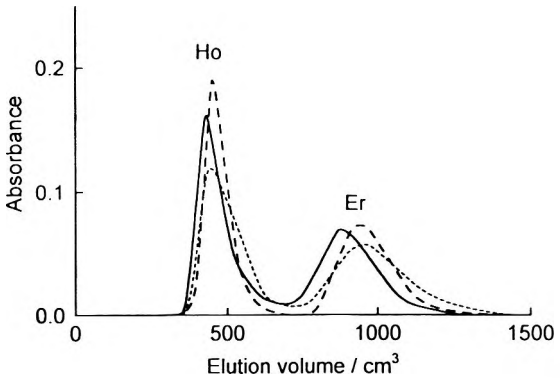


Figure 4. Chromatograms for different pH of sample solution. Mobile phase: pH 2.04-2.07. Sample: 10^{-6} M RE, 1000 cm^3; nonequilibrium injection; broken: pH 3.03, solid: pH 3.47, dotted: pH 4.63.

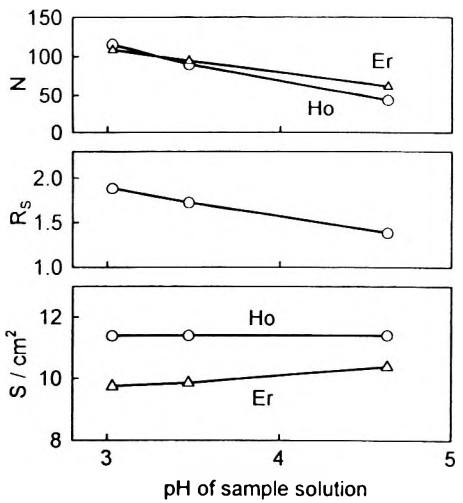


Figure 5. Effect of pH of sample solution on the chromatographic parameters.

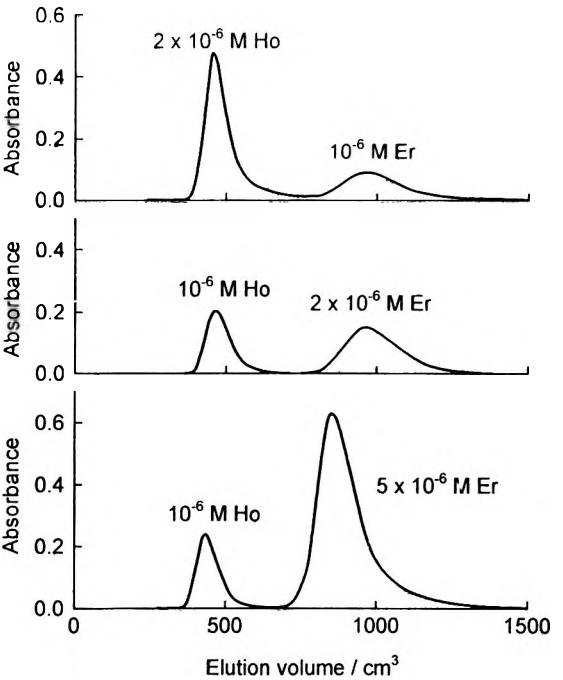


Figure 6. Chromatograms for different concentration ratios of holmium and erbium. Mobile phase: pH 2.05-2.06. Sample: pH 3.45, 1000 cm^3, nonequilibrium injection.

Effect of pH of Sample Solution

For the enrichment of rare earth elements in the column, the pH value is required to be high enough to extract these metals; that is, the distribution ratios of holmium and erbium, between the toluene solution of 0.02 M $(EHPA)_2$ and the sample solution, are expected to be larger than 100 at pH > 3. Chromatograms obtained after nonequilibrium injection of 1000 cm^3 solutions in the pH region from 3 to 4.6 are presented in Fig. 4. The elution peaks of holmium and erbium are considerably separated, but the peak profiles become slightly broader with an increase in pH values of sample solutions. The chromatographic parameters evaluated from individual elution peaks are illustrated in Fig. 5. The number of theoretical plate (N) increases in a lower pH region, and hence resolution is enhanced. When the sample solution of higher pH value is introduced into the column, EHPA in the "head" of the column may become slightly soluble in the mobile phase due to its acid dissociation. This slight decrease in EHPA concentration in the stationary phase appears to lead the broadening of the metal bands. In spite of deformed

profiles of elution peaks, the peak area (S) for holmium and erbium remains almost unaltered independent of pH value of sample solution. This implies that quantitative analysis can be performed based on the peak area measurement. Higher pH value of sample solution is not always desirable, as long as the desired rare earth elements can be sufficiently collected.

Effect of Metal Concentration in Sample Solution

The enrichment of rare earth elements from a sample solution of 1000 cm^3 was carried out by varying the concentration of each element. As Fig. 6 shows, the elution peaks containing different quantity of rare earth elements are sufficiently separated from each other. Chromatographic parameters at different concentrations of erbium are presented in Fig. 7. The retention volume of erbium slightly decreases with increasing erbium concentration.

The concentration of free EHPA in the stationary phase would be depressed due to the reaction with higher concentration of erbium, and the reduced distribution ratio of erbium leads to a decrease in its V_R value and in the separation factor. The R_S value, however remains high, around 2.

The rare earth elements of different concentrations, ranging from 2×10^{-7} to 2×10^{-6} M each, were first enriched and then chromatographed with the mobile phase of pH 2.07. The resultant typical chromatograms are illustrated in Fig. 8. The individual peaks of holmium and erbium are well resolved, even in the case of low concentration of 2×10^{-7} M. Complete separation with the resolution, around 2, was accomplished in a wide concentration region.

Collection of rare earth elements from further dilute solution would be also possible by passing a large volume of sample solution into the column; however it takes a longer time, or resolution might be lowered with increasing flow rate owing to decreasing retention of the stationary phase.

The peak area obtained varying the concentration of holmium and erbium is plotted in Fig. 9. The peak area varies in proportion to the concentration of the desired elements. Even in the very low concentration region around 10^{-7} M level, the enrichment, separation and analysis of rare earth elements would become possible by successive procedure through CCC. The depicted chromatographic procedure implies not only a good resolution in the separation of rare earth elements, but also the applicability in their analysis.

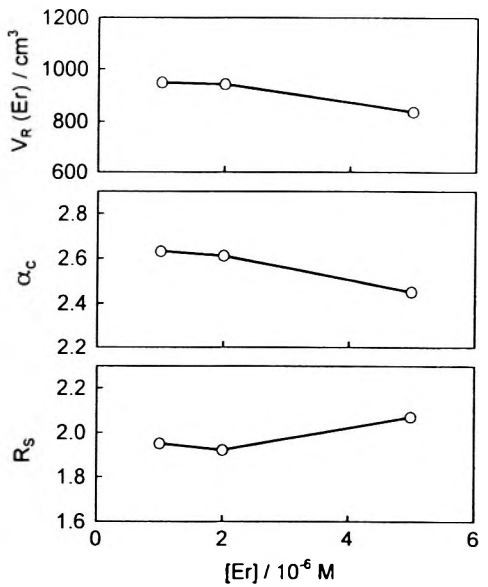


Figure 7. Effect of erbium concentration in the sample solution on the chromatographic parameters.

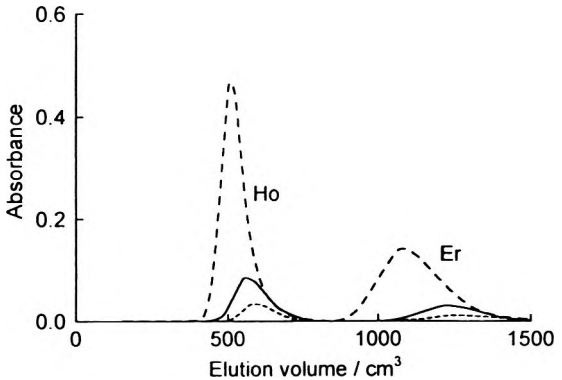


Figure 8. Chromatograms for different concentrations of holmium and erbium in the sample solution. Mobile phase: pH 2.07. Sample: 1000 cm^3, pH 3.43-3.44; equilibrium injection; broken: 2 x 10^{-6} M RE; solid: 5 x 10^{-7} M RE; dotted: 2 x 10^{-7} M RE.

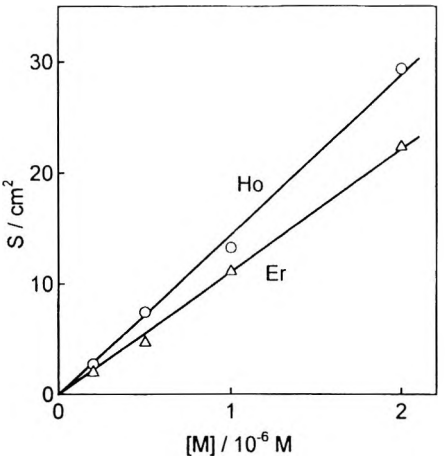


Figure 9. Effect of the metal concentration in the sample solution on peak areas of chromatograms.

CONCLUSION

The result of the present study with CCC are summarized as follows:

1. Similar chromatograms were observed through CCC by the alternative sample injections before and after equilibrium of the stationary and mobile phases.

2. Rare earth elements were efficiently enriched in the stationary phase from a large quantity of aqueous solution.

3. The rare earth elements enriched were separated from each other with sufficient resolution.

4. The area of the chromatographic peak varied in proportion to the quantity of desired metal injected.

REFERENCES

1. N. B. Mandava, Y. Ito, **Countercurrent Chromatography, Theory and Practice**, J. Cazes, ed., Marcel Dekker, Inc., New York, 1988.

2. W. D. Conway, **Countercurrent Chromatography, Apparatus, Theory, and Applications,** VCH Publishers, Inc., New York, 1990.

3. Y. Ito, W. D. Conway, **High-Speed Countercurrent Chromatography**, John Wiley & Sons, New York, 1996.

4. A. Marston, K. Hostettmann, J. Chromatogr. A, **658**, 314-341 (1994).

5. Yu. A. Zolotov, B. Ya. Spivakov, T. A. Maryutina, V. L. Bashlov, I. V. Pavlenko, Fresenius Z. Anal. Chem., **335**, 938-944 (1989).

6. E. Kitazume, N. Sato, Y. Saito, Y. Ito, Anal. Chem., **65**, 2225-2228 (1993).

7. P. S. Fedotov, T. V. Maryutina, V. M. Pukhovskaya, B. Ya. Spivakov, J. Liq. Chromatogr., **17**, 3491-3506 (1994).

8. B. Ya. Spivakov, ISEC'96, 1567-1572, 1990.

9. P. S. Fedotov, B. Ya. Spivakov, ISEC'96, 1615-1620, 1990.

10. Y. Ito, H. Oka, E. Kitazume, M. Bhatnagar, Y. W. Lee, J. Liq. Chromatogr., **13**, 2329-2349 (1990).

11. E. Kitazume, M. Bhatnagar, Y. Ito, J. Chromatogr., **538**, 133-140 (1991).

12. K. Akiba, H. Hashimoto, S. Nakamura, Y. Saito, J. Liquid Chromatogr., **18**, 2723-2741 (1995) .

13. K. Akiba, S. Sawai, S. Nakamura, W. Murayama, J. Liquid Chromatogr., **11**, 2517-2536 (1988).

14. K. Akiba, K. Yamada, S. Nakamura, Nippon Kagaku Kaishi, 555-560 (1993).

Received November 20, 1996
Accepted December 13, 1996
Manuscript 4331

J. LIQ. CHROM. & REL. TECHNOL., 20(13), 2009–2021 (1997)

COUNTING INTEGRAL NUMBERS OF γ-CARBOXYGLUTAMIC ACIDS PER PEPTIDE CHAIN USING CAPILLARY ELECTROPHORESIS

Roderick S. Black, George M. Adams,
James W. Jorgenson, Richard G. Hiskey*

Department of Chemistry
University of North Carolina at Chapel Hill
CB #3290, Venable Hall
Chapel Hill, North Carolina 27599

ABSTRACT

A method for counting the number of γ-carboxyglutamic acid (Gla) residues in peptides is presented. The product mixture generated by partial decarboxylation of a peptide containing multiple Gla residues is separated by capillary electrophoresis. A mixture derived from a peptide containing n Gla residues produces an electropherogram characterized by $n + 1$ peak groups. For a given peptide, a linear relationship ($r^2 > 0.99$) is observed between the average electrophoretic mobility of each peak group and the representative number of Gla residues contained by that group. Resolution of peptide isomers is also obtained, permitting studies of the extent and sequence-specificity of decarboxylation.

INTRODUCTION

The increasing use of capillary electrophoresis (CE) in the study of peptides and proteins is driven largely by the small minimum sample size, high separation efficiency, and the charge-to-size-based separation selectivity of the technique.[1,2] CE studies aimed at elucidating the nature of the post-translational modifications to which many proteins are subject are of special interest because the structure, origin, and location of such modifications cannot be derived from genetic sequence data. These modifications are known, frequently, to affect only a subset of the protein molecules. Furthermore, the modifications may differ in a more or less subtle way among subcomponents of an otherwise homogeneous protein sample,[3] requiring the development of characterization methods that are selective to the differing structural features. An important example of a post-translational modification is the γ-carboxyglutamic acid residue [Gla; side chain $-CH_2CH(COOH)_2$], 9 to 12 of which are located in the N-terminal "Gla domain" portion of several blood coagulation proteins including prothrombin, factor IX, and others.[4,5] In 1974, Stenflo et al. employed paper electrophoresis in the earliest characterization of Gla. They identified Gla in a tetrapeptide derived from prothrombin that exhibited an electrophoretic mobility that "was too high to be explained entirely by its amino acid composition."[6] It has since been established that a vitamin K-dependent carboxylase, via a mechanism which is presently the subject of intense study, directs the *in situ* production of Gla from Glu residues at the appropriate positions in certain protein sequences.[7,8]

The characterization of Gla-containing proteins and peptides is essential to virtually any studies that involve them. Because the Gla sidechain is acid labile, however, the presence of Gla cannot be established using many routine tools, including N-terminal Edman degradation sequencing and amino acid analysis. We have developed a CE method for counting the number of Gla residues in a peptide chain. The method relies upon the charge modification resulting from the *in situ* decarboxylation of Gla, which leaves a Glu residue.[9,10] Non-Gla amino acid side chains are unaffected. A purified *n*-Gla-containing peptide is generally subject to partial decarboxylation; that is, the reaction is terminated before quantitative decarboxylation of the peptide occurs. A family of molecules having 0, 1, 2,...*n* Gla residues is produced. At moderate to high pH, Gla contains two negative charges and Glu contains only one negative charge. The product mixture of this partial decarboxylation reaction is readily separated into $n + 1$ groups of species by CE using a pH 10.0 or 12.0 sodium tetraborate run buffer. By counting the number of peak groups in the electropherogram, a determination of the number of Gla residues can be made. Knowledge of the sample concentration and molecular weight is unnecessary.

Creighton[11] was the first to point out the general usefulness of electrophoretic analysis of a partially charge-modified sample when he employed polyacrylamide gel electrophoresis to count the number of cysteine residues in protein.

The counting of integral numbers of Gla residues is facilitated by the existence of a linear dependence between the average electrophoretic mobility of peak groups and the number of Gla residues representative of each group. In many cases the isomers produced by partial decarboxylation of peptides containing two or more Gla residues can be resolved, extending the selectivity of method to include the specific sequential position of Gla residues. This paper describes the use of this method for counting integral numbers of Gla residues in peptides and for investigating the sequence-specificity of the decarboxylation reaction. The opportunity to employ capillary electrophoresis as an investigative probe of the nature of carboxylase-mediated production of Gla from Glu is also suggested.

MATERIALS AND METHODS

Materials

Capillary electrophoresis buffer solutions were prepared from sodium tetraborate and triethylamine supplied by Sigma Chemical Co. (St. Louis, MO); sodium hydroxide pellets (Fisher Scientific, Pittsburgh, PA) were employed for pH adjustment. Deionized water for the preparation of all solutions was obtained from a Hydro (Research Triangle Park, NC) dual-cartridge purification system. 50 mM HCl was prepared by dilution of the concentrated acid obtained from Fisher Scientific. Mesityl oxide was purchased from Aldrich (Milwaukee, WI), and DPCC-treated trypsin and diisopropylfluorophosphate (DIFP) were obtained from Sigma. Gla-containing peptides were prepared using solid phase methods and were purified using reverse phase HPLC. The *N*-fluoromethoxycarbonyl-γ-carboxyglutamic acid (Fmoc-Gla) required in the synthesis of these peptides was prepared via the method of Schuerman et al.[12] Bovine prothrombin fragment 1 was isolated as described elsewhere.[13,14]

Heat-Induced Decarboxylation

The method of Poser and Price[9] was used for decarboxylation of Gla residues. Approximately 10-100 μg of Gla-containing peptide was placed in an

Eppendorf tube. dissolved in about 100 μL of 50 mM HCl, and lyophilized. The dry salt was then heated (110°C) under vacuum for 15 min to several hours to achieve partial decarboxylation, or for 10 h or more for quantitative (complete) decarboxylation. The product mixture was dissolved in a minimal amount of deionized water containing 0.005 to 0.05% mesityl oxide as a neutral marker before analysis by CE. Partial tryptic digestion of bovine prothrombin fragment 1 was accomplished by incubating an aqueous, 2 mg/mL solution of the protein with trypsin in a 20:1 (w/w) ratio at room temperature. Hydrolysis was terminated after 1.2 h by addition of an excess of DIFP.

Capillary Electrophoresis

CE was performed using a home-built system similar to that described by Jorgenson and Lucaks,[15] employing a Spellman high voltage power supply (Plainview, NY). The UV absorbance was monitored with a Linear (Reno, NV) Model UVIS 200 "on-capillary" absorbance detector set at 220 nm to detect all peptides regardless of amino acid composition. Separations were performed at room temperature in a 50 μm i.d., 360 μm o.d., untreated fused silica capillary (Polymicro Technologies, Phoenix, AZ). Unless otherwise noted, capillaries were 100 cm in length (effective length 85 cm). Runs were conducted using an aqueous buffer of 12.5 to 37.5 mM sodium tetraborate (pH 10.0 or 12.0) that occasionally contained 0.22% (v/v) triethylamine. All injections were hydrodynamic and employed a reservoir height differential of 22 cm for 5 to 30 sec followed by a constant-voltage run at 10 to 25 kV. The capillary was flushed with run buffer for 5 min between runs using aspirator suction. Several minutes of flushing with 0.1 M aqueous NaOH was performed daily to regenerate the capillary surface.

Electropherograms were acquired using an Apple Macintosh computer with a Rainin MacIntegrator (Ridgefield, NJ) data collection package. Data was filtered using a 5-point binomial filter[16] available in the Igor Pro software package (WaveMetrics, Lake Oswego, OR). For the relative peak area ratio study shown in Table 2, correction was accomplished by dividing the area of each peak by the corresponding migration time.[17] The smallest peak in each group of isomeric peaks is defined as 1 area unit; the remaining peaks are normalized accordingly.

RESULTS AND DISCUSSION

A series of electropherograms acquired after progressively longer decarboxylation periods of a 1-Gla-containing peptide is shown in Figure 1.

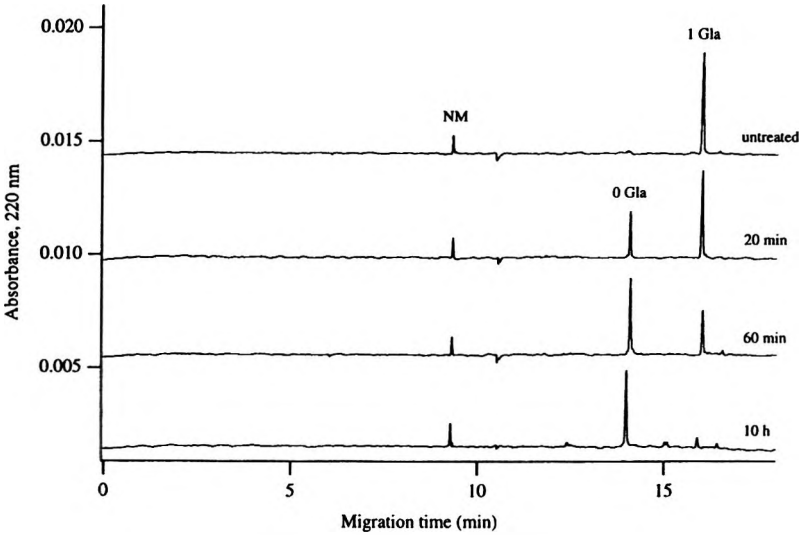


Figure 1. Electropherograms acquired after the indicated periods of decarboxylation of a peptide bearing 1 Gla residue. The peptide sequence before decarboxylation was amino-QTDγFWSKYKD-carboxyl, where γ= Gla. The removal of one carboxyl group effects a pronounced decrease in migration time. Peaks are labeled according to the number of Gla residues the peptides contain. NM indicates the neutral marker. Run buffer was 12.5 mM sodium tetraborate containing 0.22% triethylamine (pH 10.0); run voltage, 25 kV; current, 19 μA; see experimental section for additional details.

Because the high pH CE run buffer favors deprotonation of the carboxyl functionalities of Gla and Glu, the Gla-containing species contains a greater negative charge density, and subsequently exhibits a significantly greater migration time, than that of the Glu-containing product. Partial decarboxylation of a peptide containing two or more Gla residues generates a more complex mixture. The diversity of species in such a mixture results in part from the differing numbers of intact Gla residues contained by the component peptides. Due to the fundamental difference in charge of the side chain of Glu and Gla (-1 *versus* -2, respectively, at ionizing pH), high pH CE effects the separation of such a mixture into distinct groups of peaks according to the number of remaining Gla residues. Partial decarboxylation of a "parent" peptide containing *n* Gla residues will result in an electropherogram having *n* + 1 peak groups. For example, the electropherograms for a 2-Gla-containing peptide (Figure 2) show a primary separation into 3 groups based on the number of Gla residues (0, 1, or 2) remaining in each species.

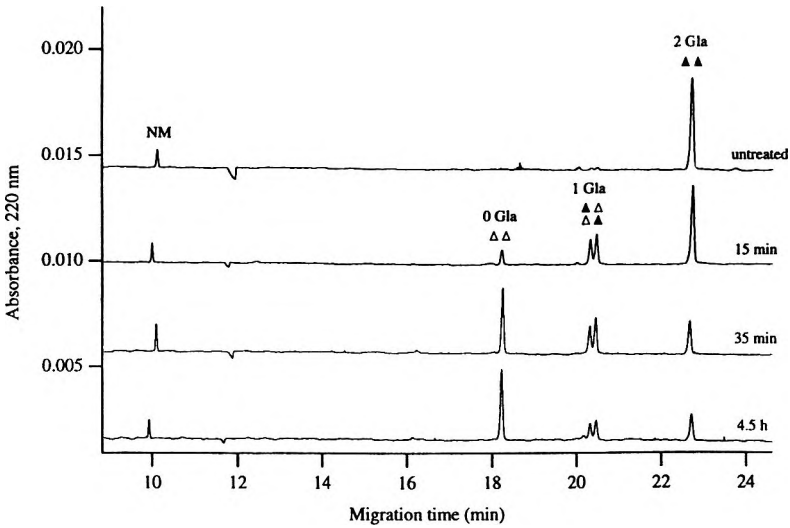


Figure 2. Separations obtained at the indicated times during decarboxylation of a 2-Gla-containing peptide of sequence N-acetyl-DAγQTDγFWSKYKYKD-carboxyl (γ = Gla). The sequence-specific Gla and Glu composition of the peptides is also indicated by icons above the peaks; filled triangles represent Gla and open triangles represent Glu. NM indicates the neutral marker. Note the resolution of the pair of peptide isomers generated by decarboxylation of one of the two Gla residues. Run buffer: 18.8 mM sodium tetraborate containing 0.22% triethylamine (pH 10.0); run voltage, 25 kV; current, 30 μA; the experimental section contains additional information.

As shown by the simple algebraic expressions in Table 1, the maximum number of groups is fixed at $n + 1$ for an n-Gla-containing peptide, but the number of peptide isomers in a given group varies as a function of both the Gla content of the parent peptide and the Gla content of the group. The electropherogram in Figure 2 exemplifies the simplest case: the doublet at about 20.5 min corresponds to the two peptide isomers produced by decarboxylation of one of the two total Gla residues present on the parent peptide. Figure 3 shows the electropherogram of a 4-Gla-containing peptide acquired after a 20-min decarboxylation. The components are separated into five groups, corresponding to 0, 1, 2, 3, and 4 Gla residues remaining per peptide. The number of peaks in each group corresponds to the number of isomers predicted using equations given in Table 1. To determine the quantitative dependence of Gla content on electrophoretic behavior, the average electrophoretic mobility *versus* number of remaining Gla residues was plotted for several partially decarboxylated multi- Gla-containing peptides (Figure 4).

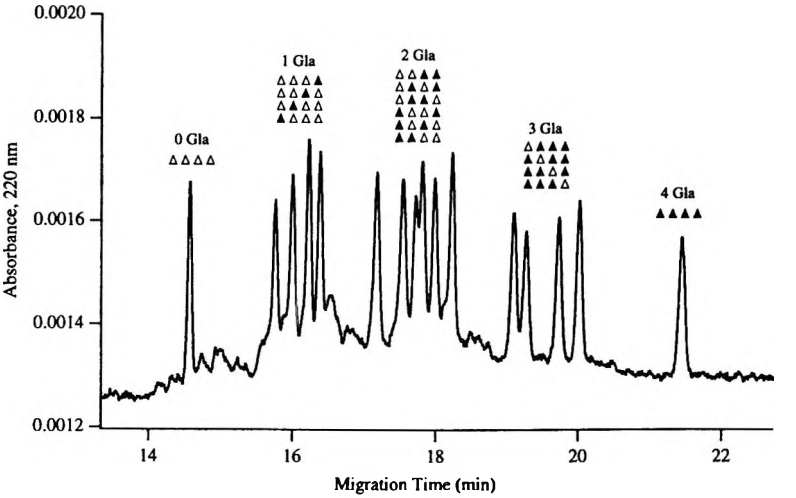


Figure 3. CE separation obtained after partial (1 h) decarboxylation of a peptide containing 4 Gla residues. The electropherogram shows separation into 5 groups (n + 1 = 5 for a 4-Gla-containing peptide). The sample was amino-AQγRIRγLNKPQYγLNRγAC-carboxyl (γ = Gla) before decarboxylation. Peak labels are explained in the caption to Figure 2. All 16 species expected from partial decarboxylation of a peptide containing 4 Gla residues are seen. Run buffer was 15 mM sodium tetraborate (pH 12.0); capillary, 60 cm long (effective length 45 cm); run voltage, 10 kV; current, 22 μA; additional details are presented in the experimental section.

Table 1

Species Produced by Partial Decarboxylation of a Peptide Containing n Gla Residues

Parameter	Algebraic Description
No. of Gla residues in parent peptide	n
Total no. of isomeric groups g	$g = n + 1$
No. of intact Gla's remaining in a given group	k
No. of isomers i in a given group	$i = n!/[k!(n-k)!]$
Total possible no. of species s generated	$s = 2^n$

The variation in the integral number of Gla residues in a partially decarboxylated sample gives rise to a corresponding variation in the charge states of the species. This incremental variation in charge state is in turn responsible for the observed incremental variation in electrophoretic mobility. The strong linear correlation ($r^2 > 0.99$) supports the assignment of decarboxylation as the source of the observed electrophoretic mobility differences. The existence of this consistent mathematical relationship can be exploited in assigning peaks in the electropherogram. For example, the presence of sample impurities may be evidenced by the appearance of one or more peaks that do not fit a linear mobility pattern. For a sufficiently pure sample, the general approach of decarboxylation followed by CE analysis provides a fast, sample-conservative count of the number of Gla residues per molecule of peptide. Such a digital count is of primary interest in the characterization of Gla-containing species including mutant proteins, genetic constructs, and synthetic peptides.

The successful separations of Gla-containing peptide isomers generated by partial decarboxylation suggest multiple opportunities for further study. In the separation shown in Figure 3, for example, the individual peaks of the quartet centered at about 38 min correspond to each of the 3-Gla-containing isomers that is generated during partial decarboxylation of a 4-Gla-containing peptide. CE analysis of synthetic versions of these decarboxylation products could be used to identify the specific isomers, providing insight into the differences in shape, and hence three-dimensional conformation, that apparently permit their successful separation. Furthermore, we believe that interests in the sequence-specificity of both heat-induced[18,19] and chemical[20-22] types of decarboxylation can be investigated using methods outlined in this report. Shown in Table 2 are preliminary data from an investigation of this sort, which suggests that the heat-induced decarboxylation of one particular 4-Gla-containing peptide is not sequence-specific under the conditions employed here. Table 2 provides the relative peak area ratios obtained as described in the Materials and Methods section for the electropherogram shown in Figure 3. The approximate equality of peak areas within each group shows there is no preferential production of any peptide isomer.

In considering the feasibility of separating Gla-containing peptide isomers, it must be noted that the theoretical maximum number of species produced from partial decarboxylation of a complete blood coagulation protein, which may contain as many as 12 Gla residues, is extremely large (e. g., 2^{10} for 10-Gla-containing prothrombin). Furthermore, the analyte/capillary wall adsorption[15] and carbohydrate microheterogeneity[23] phenomena that generally complicate CE analyses of glycoproteins would preclude resolution of partially decarboxylated blood coagulation protein mixtures.

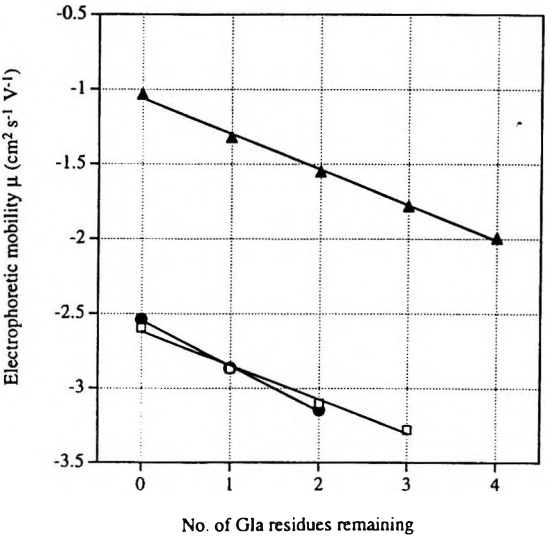


Figure 4. Plots of average electrophoretic mobility *versus* number of remaining Gla residues for three partially decarboxylated peptides that initially contained 2 Gla (circles), 3 Gla (squares), and 4 Gla (triangles) residues. The strong linear correlation (r^2> 0.99 in all cases) facilitates the counting of integral numbers of Gla residues as described in the text.

Table 2

Relative Peak Areas Within Groups of Isomers Produced by Partial Decarboxylation of a Peptide Containing 4 Gla Residues

No. of Gla Residues Remaining	Relative Peak Area Ratio[a]
1	1.0 : 1.3 : 1.5 : 1.2
2	1.6 : 1.2 : 2.0 : 1.0 : 1.3[b]
3	1.2 : 1.0 : 1.1 : 1.2

[a] This data was obtained from the electropherogram shown in Figure 3.

[b] Two isomers in this group are poorly resolved, and are thus treated as a single peak, accounting for the approximately two-fold larger area seen here.

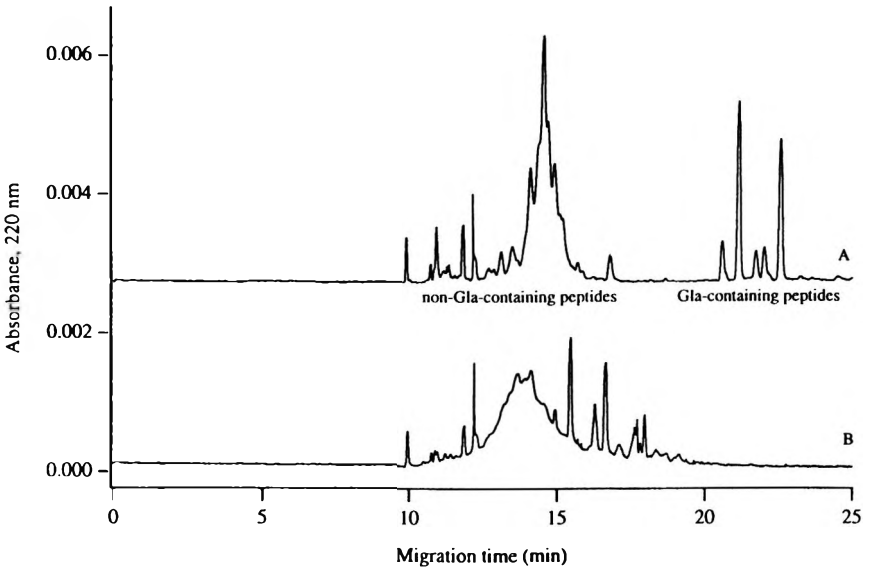


Figure 5. Electropherograms obtained after partial tryptic digestion of bovine prothrombin fragment 1, a protein containing 10 Gla residues within the N-terminal 33 amino acids of the sequence. Before decarboxylation, Gla-containing peptides are seen in the late-migrating region of the electropherogram (A). Complete decarboxylation (10 h) of Gla residues to Glu resulted in a decrease in migration time of these peptides (B). Run buffer was 25 mM sodium tetraborate (pH 10.0); run voltage, 25 kV; current, 38 μA. See main text for additional experimental details.

A primary separation into groups based strictly on the number of Gla residues remaining, even without resolution of isomers, may nevertheless be used to count the number of Gla residues in a protein. Proteolytic peptide mapping constitutes a possible approach to overcoming the limitations of analyzing intact proteins. Our results for wild type bovine prothrombin fragment 1[24] indicate that the Gla-containing peptides in enzymatic digests conveniently appear in a distinctly late region of electropherograms acquired using moderate to high pH CE run buffers (Figure 5). These studies suggest mapping as a convenient means of detecting differences in the chemical structure of Gla domains, especially the presence of uncarboxylated, mutated, or modified Gla residues that are of significance to many investigations involving blood coagulation proteins.

An application of this method that is of great biological relevance concerns the study of the nature of vitamin K-dependent, enzymatic carboxylation of Glu residues to Gla. Many investigations are aimed at determining whether or not a specific sequential order of carboxylative production of Gla exists for substrates that are destined to contain multiple Gla residues. Related studies seek to determine the number of distinct enzyme-substrate binding events required to effect complete carboxylation of one substrate molecule. Data from this work may be used to establish the processivity of vitamin K carboxylase. Complete enzymatic processivity, for example, would describe the case in which one and only one enzyme-substrate binding event occurs for production of all Gla residues in a single substrate molecule. A number of *in vitro*[25-28]and *in vivo*[29,30] investigations of both the order and processivity of vitamin K carboxylase action have appeared in the literature. Such studies invariably require characterization of samples resulting from partial or complete carboxylation of a substrate. The chemical differences in net Gla content and/or sequential position of Gla residues in these types of samples are identical to those found in the partially decarboxylated peptide samples analyzed in the research presented here. CE is apparently very well-suited for the separation of Gla-containing peptide mixtures generated during studies of vitamin K-dependent carboxylation.

ACKNOWLEDGMENTS

The authors wish to extend their thanks to Ms. Paula Berkowitz and Mr. Russ Henry for technical assistance. This work was supported by grants HL-20161 and HL-06350 from the National Institutes of Health and CHE-9215320 from the National Science Foundation..

REFERENCES

1. J. P. Landers, R. P. Oda, T. C. Spelsberg, J. A. Nolan, K. J. Ulfelder, Biotechniques, **14**, 98-111 (1993).

2. C. Schoneich, A. F. R. Huhmer, S. R. Rabel, J. F. Stobaugh, S. D. S. Jois, C. K. Larive, T. J. Siahaan, T. C. Squier, D. J. Bigelow, T. D. Williams, Anal. Chem., **67**, 155R-181R (1995).

3. R. Uy, F. Wold, Science, **198**, 890-896 (1977).

4. E. W. Davie, K. Fujikawa, W. Kisiel, Biochemistry, **30**, 10363-10370 (1991).

5. J. W. Suttie, FASEB J., 7, 445-452 (1993).

6. J. Stenflo, P. Fernlund, W. Egan, P. Roepstorff, Proc. Nat. Acad. Sci. USA, **71**, 2730-2733 (1974).

7. B. R. Hubbard, M. M. W. Ulrich, M. Jacobs, C. Vermeer, C. Walsh, B. Furie, B. C. Furie, Proc. Natl. Acad. Sci. USA, **86**, 6893-6897 (1989).

8. P. Dowd, S. W. Ham, S. J. Geib, J. Am. Chem. Soc., **113**, 7734-7743 (1991).

9. J. W. Poser, P. A. Price, J. Biol. Chem., **254**, 431-436 (1979).

10. P. M. Tuhy, J. W. Bloom, K. G. Mann, Biochemistry, **18**, 5842-5848 (1979).

11. T. E. Creighton, Nature, **284**, 487-489 (1980).

12. M. A. Schuerman, K. I. Keverline, R. G. Hiskey, Tetrahedron Lett., **36**, 825-28 (1995).

13. K. G. Mann, Methods in Enzymol., **45**, 123-156 (1977).

14. J. S. Pollock, A. J. Shepard, D. J. Weber, D. L. Olson, D. G. Klapper, L. G. Pedersen, R. G. Hiskey, J. Biol. Chem., **263**, 14216-14233 (1988).

15. J. W. Jorgenson, K. D. Lukacs, Science, **222**, 266-272 (1983).

16. P. Marchand, L. Marmet, Rev. Sci. Instrum., **54**, 1034-1041 (1983).

17. K. D. Altria, Chromatographia, **35**, 177-182 (1993).

18. A. L. Gray, R. A. Hoke, D. W. Deerfield, R. G. Hiskey, J. Org. Chem., **50**, 2189-2191 (1985).

19. S. P. Bajaj, P. A. Price, W. A. Russel, J. Biol. Chem., **257**, 3726-3731 (1982).

20. S. F. Wright, P. Berkowitz, D. W. Deerfield, P. A. Byrd, D. L. Olson, R. S. Larson, G. C. Hinn, K. A. Koehler, L. G. Pedersen, R. G. Hiskey, J. Biol. Chem., **261**, 10598-10605 (1986).

21. G. A. Zapata, P. Berkowitz, C. M. Noyes, J. S. Pollock, D. W. Deerfield, II, L. G. Pedersen, R. G. Hiskey, J. Biol. Chem., **263**, 8150-8156 (1988).

22. T. Sugo, K. Watanabe, T. Naraki, M. Matsuda, J. Biochem., **108**, 382-387 (1990).

23. P. Oefner, C. Chiesa, G. Bonn, C. Horvath, J. Cap. Elec., **1**, 5-26 (1994).

24. G. M. Adams, Ph.D., University of North Carolina, 1992.

25. P. Decottignies Le-Marechal, H. Rikong-Adie, R. Azerad, Biochem. Biophys. Res. Commun., **90**, 700-707 (1979).

26. S.-M. Wu, B. A. M. Soute, C. Vermeer, D. W. Stafford, J. Biol. Chem., **265**, 13124-13129 (1990).

27. D. P. Morris, Ph.D., University of North Carolina, 1995.

28. M. E. Benton, P. A. Price, J. W. Suttie, Biochemistry, **34**, 9541-9551 (1995).

29. M. Borowski, B. C. Furie, B. Furie, J. Biol. Chem., **261**, 1624-1628 (1986).

30. D. J. Liska, J. W. Suttie, Biochemistry, **27**, 8636-8641 (1988).

Received November 12, 1996
Accepted December 5, 1996
Manuscript 4328

J. LIQ. CHROM. & REL. TECHNOL., 20(13), 2023–2030 (1997)

DEVELOPMENT OF AN HPLC/DIODE-ARRAY DETECTOR METHOD FOR SIMULTANEOUS DETERMINATION OF SEVEN HYDROXY-CINNAMIC ACIDS IN GREEN COFFEE

P. B. Andrade,[1] R. Leitão,[1] R. M. Seabra,[1]
M. B. Oliveira,[2] M. A. Ferreira[2]

CEQUP
[1]Lab. de Farmacognosia
[2]Lab. de Bromatologia
Faculdade de Farmácia
Universidade do Porto
R. Aníbal Cunha
4050 Porto, Portugal

ABSTRACT

A simple, rapid, sensitive, reproducible, and accurate reverse phase HPLC procedure is proposed for the determination of seven phenolic acids in green coffee samples. The sample preparation was simple, involving extraction, alkaline hydrolysis, and liquid/liquid extraction. The chromatographic separation was achieved using a reverse phase column Spherisorb ODS2 (5μm; 25.0 x 0.46 cm). Gradient elution was carried out using water-formic acid (19:1) (A) and methanol (B). The effluent was monitored by a diode-array detector and chromatograms were recorded at 320 nm.

The detection limit value of the method was 0.09 μg/mL and the method was precise(CV%= 0.5%; n=10). Recovery values of caffeic acid from spiked green coffee samples were between 88.3 and 93.2%.

INTRODUCTION

Chlorogenic acids (CGA) are a group of compounds well represented in coffee beans (5-10%).[1,2] Chemically, CGAs comprise a group of esters of quinic acid with some specific phenols, mainly caffeic, ferulic, and p-coumaric acids known collectively as hydroxycinnamic acids (HCAs) The real contribution of these compounds to flavor in roasted coffee and the real importance in coffee quality remains not well understood.[1,3] By enzymatic or alkaline hydrolysis, HCAs can be obtained from endogenous CGAs in green coffee and can be of importance in its quality control.[4,5]

Monitoring hydroxycinnamic acids in coffee can be useful, particularly for the coffee industry, both for assessing their levels in raw materials before and after roasting and also to help the definition of authenticity of the commercial coffee varieties and for a possible characterisation of their geographical origins.[4-6] Due to their significative absorption and characteristic shape under UV, spectrophotometric methods are the most frequently used for global determinations of CGAs. Discrimination and specification of CGAs and their isomers are achieved by specific colorimetric reactions[1] and by HPLC techniques, respectively.[7,8,9] However, the methodologies for individual HCAs are quite scarce in literature; thus an adequate methodology is required. The aim of this paper, herein, is to describe an adequate, simple, reproducible, and accurate technique for simultaneous determination of seven phenolic acids in green coffee samples: caffeic, p-coumaric, ferulic, o-coumaric, 3,4-dimethoxycinnamic, 3,4,5-trimethoxycinnamic and 4-methoxycinnamic.

MATERIALS AND METHODS

Coffee Samples and Standards

Green Arabica (Honduras) and green Robusta (Uganda) coffee beans were supplied by the coffee industry. Green coffee beans were ground in a hammer mill to pass 0.8 mm.

Caffeic acid, p-coumaric acid, ferulic acid, o-coumaric acid, 3,4-dimethoxycinnamic acid, 3,4,5-trimethoxycinnamic acid, 4-methoxycinnamic acid were obtained from Sigma Chemical Co.

Extraction of Phenolic Acids from Coffee

A 5 g portion of green coffee bean samples, finely powdered, was blended with 60 mL of methanol/water (40/60),[9] during 24h. The mixture was filtered and the filtrate concentrated under vacuum (40°C) to a volume of 5 mL. This solution was hydrolysed with 5 mL of 2N NaOH for 240 min. The pH of the mixture was adjusted to pH 7.00 with 2N HCl and the phenolic acids were extracted by liquid/liquid extraction with ethyl acetate (20mLx3). The extracts were then combined and the ethyl acetate removed under reduced pressure. The residue was dissolved in 7 mL of methanol and 20 µl were analysed by HPLC.

HPLC Analysis of Phenolic Acids

Separation of phenolic acids was achieved with an analytical HPLC unit (Gilson), using a reverse phase Spherisorb ODS2 (5µm, particle size; 25.0 x 0.46 cm) column. The solvent system used was a gradient of water-formic acid (19:1) (A) and methanol (B). The gradient was as follows: 0'- 15% B, 10'-25% B, 25'-30 % B, 30'-35% B, 34'-50% B, 41'-70% B, 43'-75% B, 47'-80% B. Elution was performed at a solvent flow rate of 0.9 mL/min. Detection was accomplished with a diode-array detector, and chromatograms were recorded at 320 nm.

The different phenolic acids were identified by chromatographic comparisons with authentic standards and by their UV spectra. Quantification was based on the external standard method.

RESULTS AND DISCUSSION

Analytical Curve and Detection Limit

Under the assay conditions described, a linear relationship between the concentration of phenolic acids and the UV absorbance at 320 nm was obtained. This linearity was maintained over the concentration range 4-400 µg/mL. The correlation coefficient for each standard curve invariably exceeded

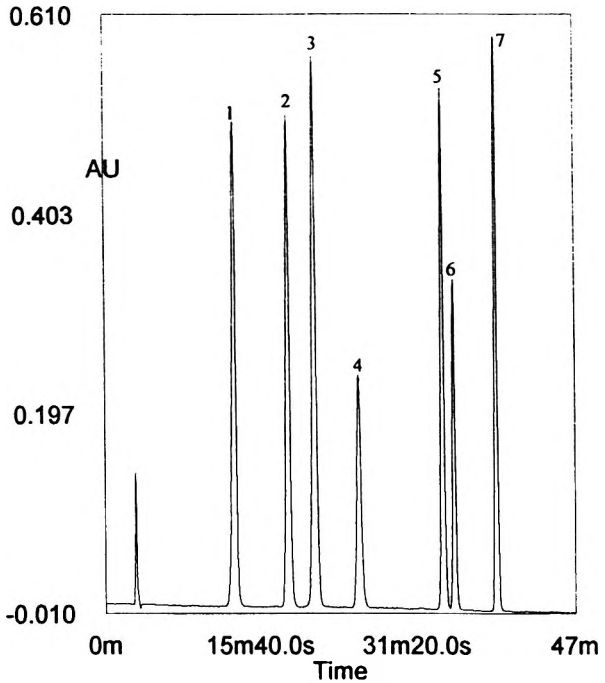


Figure 1. HPLC profile of a standard solution of phenolic acids. Detection at 320 nm. (1) Caffeic acid; (2) *p*-coumaric acid; (3)Ferulic acid; (4)*o*-coumaric acid; (5)3.4-dimethoxycinnamic acid; (6)3.4.5-trimethoxycinnamic acid; (7) 4-methoxycinnamic acid.

0.99 for all phenolic acids. The calibration curves for phenolic acids were obtained by triplicate determinations of each of the calibration standards; the peak area values (arbitrary units) were plotted as average values. The relative percent average deviations of triplicates were less than 2% in all cases. The average regression equation for caffeic, p-coumaric, ferulic, o-coumaric, 3,4-dimethoxycinnamic, 3,4,5-trimethoxycinnamic, and 4-methoxycinnamic acids were: $y=3.62 \times 10^8x + 131114.3$, $y = 3.46 \times 10^8x + 13614836$, $y = 4.10 \times 10^8x + 151863.6$, $y = 2.23 \times 10^8x + 60385.32$, $y = 2.67 \times 10^8 + 172406.5$, $y = 1.87 \times 10^8x + 68167.15$, and $y = 2.37 \times 10^8x + 140739.3$, respectively.

Given the similarity of the chemical structures of the acids analysed and, therefore their UV spectra and absortivity, the detection limit and precision of the method were determined only for caffeic acid. The detection limit value was calculated for caffeic acid as the concentration corresponding to three times the standard deviation of the background noise and was 0.09 μg/mL.

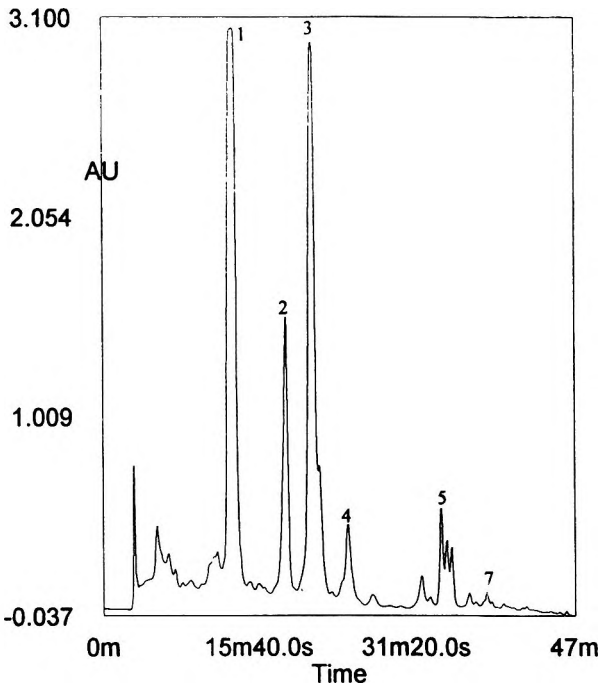


Figure 2. HPLC phenolic acids profile of a green Arabica coffee sample from Honduras. Detection at 320 nm. (1) Caffeic acid; (2) *p*-coumaric acid; (3)Ferulic acid; (4)*o*-coumaric acid; (5)3.4-dimethoxycinnamic acid; (7)4-methoxycinnamic acid.

Validation of the Method

The chromatogram obtained for standard phenolic acids solution is shown in Figure 1. The retention times (RT) obtained for phenolic acids were: RT 12m33s for caffeic acid, RT 18m33s for p-coumaric acid, RT 21m7s for ferulic acid, RT 24m56s for o-coumaric acid, RT 33m55s for 3,4-dimethoxycinnamic acid, RT 34m49s for 3,4,5-trimethoxycinnamic acid, and RT 38m13s for 4-methoxycinnamic acid.

As an example, Figure 2 shows the HPLC phenolic acid profile of a green coffee sample from Honduras. The unidentified compounds had identical UV spectra when recorded with a diode-array detector, with identical shape and maximum at 320 nm, which suggested that they could be hydroxycinnamic acids. Results from quantification applied to two samples (from Honduras and Uganda) are shown in Table 1.

Table 1

Phenolic Acids Content in Green Coffee Samples[a]

	Honduras (Coffea Arabica) g/kg ± SD	Uganda (Coffea Robusta) g/kg ± SD
Caffeic	1.051 ± 0.0007	1.125 ± 0.01
p-coumaric	0.340 ± 0.03	0.302 ± 0.007
Ferulic	0.841 ± 0.0007	1.153 ± 0.05
o-coumaric	0.200 ± 0.005	0.165 ± 0.005
3,4-dimethoxycinamic	0.056 ± 0.0042	0.489 ± 0.02
3,4,5-trimethoxycinamic	------	0.734 ± 0.05
4-methoxycinamic	nq	------

*Values are expressed as mean ± SD of three determinations.
nq - Not quantified.

Table 2

Recovery of Caffeic Acid from a Spiked Green Coffee Sample

Present (g/kg)	Added (g/kg)	Found[a] (g/kg)	Standard Deviation	CV (%)	Recovery (%)
	0.2	1.047	0.05	7.6	93.2
0.924	0.3	1.141	0.02	1.7	93.2
	0.4	1.169	0.03	2.5	88.3

[a]Mean value found for 3 assays for each studied concentration

The precision of the analytical method was evaluated by measuring the peak chromatographic area of caffeic acid, 10 times on the same sample. The standard deviation was 0.006 and the coefficient of variation was 0.5%. In order to study the recovery of the procedure, and bearing in mind that the method includes an alkaline hydrolysis, one coffee sample was added to known quantities of 5-caffeoylquinic acid and recovery percentage calculated as caffeic acid. The sample was analysed in triplicate before and after the addition

of 5-caffeoylquinic acid. Thus, this procedure demonstrated the effectiveness of the extraction and hydrolysis step and the accuracy of the proposed method. The results are listed in table 2. Recovery values were between 88.3 and 93.2%.

In conclusion, this study suggests that the technique herein is quite useful for the analysis of phenolic acids in samples, allowing the separation and quantification of the main coffee phenolic acids. This technique can be useful to help in the differentiation of coffee varieties and, hypothetically, for the characterisation of their geographical origin.

ACKNOWLEDGMENT

The authors are grateful to JNICT - Project PBIC/C/TPR/2565/95 for financial support of this work.

REFERENCES

1. M. N. Clifford, "Chlorogenic Acids", In **Coffee Vol I : Chemistry**, R. J. Clarke, R. Macrae, Eds., Elsevier Applied Science Publishers, Ltd., London 1985.

2. M. N. Clifford, T. Williams, D. Bridson, Phytochemistry, **28(3)**, 829-838 (1989).

3. C.W. Nagel, I. W. Herrick, W. R. Graber, J. Food Science, **52 (1)**, 213 (1987).

4. J. J. R. Rakotomalala, E. Cros, A. Charrier, ASIC, 15^{e} Colloque, Montpellier, 47-53, (1993).

5. J. J. R. Rakotomalala, E. Cros, A. Charrier, F. Anthony, M. Noirot, ASIC, 15^{e} Colloque, Montpellier, 637-643, (1993).

6. C. P. Bicchi, A. E. Binello, M. M. Legovich, G. M. Pellegrino, A. C. Vanni, J. Agric. Food Chem., **41**, 2324-2328 (1993).

7. L. C. Trugo, R. Macrae, Food Chemistry, **15**, 219-217 (1984).

8. L. C. Trugo, R. Macrae, Analyst, March, **109**, 263-266 (1984).

9. C. P. Bicchi. A. E. Binello, G. M. Pellegrino, A. C. Vanni, J. Agric. Food Chem., **43**, 1549-1555 (1995).

Received December 1, 1996
Accepted December 18, 1996
Manuscript 4338

J LIQ. CHROM. & REL. TECHNOL., 20(13), 2031–2039 (1997)

MOBILE PHASE FLOW PROGRAMMING IN LIQUID CHROMATOGRAPHY USING SHORT NARROW BORE COLUMNS

Y. He and H. K. Lee*

Department of Chemistry
National University of Singapore
Kent Ridge
Republic of Singapore 119260

ABSTRACT

Application of mobile phase flow programming to effect fast liquid chromatographic analysis of multi-components sample in short narrowbore columns was investigated. It was found that the generally accepted limited power of flow programming can, nevertheless, be enhanced by the right configuration of operating parameters, such as column I.D. and length, particle size of packing material, mobile phase, detector cell volume, etc. Average reproducibility of flow programming in the separation of five aromatic compounds were 0.18 % in retention time and 6.0 % in peak area. Initial flow rate was restored within 1 minute, all of which are favourable for reliable and fast analysis in routine HPLC laboratory.

INTRODUCTION

In the chromatographic separation of complicated samples containing components with great difference in capacity factor, chromatographers usually employ mobile phase gradient elution or temperature-programming (especially

in GC). Flow programming is often considered limited and has rarely been used. Among the limited number of publications on flow programming, most are on its application in GC with few in HPLC.[1-4]

Narrow bore columns (2.0 mm I.D) are columns whose diameter are between that of microbore (<1.0 mm I.D.) and conventional columns (4-5 mm I.D.). With special size, narrow bore columns have some combined characteristics possessed by microbore and conventional columns. Narrowbore-LC can be easily implemented on conventional equipment with little modification, while maintaining some advantages possessed by micro-LC, such as reduction in solvent and stationary phase consumption, and higher mass sensitivity.[5-9]

This paper reports the application of flow programming in LC using a 10 cm x 2.0 mm I.D. narrowbore column. We discuss the use of the appropriate combination of column length and I.D., particle size of packing material, mobile phase, and flow cell volume in order to achieve fast separation of some aromatic compounds.

EXPERIMENTAL

Instrumentation and Reagents

Chromatography was carried out with a JASCO (Tokyo, Japan) Model PU980 pump, a Model 7825 Rheodyne (Cotati, CA, USA) injection valve equipped with 5-μL loop, and a programmable Model 786A UV absorbance detector (Applied Biosystems, Foster City, CA, USA), connected to a Shimadzu (Tokyo, Japan) Model CR-6A integrator. A prepacked (C_{18}) short narrowbore column (100 mm x 2.0 mm I.D.) (Upchurch, WA, USA) was used.

Alkylphenols were purchased from Fluka (Buchs, Switzerland)). Benzene, toluene, ethylbenzene, acenaphthene, dibenz[*a,h*]anthracene were obtained from Sigma (St. Louis, MO, USA). LC-grade methanol, acetonitrile, and water from a MilliQ purification system (Millipore, Bedlford, MA, USA) were employed.

RESULTS AND DISCUSSION

The successful application of flow programming for faster analysis requires the appropriate configuration of the LC system. It is known that

solvent gradient elution can be easily implemented in conventional columns (e.g. 25 cm x 4.6 mm I.D.), but much more difficult in microbore columns (I.D. < 1 mm). On the other hand, temperature programming can be easily performed in microbore columns (small heat capacity), but more difficult in conventional columns (large heat capacity). With regards to flow programming, it can be more easily and effectively implemented in short narrowbore columns (e.g. 100 mm x 2.0 mm I.D.).

Firstly, a short narrowbore column with plate numbers around 4000 is efficient enough for many routine analyses whose requirements for efficiency are usually within the range of several hundred to several thousand plates. Using a long standard column with much higher efficiency is unnecessary and is sometimes a "waste" of column efficiency, analysis time, and solvent consumption. Secondly, a short narrowbore column has lower resistance which makes it possible for flow programming to be used across a wider range of flow rates and allows for a more rapid change in flow rate. Thirdly, a much lower volumetric flow rate (about 5 times lower than that in a conventional column) is required to generate high linear velocity in a narrowbore column. A high programming rate can be more easily achieved with great reduction in solvent consumption. Further, low volumetric flow rates (often less than 0.8 mL/min) cause less damage to pumps, extending their lifespans, and reducing mechanical trouble. At above the optimal flow rate, the HETP curve for a narrowbore column is flatter than for a conventional column, allowing for increasing flow rate without much compromising separation, especially for late-eluting bands.[9] In addition, a narrowbore column is compatible with most conventional HPLC equipment, unlike a microbore column putting stringent demands on many parts in HPLC.

Particle sizes of 3μm, 5μm and 10μm of LC packing materials are commonly available. Short columns packed with 3-μm particles provide greater resolving power per column length. The column can be run at higher flow rates with little reduction in the number of theoretical plates.[10] The 3-μm column, however, is not without disadvantages. It is often run at much higher back pressure, which is caused by fines present in the particle size distribution. Usually 0.5-μm porosity column inlet frits are used; these can plug easily and cause excessive back pressure, leading to short column lifetimes. In addition, the column often exhibits >3 - 3.5 d_p plate height, greater than 2 - 2.5 d_p plate height typical of well-made commercial columns packed with 5-μm particles. Finally, the sharp, low volume peaks are often degraded because of extra-column effects.[10] A short narrowbore column packed with 10-μm particles often exhibits inadequate efficiency for some routine separations. Further, the plate height increases more rapidly with

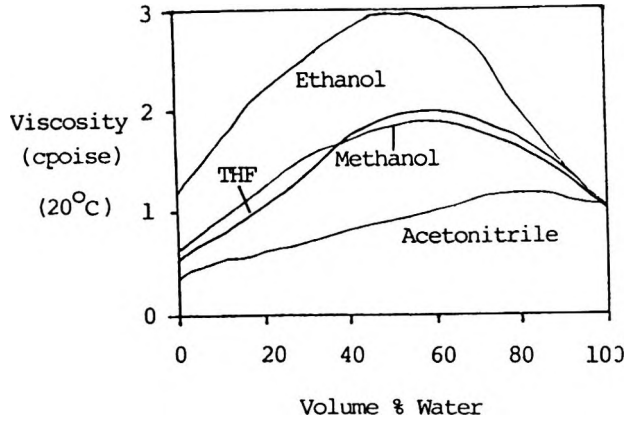


Figure 1. Viscosity curves of water:organic solvent mixture

velocity, which makes the column inappropriate for high speed analysis, although it is a preferred choice in the view of low flow resistance. In the present work, 5 μm was chosen as the size of packing material. This represents a good compromise when considering column efficiency, back pressure, and extra-column dispersion effects.

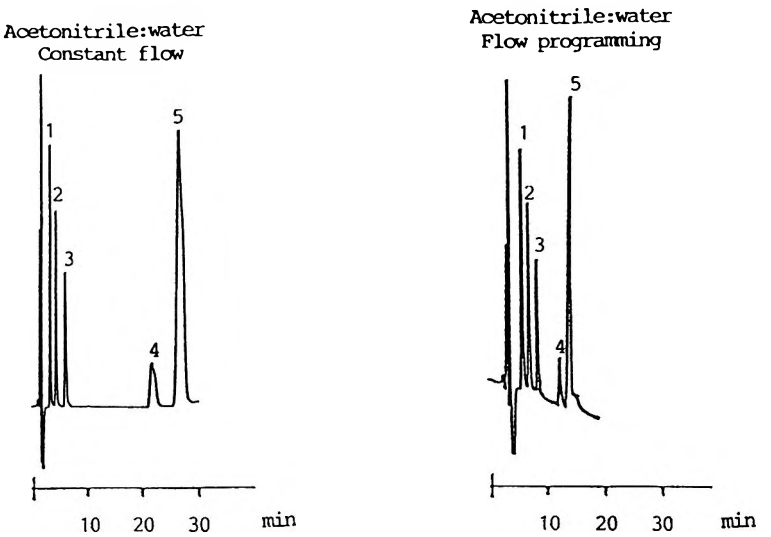
Emphasis in the following was placed on choosing an appropriate solvent system and testing the effects of detector cell volume on flow programming.

In mobile phase flow programming, the programming range is often limited by the back pressure that the pump, injector and column can handle. For routine operation, the back pressure should be less than 197 atm (2900 psi). The viscosity of the eluent has a great effect on the system back pressure. Acetonitrile has the lowest viscosity among the four most popular organic solvents (the other three being methanol, ethanol, and tetrahydrofuran), used in reversed-phase LC. Further, the viscosity of an acetonitrile-water eluent varies only slightly within the whole range of different acetonitrile-water ratios with

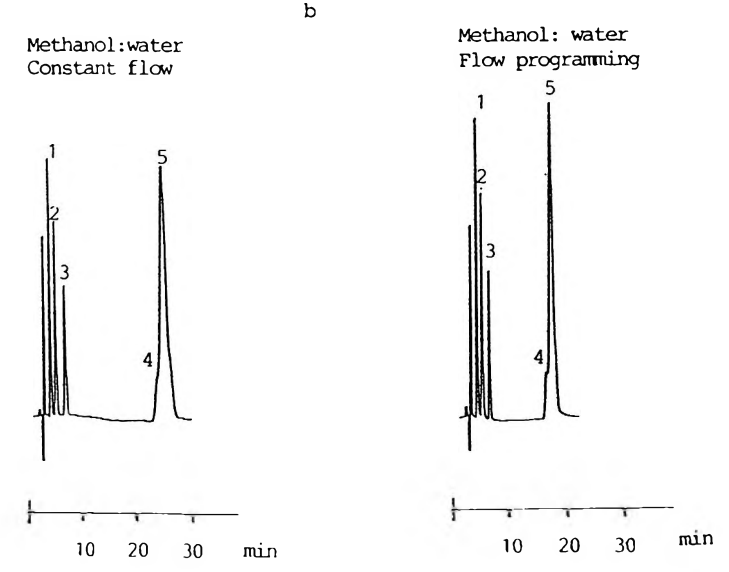
---

Figure 2. **(right)** Comparison of flow programming LC and constant flow LC. Separation of 5 alkyphenols using two different mobile phases: acetonitrile:water (54%:46%) and methanol:water (68%:32%). Column: 100 mm x 2.0mm I.D.; stationary phase: C_{18} (5- μm particle size). Peaks: 1) *p*-Ethylphenol, 2) *p*-Propylphenol, 3) *p*-Butylphenol, 4) *p*-Heptylphenol , 5) *p*-Octylphenol.

a


Acetonitrile:water
Constant flow
1
2
3
4
5
10
20
30
min
Acetonitrile:water
Flow programming
1
2
3
4
5
10
20
30
min

b


Methanol:water
Constant flow
1
2
3
4
5
10
20
30
min
Methanol: water
Flow programming
1
2
3
4
5
10
20
30
min

back pressure remaining low (Fig. 1). Hence, mobile phase flow programming can be performed satisfactorily within almost the whole range of mixing ratios without sacrificing selectivity.

Figure 2 shows the chromatograms of 5 alkylphenols obtained by constant flow and flow programming modes, for acetonitrile:water (54%:46%) and methanol:water (68%:32%) eluent mixtures. The two mobile phases □□are similar solvent strengths and selectivities for early eluting peaks. The viscosity of acetonitrile:water is, however, only about half of that of methanol:water. As shown in Fig. 2, flow programming when acetonitrile-water eluent is used can be performed across a wider flow rate range (0.1 - 0.6 mL/min) with pressure still below 200 atm (2940 psi). Flow programming is effective in reducing analysis time by half whilst maintaining the resolution of the three early- eluting peaks. On the other hand, as shown in Fig. 2b, with methanol-water, flow programming can be performed only within a narrow range of 0.1 - 0.3 mL/min when the back pressure is over 200 atm. Analysis time is only slightly reduced compared with the constant flow mode. Further, the last two bands are baseline separated when acetonitrile-water is used, but not when methanol-water is used. In this case, acetonitrile-water mobile phase is preferred to methanol-water not only insofar as the flow programming range is concerned, but also in terms of selectivity.

In the miniaturized HPLC, the achievement of theoretical limit of speed is limited by the extra-column dispersion which comes primarily from the injector, detector, and connecting tube. The external column band broadening becomes very critical, especially for early elution peaks with low k' values, when column internal diameter, length and particle size are decreased.[9] Hence, appropriate choice of detector cell volume, injection volume, and connecting tube is necessary in order to obtain the maximum performance from short narrow-bore column with flow programming. In this work, we investigated the effect of detector cell volume.

Figure 3 shows the chromatograms of five aromatic compounds. As can be seen, early-eluting peaks are unresolved using constant flow mode with a detector cell volume of 12 μL (Fig. 3a). Improvement in the resolution of these coincident peaks, and reduction in analysis time were obtained by using flow programming together with a smaller-volume flow cell (2.4 μL) (Fig. 3b).

In flow programming (Fig. 3b), the initial flow rate was restored and stabilized within 1 minute, which was much shorter than that required in solvent gradient elution and temperature programming operations. Taking this

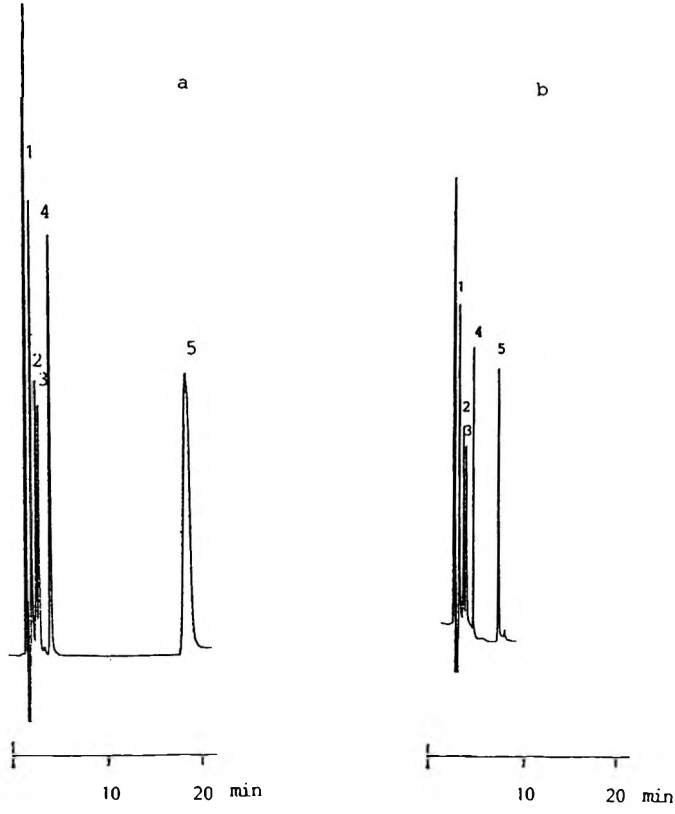


Figure 3. Effect of detector cell volume on flow programming LC. Conditions: (a) 12-μL cell with constant flow at 0.2 mL/min (b) 2-μL cell with flow programming: initial 0.20 mL/min, then increasing to 0.40 mL/min after 4 min., then further increase to 0.8 mL/min after 6 min. Peaks: 1) Benzene, 2) Toluene, 3) Ethyl benzene, 4) Acenaphthene, 5) Dibenz[*a*,*h*,]-anthracene.

into consideration, the total analysis time is in some cases even shorter than those required for solvent gradient and/or temperature programming.

The retention time and concentration reproducibilities for 12 consecutive flow-programmed runs were calculated, and showed an average relative standard deviation of 0.18 in retention time and 6.0 % in peak area, which are favourable for reliable routine analysis.

CONCLUSION

Compared with conventional or microbore columns, short narrowbore columns with flow programming provide a more practical configuration for fast analysis of multi-component samples in a routine HPLC lab. The power of flow rate programming, though still limited, can be expanded through the appropriate combination of some operating parameters, including column length, I.D, particle size of packings, solvent type, and detector cell volume. In routine analysis where the sample is not very complicated, flow programming with short narrowbore column can be used as an alternative to temperature programming and solvent gradient elution while providing simplicity in operation, better reproducibility of chromatographic behaviour, and even shorter overall analysis times in some cases.

ACKNOWLEDGMENTS

The authors thank the National University of Singapore for financial support of the work.

REFERENCES

1. S. Nygren , J. H.R.C.&.C.C., **2**, 319 (1979).

2. S. Nygren, J. Chromatogr., **142**, 109 (1977).

3. L. R. Snyder, **Introduction to Modern Liquid Chromatography**, John Wiley, Inc, N.Y., 1979, Chapter 16.

4. R. C. Nielson, J. Liq. Chromatogr., **16**,1625 (1993).

5. R. P.W. Scott, J. Chromatogr., **23**, 233 (1985).

6. K. Slais, D. Kourilova, J. Chromatogr., **258**, 57 (1983).

7. M. Krejci, K. Slais, A. Kunath, Chromatographia, **22**, 311(1986).

8. M. Krejci, V. Kahle, J. Chromatogr., **392**,133 (1987).

9. N. Vonk, W. P. Verstaeten, J. W. Marinissen, J. Chromatogr. Sci., **30**, 296 (1992).

10. J. J. Kirkland, J. Chromatogr. Sci., **31**, 493 (1993).

Received June 26, 1996
Accepted July 17, 1996
Manuscript 4218

J. LIQ. CHROM. & REL. TECHNOL., 20(13), 2041-2051 (1997)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR CISATRACURIUM AND ITS METABOLITES IN HUMAN URINE

B. J. Bryant, Charles D. James, Jr.,
D. Ryan Cook, Jane Croft Harrelson

Bioanalysis and Drug Metabolism
GlaxoWellcome, Inc.
Research Triangle Park, NC 27709

ABSTRACT

An HPLC assay for the analysis of cisatracurium and its metabolites, laudanosine and the cis-monoquaternary alcohol (MQA), in human urine was developed. The assay involved solid-phase extraction followed by HPLC analysis on a strong cation exchange column with fluorescence detection. Linear regression was used to quantitate cisatracurium and its metabolites over the concentration ranges 10 to 2000 ng/mL for cisatracurium and 10 to 1000 ng/mL for the metabolites. The lower limit of quantitation for the assay was set at 10 ng/mL. The accuracy of the assay ranged from 6.6 to 17.0% for cisatracurium, -1.3 to 2.7% for laudanosine, and 0.2 to 3.4% for MQA. The precision of the assay ranged from 4.7 to 14.0% for cisatracurium, 4.6 to 7.7% for laudanosine, and 3.3 to 7.4% for MQA. The assay is specific for cisatracurium and its metabolites and no interfering endogenous peaks were observed in human urine. Cisatracurium, laudanosine and MQA were stable in acidified human urine (-20°C) for at least 9 months.

Cisatracurium and its metabolites were also stable in acidified stock solutions and in urine after three freeze-thaw cycles. The mean recoveries for cisatracurium, laudanosine, MQA, and the internal standard from human urine were 104.4, 91.9, 91.9 and 93.4%, respectively.

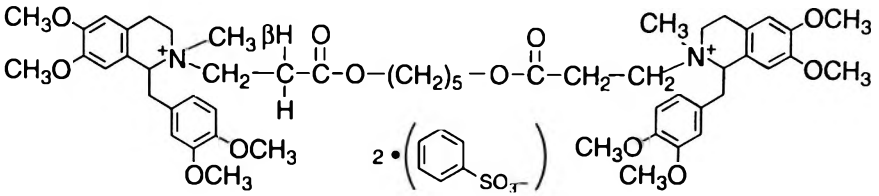
INTRODUCTION

Cisatracurium, (1*R*, 2*R*)-2,2'-[pentamethylenebis(oxycarbonylethylene)] bis- (1,2,3,4-tetrahydro-6,7-dimethoxy-2-methyl-1-veratrylisoquinolinium) dibenzenesulfonate, (Figure 1), is an investigational intermediate-acting nondepolarizing neuromuscular blocking agent.[1] Cisatracurium is one of ten isomers contained in atracurium and represents approximately 15 percent of the atracurium mixture. Cisatracurium has similar neuromuscular blocking properties to atracurium.[2] However, it is more potent and is significantly weaker as a histamine releaser than atracurium. *In vitro* studies have shown that cisatracurium spontaneously degrades at physiological pH via Hofmann elimination to form laudanosine (Figure 1).[1] In human plasma, cisatracurium is also metabolized by esterases to the monoquaternary alcohol. The other potential product of ester hydrolysis, the monoquaternary acid, has not been detected *in vitro* in human plasma.[1] A high-performance liquid chromatography (HPLC) method was developed to quantitate cisatracurium and two of its metabolites in human plasma.[3,4] The assay was adapted for human urine to support clinical studies designed to investigate the excretion of cisatracurium and the two metabolites. This report describes the accuracy, precision, and specificity of the assay and the stability of cisatracurium and its metabolites in human urine.

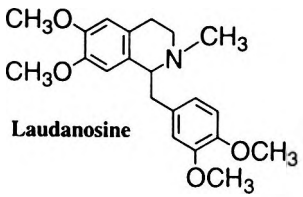
MATERIALS AND METHODS

Chemicals

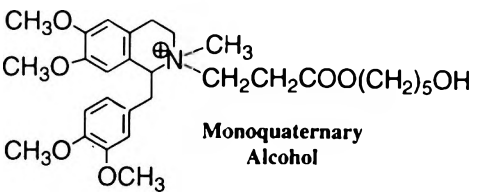
Cisatracurium and the cis-monoquaternary alcohol (MQA) were synthesized at the Wellcome Foundation, Dartford, Kent, UK. Laudanosine was purchased from Aldrich Chemical Co., (Milwaukee, WI). The internal standard, N-methyl laudanosine, was synthesized at the Wellcome Foundation, Dartford, Kent, UK. High purity sulfuric acid was purchased from GFS Chemicals (Columbus, OH). All solvents used were HPLC grade (Omnisolv, EM Science, Cherry Hill, NJ).



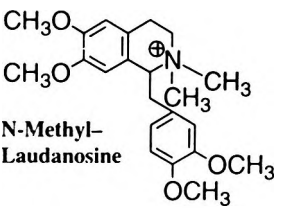
Cisatracurium



Laudanosine



Monoquaternary Alcohol



N-Methyl–Laudanosine

Figure 1. HPLC chromatograms of cisatracurium, its metabolites and internal standard.

Instrumentation

The HPLC system used consisted of either a Hewlett Packard model 1090 with autosampler and column heater (Hewlett Packard, Avondale, PA) or a Waters Model 600 multisolvent delivery system with a model 712 WISP injector (Waters Associates., Milford, MA) and a Systek thin foil heating system (Systek, Minneapolis, MN). A Hewlett Packard model 1046A fluorescence detector was used with both systems. The fluorescence excitation and emission wavelengths were optimized at 230nm for excitation and 315nm for emission. A Spherisorb® strong cation exchange (SCX) HPLC column (250 mm x 4.6 mm, Phase Separations, Norwalk,CT) was used for the analyses. The column was maintained at 50°C. The HPLC system was programmed to deliver acetonitrile:7 mM sodium sulfate in 0.5 mM sulfuric

acid (6:4,v:v) for 18 min, followed by acetonitrile:56 mM sodium sulfate in 0.5 mM sulfuric acid (6:4,v:v) for 11 min, returning to the initial condition to re-equilibrate for 5.5 min. The flow rate was 2 mL/min and the column temperature was 50°C. Data were collected and analyzed with VG Multichrom software (VG data systems, Altrincham, Cheshire, UK) on a VAX 6000-320 computer (Digital Equipment Corp., Maynard, MA).

Preparation of Calibration Standards and Control Samples

Urine samples containing cisatracurium must be acidified to prevent the degradation by Hofmann elimination and ester hydrolysis.[2] Calibration standards and quality control samples were prepared by adding aliquots of the standard solutions to acidified control urine. For every 1 mL of urine, 4 mLs of 0.05M citrate buffer (pH 3.05) were added. Calibration standards ranged from 10 to 2000 ng/mL for cisatracurium and 10 to 1000 ng/mL for laudanosine and MQA. Urine quality control samples spiked at five concentrations (50, 500, 1000, 5000, and 10,000 ng/mL for cisatracurium and 50, 250, 500, 2500, and 5000 ng/mL for laudanosine and MQA) were prepared by adding aliquots of standard solutions (prepared separately from the calibration standards) to acidified control urine. The control samples were divided into 3.5 mL portions and stored in polypropylene tubes at -20°C. Prior to analysis, the two highest concentrations were diluted 1:10 with citrate buffer to fit the calibration curve range.

Extraction Procedure

Calibration standards, spiked control samples, and study samples were processed as follows. Acidified urine samples (1.5 mL) and internal standard (75 μL of a 1 mg/mL solution) were combined in a polypropylene tube. Samples were mixed, allowed to equilibrate for 5 minutes and then centrifuged at 3000 x g for 10 minutes. Phenyl Bond Elut® cartridges were conditioned with acetonitrile and 5 mM sulfuric acid (1 mL of each).

Acidified urine samples (1 mL of the supernatant) were loaded onto the cartridges. Cartridges were washed with 5 mM sulfuric acid and 1:1 methanol:water (1 mL each), and cisatracurium and its metabolites eluted with acetonitrile:80 mM sodium sulfate in 5 mM sulfuric acid (6:4,v:v), (0.6 mL). The eluate volumes were reduced under nitrogen and then transferred to polypropylene vial inserts for HPLC analysis.

Calculations

The peak height ratios of each compound and internal standard were calculated from the calibration standards and the data fitted to a least-squares linear-regression model. The concentrations of the analytes were calculated from the regression parameter estimates obtained from the calibration curve.

Assay Validation

The appropriate regression model for the assay was determined by analyzing replicate spiked calibration standards (10 to 2000 ng/mL for cisatracurium; 10 to 1000 ng/mL for laudanosine, n=6 at each of 6 concentrations). The concentration-peak height data were fitted to a least-squares linear regression model (LSLR) with four weighting schemes (unweighted, $1/c$, $1/c^2$, and log-log transformed). The residuals at each concentration were calculated, and a plot of the Studentized residuals versus concentration was used to determine the appropriate model and weighting.

The accuracy and precision of the method were estimated by assaying spiked urine samples at five concentrations (50, 500, 1000, 5000, and 10000 ng/mL for cisatracurium and 50, 250, 500, 2500, and 5000 ng/mL for laudanosine and MQA).

One-way analysis of variance (ANOVA) was used to partition the total observed variance of the assay into its two components, within-assay variance (random error) and between-assay variance (error associated with differences in day-to-day conditions). Precision was expressed as the coefficient of variation (CV) of the means of these runs. Accuracy was calculated as the percentage difference between the mean calculated concentration and the amount added (% bias).

Precision: CV = Standard deviation/mean x 100

$$\text{Accuracy: \%Bias} = \frac{\text{Mean measured value - Theoretical value}}{\text{Theoretical value}} \times 100$$

The stability of cisatracurium and its metabolites in acidified human urine stored at -20°C was determined. Urine spiked at five concentrations (50, 500, 1000, 5000, and 10000 ng/mL for cisatracurium and 50, 250, 500, 2500, and 5000 ng/mL for laudanosine and MQA) was assayed on the day of preparation and at various time intervals after storage at -20°C.

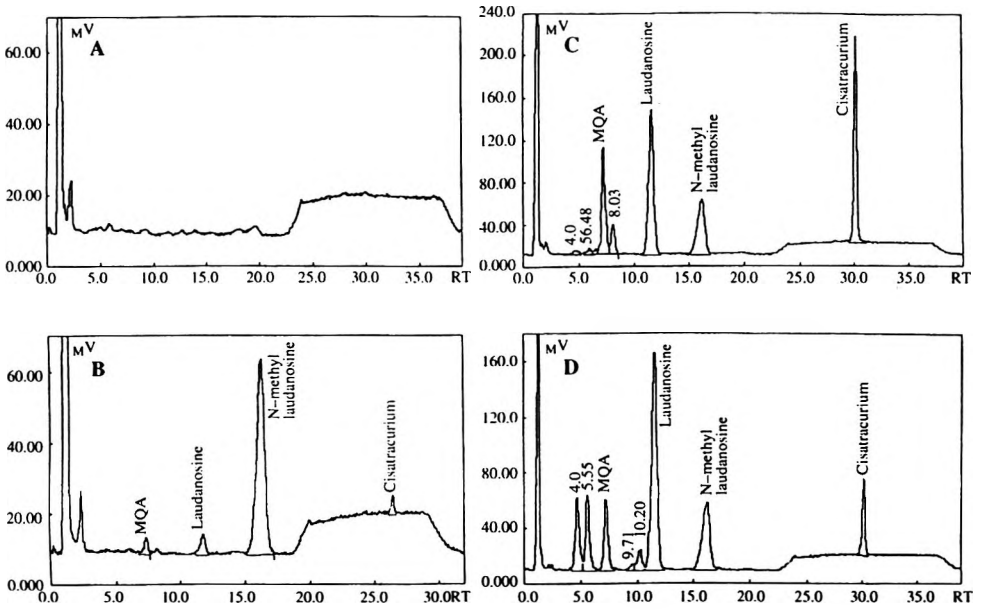


Figure 2. HPLC Chromatograms of Cisatracurium and Its Metabolites. a) Chromatogram of extracted acidified blank human urine. b) Chromatogram of extracted acidified blank urine spiked with Cisatracurium and its metabolites (10 ng/mL of each). c) Chromatogram of extracted QC sample spiked with 500 ng/mL of Cisatracurium and 250 ng/mL of each metabolite. d) Chromatogram of extracted patient urine sample 109-203. Sample collected from 5 t 10 hours after a 7.2 mg dose was received.

The stability of cisatracurium and its metabolites was also determined in stock solutions stored at 4°C, and in urine samples after three freeze-thaw cycles. The extraction efficiency of the assay was determined by comparing the peak heights for each analyte in extracted standards with those obtained by the injection of unextracted standards.

Biomedical Application

A clinical study was designed to investigate the safety, efficacy and pharmacokinetics of cisatracurium in human volunteers.[5] During the surgical procedure, volunteers received an intravenous bolus dose of 0.1 mg/kg cisatracurium.

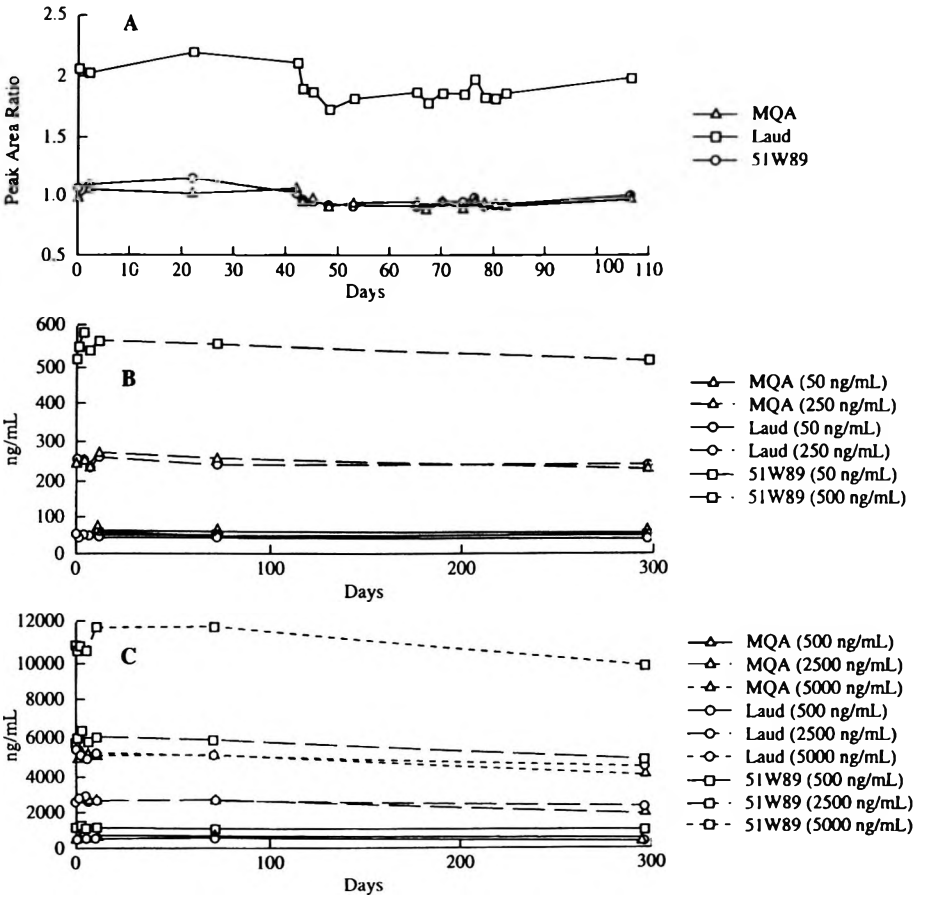


Figure 3. Stability of 51W89, laudanosine, and MQA. a) Stock solutions (2 μL of a 10μL solution). b) Human urine. c) Human urine.

Urine samples were collected at 5-hour intervals for 10 hours after cisatracurium administration via an in-dwelling urinary catheter. Urine samples were stored at -20°C until analyzed. Urine was collected into bottles containing sodium citrate buffer, pH 3.1.

At the end of the collection period, the total volume was measured and a 50 mL aliquot removed for the determination of cisatracurium and its metabolites.

Table 1

Recovery of Cisatracurium, Its Metabolites, and the Internal Standard from Human Urine

Compound	Concentration (ng/mL)	% Recovery (mean ± S.D.)
Cisatracurium	1.00.0	108.3 ± 5.3
	1000.0	100.4 ± 2.2
Laudanosine	100.0	92.1 ± 5.9
	250.0	93.3 ± 8.6
	500.0	90.2 ± 8.2
MQA	100.0	92.7 ± 5.0
	250.0	94.7 ± 9.2
	500.0	88.4 ± 10.7
N-methyl-laudanosine	25.0	90.7 ± 1.7
	50.0	96.0 ± 10.2
	100.0	93.4 ± 5.5

(n=3 at each concentration)

RESULTS AND DISCUSSION

Figures 2a - d show representative chromatograms of blank urine, urine spiked with cisatracurium and its metabolites near the lower limit of quantitation (10ng/mL), urine quality control, and urine from a patient who received cisatracurium. The retention times of cisatracurium, the internal standard, laudanosine and MQA were typically 30, 16, 11.5, and 7.5 min, respectively, and no endogenous interferences were noted.

Residual plots from the calibration curve data fitted to an LSLR model indicated that the variance associated with the response (peak height) was homogeneous throughout the concentration range when the data were weighted by 1/(concentration).[2]

Table 2

Accuracy and Precision Data for the Assay in Human Urine - Results of ANOVA

Conc. Nominal ng/mL	Conc. Assayed ng/mL	Std. Dev.	Overall CV(%)	Within-Day CV(%)	Between Day CV(%)	Bias (%)
Cisatracurium						
50	53.1	3.6	6.8	5.0	4.6	6.2
500	551.7	10.5	5.1	3.0	4.2	10.3
1000	1091.0	127.9	11.7	11.7	0.0[a]	9.1
5000	5848.4	274.8	4.7	3.7	2.9	17.0
10000	11137.3	1554.4	14.0	14.0	0.0[a]	11.4
Laudanosine						
50	49.9	3.8	7.7	7.7	0.0[a]	-0.2
250	250.9	11.5	4.6	1.8	4.2	0.4
500	493.3	22.8	4.6	4.3	1.6	-1.3
2500	2566.6	134.6	5.3	5.3	0.0[a]	2.7
5000	5026.9	375.2	7.5	7.5	0.0[a]	0.5
MQA						
50	51.7	3.2	6.2	5.9	1.7	3.4
250	255.6	14.1	5.5	5.3	1.5	2.2
500	501.1	16.4	3.3	2.1	2.5	0.2
2500	2581.1	146.3	5.7	5.7	0.0[a]	3.2
5000	5068.6	373.9	7.4	7.4	0.0[a]	1.4

(n=5 assays)

[a]ANOVA estimated this as zero because within-day variability was significantly larger than between-day variability.

Results from the residual plots also indicated the assignment of the upper and lower limits of quantitation which were 2000 and 10 ng/mL for cisatracurium and 1000 and 10 ng/mL for laudanosine and MQA. The concentrations of cisatracurium, laudanosine and MQA in acidified stock solutions stored at 4°C were stable for at least 3 months.

Table 3

Urinary Excretion Data from a Clinical Study

Patient Group	Cis-atracurium Dose (mg/kg)	N	Mean % of the Dose Recovered Unchanged in Urine*	Amount of Dose Recovered in Urine as Cistracurium* (ug)	Amount of Dose Recovered in Urine as Landanosine* (ug)	Amount of Dose Recovered in Urine as MQA* (ug)
End-Stage Liver Disease	0.1	11	11.0 ± 6..9 (5.1 - 27.9)	909.9 ± 460.1 (341.6 - 1719.5)	602.4 ± 280.1 (79.1 - 1245.9)	385.9 ± 232.4 (142.4 - 758.5)
Healthy Adults	0.1	8	14.3 ± 4.0 (6.4 - 18.9)	1302.8 ± 458.8 (398.5 - 1912.0)	503.5 ± 259.7 (61.2 - 803.2)	391.3 ± 138.5 (86.7 - 551.0)

* Data presented as mean ± S.D. (range)

Cisatracurium and its metabolites were also stable in urine frozen at -20°C over a period of at least 9 months, and in acidified urine after three freeze-thaw cycles. No trends in concentration were observed in any of these stability studies (Figure 3).

The recovery results of cisatracurium, laudanosine, MQA and the internal standard from human urine are shown in Table 1. The mean recoveries ranged from 100.4 to 108.3% for cisatracurium, 90.2 to 93.3% for laudanosine, 88.4 to 94.7% for MQA and 90.7 to 96.0% for the internal standard, respectively.

The accuracy of the assay, expressed as % bias, and the within-day and between-day precision data determined by ANOVA are shown in Table 2. The % bias of the assay ranged from 6.2 to 17.0% for cisatracurium, -1.3 to 2.7% for laudanosine, and 0.2 to 3.4% for MQA.

The precision of the assay ranged from 4.7 to 14.0% for cisatracurium, 4.6 to 7.7% for laudanosine, and 3.3 to 7.4% for MQA.

The average % bias over the entire concentration range was 10.0, 0.25, and 1.9 for cisatracurium, laudanosine, and MQA, respectively. The average precision over the entire concentration range was 8.9, 5.9, and 5.6% for cisatracurium, laudanosine, and MQA, respectively

The assay has been used to quantitate urine concentrations of cisatracurium and its metabolites in volunteers after an intravenous bolus dose of cisatracurium. As an example of the utility of the assay, urinary excretion data in healthy adults and end-stage liver disease patients are shown in Table 3. The data indicate that urinary excretion is a minor elimination pathway for cisatracurium in humans.

In summary, a sensitive and selective HPLC assay has been developed and validated for the simultaneous quantitation of cisatracurium and two of its major metabolites in urine. The method has been used to study the pharmacokinetics of cisatracurium and its metabolites in humans.

REFERENCES

1. R. M. Welch, A. R. Brown, R. Dahl "The *In Vitro* Degradation of Cisatracurium, the R,Cis-R',Cis Isomer of Atracurium, in Human and Rat Plasma," Clin. Pharm. & Therap., **58(2)**, 132-142 (1995).

2. R. L. Stiller, D. R. Cook, S. Chakravorti, "*In vitro* Degradation of Atracurium in Human Plasma," Br. J. Anaesth., **57**, 1085-1088 (1985).

3. C. D. James, M. O'Mara, C. Rawls, B. J. Bryant, L. Kanics, G. Ridout, G. Lovell, J. C. Harrelson "A High-Performance Liquid Chromatographic Assay for the Quantitation of Cisatracurium and its Metabolites in Human Plasma," Submitted for internal review; Prepared for Journal of Chromatography.

4. G. Lovell, J. Morgan, "The Analysis of the Geometric Isomers of Atracurium and Principal Metabolites in Biological Fluids Using Solid-Phase Extraction and Gradient Cation Exchange HPLC with Fluorescence Detection," 2nd International Symposium on Pharmaceutical and Biomedical Analysis, 4-7 Apr 1990.

5. M. R. Belmont, R. B. Maehr, W. B. Wastila, J. J. Savarese, "Pharmacodynamics and Pharmacokinetics of Benzylisoquinolinium (Curare-Like) Neuromuscular Blocking Agents," Anes. Clin. of North America, Quarterly Review, **2(2)**, June 1993.

Received October 12, 1996
Accepted December 16, 1996
Manuscript 4299

J. LIQ. CHROM. & REL. TECHNOL., 20(13), 2053–2061 (1997)

REVERSE PHASE HPLC ANALYSIS OF ALKYL SULFONATES WITH NON-SUPPRESSION CONDUCTIVITY DETECTION

S. X. Jiang,* X. Liu

Analytical Chemistry Division
Lanzhou Institute of Chemical Physics
Academia Sinica
730000 Lanzhou, P. R. China

ABSTRACT

C_{10}-C_{16} alkyl sulfonates were used as surface active reagents in a micellar flooding study for enhancing oil recovery. They were analyzed by reverse phase high performance liquid chromatography (RP-HPLC). Sodium dihydrogen phosphate of low concentration was used as eluent component and conductivity detector was used for detection. The eluted alkyl sulfonate was detected directly and no suppressor column was required. This method was characterized by its simplicity, rapidity and sensitivity.

INTRODUCTION

Surface active petroleum sulfonate has been widely used as a chemical flooding agent in the petroleum industry. This sulfonate is obtained by sulfonation of a certain petroleum fraction, and its composition is rather complex.

Depending upon the degree of sulfonation, there may be mono-, di- and poly-sulfonates. According to the parent compound used for the sulfonation, there may be alkyl sulfonates and aryl sulfonates.

Clarification of the composition of the sulfonate is very important in studying the flooding mechanism, guiding the synthesis of petroleum sulfonate, and understanding the chromatographic separation phenomena in the flooding process. In this paper, the analysis of the alkyl sulfonate with liquid chromatography is studied.

The alkyl sulfonate exhibits only a weak ability to absorb ultraviolet light; it cannot be detected with the UV detector usually used in HPLC. The differential refraction detector cannot be used either; its sensitivity is too low and cannot meet the requirement of the analysis of low sulfonate concentration. Thus, besides the search for good separation conditions in the analysis of alkyl sulfonate with HPLC, it is also important to choose the proper detection system. Eppert, et. al.[1,2] and Larson[3] used indirect photometric detection for the analysis of alkyl sulfonate with ion pair chromatography (IPC). Pan et. al.[4] used ion chromatography and Weiss[5] adopted the mobile phase ion chromatography (MPIC) with ion pair reagent added to the mobile phase for the analysis; a suppressing conductivity detector was used in their system. The whole method is rather complex and it is necessary to regenerate the suppressor column, periodically; the entire operation is tedious.

In this paper, RP-HPLC is used for the analysis of C_{10} - C_{16} alkyl sulfonate, usually encountered in enhanced oil recovery processes. The mobile phase used contains sodium dihydrogen phosphate and a conductivity detector is used directly for the detection. No suppressor column is required. The inorganic salts and alcohol mixed in the sample do not interfere with the detection, and there is no necessity to remove them in advance. Thus, the operation is simple and the sensitivity is higher.

EXPERIMENTAL

Apparatus

The liquid chromatographic system was comprised of a Gilson 302C pump, a DDJ-01 conductivity detector (Sichuan Analytical Instrument Plant, China), two Rheodyne 7125 injection valves (10 and 20 μL) and a XWT 200 recorder (Shanghai Dahua Instrument and Meter Plant, China).

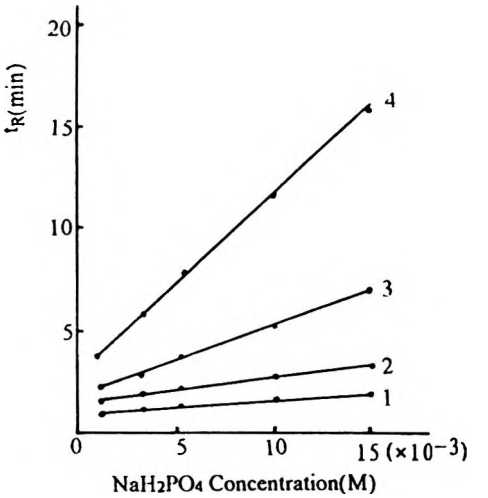


Figure 1. Relationship between alkyl sulfonate retention and phosphate concentration in the mobile phase. Mobile phase: 0.001M. NaH_2PO_4 : MeOH = 35:65; Flow rate: 1mL/min; 1. C_{10} alkyl sulfonate, 2. C_{12} alkyl sulfonate, 3. C_{14} alkyl sulfonate; 4. C_{16} alkyl sulfonate.

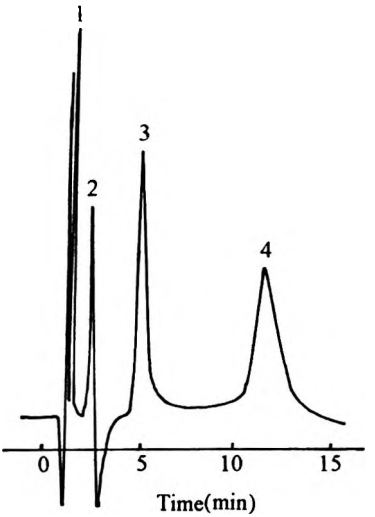


Figure 2. Chromatogram of alkyl sulfonates. Mobile phase: 0.001M. NaH_2PO_4 : MeOH = 65:35; Flow rate: 1mL/min; 1. C_{10} alkyl sulfonate, 2. C_{12} alkyl sulfonate, 3. C_{14} 10^7 Pa. alkyl sulfonate; 4. C_{16} alkyl sulfonate.

The chromatographic column was a 4.6mm x 150mm stainless steel tube, slurry packed with ODS (3-5μm, Shanghai First Reagent Plant, China) under 3.5×10^7 Pa.

Reagents

C_{10}-, C_{14}-, and C_{16}- alkyl sulfonates were supplied by Xinjiang Institute of Chemistry, Chinese Academy of Sciences.

C_{12}- alkyl sulfonate was a chemically pure reagent (Bei He Chemical Plant, China), and was recrystallized before use.

Sodium dihydrogen phosphate, analytically pure (Xian Chemical Reagent Plant , China).

Methanol, analytical pure reagent. Both methanol and freshly distilled and deionized water were filtered through a sintered glass filter before use.

The mobile phases were composed of methanol and aqueous sodium dihydrogen phosphate of various concentrations.

RESULTS AND DISCUSSION

Effect of Sodium Dihydrogen Phosphate Concentration on Retention

The retention of alkyl sulfonate is affected by the concentration of sodium dihydrogen phosphate in the mobile phase. When the concentration is very low, the alkyl sulfonate is not retained at all. By contrast, when the concentration is higher, the alkyl sulfonate is difficult to elute. This is caused by the salting out effect.

When the amount of phosphate in the mobile phase is increased, the distribution of alkyl sulfonate in water-containing mobile phase will decrease and its distribution in the hydrophobic stationary phase will enhance accordingly, so that its retention will increase.

The experimental results showed that the mobile phase containing a proper concentration of sodium dihydrogen phosphate can elute and separate C_{10} - C_{16} alkyl sulfonates; the retention increased with the increasing of phosphate concentration.

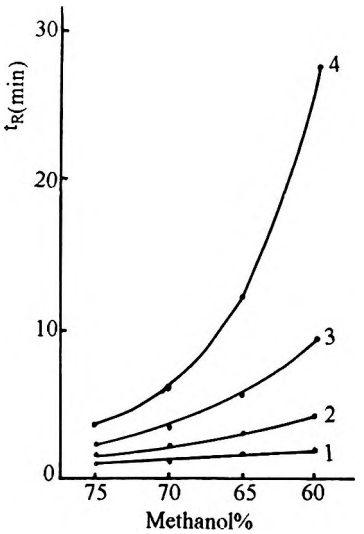


Figure 3. Relationship between alkyl sulfonate retention and methanol content in the mobile phase. Mobile phase: 0.001M NaH_2PO_4/MeOH; Flow rate: 1 mL/min; 1. C_{10} alkyl sulfonate, 2. C_{12} alkyl sulfonate, 3. C_{14} alkyl sulfonate; 4. C_{16} alkyl sulfonate.

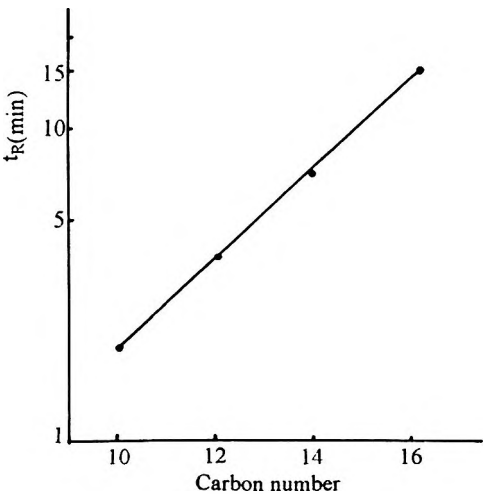


Figure 4. Relationship between alkyl sulfonate retention and number of carbon atoms. Mobile phase: 0.001M. NaH_2PO_4: MeOH = 65:35; Flow rate: 1mL/min.

Figure 1 shows the relationship between retention and phosphate concentration at a certain aqueous phosphate-methanol proportion. The complete separation of C_{10}, C_{12}, C_{14}, and C_{16} alkyl sulfonates, under proper elution condition, is given in Figure 2.

Effect of Methanol Content

The distribution of alkyl sulfonate in mobil phase is enhanced with the increase of methanol percentage in mobile phase; the retention is also decreased (Figure 3).

Effect of Carbon Number on Alkyl Sulfonate Retention

From the above experimental results, it can be seen that, under same operating condition, the retention of alkyl sulfonate which contains a greater number of carbon atoms is longer. This is due to the strengthening of lipophilic behaviour. By plotting the logarithm of the studied alkyl sulfonate retentions versus the number of carbon atoms contained in their carbon chains, a good linear relationship is obtained (Figure 4). Thus, qualitative ascertainment of the alkyl sulfonate can be obtained by determining the carbon atoms contained in the carbon chain from the retention of the sample.

Determination of Dodecyl Sulfonate in Micellar Fluid

In enhanced oil recovery studies, a micellar fluid composed of alkanes, fatty alcohols, inorganic salts, and dodecyl sulfonate was used for oil flooding. In order to take concerted study, the authors carried out the quantitative work with HPLC. Because of the fact that the reverse phase system was used for the separation and the conductivity detector was used for the detection, both alkanes and alcohols gave no signal, so they did not interfere with the detection. Besides, the retention of the inorganic salts was very short; these salts eluted before the alkyl sulfonate and did not interfere with the detection either.

For the determination in this work, a mixture composed of low concentration of aqueous sodium dihydrogen phosphate and certain amount of methanol (0.001M NaH_2PO_4:CH_3OH = 50:50) was used as the mobile phase. Also, no suppressor was used; the background conductivity was very low, so that the detection could be conducted at rather higher sensitivity.

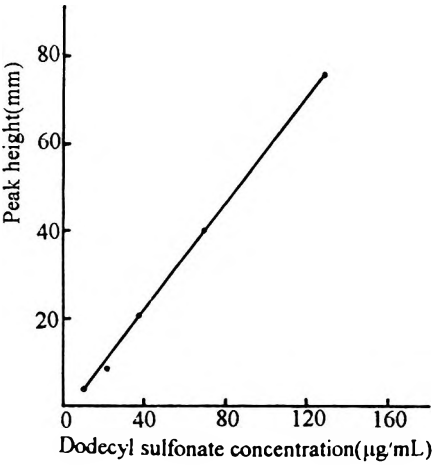


Figure 5. Calibration curve of dodecyl sulfonate. Mobile phase:MeOH : 0.001M NaH_2PO_4 = 50:50; Flow rate:1mL/min

When the sample volume was 20μL, the plot of peak height with sodium dihydrogen phosphate concentration maintained good linearity when sample concentration went up to 120ppm (Figure 5). So, this chromatographic condition was used for the determination of dodecyl sulfonate.

During the preparation of micellar fluids for enchanced oil recovery study, three phases, the upper oil phase, the middle micellar phase, and the lower aqueous phase were obtained. The contents of dodecyl sulfonate in these phases were analyzed. The result is shown in Table 1.

As shown in the table, some errors are rather high. This is caused, mainly, by the larger reading error of volume, especially the reading of the micellar phase of smaller volume. In general, the results obtained can meet the requirement of enhanced oil revovery studies.

CONCLUSION

1. RP-HPLC with conductivity detection was successfully used for the detection of alkyl sulfonate. The mobile phase contained low concentration of dihydrogen sodium phosphate; no suppressor column was necessary.

Table 1

Dodecyl Sulfonate in Micellar Fluid

	Sample Number		
	1	**2**	**3**
Upper Phase			
Volume, mL	4.4	3.3	3.7
Conc., μg/mL	---	1.05×10^3	1.79×10^3
Content, mg	---	3.47	6.62
Middle Phase			
Volume, mL	2.9	1.9	2.1
Conc., μg/mL	6.68×10^4	1.10×10^5	8.81×10^4
Content, mg	194	209	185
Lower Phase			
Volume, mL	2.7	4.6	4.0
Conc., μg/mL	2.05×10^3	1.10×10^3	---
Content, mg	**5.5**	**5.06**	---
Total Amount, mg	199	218	192
Recovery, %	99.5	109	96.6

2. Although no suppressor column was used, the results of quantitative analysis of dodecyl sulfonate show the rather higher sensitivity of the described method.

3. The separation of C_{10} - C_{16} alkyl sulfonates used in enhanced oil recovery studies can be accomplished by varying the sodium dihydrogen phosphate concentration in water and the amount of methanol in the mobile phase.

ACKNOWLEDGMENT

The authors express their thanks to Prof. Li Zhi Ping for kindly supplying the alkyl sulfonates.

REFERENCES

1. G. Eppert, G. Liebscher, J. Chromatogr., **356**, 372-378 (1986).

2. G. Eppert, G. Liebscher, J. Chromatogr. Sci., **29(1)**, 21-25 (1991).

3. J. R. Larson, J. Chromatogr., **356**, 379-381 (1986).

4. N. Pan, D. J. Dietrzyk, J. Chromatogr., **706**, 327-337 (1995).

5. J. Weiss, J. Chromatogr., **353**, 303-307 (1986).

Received August 5, 1996
Accepted November 27, 1996
Manuscript 4249

J. LIQ. CHROM. & REL. TECHNOL., 20(13), 2063-2073 (1997)

ANALYSIS OF COMMERCIAL NEOHESPERIDIN DIHYDROCHALCONE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

M. R. Castellar,[1] J. L. Iborra,[1]* I. Canales[2]

[1]Department of Biochemistry and Molecular Biology B
and Immunology
Faculty of Chemistry, University of Murcia
P.O. Box 4021
E-30001 Murcia (Spain).

[2]Zoster S.A. Raiguero
143, Zeneta
E-30588, Murcia (Spain).

ABSTRACT

An HPLC method for checking the purity of commercial neohesperidin dihydrochalcone has been developed and validated. The optimised method was conducted by using an RP C_{18} stationary phase, 20% acetonitrile acidified with acetic acid as mobile phase, 1 mL/min flow rate, spectrophotometric detection at 282 nm, and a temperature of 25°C. Under these conditions, neohesperidin DC showed a retention time of 17.4 min. Different structurally related flavonoids were added to the sample for selectivity determination. Linearity of the method was proved in the range 0-500 mg/L. Sample preparation was optimised and relevant analytical parameters (accuracy, precision, repeatability, reproducibility) determined.

INTRODUCTION

Neohesperindin dihydrochalcone (neohesperidin DC) is an intense sweetener which is about 1800 times sweeter than sucrose at threshold levels and about 400 times sweeter in comparison to a 6% sucrose solution.[1]

Its sweetening profile in water is characteristical by a delay before its maximum intensity is reached, followed by a menthol- or licorice-like lingering aftertaste.[2] When used at low concentrations in combination with other intense or bulk sweeteners, neohesperidin DC enhances the quality of the sweetness given to the food, contributing beneficially to its flavour and mouthfeel and providing synergistic effects.[3,4]

International acceptance of neohesperidin DC was manifested by a favourable assessment and the allocation of an ADI by the Scientific Committee for Food of the European Union. In fact, it has been recently authorised in the EU as an intense sweetener[5] and flavour enhancer.[6]

Although official purity criteria have also been established,[7] no mention is made of the analytical method proposed to control the purity of the sweetener. Therefore, the development of a validated method to ensure that commercial products meet official specifications is of industrial and scientific interest.

The objective of the present work has been the development and validation of such a procedure using HPLC, for use in the analysis of the commercial product's purity.

MATERIALS AND METHODS

Materials

Neohesperidin DC (NHDC) and related compounds were supplied by Zoster S.A. (Raiguero, 143, Zeneta, E-30588, Murcia, Spain). The related compounds were: phloroacetophenone neohesperidoside (FANH), naringin (NA), neohesperidin (NH), naringin dihydrochalcone (NADC), hesperidin dihydrochalcone (HEDC), hesperetin dihydrochalcone glucoside (HPDCG), and hesperetin dihydrochalcone (HPDC). Acetonitrile (ACN) and methanol were from Merck and dimethyl sulphoxide (DMSO) was from Romil. All solvents were HPLC grade. Acetic and phosphoric acids employed for mobile phase acidification were from Fluka and Merck, respectively. Water was double distilled and purified through a Millipore system (Milli-Q).

Equipment

HPLC analysis was performed on a Shimadzu HPLC system equipped with two LC-6A pumps, a UV-Vis SPD-M6A diode array detector and a SIL 9A automatic injector. For reproducibility determination two HPLC systems from different laboratories were used: i) a Hitachi HPLC system equipped with a L6200A pump, UV-Vis L 4250 detector, AS 2000A automatic injector and D 2500 integrator; and ii) a Shimadzu HPLC equipped with two LC8A pumps, UV-vis SPD-6AV detector and C-R4A integrator. The columns employed with this equipment were Lichrospher 100 RP-C_{18} 5μm (120 x 4 mm) columns from Merck. Assay temperature was 25°C.

Sample Preparation

A standard sample of neohesperidin DC (200 mg/L) was prepared by dissolving 50 mg of commercial neohesperidin DC (L29024) in 250 mL of solvent (DMSO, 50% methanol or 50% ACN, depending on the assay). The other related compounds were added at a lower concentration (4 mg/L) to simulate the presence of impurities. 4 mg/L was sufficient for quantitation and identification of each added compund. Four different samples were analysed: neohesperidin DC dissolved in DMSO (sample P); and neohesperidin DC plus all related compounds dissolved in DMSO (sample A), in 50% methanol (sample B), and in 50% ACN (sample C). For calibration, precision, and accuracy determinations, neohesperidin DC dissolved in DMSO was analysed at different concentrations. For selectivity determinations, a standard of neohesperidin DC purified by preparative HPLC was employed as reference.

Method Development

The mobile phases assayed were binary mixtures of methanol:water and ACN:water, acidified with acetic acid (50 mM). Samples B and C were assayed only with methanol:water and ACN:water, respectively, while samples prepared in DMSO (P and A) were assayed with all the elution systems. All analyses were performed in triplicate. Chromatographic parameters were calculated according to.[8,9] Optimum mobile phase acidification was selected from the analysis of neohesperidin DC in 20% ACN acidified with both acetic (50 mM) and phosphoric (50 mM) acids. All analyses were performed with a flow rate of 1 mL/min and temperature of 25°C. Detection was at 282 nm and a 20μl sample size was used.

Dilution and Weight Errors

Dilution error was determined by analysis of three samples (200 mg/L neohesperidin DC, analysed six times) obtained from a 800 mg/L neohesperidin DC stock solution in DMSO. For weight error determination, four different amounts of neohesperidin DC (10, 20, 40 and 50 mg) were weighed three times each, and dissolved in DMSO until a final concentration of 200 mg/L. Analyses were carried out six times.

Straight Line Calibration

Three series (six samples each, at 100, 150, 200, 300, 400, and 500 mg/L neohesperidin DC dissolved in DMSO) were injected on three different days. Each sample was analysed six times. The final calibration straight line was taken as the mean of the slopes, independent of terms and correlation values of the three straight lines obtained, in accordance with reference 10.

Accuracy

Samples of neohesperidin DC were prepared in triplicate at 100, 150, 200, 300, 400, and 500 mg/L and were analysed six times. Accuracy was checked by the straight line obtained from the measured versus the theoretical neohesperidin DC concentrations, in accordance with reference 11.

Precision, Repeatability, and Reproducibility

The system precision was measured by the R.S.D. value of ten replicate injections of a 100 mg/L neohesperidin DC sample, according reference 11. The precision of the method was determined,[11] with five 100 mg/L neohesperidin DC samples analysed six times at the same session. Additionally, for repeatability determination, four 200 mg/L neohesperidin DC samples were analysed on four different days, six times every day. Reproducibility was tested, employing three different HPLC equipments, in each of which three 200 mg/L neohesperidin DC samples were analysed six times.

To ascertain the method precision, the repeatability and the reproducibility, R.S.D. values were determined; repeatability and reproducibility values were obtained.[12]

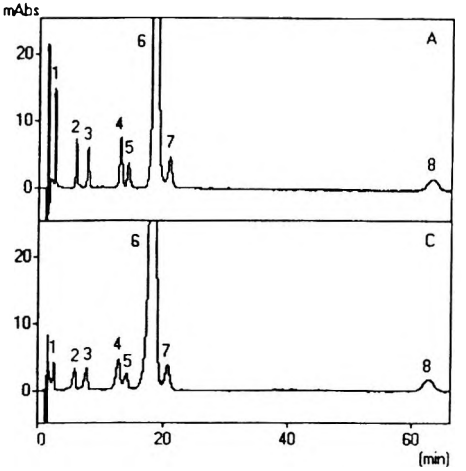


Figure 1. Chromatograms of samples A and C with 20% ACN and RP-C18 column as mobile and stationary phases, respectively, 1 mL/min flow rate, 25°C, 282 nm, 20μL sample size, 200 mg/L neohesperidine DC concentration and 4 mg/L other compounds concentration. The following compounds are present: FANH (1), NA (2), NH (3), NADC (4), HEDC (5), NHDC (6), HPDCG (7) and HPDC (8)

RESULTS AND DISCUSSION

Effects of Mobile Phase and of Sample Preparation Solvent

Of the different methanol:water and ACN:water elution systems assayed, 20% ACN gave the best results. Chromatograms of samples A and C eluted with 20% ACN are shown in Figure 1, and Table 1 reports the chromatographic parameters obtained from these analyses. Longer retained compounds showed higher than recommended k′ values (≈10),[9] because it was necessary to prolong the operation time to separate the eight different compounds, contained in samples A and C, some of them with very similar chromatographic behaviour. All α values were higher than the minimum accepted (1.05).[8] Resolution values were, in general, higher for sample A (with DMSO as solvent) than for sample C (with ACN as solvent).

This observation and the high As value (0.9-1.1 accepted range)[8] of sample C determined the selection of DMSO as standard solvent for sample preparation.

Table 1

Chromatographic Parameters Obtained for Samples A (with DMSO as Solvent) and C (with 50% ACN S solvent) Under Selected Standard Conditions

Compound	Sample A				Sample C			
	As	K′	α	Rs	As	K′	α	Rs
FANH	1.62	0.99			1.69	1.04		
			4.31	8.65			3.63	5.64
NA	1.07	4.28			2.38	3.78		
			1.21	3.86			1.43	2.96
NH	1.13	5.19			1.90	5.39		
			1.75	7.37			1.76	6.20
NADC	0.93	9.07			1.53	9.48		
			1.12	1.73			1.12	1.51
HEDC	1.00	10.15			1.44	10.61		
			1.33	4.39			1.32	3.98
NHDC	1.06	13.45			1.62	14.06		
			1.13	2.12			1.13	2.95
HPDCG	0.97	15.24			1.47	15.93		
			3.14	20.87			3.13	20.96
HPDC	1.02	47.86			1.00	49.88		

No differences were observed between acetic and phosphoric acids when used for mobile phase acidification. The selected conditions for neohesperidin DC analysis were, therefore, 20% ACN as mobile phase, acidified with 50 mM acetic acid, 1 mL/min flow rate, and neohesperidin DC dissolved in DMSO.

Optimisation of Sample Preparation.

It was necessary to use a neohesperidin DC concentration high enough to detect possible impurities present at the sample. Thus, 200 mg/L neohesperidin DC was selected as a standard concentration for sample preparation to allow detection of structurally related compounds. Dilution and weight errors were determined as reported in Materials and Methods. The R.S.D. for dilution error was 0.17%, thus making no significant contribution to the total error of the method.

Table 2

Linearity of Recovery Results for Neohesperidin DC Analysis

Assay Day	Added (mg/L)	Mean Found (n=6) ± SD (mg/L)	Recovery (%)	Mean Recovery (%)	R.S.D. Recovery (%)
1	98.9	98.6 ± 0.9	99.7		
2	95.8	95.1 ± 0.7	99.3	99.7	0.35
3	92.5	92.5 ± 1.2	100.0		
1	147.0	148.9 ± 1.2	101.3		
2	137.9	134.8 ± 1.0	97.7	99.2	1.87
3	139.7	137.9 ± 1.6	98.7		
1	182.4	181.9 ± 1.0	99.7		
2	181.5	180.0 ± 0.6	99.2	99.5	0.25
3	187.8	186.8 ± 0.3	99.5		
1	273.1	284.1 ± 3.3	104.0		
2	272.2	269.9 ± 1.2	99.2	101.7	2.36
3	274.0	278.8 ± 0.5	101.8		
1	368.4	376.2 ± 3.1	102.1		
2	363.8	361.4 ± 1.4	99.3	99.8	2.14
3	374.7	366.8 ± 2.9	97.9		
1	457.3	459.5 ± 3.1	100.5		
2	457.3	453.0 ± 2.5	99.1	99.8	0.70
3	460.0	458.8 ± 2.4	99.7		
		Mean:		99.9	1.28
		Slope:			0.9991
		Intercept:			0.48
		Correlation coefficient:			0.9998

The four amounts of neohesperidin DC assayed for weight error determination showed R.S.D. values ranging from 0.22% to 2.05%, the lowest values being for 50 mg of neohesperidin DC. Taking into consideration the best solvent as described above, the standard sample was prepared at 200 mg/L, by dissolving 50 mg of neohesperidin DC in 250 mL of DMSO.

Table 3

Method Precision of Neohesperidin DC Analysis

Sample (n°)	Mean Found (n=6) ± SD (mg/L)
1	89.9 ± 0.7
2	89.9 ± 0.3
3	89.9 ± 0.4
4	90.3 ± 0.4
5	90.6 ± 0.5
Mean ± SD (mg/L):	90.2 ± 0.3
R.S.D. (%):	0.33

Table 4
Repeatability of Neohesperidin DC Analysis

Sample (n°)	Mean Found (n=6) ± SD (mg/L)
1	188.3 ± 1.4
2	186.3 ± 0.9
3	184.7± 1.4
4	186.4 ± 2.0
Mean ± SD (mg/L):	186.4 ± 1.3
R.S.D. (%):	0.68
r (mg/L):	3.6

Validation Parameters

Linearity

The straight line obtained is expressed by the equation: Y= 34025 X - 1149 with r=0.9998, where X is neohesperidin DC concentration in mg/L and Y is the peak area. A linearity range 0-500 mg/L was demonstrated.

Table 5

Reproducibility of Neohesperidin DC Analysis

Equipment (n°)	Sample (n°)	Mean Found (n=6) ± SD (mg/L)	Mean (n=3) ± SD (mg/L)
1	1	173.5 ± 0.4	
	2	176.2 ± 1.1	175.5 ± 1.8
	3	176.8 ± 0.5	
2	1	178.9 ± 0.6	
	2	181.2 ± 1.6	180.0 ± 1.2
	3	179.8 ± 0.9	
3	1	178.8 ± 0.5	
	2	176.9 ± 1.0	177.7 ± 1.0
	3	177.6 ± 0.8	
	Mean ± SD (mg/L):		177.7 ± 1.8
	R.S.D. (%):		1.03
	R (mg/L):		5.1

Accuracy

The accuracy of the described method is shown by the linearity of the recovery data for the neohesperidin DC analyses.[11] The results are shown in Table 2. The slope of the straight line obtained from the theoretical vs the measured data was 0.9991. The highest R.S.D. was 2.36% and mean recovery was 99.9%. with an acceptable mean R.S.D. of 1.28%;

Precision, Repeatability, and Reproducibility

The precision of the system was acceptable with an R.S.D. of 0.74%. The results shown in Table 3 reveal the high precision of the method with an R.S.D. of 0.33% between the different samples. Additionally, repeatability and reproducibility were also determined.

The results are shown in Tables 4 and 5, respectively. R.S.D. values were 0.68% in determinations performed on different days, and 1.03% between the three HPLC equipments assayed. Repeatability and reproducibility values were 3.57 and 5.12 mg/L, respectively.

Selectivity

In previous research, neohesperidin DC was purified by preparative HPLC, and its structure confirmed by RMN, IR and MS analyses (unpublished results). Purified neohesperidin DC (99.9% chromatographic purity) was employed as an external standard to check the HPLC profile of commercial neohesperidin DC (L29024). The same retention time (17.4 min) was observed for both purified and commercial neohesperidin DC samples. No analytical interferences between neohesperidin DC and the added related compounds was detected. Resolution values were above the minimum recommended (Rs > 1.5, Table 1).[8] The analytical method used could therefore, be considered specific for checking the purity of neohesperidin DC.

CONCLUSIONS

In conclusion, this HPLC method is suitable and selective for neohesperidin DC analysis, regardless of the equipment used and laboratory conditions. The method shows very high accuracy, precision and repeatability values.

REFERENCES

1. R. M. Horowitz, B. Gentilli, "Dihydrochalcone Sweeteners from Citrus Flavanones" in **Alternative Sweeteners**, L. O'Brien, R. C. Gelardi, eds., Marcel Dekker, Inc. New York, 1991, pp. 97-115.

2. M. G. Lindley, I. Canales, F. Borrego, FIE 91 Conference Proceedings, Paris, Expoconsult, Maarsen, 1991, pp. 48-51.

3. F. Borrego, I. Canales, M. G. Lindley, Z. Lebens. Unters. Forsch, **200**, 32-37 (1995).

4. M. G. Lindley, P. K. Beyts, I. Canales, F. Borrego, J. Food Sci., **58**, 592-596 (1993).

5. Anonymous, European Parliament and Council Directive 94/35/, Off. J. Eur. Comm., September 16, Nº C 237/12 (1994).

6. Anonymous. European Parliament and Council Directive 95/2/EC, Off. J. Eur. Comm., March 18, Nº L 61/1 (1995).

7. Anonymous, Commission Directive 95/31/EC, Off. J. Eur. Comm., July 28, Nº L 178/1 (1995).

8. L. R. Snyder, J. L. Glajch, J. J. Kirkland, **Practical HPLC Method Development**, John Wiley & Sons Inc. New York, 1988.

9. S. Lindsay, **High Performance Liquid Chromatography**, John Wiley & Sons Ltd., Chichester, 1992.

10. M. A. Camacho, A. I. Torres, M. E. Gil, M. M. Obregón, Tecnifarma, **4**, 10-12 (1993).

11. T. D. Wilson, W. G. Jump, W. C. Newmann, T. San Martín, J. Chromatogr., **641**. 241-248 (1993).

12. Anonymous, J. Assoc. Off. Anal. Chem., **71**, 161-173 (1988).

Received November 3, 1996
Accepted November 30, 1996
Manuscript 4324

J. LIQ. CHROM. & REL. TECHNOL., 20(13), 2075–2087 (1997)

HPLC METHOD WITH PRECOLUMN PHENACYLATION FOR THE ASSAY OF VALPROIC ACID AND ITS SALTS IN PHARMACEUTICAL DOSAGE FORMS

C. A. Lau-Cam, R. W. Roos[†]

College of Pharmacy and Allied Health Professions
St. John's University
Jamaica, New York 11439

ABSTRACT

A simple, accurate and reproducible HPLC method is described for the assay of valproic acid and its sodium salts in commercial dosage forms. The analyte and sodium caproate, the internal standard, were detected in the UV range after formation of the corresponding phenacyl ester derivatives with a mixture of phenacyl bromide-triethylamine in acetone. The ester derivatives were analyzed directly on a Microsorb-MV C18 column with a mobile phase composed of acetonitrile-methanol-water (50:20:30) and detection at 245 nm. At a flow rate of 2 mL/min, phenacyl caproate and phenacyl valproate eluted at about 4.5 min and 8.5 min, respectively. Peak height ratios were linearly related to amounts of valproic acid, or its equivalent in sodium valproate, in the range 30-750 μg ($r = 0.9997$). Based on peak height ratios, the RSD for a set of replicate injections was 1.10% ($n = 6$).

Recoveries of valproic acid or sodium valproate from spiked commercial dosage forms were in the range 101.0-102.6% of added (n = 2). The proposed HPLC method yielded assay values that were in good agreement with those obtained by the GC method for valproic acid in USP 23.

INTRODUCTION

Valproic acid (2-propylpentanoic acid) is a simple aliphatic carboxylic acid endowed with anticonvulsant properties that are found useful to suppress epileptic seizures while causing only minimal sedation and other effects in the central nervous system.[1] By the oral or rectal routes of administration, valproic acid is indicated for use as sole or adjunctive therapy to manage simple and complex absence seizures, mixed seizure types, and myoclonic seizures coexisting with tonic-clonic or absence seizures.[1-3]

As such, valproic acid is commercially available in the form of soft gelatin capsules; as the sodium salt it is formulated as a syrup; and as divalproex sodium (a 1:1 coordination compound with the sodium salt) it is marketed as a delayed release tablet.[3]

Owing to the widespread use and clinical importance of valproic acid and its salts, numerous analytical methods have been reported for monitoring their therapeutic circulating levels in human subjects.[4-22] Most of these methods have been based on either gas chromatography (GC)[4-9] or high performance liquid chromatography (HPLC);[10-20] but thin-layer chromatography,[19] enzyme immunoassay,[9,12] immunoassay,[21] and chemical ionization-mass spectrometry[22] have also been proposed. In contrast, methods for the assay of these drugs in pharmaceutical samples are fewer in number, and have relied on spectrophotometry,[23] isotachophoresis,[24] GC,[25] HPLC with conductivity[20] or fluorescence[26] detection, and potentiometry.[25,27] In USP 23, the assay of valproic acid in dosage forms entails a GC approach whereas that for the drug substance is accomplished by a nonaqueous potentiometric titration.[28]

The purpose of this communication is to describe a simple and rapid reverse phase HPLC method with UV detection for the determination of valproic acid and its salts in pharmaceutical products after precolumn derivatization to phenacyl esters. This HPLC method was found to be well suited for the analysis of the title drugs in both liquid and solid commercial dosage forms.

EXPERIMENTAL

Materials

Dosage forms

Various lots of valproic acid capsules (250 mg), sodium valproate syrup (250 mg/5 mL), and divalproex sodium tablets (250 and 500 mg) were obtained from local commercial sources.

Chemicals

Valproic acid, sodium valproate, sodium caproate (Sigma Chemical Co.) and phenacyl bromide (Fluka) were used as received. Triethylamine and acetone (J.T. Baker) were of analytical reagent grade. The acetonitrile, methanol and water (EM Science) were of HPLC grade.

Reagents

Phenacyl bromide solution

A solution of phenacyl bromide in acetone was prepared to contain about 12.8 mg/mL. This solution was stable for at least 3 weeks when stored in an amber glass bottle and in the refrigerator.

Triethylamine solution

A solution of freshly distilled triethylamine in acetone was prepared to contain about 10 mg/mL. This solution was stable for at least 3 weeks when stored in an amber glass bottle and in the refrigerator.

Internal standard solution

It was prepared by dissolving sodium caproate, previously dried to constant weight, in acetone-water (45:55) to a concentration of about 400 μg/mL. This solution was diluted with acetone to a final concentration of about 200 μg/mL.

Sample Preparations

Valproic acid standard preparation

A solution of valproic acid in acetone was prepared to contain about 2.5 mg/mL of valproic acid.

Sodium valproate standard preparation

An accurately weighed quantity of sodium valproate, previously dried to constant weight and equivalent to 250 mg of valproic acid, was transferred to a 100 mL volumetric flask, and dissolved in, and diluted with, acetone-water (45:55) to volume. This solution contained the equivalent of 2.5 mg/mL of valproic acid.

Capsules

The contents of 10 capsules were squeezed into a 200 mL volumetric flask after puncturing each capsule with a pair of scissors. Then each capsule was cut in half and dropped into the flask. The scissors were rinsed with acetone, and the rinsings were quantitatively collected in the flask. After diluting with acetone to volume, and mixing, 20.0 mL of the solution was transferred to a 100 mL volumetric flask, to be diluted with acetone-water (45:55) to volume, and mixed. This solution contained about 2.5 mg/mL of valproic acid.

Syrups

An accurately measured volume of syrup (5 mL) was transferred to a 100 mL volumetric flask, diluted with acetone-water (45:55) to volume, and mixed. This solution contained the equivalent of about 2.5 mg/mL of valproic acid.

Tablets

A group of 20 tablets was accurately weighed, and reduced to a fine powder with the aid of an electric mill. A portion of powder, equivalent to 250 mg of valproic acid, was transferred to a 100 mL volumetric flask, mixed with about 50 mL of acetone-water (45:55), and sonicated for about 10 min.

After diluting with acetone-water (45:55) to volume, and mixing, a portion of the suspension was centrifuged at 4000 rpm for 5 min. The clear solution contained the equivalent of about 2.5 mg/mL of valproic acid.

Derivatization Method

A 1.0 mL volume of sample preparation was transferred to a 10 mL volumetric flask, diluted with acetone to volume, and mixed. To a glass test tube with a Teflon-lined screw cap, 1.0 mL of diluted sample preparation, 1.0 mL of internal standard solution, 50 µL of phenacyl bromide solution, and 50 µL of triethylamine solution, were added in succession. After mixing with gentle swirling, and stoppering tightly, the mixture was heated to 50°C for 2 hr on a heating block. After allowing to cool to room temperature, a portion of the reaction mixture was injected into the liquid chromatograph.

HPLC Method

Apparatus

An isocratic HPLC system was used, consisting of a Series 10 liquid chromatograph and LC 90 UV spectrophotometric detector (Perkin-Elmer), connected to a ChromJet integrator (Spectra-Physics). Samples were introduced through a high pressure injection valve fitted with a 50 µL sample loop (Rheodyne).

Chromatographic conditions

Analyses were performed at ambient temperature on a Microsorb-MV C18, 25 cm x 4.6 mm i.d., 5 µm, column (Rainin). The mobile phase was a mixture of acetonitrile-methanol-water (50:20:30), filtered and degassed prior to use, and flowing at the rate of 2 mL/min. The detection wavelength was 245 nm.

Calculations

The quantity of valproic acid, or the equivalent in sodium valproate, in the dosage form analyzed was calculated from one of the following equations:

$$\text{mg/tablet} = (R_1/R_2) \times C \times (W/S)$$

$$\text{mg/capsule} = (R_1/R_2) \times C$$

$$\text{mg/mL syrup} = (R_1/R_2) \times (C/V)$$

where R_1 and R_2 are the detector response ratios (i.e.,, peak height of analyte/peak height of internal standard) for the sample preparation and standard preparation, respectively; C is the concentration of the standard preparation, mg; W is the average tablet weight, mg; S is the quantity of powdered tablet taken for the assay, mg; and V is the volume of syrup taken for the assay, mL.

RESULTS AND DISCUSSION

Ionization of a saturated fatty acid followed by reaction of the carboxylate anion with a phenacyl halide in an aprotic solvent is a chemical reaction that has been widely used for the prechromatographic formation of ester derivatives with excellent UV light absorbing properties.[10-18,29-33] In general, phenacyl esters can be obtained either by (a) forming an electron-deficient carboxylate ion with the aid of an inorganic base prior to alkylation in the presence of a crown ether;[7,11,13,14,16,31] or (b) direct alkylation following abstraction of the acidic proton with an organic base[10,12,15,18] or alkali fluoride.[32] In the present study, the phenacylation of valproic acid, its sodium salt, and the internal standard, was carried out essentially as described by Borch[33] for the derivatization of long chain fatty acids. This direct approach requires neither the strict anhydrous conditions nor the tedious and time-consuming evaporation step of those methods where the free acid is neutralized with an aqueous alkali prior to alkylation,[31] and in which solvation might slow down the rate of alkylation by decreasing nucleophilicity.[29] Moreover, the use of an amine such as triethylamine represents a more convenient alternative to the more expensive and hygroscopic crown ether catalysts.[31]

Preliminary studies with various aromatic alkyl bromides, including phenacyl, 4-bromophenacyl, 4-nitrophenacyl, 4-methoxyphenacyl, and 4-phenylphenacyl, indicated that phenacyl bromide was the most suitable one in view of the shorter retention times of the esters obtained with this reagent. The formation of potentially bothersome thermal degradation products was minimized by conducting the derivatization at the relatively low temperature of 50°C, and the addition of only a slight excess of reagents insured good reproducibility and minimal peak tailing. Typically, 1.73 μmol of analyte and 1.45 μmol of internal standard were reacted with 3.20 μmol of alkyl halide and 4.94 μmol of triethylamine. The reaction mixture was analyzable without the need for a purification or isolation step since no interfering peaks were observed in the chromatograms. The derivatives were stable in solution and at ambient temperature for at least two weeks when stored in tightly capped test tubes and away from light.

Table 1

Results of Recovery of Valproic Acid, or its Equivalent in Sodium Valproate, from Commercial Dosage Forms by Proposed HPLC Method[a]

	Amount of Valproic Acid Found, as % of Added			
Matrix	**Run 1**	**Run 2**	**Mean**	**SD**
Capsule, 250 mg[b]	101.1	101.0	101.1	0.05
Syrup, 250 mg/5mL[c]	102.8	102.5	102.6	0.15
Tablet, 250 mg[b]	100.4	101.7	101.1	0.67

[a] All experiments were conducted at a spike concentration of valproic acid, or its equivalent in sodium valproate, equal to 50% of the declared amount.
[b] Spiked with valproic acid.
[c] Spiked with sodium valproate.

Figure 1 shows the time course of the derivatization reaction for valproic acid and sodium caproate, the internal standard. For both compounds, the reaction rate became nearly constant after 2 hr of heating at 50°C. An evaluation of detector response as a function of detection wavelength indicated that, although peak heights were maximal at 245 nm, comparable analytical results could be obtained at wavelengths in the range 210-254 nm. On the other hand, detector responses dropped significantly at ≥ 260 nm.

The linearity of the proposed HPLC method was verified by serially diluting a stock solution of valproic acid in acetone with the same solvent, mixing each dilution with the internal standard solution, and subjecting the mixtures to derivatization. Peak height ratios were found to be linearly related to amounts of derivatized valproic acid in the approximate range of 30-750 μg. A similar linearity study was also conducted on the internal standard but keeping the concentration of valproic acid constant. Line equations for valproic acid and sodium valproate were $y = 0.00328x + 0.0124$ ($r = 0.9997$) and $y = 0.00486x - 0.00102$ ($r = 0.9999$), respectively, with both lines passing through the origin. Recommended reaction conditions were set to analyze 250 μg of drug and 200 μg of internal standard in a total volume of reaction mixture of about 2.1 mL.

The reproducibility of the method was assessed on the basis of peak height ratios for a set of 6 replicate injections of a standard phenacylated valproic acid preparation containing 250 μg/mL of analyte. The RSD value was 1.10%. As

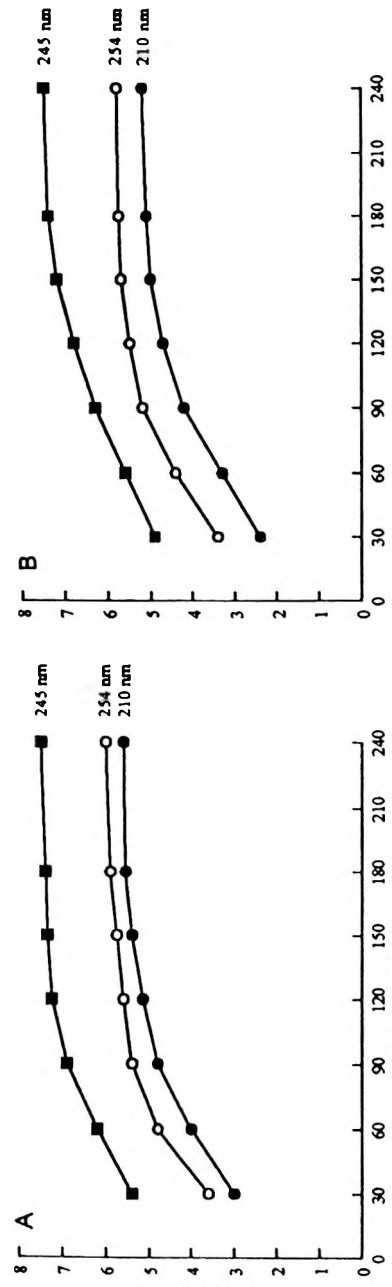


Figure 1. Time course of the derivatization of (A) valproic acid (1.73 µmol) and (B) sodium caproate, the internal standard (1.45 µmol) with phenacyl bromide (3.20 µmol) - triethylamine (4.94 µmol) in acetone at 50°C. For comparative purposes, curves obtained at 210, 245 and 254 nm are shown for each reacting compound.

Table 2

Results of the Assay of Valproic Acid in Commercial Dosage Forms by Proposed HPLC Methd and USP 23 GC Method[a,b]

	Valproic Acid Found, as % of Declared					
	HPLC			GC		
Lot No.	Run 1	Run 2	Mean	Run 1	Run 2	Mean
			Capsules, 250 mg/Capsule			
1	104.5	103.8	104.2	104.8	103.8	104.3
			Syrup, 250 mg/5mL[c]			
1	103.8	103.1	103.5	100.2	100.5	102.9
2	102.3	103.2	102.3	101.8	99.7	100.8
3	103.7	103.8	103.8	100.5	100.5	102.8
			Tablets, 250 mg/Tablet[d]			
1	99.3	100.7	100.0	100.5	100.8	100.7
			Tablets, 500 mg/Tablet[d]			
1	99.7	100.3	100.0	101.1	100.8	100.9

[a] USP 23 requirement: not less than 90.0 percent and not more than 110.0 percent the labeled amount of $C_8H_{16}O_2$.

[b] USP 23 does not list the tablets.

[c] Contains sodium valproate in an amount equivalent to 250 mg/5mL of valproic acid.

[d] Contains divalproex sodium, a 1:1 coordination of valproic acid with sodium valproate.

a verification of accuracy, commercial dosage forms whose drug content had been previously determined by the GC method for valproic acid in USP 23,[32] were spiked with valproic acid (capsules, tablets), or the equivalent in sodium valproate (syrup), in an amount equal to one-half the declared amount, diluted as described for the dosage form preparations, and put through the proposed derivatization method. As presented in Table 1, the mean recovery of valproic acid from a tablet or capsule matrix was, in both instances, 101.1%, whereas that of sodium valproate from a syrup matrix was 102.6% (n = 2).

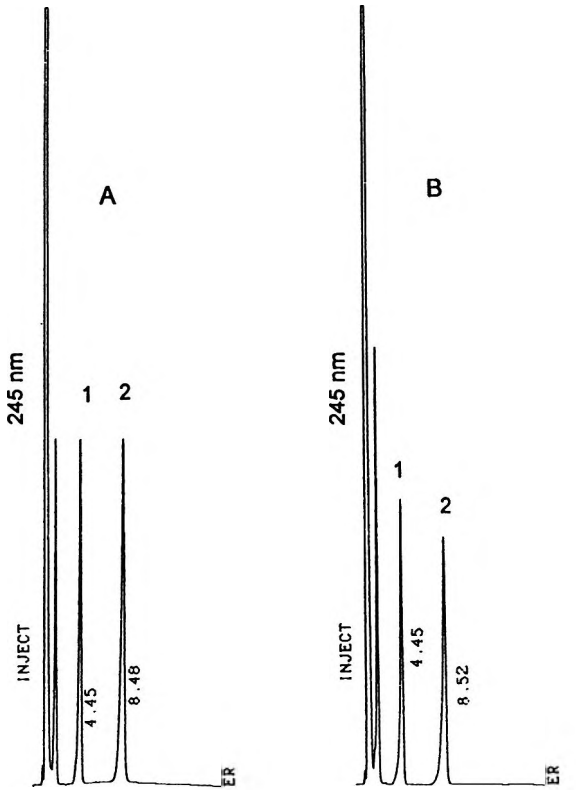


Figure 2. Typical high performance liquid chromatograms of 1, phenacyl caproate, the internal standard, and 2, phenacyl valproate in (A) a standard preparation and (B) a capsule preparation. Flow rate was 2 mL/min.

Table 2 summarizes the results of the assay of commercial dosage forms, comprising capsules, tablets, and syrups, by the proposed method, and they represent the means of duplicate analyses. Typical chromatograms of a standard preparation and a dosage form preparation are shown in Figures 2A and 2B, respectively, with phenacyl caproate eluting ahead of phenacyl valproate. A run was completed in about 10 min. Assay values by the HPLC method were compared with those obtained by the compendial GC assay method for valproic acid tablets and capsules.[28] Intermethod differences were about 0.1 % of labeled for capsules, ≤0.9% of labeled for tablets, and 0.6-1.5% of labeled for syrups. All samples were found to meet the official requirements for labeled drug content. No interferences were noted from excipients or other inert ingredients present in the formulations tested.

In summary, the conversion of valproic acid, sodium valproate or divalproex sodium to phenacyl ester derivatives served as the basis for a specific, accurate, and reproducible means of analyzing these antiepileptic agents in commercial pharmaceutical products by HPLC with spectrophotometric detection. In addition to its simplicity and rapidity, this method was equally applicable to the quantitative analysis of liquid and solid dosage forms, and yielded results that were in close agreement with those obtained by the GC method for valproic acid in USP 23.

REFERENCES

† Present address: Food and Drug Administration, Northeast Regional Laboratory, 850 Third Avenue, Brooklyn, NY 11232.

1. T. W. Rall, L. S. Schleifer, "Drugs Effective in the Therapy of the Epilepsies," in **Goodman and Gilman's The Pharmacological Basis of Therapeutics**, 7th ed., A. G. Gilman, L. S. Goodman, T. W. Rall, F. Murad, eds., Macmillan Publishing Co., New York, NY, 1985, pp. 461-462.

2. J. O. McNamara, "Drugs Effective in the Therapy of the Epilepsies," in **Goodman and Gilman's The Pharmacological Basis of Therapeutics**, 9th ed., J. G. Hardman, L. E. Limbird, eds., McGraw-Hill, New York, NY, 1996, p. 483.

3. **Physicians' Desk Reference**, 48th ed., Medical Economics Data Production Co., Montvale, NJ, 1994, pp. 412-414.

4. C. J. Jensen, R. Gugler, J. Chromatogr., **137**, 188-193 (1977).

5. S. Willox, S. E. Foote, J. Chromatogr., **151**, 67-70 (1978).

6. R. N. Gupta, F. Eng, M. L. Gupta, Clin. Chem., **25**, 1303-1305 (1979).

7. S. C. Chan, Clin. Chem., **26**, 1528-1530 (1980).

8. A. R. Rege, J. J. L. Lertora, L. E. White, W. J. George, J. Chromatogr., **309**, 397-402 (1984).

9. S. L. Braun, A. Tausch, W. Vogt, K. Jacob, M. Knedel, Clin. Chem., **27**, 169-172 (1981).

10. R. N. Gupta, P. M. Keane, M. L. Gupta, Clin. Chem., **25**, 1984-1985 (1979).

11. R. Alric, M. Cociglio, J. P. Blayac, R. Puech, J. Chromatogr., **224**, 289-299 (1981).

12. W. Ehrenthal, M. Rochel, Arzneim.-Forsch., **32**, 449-452 (1982).

13. W. F. Kline, D. P. Enagonio, D. J. Reeder, W. E. May, J. Liq. Chromatogr., **5**, 1697-1709 (1982).

14. J. P. Moody, S. M. Allan, Clin. Chim. Acta, **127**, 263-269 (1983).

15. M. Nakamura, K. Kondo, R. Nishioka, S. Kawai, J. Chromatogr., **310**, 450-454 (1984).

16. C. Lucarelli, P. Villa, E. Lombaradi, P. Prandini, A. Brega, Chromatographia, **33**, 37-40 (1992).

17. M. Monari, G. Sanchez, I. Cadiz, S. Mera, P. Castaneda, Gaz. Ital. Chim. Clin., **17**, 353-360 (1992); Anal. Abstr., **56**, 1G58 (1994).

18. P. Corti, A. Cenni, G. Corbini, E. Dreassi, C. Murratzu, A. M. Caricchia, J. Pharm. Biomed. Anal., **8**, 431-436 (1990).

19. H. Liu, L. J. Forman, J. Montoya, C. Eggers, C. Barham, M. Delgado, J. Chromatogr., **576**, 163-169 (1992).

20. H. Itoh, Y. Shinbori, N. Tamura, Bull. Chem. Soc. Jpn., **59**, 997-1000 (1986).

21. H. Feinstein, H. Hovav, B. Fridlender, D. Inbar, Clin. Chem., **28**, 1665-1666 (1982).

22. G. M. Schier, I. E. T. Gan, B. Halpern, J. Korth, Clin. Chem., **26**, 147-149 (1980).

23. R. Matsuda, Y. Takeda, M. Ishibashi, M. Uchiyama, M. Susuki, S. Takitani, Bunseki Kagaku, **35**, 151-156 (1986); Anal. Abstr., **48**, 9E11 (1986).

24. T. Tatsuhara, H. Muro, F. Ozoe, Yakugaku Zasshi, **102**, 988-991 (1982); Anal. Abstr., **44**, 6E25 (1983).

25. Z. L. Chang, "Sodium Valproate and Valproic Acid", in **Analytical Profiles of Drug Substances**, vol. 8, K. Florey, ed., Academic Press, New York, NY, 1979, pp. 552-553.

26. R. Gatti, V. Cavrini, P. Roveri, Chromatographia, **33**, 13-18 (1992).

27. H. Suzuki, K. Akimoto, H. Nakagawa, Chem. Pharm. Bull., **39**, 133-136 (1991).

28. United States Pharmacopeia 23, U.S. Pharmacopeial Convention, Inc., Rockville, MD, 1995, pp. 1619-1620.

29. L. A. Sternson, "General Aspects of Precolumn Derivatization with Emphasis on Pharmaceutical Analysis", in **Chemical Derivatization in Analytical Chemistry,** vol.1, R. W. Frei, J. F. Lawrence, eds., Plenum Press, New York, NY, 1981, pp. 173-175.

30. J. A. P. Meulendijk, W. J. M. Underberg,"Ultraviolet-Visible Derivatization", in **Detection-Oriented Derivatization Techniques in Liquid Chromatography**, H. Lingeman, W. J. M. Underberg, eds., Marcel Dekker, Inc., New York, NY, 1990, pp. 252-253.

31. H. D. Durst, M. Milano, E. J. Kikta, Jr., S. A. Connelly, E. Grushka, Anal. Chem., **47**, 1797-1801 (1975).

32. L. Nagels, C. Debeuf, E. Esmans, J. Chromatogr., **190**, 411-417 (1980).

33. R. F. Borch, Anal. Chem., **47**, 2437-2439 (1975).

Received June 7, 1996
Accepted June 18, 1996
Manuscript 4205

J. LIQ. CHROM. & REL. TECHNOL., 20(13), 2089–2098 (1997)

CHROMATOGRAPHIC SEPARATION OF SOME COUMARINS AND FLAVONOIDS ON DIOL-MODIFIED SILICA GEL PHASE

Maria L. Bieganowska

Department of Inorganic and Analytical Chemistry
Medical Academy
Staszica 6
20-081 Lublin, Poland

ABSTRACT

The chromatography of polar compounds, such as coumarins and flavonoids, has been investigated on diol-bonded silica gel layers using various organic modifiers (2-propanol (2PrOH), ethyl acetate (EtOAc), methyl ethyl ketone (MeEtCO), 2-propyl ether $(2Pr)_2O$, 1,4-dioxane (DX) and tetrahydrofuran (THF)) in n–heptane as the mobile phase. It was found that diol phases are very suitable for separation of the above-mentioned compounds in normal phase thin-layer chromatography. The selectivity of the systems, the adsorptive properties of the diol–modified phases, and the influence of the organic modifiers on retention are discussed. Some results, obtained on diol-bonded silica gel, are compared to underivatized silica gel as R_M diol vs. R_M silica relationship.

INTRODUCTION

In recent years, high performance liquid chromatography (HPLC) has become very important as an analytical technique well established in the

Table 1

The Compounds Studied

No.	Popular Name	Systematic Name
1	Coumarin	
2	4-Hydroxycoumarin	
3	Umbelliferon	7-Hydroxycoumarin
4	4-Methylesculetin	4-Methyl-6,7-dihydroxycoumarin
5	Isopimpinellin	5,8-Dimethoxypsoralen
6	Esculin	6,7-Dihydroxycoumarin-6-glucoside
7	Flavone	
8	α-Naphthoflavone	
9	Kaempferol	3,4',5,7-Tetrahydroxyflavone
10	Quercetin	3,3',4',5,7-Pentahydroxyflavone
11	Isoquercitrin	Quercetin-3-O-glucoside
12	Robinetin	3,3',4',5',7-Pentahydroxyflavone
13	Robinin	Kaempferol-3-O-robinoside-7-O-rhamnoside
14	Myricetin	3,3',4',5,5',7-Hexahydroxyflavone
15	Luteolin-7-O-glucoside	3',4',5,7-Tetrahydroxyflavone-7-O-glucoside
16	Rutin	3,3',4',5,7-Pentahydroxyflavone-3-O-rhamnoglucoside
17	Hesperetin	3',5,7-Trihydroxy-4'-methoxyflavanone
18	Hesperidin	Hesperetin-7-O-rhamnoglucoside
19	Naringin	4',5,7-Trihydroxyflavanone-7-O-rhamnoglucoside
20	Pelargonidin chloride	3,4',5,7-Tetrahydroxyflavylium chloride
21	Malvin chloride	3,4',5,7-Tetrahydroxy-3',5'-dimethoxyflavylium-3,5-diglucoside

separation of coumarins and flavonoids.[1-8] At present, after the introduction of HPTLC plates with various chemically bonded stationary phases (aminopropyl, phenyl, alkyl, cyanopropyl, diol, and others), they are applied even to difficult separations of closely related compounds in mixtures. The most frequent silica and silanized silica gel plates were used,[9-15] but the polar bonded phases with amino[16] or diol functional groups were used considerably less. Despite many publications on TLC of coumarins and flavonoids, no publication on the separation of investigated compounds on diol phases could be found. These very polar compounds are strongly retained on silica gel plates, but diol phases which behave like a deactivated silica gel with a similar retention mechanism should be more suitable for chromatographic analysis.[17,19]

In this paper, the retention of coumarins and flavonoids on HPTLC-Diol plates, in normal-phase systems, was investigated. The experimental data obtained on diol-phases was compared to the data obtained with bare silica gel phases.

EXPERIMENTAL

Thin-layer chromatography was performed with 10 x 10 cm glass HPTLC-DIOL F_{254} precoated plates (E. Merck, Darmstadt, Germany) in a sandwich chamber with an eluent distributor. Samples (2 μL) of solution (0.2% w/v) from the solutes in methanol were spotted and developed to a distance of 8 cm. The location of the spots was determined under UV light (λ = 254 nm). The results (an average of three measurements, differing by no more than 0.02 R_F units) and chromatographic conditions used are given either in the figures or in the text. The temperature was maintained at 20±1°C. All reagents were of analytical reagent grade from E. Merck. The investigated coumarins and flavonoids are listed in Table 1.

RESULTS

Plots showing the effect of organic modifier concentration on the retention of a series of coumarins and flavonoids on HPTLC-DIOL plates as adsorbent are presented in Figures 1-3. In most instances, the linear relationship R_M = f(log C%) was obtained in accordance with the equation $R_M = R^0_M - m \log C\%$. The experimental data obtained for dioxane and 2-propanol are presented in Figs. 1 and 2. The lines sometimes intersect, giving different selectivities relative to the concentration of the stronger component in the mobile phase. In Fig. 1A and 1B, the results obtained for dioxane in n-heptane as the mobile phase are presented. The very high slopes obtained on the diol-silica phase indicate a multipoint adsorption, as well as solvation in the mobile phase. An increased number of the active sites (e.g.OH groups) in the solute molecule causes a considerably stronger adsorption (lower R_F values) and also increased intercept (R^0_M) values. The glucosides, as very polar compounds, have a lower R_F value on the diol-phase than do their aglycones. Methylation of the hydroxyl group decreases the retention and, also, the slopes and intercepts are lower. Generally, the order of R_F values is in accordance with their hydrophobicity $ROCH_3$ >ROH > $R(OH)_2$ > RO-sugar.[12] Malvine chloride, hesperetin and isopimpinellin exhibit lower slope and intercept values according to their hydrophobic methoxyl groups; they have higher R_F values. Coumarins and flavonoids possessing hydroxyl groups have the possibility of forming hydrogen bonds with alcoholic hydroxyl groups on the diol-adsorbent.

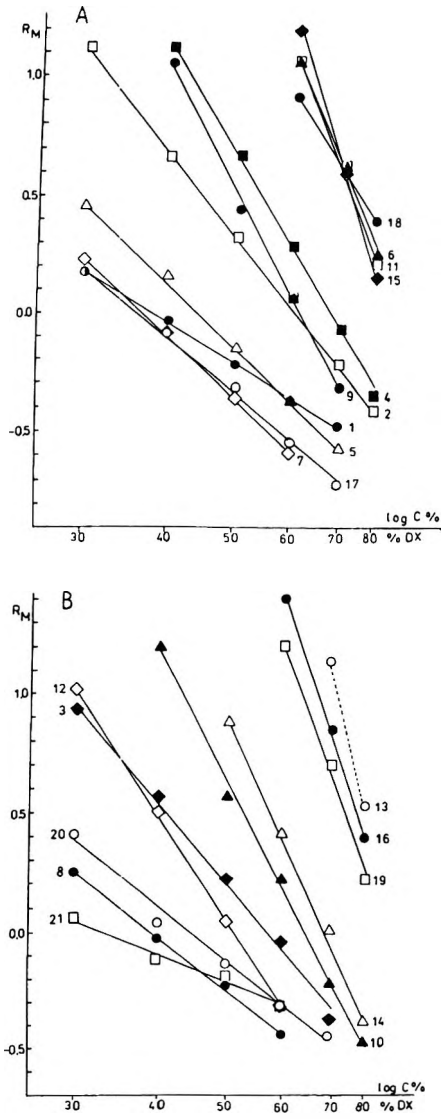


Figure 1. Dependence of coumarins and flavonoids R_M values on log C (% v/v) for HPTLC on diol-modified silica with dioxane - n-heptane as mobile phase. Compound identities are as in Table 1.

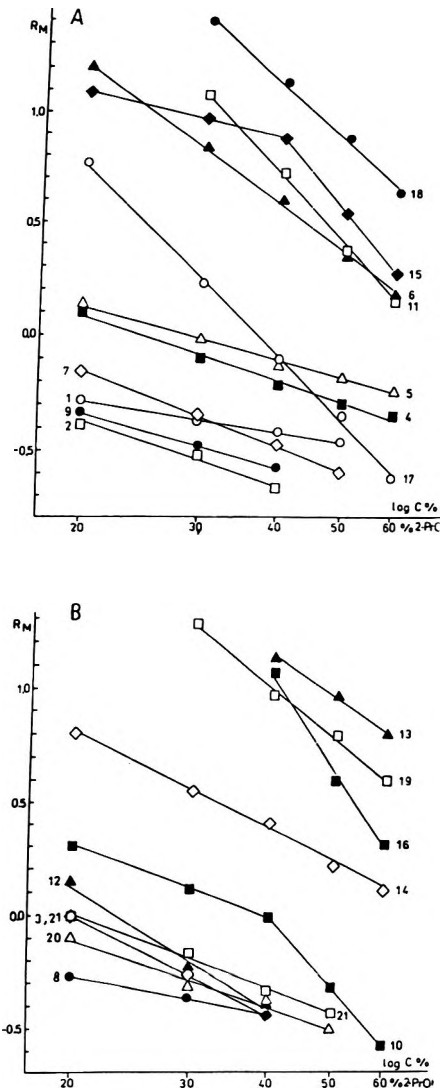


Figure 2. Dependence of coumarins and flavonoids R_M values on log C (% v/v) for HPTLC on diol-modified silica with 2-propanol - n-heptane as mobile phase. Compound identities are as in Table 1.

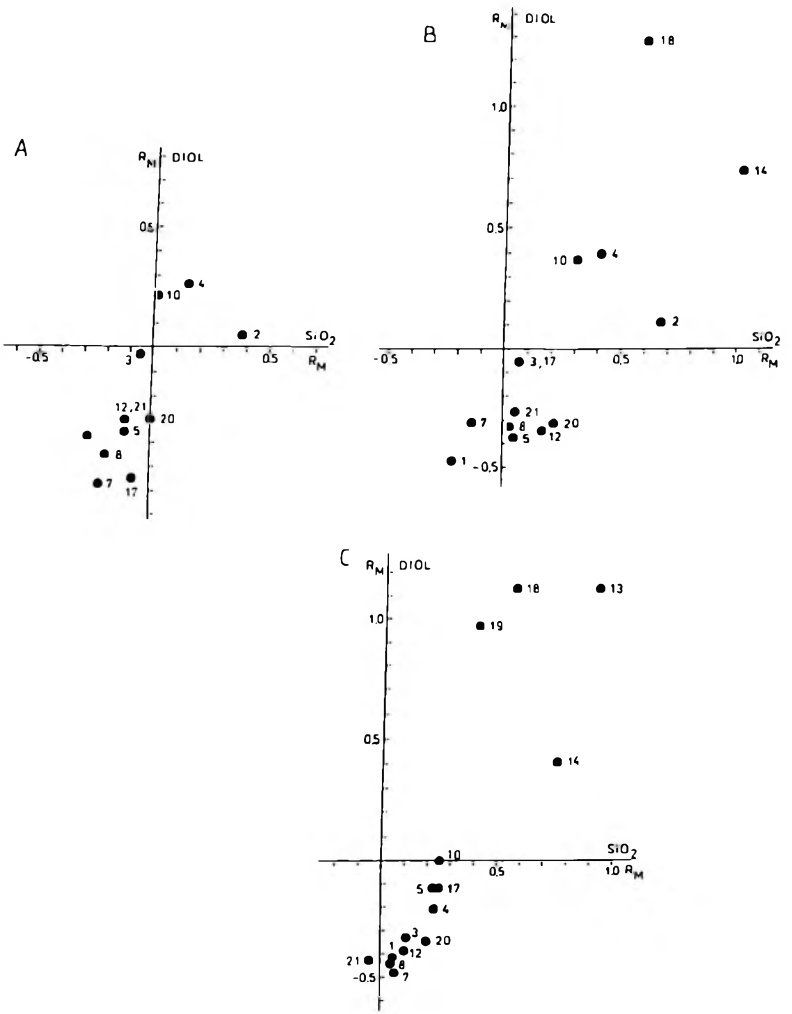


Figure 3. Correlation between R_M values of coumarins and flavonoids obtained on diol and silica layers. Mobile phases: 3A, 60:40 (v/v) dioxane - n-heptane for diol and 80:20 (v/v) dioxane - n-heptane for silica; 3B, 60:40 (v/v) ethyl-acetate - n-heptane for diol and 80:20 (v/v) ethyl-acetate - n-heptane for silica; 3C, 40:60 (v/v) 2-propanol for both adsorbents. For identification of solutes, see Table 1.

Table 2

R_M(HPTLC$_{DIOL}$) and ΔR_M Values Relative to Coumarin*

No.	40% 2-PrOH + Heptane		60% EtOAc + Heptane		60% MeEtCO + Heptane		50% THF + Heptane		60% DX + Heptane		80% $(2\text{-Pr})_2O$ + Heptane	
	R_M	ΔR_M	R_M	ΔR_M	R_M	ΔR_M	R_M	ΔR_M	R_M	ΔR_M	R_M	ΔR_M
1	-0.44	---	-0.51	---	-0.43	---	-0.40	---	0.37	---	0.08	---
2	-0.67	-0.23	0.10	0.61	0.00	0.43	-0.12	0.28	0.04	0.41	0.73	0.65
3	-0.33	0.11	-0.04	0.47	-0.04	0.39	-0.13	0.27	-0.03	0.34	0.55	0.47
4	-0.21	0.23	0.40	0.91	0.31	0.74	0.06	0.46	0.27	0.64	1.07	0.99
5	-0.13	0.31	-0.38	0.13	-0.31	0.12	0.29	0.11	-0.37	0.00	0.37	0.29
6	0.60	-0.16	---	---	1.20	1.63	1.08	1.48	1.08	1.45	---	---
7	-0.52	-0.08	-0.31	0.20	-0.21	0.22	-0.40	0.00	-0.57	-0.20	0.51	0.43
8	-0.47	-0.03	-0.34	0.17	-0.23	0.20	-0.38	0.02	-0.47	-0.10	0.48	0.40
9	-0.58	-0.14	-0.31	0.20	0.06	0.49	-0.15	0.25	0.06	0.43	0.41	0.33
10	0.00	0.44	0.37	0.88	0.41	0.84	0.06	0.46	0.22	0.59	1.14	1.06
11	0.73	1.17	---	---	---	---	1.08	1.48	1.08	1.45	---	---
12	-0.40	0.04	-0.23	0.28	-0.25	0.18	-0.34	0.06	-0.32	0.05	0.36	0.28
13	1.13	1.57	---	---	---	---	---	---	---	---	---	---
14	0.41	0.85	0.73	1.24	0.84	1.27	0.17	0.57	0.40	0.77	1.38	1.30
15	0.88	1.32	---	---	---	---	1.20	1.60	1.20	1.57	---	---
16	1.06	1.50	---	---	---	---	---	---	---	---	---	---
17	-0.11	0.33	-0.05	0.46	0.06	0.49	-0.18	0.22	-0.53	-0.16	0.88	0.80
18	---	---	---	---	---	---	1.13	1.53	0.93	1.30	---	---
19	0.98	1.42	---	---	---	---	---	---	1.20	1.57	---	---
20	-0.36	0.08	-0.34	0.17	-0.25	0.18	-0.29	0.11	-0.32	0.05	0.43	0.35
21	-0.43	0.01	-0.28	0.23	-0.08	0.35	-0.37	0.03	-0.32	0.05	0.22	0.14

* Compound No. 1 in Table 1.

surface. Thus, the more OH groups in the molecule, the stronger binding with polar groups of the adsorbent and the lower the R_F values. Coumarins and flavonoids act as proton donors or proton acceptors towards the adsorbent-active sites that greatly influence retention and separation selectivity.

The results presented in the figures as plots R_M = f(log C%) clearly visualize the differences of the selectivity. 2-Propanol, with a higher eluent strength than dioxane, gave comparable R_F values at lower concentrations

(Figs. 2A and 2B). The elution order of the solutes changed, somewhat, but generally, the more polar compounds (similarly with dioxane as modifier) are more strongly retained. The dependence of retention and selectivity on the type of organic modifier in the mobile phase is shown in Table 2. Selectivities of modifiers are compared, and the best composition of the mobile phase to be chosen as complementary solvent systems for rechromatography or for two-dimensional development of the compounds not separated in one system. Some compounds had too low or too high R_F values (beyond the range 0.2 - 0.8 RF units). Robinine, luteolin 7-O-glucoside, rutin and pelargonidin, the more polar compounds, did not migrate from the origin, but α-naphthoflavone, a more hydrophobic compound, migrated at the solvent front. Diisopropyl ether is a more selective modifier of low eluent strength. An 80% concentration of 2-propanol in n-heptane was used to elute the majority of the investigated compounds in a reasonable range, in approximately the same order as was obtained for other systems used.

The selectivity is very different from one modifier to another. Figs. 3A, 3B, 3C show $R_{M(diol)}$ vs. $R_{M(silica)}$ relationships in dioxane, ethyl acetate and 2-propanol systems. The compounds are more strongly retained on silica than on the diol-phases (Fig. 3A). The selectivity on both types of adsorbent is very similar; for quercetin, 4-hydroxycoumarin, and 4-methylesculetin, a better selectivity on silica plates was observed and, for naringin, umbelliferon, malvin chloride, and hesperetin, on diol plates.

The second electron-donor solvent, ethyl acetate, was also compared on both adsorbents. For some compounds, the diol-phase is more selective; for other compounds, silica is more selective. In the 2-propanol - n-heptane systems (Fig. 3C), the solutes were adsorbed less strongly than in dioxane and ethyl acetate - n-heptane. 2-Propanol, a proton donor-acceptor solvent with the highest eluent strength, at 40% concentration in the mobile phase, gave comparable R_F values whereas, for dioxane and ethyl acetate, 60% concentrations were required. For many compounds (robinine, myricetin, quercetin, 4-methylesculetin and pelargonidin chloride), the diol phase was more selective than the silica gel phase; however, in both cases, hydroxyl groups were the active centers influencing the retention.

CONCLUSION

The diol-phases are very suitable for separation of coumarins and flavonoids. The investigated compounds are less strongly adsorbed on diol-modified than on underivatized silica gel. Differences in the selectivity of adsorbents and eluent systems can be utilized in the difficult separation of

closely related compounds and they offer the possibility of choosing suitable phase systems to identify the substances and they are useful in two-dimensional development.

REFERENCES

1. K. Głowniak, M. L. Bieganowska, J. Chromatogr., **370**, 281 (1986).

2. K. Głowniak, M. L. Bieganowska, J. Liq. Chromatogr., **8(16)**, 2927 (1985).

3. R. V. Tamma, G. Miller, R. Everett, J. Chromatogr., **322**, 236 (1985).

4. M. C. Pietrogrande, C. Bighi, G. Blo, Y. D. Kahie, P. Reschiglian, F.Dondi, Chromatographia, **27**, 625 (1989).

5. J. F. Goossens, A. J. Van Laere, J. Chromatogr., **267**, 439 (1983).

6. F. Siwek, R. Galensa, J. Chromatogr., **294**, 395 (1984).

7. R. N. Stewart, S. Asen, D. R. Massie, K. H. Norris, Biochem. Syst. Ecol., **8**, 119 (1980).

8. J. Gudej, M. L. Bieganowska, Chromatographia, **30**, 333 (1990).

9. M. L. Bieganowska, K. Glowniak, Chromatographia, **25**, 111 (1988).

10. M. L. Bieganowska, A. Petruczynik, A. Zobel, J. Planar Chromatogr., **9**, 273 (1996).

11. K. Głowniak, G. Matysik, M. L. Bieganowska, E. Soczewinski, Chromatographia, **22**, 307 (1986).

12. E. Menziani, B. Tosi, A. Bonora, P. Reschiglian, G.Lodi, J. Chromatogr., **511**, 396 (1990).

13. P. Harmala, L. Botz, O. Stiicher, R. Hiltunen, J. Planar Chromatogr., **3**, 515 (1990).

14. D. Heimler, V. Boddi, J. Chromatogr., **466**, 371 (1989).

15. F. Dondi, G. Grassini-Strazza, Y. D. Kahie, G. Lodi, C. Pietrogrande, P. Reschiglian, C. Bighi, J. Chromatogr., **462**, 205 (1989).

16. M. L. Bieganowska, A. Petruczynik, Chem. Anal., **40**, 869 (1995).

17. T. Cserhati, H. Hauck, J. Chromatogr., **514**, 45 (1990).

18. H. E. Hauck, M. Mack, W. Yost, in J. Sherma and B. Fried (Eds.) **Handbook of Thin Layer Chromatography**, Chromatographic Science Series, Vol. 55, Marcel Dekker, Inc., New York, 1991, p. 87.

19. P. Martin, J. Taberner, R. J. Throp, I. D. Wilson, J. Planar Chromatogr., **5**, 99 (1992).

20. L. Witherow, R. J. Throp, I. D. Wilson, A. Warrander, J. Planar Chromatogr., **3**, 169 (1990).

21. L. R. Snyder, J. W. Dolan, J. R. Gant, J. Chromatogr., **165**, 3 (1979).

22. L. R. Snyder, J. L. Glajch, J. J. Kirkland, J. Chromatogr., **218**, 229 (1981).

23. P. J. Schoenmakers, H. A. H. Billiet, L. deGalan, J. Chromatogr., **185**, 179 (1979).

24. E. Soczewinski, Anal. Chem., **41**, 179 (1969).

Received June 18, 1995
Accepted January 3, 1997
Manuscript 3895

J. LIQ. CHROM. & REL. TECHNOL., 20(13), 2099-2108 (1997)

DEVELOPMENT OF AUTOMATED HIGHLY SENSITIVE ANALYTICAL SYSTEM FOR GUANETHIDINE SULFATE IN SERUM

Yukiko Inamoto,[1] Shigeyuki Inamoto,[1] Toshihiko Hanai,[2]
Yuichiro Takahashi,[3] Kazuomi Kadowaki,[4] Toshio Kinoshita[5]

[1]Research & Development
Teikoku Seiyaku Co., Ltd.
567 Sanbonmatu, Ochi-Cho, Okawa-Gun
Kagawa 769-26 Japan

[2]International Institute of Technological Analysis
Health Research Foundation
Institut Pasteur de Kyoto, 5F
Hyakumanben, Sakyo-Ku
Kyoto 606 Japan

[3]Jinken Clinic
1-8-6 Chuo, Ebina-shi
Kanagawa, 243-04 Japan

[4]Kitasato Institute Hospital
Department of Urology
9-1 Shirokane-5, Minato-ku
Tokyo 108 Japan

[5]School of Pharmaceutical Sciences
Kitasato University
9-1, Shirokane-5, Minato-Ku
Tokyo 108 Japan

ABSTRACT

An automated trace analytical system for guanethidine sulfate in sera was developed using a liquid chromatograph with column-switching and post-column reaction detection systems. Guanethidine sulfate was post-labeled with ninhydrin in alkaline solution, and the reaction products were monitored with a fluorescence detector. Guanethidine sulfate, an antihypertensive agent, in rat and human sera was analyzed using this automated analyzer. The detection limit of guanethidine sulfate was 1.0 ng/mL, and the calibration curve was linear from 3.1 ng/mL to 1000 ng/mL with relative standard deviation of less than 3%. The recovery from sera was 99.7 ± 3.9%

INTRODUCTION

Guanethidine sulfate, 1-[2-(1-perhydroazocinyl) ethyl] guanidine monosulfate, is an antihypertensive agent usually administered orally to patients with essential hypertension. Also, this drug is a selective inhibitor of the sympathetic nervous system at the postganglionic terminal of the adrenergic neuron. Hannington-Kiff[1] first reported the use of guanethidine for the treatment of reflex sympathetic dystrophy. Since then, there have been numerous reports regarding the effectiveness of intravenous regional block of guanethidine.[2-4] Sharpe et al.[5] reported a case of severe and persistent hypotension which occurred after a repeated guanethidine block, and concluded that a careful monitoring of the cardiovascular system is important to prevent a prolonged hypotension. Therefore, a rapid, and sensitive analytical method for guanethidine sulfate is required for monitoring its concentration in patients' sera and for determination of bioavailability of different preparations.

Several methods have been reported for the determination of guanidine such as oxidimetry,[6] complexometry,[7] calorimetry,[8-10] fluorometry,[11-12] and liquid chromatography.[13-16] Gas chromatography, after selective extraction, was used for determination in biological fluids.[17] Guanethidine sulfate, in tablets and biological fluids, was analyzed by a spectrofluorometric method using the condensation reaction of the guanidino group with 9,10-phenanthraquinone,[18] and with benzoin.[19] The official method[20] for assaying of guanethidine sulfate is based on colorimetry of the guanidino group with sodium nitroferricyanide and potassium ferricyanide. However, the disadvantage of this method is the time-consuming, selective extraction procedures and the low sensitivity.

Hiraga and Kinoshita[21] developed a post-column high performance liquid chromatography method with fluorometric detection, based on the detection of intense fluorescence produced using a reaction of guanidino base and ninhydrin under alkaline conditions. We modified this method and connected an on-line pre-treatment column for the selective extraction of guanethidine sulfate from sera.

Here, we describe the details and performance of our newly developed analytical system for guanethidine sulfate analysis, based on a liquid chromatograph with automated column-switching and post-column reaction fluorometric detection. This system was applied for analysis of guanethidine sulfate in rat and human sera.

EXPERIMENTAL

Materials and Methods

Guanethidine monosulfate was purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, U.S.A.). Ion pair reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals were purchased from Wako Chemical Co. Ltd. (Tokyo, Japan). Blood samples, collected from rat and human, were centrifuged at 1,000 g for 15 min at room temperature and the supernatant was directly injected into this analytical system.

Chromatography

Figure 1 shows the flow diagram of the automated analytical system based on a liquid chromatograph with a column-switching and a post-column reaction detector. Eluent 1 consisted of 50 mM sodium dihydrogenphosphate (pH 3.0 adjusted with 50 mM phosphoric acid). Eluent 2 consisted of acetonitrile and 50 mM sodium dihydrogenphosphate (pH 3.0 adjusted with 50 mM phosphoric acid) (30:70, v/v) containing sodium 1-octanesulfonate (7 g/L). Eluent 3 was aqueous acetonitrile (500 ml/L).

Eluent 1 was discarded after passing through the column switching valve MV, Model MV-8010 (Tosch, Tokyo, Japan) and pre-column I (TSK precolumn BSA-ODS; 35 mm x 4.6 mm I.D., 10 μm, Tosoh).

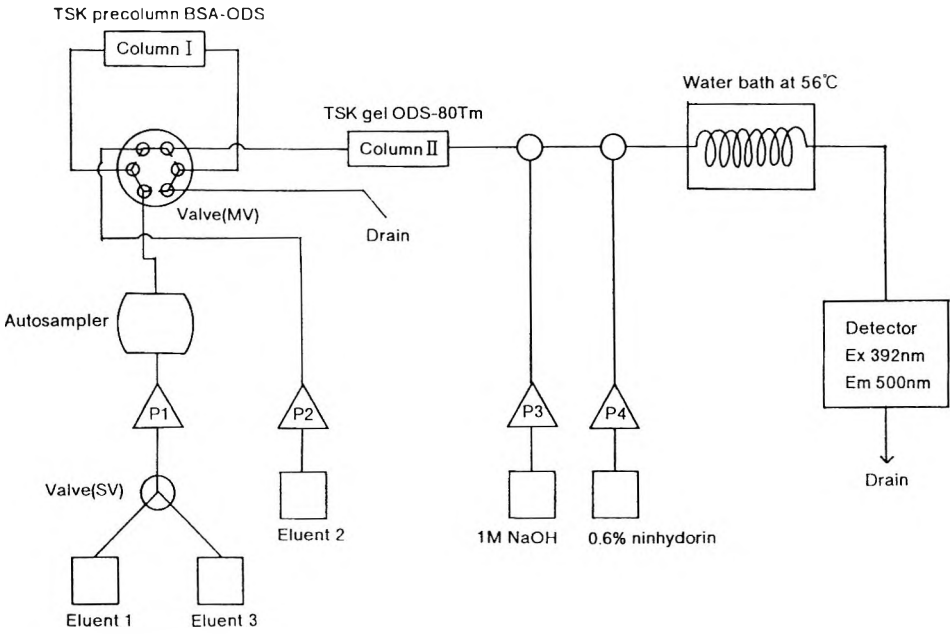


Figure 1. Flow diagram of the automated guanethidine analyzer based on a liquid chromatograph with column-switching and post-column reaction systems.

Eluent 2 flowed through the switching valve to the analytical column II (TSKgel ODS-80TM; 150 mm x 4.6 mm I.D., 5 μm, Tosoh) and a spectrofluorometric detector, Model FS-8010 (Tosoh), then to waste in that order. The flow rate of eluents 1 and 2 were 1.0 mL/min regulated with two Model CCPM (Tosoh) pumps.

Aliquots of serum samples, each 200 μL, were directly injected into column I using an autosampler, Model AS-8000 (Tosoh), where they were trapped and rinsed with eluent 1. Guanethidine sulfate was separated from the interfering substances and proteins in serum on pre-column I. Two minutes after injection, samples were transferred from pre-column I to column II with eluent 2 by turning valve MV. Valve MV was then returned to the original position after 5 min. At 15 min after sample injection, precolumn I was rinsed with eluent 3 at a flow-rate of 1.0 mL/min. For 15-22 min, eluent 3 in pre-column I was replaced with eluent 1, returning to the initial conditions, by switching valve SV (Model SV-8010, Tosoh).

On the other hand, eluent 2 passed through MV and column II during analysis, and passed from pre-column I to column II over a period of 2-5 min via MV. Guanethidine sulfate was separated on column II.

Post-column derivatization was accomplished as follows: Aqueous ninhydrin solution (6 g/L) and 1 M sodium hydroxide were delivered from two reagent pumps (Model 885-PU, Japan Spectroscopic, Tokyo, Japan), at flow-rates of 0.3 mL/min. The effluent from column II was first mixed with 1 M sodium hydroxide, then mixed with aqueous ninhidrin solution (6 g/L). The mixture was heated in a reaction coil (Teflon™ tube, 10 m x 0.5 mm I.D.), placed in a water-bath at 56°C, and the reaction products were monitored at Ex 392 nm and Em 500 nm with a spectrofluorometric detector.

Animal Experiments

Male wistar rats, weighing 250-280 g, were fasted for 18h before experiments. Guanethidine sulfate in saline was administered to rats intramuscularly. Blood samples were taken from the right jugular vein before, as well, as 15, 30, 60 and 120 min after administration. The amounts of guanethidine sulfate in their sera were analyzed quantitatively.

RESULTS

The excitation and fluorescence maxima obtained from the reaction products were 392 nm and 500 nm, respectively. Post-column derivatization was performed by injecting 10 μL of standard solution of guanethidine sulfate (5 μg/L) using a flow injection method. The effluent was allowed to react with reagents containing various concentrations of ninhydrin (1-10 g/L) and sodium hydroxide (0.5-1.5 M) at a flow rate of 0.3 mL/min, passing through a heating device set at various temperatures (30-70°C), and the fluorescence intensity of the reaction products was measured with a spectrofluorometric detector. The fluorescence intensities reached plateaus at the ninhydrin and sodium hydroxide concentrations of 5 g/L and 0.8 M, respectively. The fluorescence increased with increasing reaction temperature and reached a maximum at 56°C. In the standard procedure, an aqueous solution containing ninhydrin (6 g/L) and 1 M sodium hydroxide was adopted as the reaction reagent, and the reaction temperature was 56°C.

A pretreatment method, to clean up samples and for separation of guanethidine sulfate, was examined using rat serum. A TSK precolumn BSA-ODS (bovine serum albumin-coated ODS column; 35 mm x 4.6 mm I.D.,

particle size 10 μm) and a TSK precolumn SW (hydrophilic silica gel column; 35 mm x 4.6 mm I.D., particle size 15 μm) were examined as pre-column I for sample clean-up. A good separation of guanethidine sulfate from the interfering substances and proteins in serum was achieved using the TSK precolumn BSA-ODS. Retention of guanethidine sulfate on pre-column I and separation from the interfering substances were influenced by the pH of sodium phosphate buffer. The final solution for eluent 1 was 50 mM sodium phosphate solution, pH3.0.

Guanethidine sulfate concentrated on pre-column I was eluted with eluent 2, and the effluent was transferred to column II (TSKgel ODS-80TM) for further separation. The effects of ion-pair reagents were examined to separate the drug from the interfering substances in the effluent on column II. The best separation was achieved in acetonitrile and 50 mM sodium dihydrogenphosphate (pH 3.0, adjusted with 50 mM phosphoric acid) 30:70, v/v mixture containing sodium 1-octanesulfonate (7 g/L).

Under these conditions, a chromatogram of human serum was shown in Fig. 2. No compounds were found to interfere in the quantitative analysis of guanethidine. The calibration curve for guanethidine sulfate was linear, in the range from 3.1 to 1000 ng/mL in human serum. The peak area was related to the amount of guanethidine sulfate in human serum. The relationship was $y = 0.8424x-0.7818$ ($r = 0.9999$). The detection limit of guanethidine sulfate was 1.0 ng/mL (S/N = 3). The reproducibility of this procedure was sufficient, and the coefficient of variation for 50 ng/mL guanethidine sulfate in human serum was 2.6 % (n=100). The recoveries from human serum were around 99.7 ± 3.9% (n=40).

The solutions of guanethidine sulfate in saline were administered to rats intramuscularly. Figure 3 showed the dose-response profiles of serum guanethidine sulfate levels in rats. The changes in guanethidine sulfate in rat serum were dose-dependent.

DISCUSSION

In the newly developed analytical system for the determination of guanethidine sulfate in serum, sample treatment is very simple requiring only one centrifugation step. The supernatant is automatically cleaned up with a column-switching method, separated, and the guanethidine derivatives undergo post-column derivatization and are quantified with a fluorescence detector.

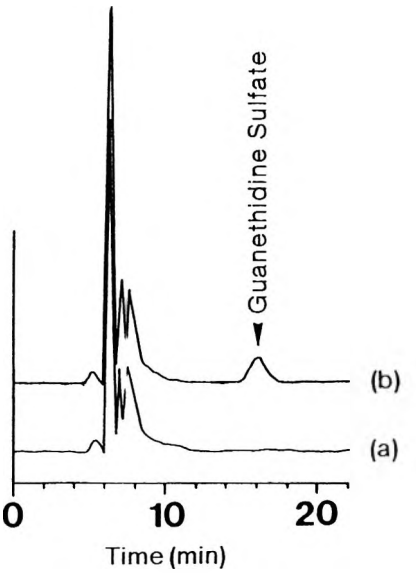


Figure 2. Chromatograms of guanethidine sulfate spiked to human serum: (a) control and (b) spiked.

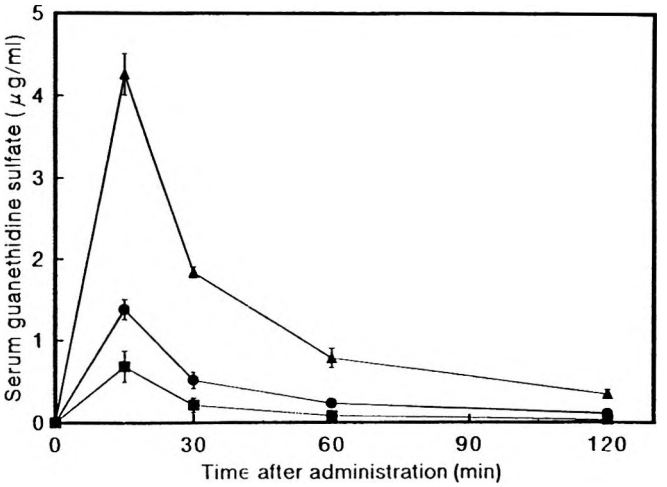


Figure 3. Dose-dependency of serum guanethidine sulfate levels following intramuscular administration to rats. Intramuscular administration: (■) 2, (●) 4, (▲) 10 mg/kg. Each point is the mean ± S.D. of 5 animals.

Guanethidine sulfate uniquely targets to the peripheral sympathetic nervous system. The adrenergic blocking effects of guanethidine sulfate requires presence of the drug in adrenergic neurons; the degree of adrenergic blockade is a function of the plasma concentration of guanethidine under normal conditions.[22] Adrenergic neuronal blockade is generally accomplished by plasma concentrations greater than 10 μg/L. After a single oral dose of 41mg guanethidine-^{14}C, the maximum blood concentration of uncharged guanetidine was 22 μg/L,[23] and half-life was diphasic; 1.5 days and from 4.1 to 7.7 days.[24] Guanethidine bioavailability was incomplete and variable among individual subjects. Only 3 to 50 % of an oral-dose reached the systemic circulation.[23,25,26] Such variation might be attributable to individual differences in enteric absorption, first-pass metabolism, or renal clearance of guanethidine.

A significant correlation was also observed between the area under the plasma level curve during the dose interval and the oral maintenance dose.[24] With regard to administration of guanethidine sulfate in patients, those with renal failure show orthostatic hypotension because of decreased renal blood flow and depression of renal excretion. A stronger antihypertensitive action causes a depression of metabolic function in aged patients. Therefore, it is desirable to quantify serum concentration of guanethidine sulfate in patients to determine appropriate dosage and dosing interval as well as to avoid side-effects.

In conclusion, this method is simple, rapid, and sensitive, and is therefore expected to be clinically useful for monitoring the concentration of guanethidine sulfate in patients.

REFERENCES

1. J. G. Hannington-Kiff, Lancet, 8022, 1132-1133 (1977).

2. J. Driessen, C. Van Der Werken, P. A. Nicolai, J. F. Crul., Acta Anaethesiol. Scand., **27**, 505-509 (1983).

3. A. G. Rocco, A. F. Kaul, R. M. Reisman, J. P. Gallo, P. A. Lief, Clin. J. Pain, **5**, 205-209 (1989).

4. S. Ramamurthy, J. Hoffman, the Guanethidine Study Group, Anesth. Analg., **81**, 718-723 (1995).

5. E. Sharpe, R. Milaszkiewicz, F. Carli, Anaesthesia, **42**, 1081-1084 (1987).

6. I. Ganescu, G. Brizan, C. Verhelyi, Chem. Anal. (Warsaw), **29**, 549-553 (1984).

7. E. Zollnernevan, Acta Pharm. Hung.,**48**, 76-84 (1978).

8. I. Ganescu, I. Papa, M. Preda, Pharmazie, **40**, 495 (1985).

9. G. R. Rao, S. Raghuveer, Indian J. Pharm. Sci., **42**, 141-142 (1980).

10. S. Tanabe, T. Oya, T. Sakaguchi, Chem. Pharm. Bull., **23**, 1657-1663 (1975).

11. M. A. Parniak, G. Lange, T. Viswanatha, J. Biochem. Biophys. Methods, 7, 267-276 (1983).

12. M. Kai, T. Miura, K. Kohashi K., Y. Ohkura, Chem. Pharm. Bull., **29**, 1115-1120 (1981).

13. Y. Kobayashi, H. Kubo, T.Kinoshita, Anal. Sci., **3**, 363-367 (1987).

14. M. Kai, T. Miyazaki, Y. Ohkura, J. Chromatogr., **311**, 257-266 (1984).

15. J. T. Stewart, S. S. Clark, J. Pharm. Sci., **75**, 413-415 (1986).

16. Y. Golander, U. Schurrath, R. J. Luck, J. Pharm. Sci., 77, 902-905 (1988).

17. J. H. Hengstmann, F. C. Falkner, J. T. Watson, J. Oates, Anal. Chem., **46**, 34-38 (1974).

18. M. H. Abdel-Hay, S. M. Galal, M. M. Bedair, A. A. Gazy, A. A. M. Wahbi, Talanta **39**, 1369- 1375 (1992).

19. A. M. Wahbi, M. M. Bedair, S. M. Galal SM., M. H. Abdel-Hay, A. A. Gazy, Mikrochim. Acta, **111**, 83-91 (1993).

20. Guanethidine monosulfate, USP XXII, 1990, pp. 623-624.

21. Y. Hiraga, T. Kinoshita, J. Chromatogr., **342**, 269-275 (1985).

22. I. E. Waiter, J. Khandelwal, F. Falkner, A. S. Nies, Clin. Pharmacol. Ther., **18**, 571-580 (1975).

23. K. H. Rahn, L. I. Goldberg, Clin. Pharmacol. Ther., **10**, 858-866 (1969).

24. J. H. Hengstmann, F. C. Falkner, Europ. J. Clin. Pharmacol., **15**, 121-125 (1979).

25. C. Mcmartin C., P. Simpson, Clin. Pharmacol. Ther., **12**, 73-77 (1971).

26. G. Lukas, Drug Metab. Rev., **2**, 101-116 (1973).

Received August 20, 1996
Accepted September 6, 1996
Manuscript 4265

J. LIQ. CHROM. & REL. TECHNOL., 20(13), 2109-2121 (1997)

VALIDATION OF A STABILITY-INDICATING HPLC METHOD FOR THE DETERMINATION OF DIPYRIDAMOLE IN DIPYRIDAMOLE INJECTION

John Zhang, R. Brent Miller, Susan Russell, Robert Jacobus*

Fujisawa USA, Inc.
Pharmaceutical Sciences Department
2045 N. Cornell Avenue
Melrose Park, IL, 60160, USA

ABSTRACT

The validation of an isocratic high performance liquid chromatographic (HPLC) procedure employing ultraviolet (UV) detection for the analysis of dipyridamole in Dipyridamole Injection is reported. The method is simple, reproducible, accurate, and selective. The peak area versus dipyridamole concentration is linear over the range of 50-150% of its label claim of 5 mg/mL. The mean absolute recovery of dipyridamole using the described method is 101.0 ± 0.6%, (mean ± SD, n = 9). The precision (relative standard deviation, RSD) of label claim amongst five independent sample preparations, is not more than 1.4%. Intermediate precision, as determined from fifteen sample preparations, generated by two Analysts on different HPLC systems over three days, exhibits an RSD of 1.0%. The Standard and Assay Preparations are stable for up to 48 hours at room temperature.

The selectivity was evaluated by subjecting the finished product (Dipyridamole Injection) to thermal, acidic, basic, oxidative, and fluorescent radiation stress conditions. No interference in the analysis of dipyridamole was observed from degradation products, showing the method is stability-indicating.

INTRODUCTION

Dipyridamole is a coronary vasodilator used as an alternative to exercise in thallium myocardial perfusion imaging for the evaluation of coronary artery disease in patients who cannot exercise adequately.[1,2] Dipyridamole Injection is a parenteral solution consisting of dipyridamole (active), tartaric acid, and polyethylene glycol 600 in sterile water for injection.

The analysis of dipyridamole has been determined by spectrophotometry,[3] adsorptive stripping voltammetry,[4] and HPLC with UV[5-7] and electrochemical detection.[8,9] However, none of these methods are selective for potential degradation products in the finished product, although two methods[5,6] do demonstrate selectivity with the raw material. This manuscript describes the validation of a reverse phase HPLC method that is sensitive, accurate, and reproducible for the determination of dipyridamole in Dipyridamole Injection. Moreover, this method was determined to be stability-indicating.

According to the USP 23 <1225> guidelines, analytical methods for the quantitation of major components of bulk drug substance or preservatives in finished pharmaceutical products fall under Assay Category I.[10] Validation data elements required for Assay Category I include precision, accuracy, specificity, range, linearity, and ruggedness. The method for dipyridamole in Dipyridamole Injection satisfies all of these requirements.

EXPERIMENTAL

Chemical and Reagents

Dipyridamole Injection was formulated at Fujisawa USA, Inc. (Melrose Park, IL, USA). Dipyridamole was a USP reference standard. Polyethylene glycol 600 was purchased from Dow Chemical (Freeport, TX, USA). ACS reagent grade hydrochloric acid, acetic acid, sodium acetate, sodium hydroxide, hydrochloric acid, hydrogen peroxide, and NF grade tartaric acid were purchased from Mallinckrodt (Paris, KY, USA).

HPLC grade methanol was purchased from Baxter (Deerfield, IL, USA). The water was deionized and distilled. All reagents were used without further purification.

Apparatus

The chromatographic system consisted of a solvent delivery system, variable wavelength UV-visible detector set at 276 nm, variable volume injector (all HP Model 1050, Hewlett-Packard, Palo Alto, CA, USA). A Waters μBondapak C18 column (3.9 x 300 mm, 10 μm, Waters Associates, Milford, MA, USA) was maintained at ambient temperature. The flow rate was 1.0 mL/minute with a typical operating pressure of *ca.* 1300 psi. Under these conditions, the retention time of dipyridamole was 23 minutes.

Preparation of Solutions

Mobile phase

Prepare an acetate buffer by dissolving 2.38 g of sodium acetate in 350 mL water, adjust to pH 5.1 ± 0.1 with 36% acetic acid and mix. Add 650 mL methanol to the acetate buffer, mix and filter through a 0.5 μm filter, and degas.

Standard preparation

Accurately weigh Dipyridamole, USP reference standard and dilute to volume with Mobile Phase and mix to yield a concentration of 1.0 mg/mL.

Assay preparation

Accurately transfer Dipyridamole Injection (label claim 5.0 mg/mL dipyridamole) and dilute to volume with Mobile Phase and mix to yield a dipyridamole concentration of 1.0 mg/mL.

System Suitability

The system suitability results were calculated according to the USP 23 <621> from typical chromatograms.[11] The instrument precision, as determined by five successive injections of the Standard Preparation, should provide an

RSD not more than (NMT) 2.0%. The column efficiency should be greater than 1000 theoretical plates. The tailing factor should not exceed 1.5 at 5% peak height.

Specificity

The specificity of the method was studied through the analysis of stressed Dipyridamole Injection (finished product) and stressed Placebo Solutions (finished product without dipyridamole). The finished product was subjected to thermal, acidic, basic, oxidative, and fluorescent light environments for set periods of time or until dipyridamole degradation of 10-30%, as determined by peak area percent, was obtained.

Five mL aliquots of the finished product and Placebo Solution were sealed in transparent glass containers with equal head space and exposed to various stress conditions. Thermal stressed samples were stored at 70°C. Acid stressed samples were adjusted to pH 2 with concentrated HCl. Base stressed samples were adjusted to pH 12 with 50% NaOH. Oxidative stressed samples were subjected to 30% H_2O_2. Fluorescent stressed samples were subjected to 500-700 foot-candles of radiation.

Data Acquisition

The peak areas of dipyridamole were measured using HP Chemserver Model 4930 (Hewlett-Packard, Palo Alto, CA, USA). The chromatographic data was automatically processed for peak area followed by an unweighted linear regression analysis.

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms obtained from a 15 μL injection of a Standard Preparation, Assay Preparation, and Placebo Solution are illustrated in Figures 1 (a-c), respectively. The retention time of dipyridamole was 23 minutes. The overall chromatographic run time was 30 minutes.

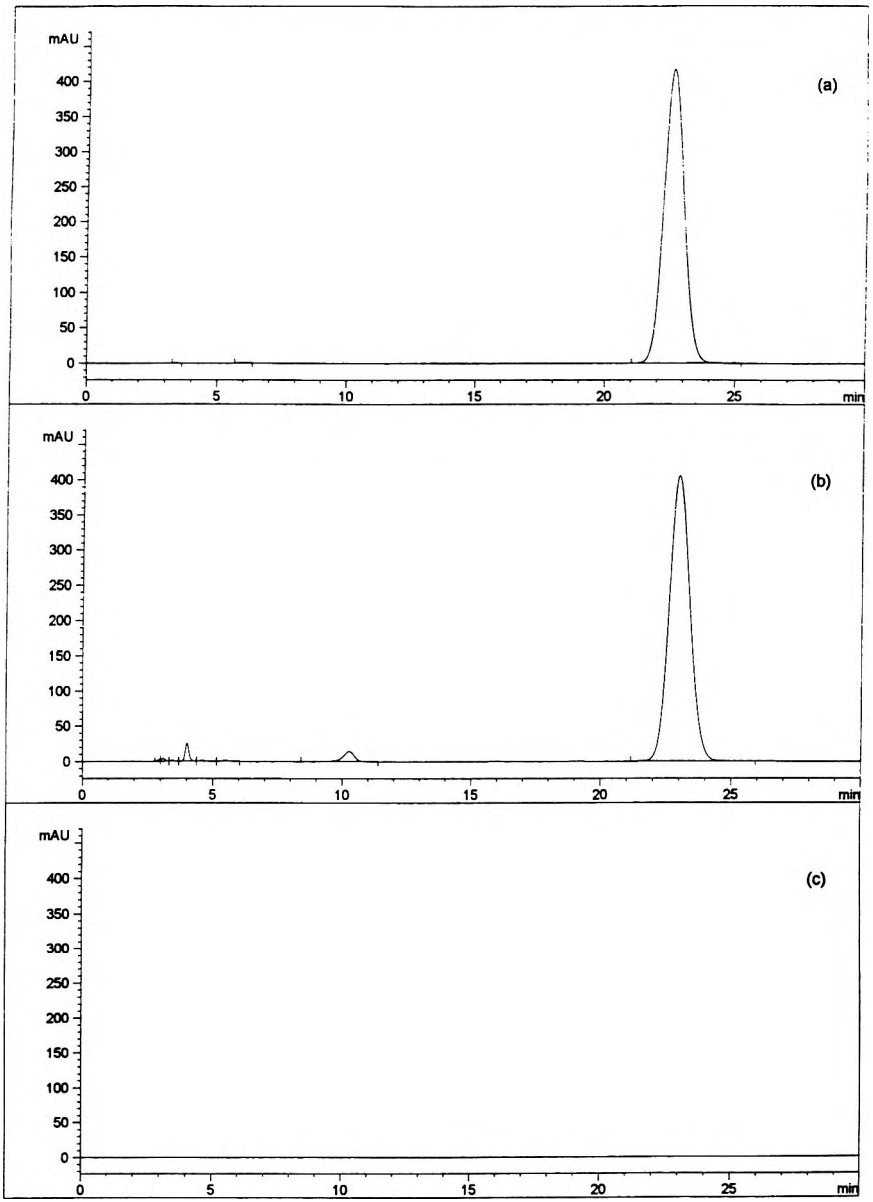


Figure 1. Typical chromatograms of (a) a Standard Preparation, (b) Assay Preparation and (c) Placebo Solution (Dipyridamole Injection not containing dipyridamole). The retention time of dipyridamole is 23 minutes.

Table 1

System Suitability*

Run	% RSD (n=5)	Tailing Factor Limit: NMT 2.0	Column Efficiency Limit: NLT 1000
1	0.3	1.3	2857
2	0.4	1.1	2704
3	0.1	1.2	3407
4	0.1	1.2	3079
5	0.2	1.0	3352
6	0.3	1.1	3021
7	0.1	1.0	3015
8	0.7	1.1	3202
9	0.2	1.1	2655
10	0.4	1.0	3480

* Tailing factor and column efficiency are calculated from the dipyridamole peak.

System Suitability

In all cases, the column efficiency for dipyridamole was greater than 2650 theoretical plates. The tailing factors of dipyridamole were not more than 1.3. The instrument precision, determined by 5 replicate injections of the Standard Preparation, exhibited a maximum RSD of 0.7%. Table 1 illustrates the system suitability results obtained over 10 independent runs spanning 3 months.

Precision

The precision (repeatability and intermediate precision) of the method was determined from one lot of finished product.

Repeatability

Five Assay Preparations were analyzed in a single session by Chemist I with HPLC System I. The RSD of the five results were within the 2% limit (Table 2).

Table 2

Assay Precision*

Run	Assay Value (%)	Average Assay (%)	RSD (%)
1 Chemist I HPLC System I	99.8 100.3 100.3 100.4 97.2	99.6	1.4
2 Chemist I HPLC System I	100.9 100.8 100.7 100.7 100.0	100.6	0.4
3 Chemist II HPLC System II	101.4 101.3 101.4 100.1 100.5	100.9	0.6
Intermediate Precision (n=15)		100.4	1.0

*Repeatability acceptance criteria: RSD NMT 2%.

Intermediate Precision

Intermediate precision was evaluated using Chemist I/HPLC System I to independently analyze five Assay Preparations from the same lot of finished product, and to have another analyst using a different chromatographic system (Chemist II/HPLC System II) analyze five Assay Preparations from the same lot. The RSD of each individual precision run was not more than 2% (Table 2).

Table 3

Assay Accuracy*

Approximate % Claim of Sample	Amount Determined (mg/mL)	Theoretical Amount (mg/mL)	Amount Recovered (%)	Average Recovery (%) (n=3)	RSD (%)
50	0.5104	0.5000	102.1	101.5	0.6
	0.5065	0.5000	101.3		
	0.5049	0.5000	101.0		
100	1.008	1.000	100.8	101.0	0.4
	1.007	1.000	100.7		
	1.014	1.000	101.4		
150	1.520	1.500	101.3	100.6	0.7
	1.500	1.500	100.0		
	1.506	1.500	100.4		
	Overall Recovery (n=9)			101.0	0.6

*Accuracy acceptance criteria: 97.0 to 103.0%. Precision acceptance criteria: 2% within each level.

Table 4

Linearity of Dipyridamole*

% Label Claim	Final Concentration (mg/mL)	Average Peak Area Response
50	0.50	12891651
80	0.80	20654643
100	1.00	25685373
120	1.20	30784865
160	1.61	40631288
	slope, **m** = 2.50×10^7	
	y-intercept, b = 5.81×10^5	
	correlation, r = 1.000	
	bias = 0.02%	

* Coefficient of Correlation Acceptance Criteria: NLT 0.999. Bias acceptance criteria: ± 3.0%.

Table 5

Stability of Analytical Solutions*

Time (Hours)	Peak Area Response and % Change			
	Standard Preparation	% Change	Assay Preparation	% Change
Zero Time	25622948	NA	25795058	NA
25	25480425	-0.6	25677676	-0.5
48	25606819	-0.1	25803515	0.0

* Stability Criteria: stable over the interval where the % change from zero time is within 2%.

Furthermore, the average percent assay values obtained were 99.6, 100.6, and 100.9% for runs 1, 2, and 3, respectively (Table 2). This yields an intermediate precision RSD value of 1.0% (mean=100.4% dipyridamole, n=15) amongst the three runs. The low scatter in the data supports the high degree of ruggedness of the analytical method.

Accuracy

The accuracy of the method was shown by analyzing Placebo Solutions spiked with known amounts of dipyridamole and comparing the analytical result to the known added value. The average percent recovery was calculated at each concentration level. The average amounts recovered were 101.5, 101.0, and 100.6% for concentrations of about 50, 100, and 150% of label claim, respectively.

This yields an overall average recovery of 101.0% (n=9) for the analytical method (Table 3). Since the results obtained are within the acceptable range of 97.0 to 103.0%, the method is deemed to be accurate.

Linearity

A linear response in peak area for dipyridamole over the range of 50-160% of its label claim was observed. The correlation coefficient was 1.000 and the bias was 0.02% (Table 4).

Table 6

Specificity Results

Stress Condition of Finished Product	% Degradation	Peak Homogeneity Limit: NLT 990
Thermal (70°C, 110 hrs)	16.0	999.99
Acid (2 weeks)	0.2	999.95
Base (2 weeks)	1.1	999.98
Oxidation (110 hrs)	11.0	999.98
Fluorescence (500-700 ft-candles, 72 hrs)	6.0	999.99

Range

The range of the assay method has been set at 50 t0 150% of the finished product label claim (5mgmL dipyridamile), since the method has been shown to be precise, accurate, and linear within this range.

Stability of Analytical Solutions

The stability of the analytical solutions was determined from the Standard Preparation (prepared from USP Reference Standard) and Assay Preparation (prepared from finished product) at room temperature. These solutions were analyzed at 0, 24, and 48 hours and analyzed against a freshly prepared standard at each time interval. The dipyridamole concentrations were examined as a function of time (Table 5). These data were evaluated for percent change from time zero. The Standard Preparation and Assay Preparation were found to be stable for 48 hours, respectively. Since the percent change is within ± 2%, the solutions are considered stable at room temperature.

Specificity

Dipyridamole Injection was stressed by thermal, acidic, basic, oxidative, and fluorescent radiation for up to 2 weeks or until approximately 10-30% degradation of dipyridamole was achieved, as determined by peak area percent. The results of the stress studies are presented in Table 6.

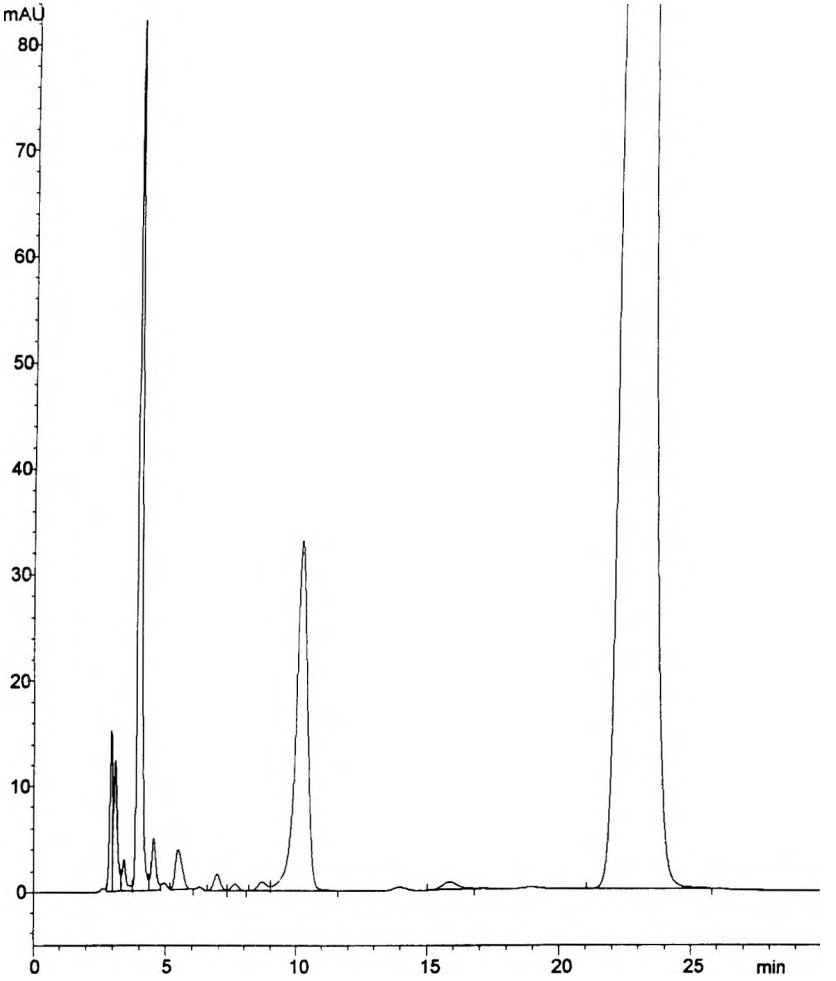


Figure 2. Specificity chromatogram of stressed Dipyridamole Injection. The retention time of dipyridamole is 23 minutes.

No interfering peaks at the retention time of dipyridamole were observed in any of the stressed samples. A chromatogram illustrating the specificity from a combination of thermal, oxidation and light stress is provided in Figure 2.

Peak Homogeneity

The control sample, stress samples, and placebo samples were analyzed using an HPLC equipped with a photodiode array detector. The dipyridamole peak was determined to be homogeneous since a purity value ≥ 990 was obtained in all cases (Table 6)

CONCLUSION

The described isocratic HPLC method for the analysis of dipyridamole has been evaluated for system suitability, linearity, precision, accuracy, stability of solutions, and specificity. The dipyridamole peak response has been shown to be precise, accurate, and linear, in the range of 50 to 150% label claim. Precision between two chemists on two different chromatographic systems was demonstrated to be within 1.0%. The Standard and Assay Preparations were found to be stable for 48 hours, at room temperature. Finally, the method has proven to be specific under a variety of stress conditions, while maintaining peak homogeneity. Consequently, the validated method for the determination of dipyridamole in Dipyridamole Injection is regarded as stability-indicating.

REFERENCES

1. P. Needleman, P. B. Corr, E. M. Johnson, Jr, in **The Pharmacological Basis of Therapeutics**, 7th edition, A. G. Gilman, L. S. Goodman, T. W. Rall, F. Murad, eds., p. 822, Macmillian, New York, NY, USA (1985).

2. USP Dispensing Information, Volume IA, pp. 1200-1202, U. S. Pharmacopeial Convention, Rockville, MD (1990).

3. I. E. Borisevitch, P. G. Pasutti, M. Tabak, Spectrochimica Acta, **48**, 1427-1436 (1992).

4. X. Zeng, S. Lin, N. Hu, Talanta, **40**, 1183-1187 (1993).

5. F. Fontani, G. P. Finardi, G. Targa, G. P. Besana, M. Ligorati, J. Chromatogr., **280**, 181-187 (1983).

6. J. H. Bridle, M. T. Brimble, Drug Dev. Ind. Pharm., **19**, 371-381 (1993).

7. The United States Pharmacopeia 23, Dipyridamole Monograph, pp. 539-540, U. S. Pharmacopeial Convention, Rockville, MD (1995).

8. M. Barberi-Heyob, J. L. Merlin, L. Pons, M. Calco, B. Weber, J. Liq. C Chromatogr., **17**, 1837-1848 (1994).

9. C. Deballon, M. Guernet, J. Pharm. Biomed. Anal., **6**, 1045-1048 (1988).

10. The United States Pharmacopeia 23, pp. 1982-1984, U.S. Pharmacopeial Convention, Rockville, MD (1995).

11. The United States Pharmacopeia 23, pp. 1768-1778, U .S. Pharmacopeial Convention, Rockville, MD (1995).

Received December 1, 1996
Accepted February 13, 1996
Manuscript 4337

J. LIQ. CHROM. & REL. TECHNOL., 20(13), 2123-2133 (1997)

AN HPLC METHOD FOR THE DETERMINATION OF VITAMIN B1, CAFFEINE, ACETYLSALICYLIC ACID, AND THE IMPURITIES OF SALICYLIC ACID IN A PHARMACEUTICAL PREPARATION

L. Gámiz-Gracia, M. D. Luque de Castro*

Department of Analytical Chemistry
Faculty of Sciences
University of Córdoba
E-14004 Córdoba, Spain

ABSTRACT

An HPLC method for the determination of Vitamin B1, caffeine, acetylsalicylic acid and salicylic acid in tablets containing all four drugs is reported. After optimization of the variables involved, the method has been characterized and validated in terms of calibration (Standard Addition Methodology), repeatability and selectivity, and finally, applied to the quality control of the final product.

INTRODUCTION

Calmante Vitaminado is a Spanish pharmaceutical with analgesic effects for mild and moderate pain, which contains acetylsalicylic acid (ASA), caffeine and vitamin B1 as active compounds. In addition, salicylic acid (SA) is present as an impurity, while wheat and magnesium stearate are found as excipients in the tablets. Despite the fact that methods for the individual determination of

these compounds in mixtures have been reported so far, there are no methods for the joint determination of these three active drugs. Various HPLC methods for the determination of thiamine, together with other water-soluble and liposoluble vitamins in multivitamin preparations, can be found in the analytical literature.[1-4] Pharmaceuticals containing ASA and caffeine in binary or higher multianalgesic mixtures are very common, so a pleiade of methods, mainly based on HPLC,[5-9] have been reported so far. Nevertheless, there are no methods in the pharmaceutical literature for the determination of ASA, caffeine, and vitamin B1.

We have developed and validated an HPLC method for the joint determination of the active compounds of Calmante Vitaminado, in order to use it for the quality control of the final product (tablets). As, salicylic acid is common in formulations containing ASA as a result of both its presence as an impurity of SAS and its formation during the preparation of aspirin products,[10] the method also involves the determination of SA.

MATERIALS

Reagents

HPLC-grade methanol (Scharlau Chemicals), orthophosphoric acid (85% w/w, Merck), potassium dihydrogen phosphate (Merck), and ultrapure water were used to prepare the HPLC mobile phase. Standard solutions were prepared from Vitamin B1, caffeine, acetylsalicylic acid, magnesium stearate, wheat starch (kindly provided by Calmante Vitaminado, S.A., Spain), and salicylic acid (Aldrich). Albet filter-paper (No 1305) was used for sample preparation.

Apparatus and Instruments

The HPLC system consisted of a Knauer HPLC pump-64, a Rheodyne 7125 high-pressure injection valve furnished with a 135 μL loop, a Waters 490 programmable multiwavelength uv/vis detector, and a Knauer recorder. The 250×4.6 mm i.d. column was packed with 5 μm Ultrabase C_{18} (Scharlau Science, Spain). A Bandelin Sonorex K 52 ultrasonic bath was used to dissolve the samples.

METHODS

Chromatographic Conditions

The mobile phase was methanol-0.02 M potassium dihydrogen phosphate (30:70, v/v) (pH 4.0) at a flow-rate of 1.5 mL min^{-1}. The injection volume was 135 μL and the UV detector was operated at 246 nm (vitamin B1), 274 nm (caffeine), and 224 nm (acetylsalicylic acid and salicylic acid). These wavelengths provided the maximum absorbance for each compound. The retention times of vitamin B1, caffeine, SA and SAS acid were 2.3, 6.6, 9.7, and 12.8 min, respectively. Figure 1 shows a typical HPLC chromatogram.

Preparation of the Mobile Phase

A 0.02 M buffer solution was prepared from potassium dihydrogen phosphate and ultrapure water. HPLC grade methanol and the buffer solution, filtered through a 0.45 μm Nylon filter, were mixed in a 30:70 (v/v) ratio. The solution was degassed in an ultrasonic bath for 15 min and the pH adjusted to 4.0 by adding orthophosphoric acid (1% in ultrapure water).

Preparation of the Standard Solutions

Calibration solutions were prepared by diluting the standard stock solution (100 mg l^{-1} of each compound prepared in the mobile phase) with the mobile phase. The solutions were sonicated for 5 min.

Preparation of the Tablet Solutions and Procedure

10 tablets accurately weighed were ground and mixed in a mortar. 75 mg of the powder was transferred to a 25 mL flask and made to volume with mobile phase, sonicated for 15 min, and filtered through filter-paper.

For vitamin B1 determination, the solution was injected directly, whereas for the determination of the other compounds, a dilution (200 μL in 10 mL) with the mobile phase was necessary. Duplicate injections were performed in all instances. Measurements were based on peak height.

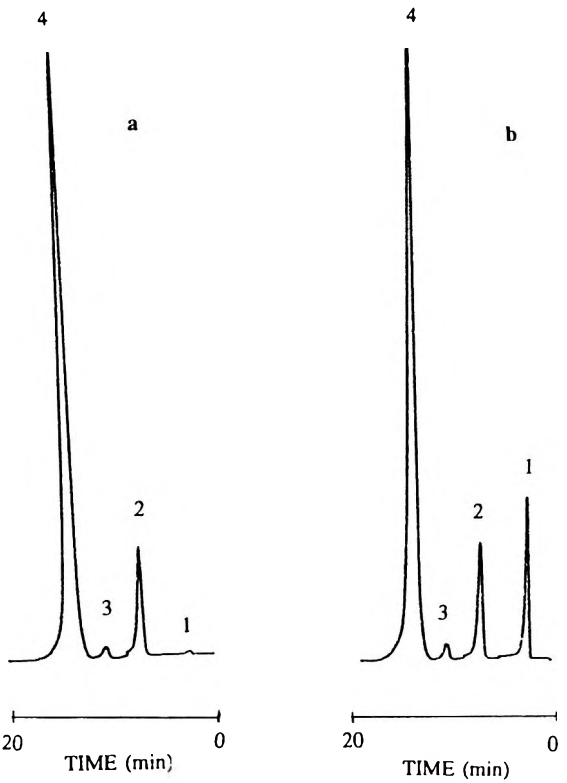


Figure 1. HPLC chromatogram **(a):** Tablet solution (60 μg mL^{-1}). **(b):** Tablet solution (60 μg mL^{-1}) "spiked" with Vitamin B1 (10 μg mL^{-1}). Peak identification: (1): Vitamin B1; (2): caffeine; (3): salicylic acid; (4): acetylsalicylic acid. See text for chromatographic conditions.

RESULTS AND DISCUSSION

Optimization of the Chromatographic Conditions

As the vitamin B1-caffeine-ASA ratio in the tables is 1:25:250 (by weight), the optimization of the chromatographic conditions was carried out using similar amounts of the three active compounds, as well as of SA in order to clearly distinguish the behaviour of each of the compounds whilst changing the variables.

The experimental variables, optimized to obtain adequate separation of the eluted analytes, were the composition of the mobile phase, the flow-rate, and the injection volume.

The influence of the percentage of methanol in the binary methanol-phosphate buffer mixtures used as mobile phase to separate the analytes in a Ultrabase C_{18} column, was studied in the range 0-100% v/v methanol. A 30:70 methanol-buffer mixture at pH 4.0 was selected as optimal.

The influence of the flow-rate of the mobile phase was studied in the range 0.5-2.0 mL min^{-1}, providing an optimum value at 1.5 mL min^{-1}.

An injection volume of 135 μL was selected as optimum. Above this value, the increase in sensitivity did not compensate for the increase in pressure.

A mixture of the analytes at the concentration ratio in the tablets was injected after optimization. Figure 1 shows the cromatogram obtained, which justified the use of two injections of the sample at different dilutions.

Validation of the Method

Calibration

The standard addition methodology[11] was used for calibration. The sets of data obtained in 3 calibration experiments with standard solutions (namely: Standard Calibration, SC), standard additions (Standard-addition calibration, AC), and portions of sample (Youden Calibration, YC) were used for each compound. The accuracy of the analytical results was checked by comparing both the analytes content in the different calibrations and the recoveries, calculated by dividing the net content found by that added for each addition. The Alamin program was used for calculation.[12]

The SC were run with triplicate injections of the standard solutions and the responses versus concentrations were linear in the following ranges:

Vitamin B1	1.9-25 mg L^{-1}
Caffeine	2.2-25 mg L^{-1}
Salicylic acid	1.5-25 mg L^{-1}
Acetylsalicylic acid	3.6-50 mg L^{-1}

Table 1

Features of the Proposed HPLC Method for the Determination of Vitamin B1, Caffeine, Salicylic Acid, and Acetylsalicylic Acid

	r^2(%)	s_{xy}	s_a	s_b	Calibration Curve
Vitamin B1					
SC	99.91	$2.16 \cdot 10^{3}$	$9.02 \cdot 10^{-4}$	$5.96 \cdot 10^{-5}$	$Y=8.57 \cdot 10^{-4}+8.08 \cdot 10^{-3} \cdot X$
AC	99.28	$5.91 \cdot 10^{-3}$	$4.94 \cdot 10^{-3}$	$5.28 \cdot 10^{-4}$	$Y=55.30 \cdot 10^{-3}+8.76 \cdot 10^{-3} \cdot X$
YC	99.86	$1.73 \cdot 10^{-3}$	$2.12 \cdot 10^{-3}$	$8.00 \cdot 10^{-7}$	$Y=1.00 \cdot 10^{-3}+2.90 \cdot 10^{-5} \cdot X$
Caffeine					
SC	99.89	$2.24 \cdot 10^{-3}$	$9.35 \cdot 10^{-4}$	$6.18 \cdot 10^{-5}$	$Y=-1.43 \cdot 10^{-4}+7.30 \cdot 10^{-3} \cdot X$
AC	99.86	$2.31 \cdot 10^{-3}$	$1.94 \cdot 10^{-3}$	$2.07 \cdot 10^{-4}$	$Y=60.60 \cdot 10^{-3}+7.82 \cdot 10^{-3} \cdot X$
YC	99.99	$6.32 \cdot 10^{-4}$	$7.75 \cdot 10^{-4}$	$5.70 \cdot 10^{-6}$	$Y=-3.00 \cdot 10^{-3}+7.16 \cdot 10^{-1} \cdot X$
Salicylic Acid					
SC	99.95	$1.50 \cdot 10^{-3}$	$6.26 \cdot 10^{-4}$	$4.13 \cdot 10^{-5}$	$Y=-1.17 \cdot 10^{-3}+7.10 \cdot 10^{-3} \cdot X$
AC	99.96	$1.16 \cdot 10^{-3}$	$9.72 \cdot 10^{-4}$	$1.04 \cdot 10^{-4}$	$Y=29.40 \cdot 10^{-3}+6.98 \cdot 10^{-3} \cdot X$
YC	1.00	0	0	0	$Y=-1.0 \cdot 10^{-3}+8.00 \cdot 10^{-5} \cdot X$
Acetylsalicylic Acid					
SC	99.92	$3.16 \cdot 10^{-3}$	$1.32 \cdot 10^{-3}$	$4.37 \cdot 10^{-5}$	$Y=-3.03 \cdot 10^{-3}+6.10 \cdot 10^{-3} \cdot X$
AC	99.95	$2.14 \cdot 10^{-3}$	$1.79 \cdot 10^{-3}$	$9.59 \cdot 10^{-5}$	$Y=68.40 \cdot 10^{-3}+6.04 \cdot 10^{-3} \cdot X$
YC	99.99	$3.87 \cdot 10^{-4}$	$4.74 \cdot 10^{-4}$	$1.73 \cdot 10^{-5}$	$Y=-5.00 \cdot 10^{-4}+3.43 \cdot 10^{-3} \cdot X$

SC: Standard calibration
AC: Standard addition calibration
YC: Youden calibration
Y = Absorbance (peak height)
X = Concentration in $\mu g\ mL^{-1}$

Only one injection of each solution was made in the two other calibration procedures. The numerical values of the parameters of these calibrations are shown in Table 1. The values of the slopes (SC and AC) are similar in all instances.

Table 2

Results of Recovery Assays (from the Standard-Addition Calibration) to Check Accuracy

Vitamin B1[a]	Sample [b]	R[c] (%)	R[c] (Average)
5	2	98.40	
10	2	93.43	99.73
15	2	107.35	
Caffeine[b]	**Sample[a]**		
5	100	89.45	
10	100	103.23	98.66
15	100	103.29	
Salicylic Acid	**Sample[a]**		
5	400	97.12	
10	400	100.73	98.99
15	400	99.11	
Acetylsalicylic Acid[a]	**Sample[a]**		
10	20	98.11	
20	20	98.51	98.78
30	20	99.73	

[a] In $\mu g\ mL^{-1}$
[b] In $mg\ mL^{-1}$
[c] Recovery

The results from SC and AC are not significantly different, so the method is accurate. The average recoveries from the AC are shown in Table 2. This supports the accuracy of the method. The conclusion obtained is that the determination of the four compounds in tablets can be carried out directly by the SC methods. The figures of merit of the method, calculated from SC data sets,[13] are shown in Table 3.

Table 3

Features of the Analytical Method from the Standard Calibration Data Set

	Vitamin B1	Caffeine	Salic. Acid	Acetylsalic. Acid
Sensitivity[a]	0.267	0.306	0.211	0.512
Precision (RSD, %)	1.13[b]	1.25[b]	0.88[b]	1.08[c]
Detect. limit[a]	0.572	0.655	0.449	1.090
Determ. limit[a]	1.908	2.182	1.498	3.632
Linearity (%)	99.26	99.16	99.42	99.29

[a] In $\mu g\ mL^{-1}$
[b] At $15 \mu g\ mL^{-1}$
[c] At $30\ \mu g\ mL^{-1}$

Selectivity

The excipients of the commercial tablets (magnesium stearate and wheat starch) caused no effect in the determination of the compounds, as they did not absorb in the uv region where measurements were performed, and they were not retained by the column, so the determination of the active compounds of this pharmaceuticals is free from interferences.

Reproducibility

The method was applied, under the optimal working conditions, to 10 samples from powder obtained from 10 commercial tablets, and injected in duplicate. The samples were prepared as described previously. The results are shown in Table 4. The highest RSD value corresponds to the analyte at lower concentration (SA: RSD 12%); while more acceptable RSDs are obtained for ASA and vitamin B1 (note that a more concentrated sample is used for the latter).

Application of the Method

The performance of the method was tested by applying it to the determination of the target compounds in 12 samples each obtained from 10 tablets of 12 different batches of commercial tablets.

Table 4

Reproducibility of the Method: Results from 10 Different Samples, Injected in Duplicate

	Retention Time (Min)	Mg/Tablet
Vitamin B1	2.29±0.06 (RSD=2.52%)	2.17±0.09 (RSD=3.96%)
Caffeine	6.63±0.13 (RSD=1.94%)	59±4 (RSD=6.63%)
Salicylic Acid	9.39±0.17 (RSD=1.76%)	13.9±1.7 (RSD=12.11%)
Acetylsalicylic Acid	12.58±0.16 (RSD = 1.27%)	446±11 (RSD = 2.47%)

Table 5

Application of the Proposed Method to the Analysis of Tablets from Different Batches, Injected in Duplicate

Batch Number	Vitamin B1		Caffeine		Salicyclic Acid		Acetylsalicylic Acid	
	mg/Tablet	t_r	mg/Tablet	t_r	mg/Tablet	t_r	mg/Tablet	t_r
1	2.17	2.29	59.09	6.63	13.95	9.39	445.93	12.58
2	2.23	2.22	63.09	6.60	12.02	9.85	517.07	12.58
3	2.24	2.31	46.71	6.70	9.23	10.08	316.16	13.05
4	2.35	2.36	53.82	6.57	7.51	10.08	392.05	12.72
5	2.53	2.34	57.70	6.63	8.70	10.13	466.22	13.15
6	3.23	2.26	64.39	6.53	9.06	10.13	495.83	13.07
7	2.66	2.25	55.18	6.62	7.50	10.30	414.99	13.28
8	2.48	2.37	51.92	6.47	8.88	10.19	386.71	12.94
9	3.13	2.34	49.84	6.58	10.47	9.91	409.16	12.97
10	3.22	2.32	47.07	6.72	7.56	10.20	376.17	13.21
11	3.00	2.34	48.92	6.65	9.10	10.06	387.97	13.14
12	3.23	2.32	49.18	6.71	6.18	10.15	337.23	13.21
Average	2.7	2.31	53.9	6.62	9.2	10.04	412.1	12.99
s	0.4	0.05	6.1	0.06	2.1	0.24	60.2	0.24
RSD (%)	15.8	2.0	11.2	1.1	23.2	2.4	14.6	1.9

Samples were prepared as described previously and injected in duplicate. The results are shown in Table 5. The analysis revealed some shortcomings in the manufacturing process, both because the concentrations found did not always agree with the nominal content of the pharmaceutical preparation, and because of the differences between batches.

CONCLUSIONS

The method proposed here does not enable the determination of the four analytes in a single injection owing to the large differences in their concentrations in the target pharmaceuticals. The two-injection determination provides excellent precision for the most dilute active compound (vitamin B1).

The content of the decomposition product of ASA is below the limit permitted by the European Pharmacopeia in ASA-containing tablets (less than 3.2%). The validation study demonstrated the accuracy of the method, which can be safely applied for quality control of the final product.

AKNOWLEDGMENT

The Comisión Interministerial de Ciencia y Tecnología (CICyT) is thanked for financial support (Project No. ABM96-1086).

REFERENCES

1. N. R. Raju, P. Srilakshmi, G. A. Vinolia, G. J. Bhounsule, V. D. Gaitonde, Indian Drugs, **26**, 697-701 (1989).

2. A. Kozik, M. Rapala-Kozik, J. Chomatogr., **648(2)**, 349-356 (1993).

3. M. C. Genaro. J. Chromatogr. Sci., **29(9)**, 410-415 (1991).

4. M. Amin, J. Reusch, J. Chromatogr., **390(2)**, 448-453 (1987).

5. M. A. S. Salem, H. N. Alkaysi, Drug. Dev. Ind. Pharm., **16(7)**, 1185-1201 (1990).

6. M. A. Abuirjeie, M. E. Abdel-Hamid, E. A. Ibrahim, Anal. Lett., **22(2)**, 365-375 (1989).

7. K. K. Verma, S. K. Sanghi, A. Jain, D. Gupta, J. Pharm. Sci., **76(7)**, 551-553 (1987).

8. I. M. Jalal, S. I. Sa'-sa', Talanta, **31(11)**, 1015-1017 (1984).

9. V. Das-Gupta, J. Pharm. Sci., **69(1)**, 110-113, (1980).

10. R. N. Bevitt. J. R. Mather. D. C. Sharman, Analyst, **109(10)**, 1327-1329, (1984).

11. L. Cuadros Rodríguez, A. M. García Campaña, F. Alés Barrero, C. Jiménez Linares. M. Román Ceba. J. A. O. A. C., Int'l., **78(2)**, 471-476 (1995).

12. A. M. García Campaña. J. L. Sierra Fernández, L. Cuadros Rodríguez, F. Alés Barrero. M. Román Ceba, Trends Anal. Chem., submitted for publication.

13. L. Cuadros Rodríguez. A. M. García Campaña. C. Jiménez Linares, M. Román Ceba. Anal. Lett., **26(6)**, 1243-1258 (1993).

Received October 20, 1996
Accepted December 16, 1996
Manuscript 4314

J. LIQ. CHROM. & REL. TECHNOL., 20(13), 2135–2142 (1997)

DETERMINATION OF BENAZEPRIL AND CILAZAPRIL IN PHARMACEUTICALS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

A. Gumieniczek, L. Przyborowski

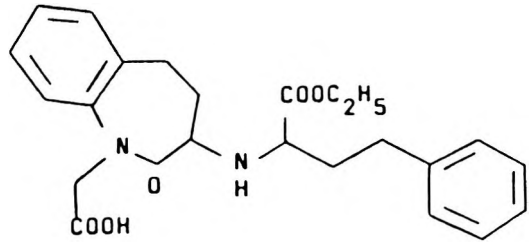
Department of Medicinal Chemistry
Medical Academy in Lublin
Chodźki Str. 6
20-093 Lublin, Poland

ABSTRACT

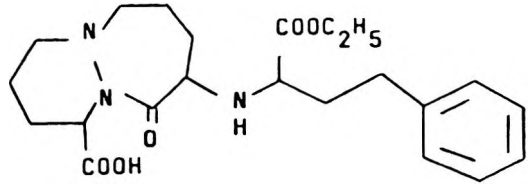
A high performance liquid chromatography method was developed for the specific determination of new angiotensin-converting enzyme inhibitors benazepril and cilazapril in pharmaceutical dosage forms. The proposed method was conducted using a reverse phase technique, UV monitoring at 211 nm and enalapril as an internal standard. The detector response was linear in the range of 10-50 μg/mL for benazepril and 40-200 μg/mL for cilazapril. The drugs were extracted from tablets with methanol. The percentage recoveries ranged from 96.34 to 102.04 and from 103.08 to 107.96 for benazepril and cilazapril, respectively.

INTRODUCTION

Benazepril-HCl, 3-[(1-ethoxycarbonyl-3-phenyl-(1S)-propyl)-amino]-2,3,4,5-tetrahydro-2-oxo-1-(3S)-benzazepine-1-acetic acid hydrochloride and cilazapril, 9(S) - 1(S)-[(ethoxycarbonyl)-3-phenylpropylamino]-octahydro-10-oxo-6H-pyridazo[1,2a] [1,2]diazepine-1(S)-carboxylic acid (Figure 1), the



BENAZEPRIL



CILAZAPRIL

Figure 1. Chemical structures of investigated drugs.

angiotensin-converting enzyme inhibitors are the new drugs used in the treatment of hypertension. So far a few analytical procedures have been described for determination of benazepril, cilazapril and their active metabolites in plasma. These methods were based on gas chromatography-mass spectrometry,[1] radioimmunoassay[2] and enzymeimmunoassay.[3] Barbato et al. examined the chromatographic behaviour of several ACE inhibitors by high performance liquid chromatography.[4] The influence of different organic modifiers and of counter-ions in the mobile phase allowing the determination of the best experimental conditions for the analysis of these compounds, was investigated. However, there are no publications concerning the analysis of benazepril and cilazapril in their dosage forms. So, we decided to work out an assay procedure which would serve as a rapid and reliable method for the quality control of benazepril and cilazapril in pharmaceutical formulations.

MATERIALS

Reagents

Benazepril hydrochloride was received from Ciba-Geigy, Ltd. (Switzerland), cilazapril was received from Hoffmann-La Roche, Ltd. (Switzerland). Enalapril maleate was obtained from Polpharma S.A.(Poland). Tablets of Lotensin® (20 mg) from Ciba-Geigy and tablets of Inhibace® (5 mg) from Hoffmann-La Roche were used. HPLC grade acetonitrile and methanol were purchased from E.Merck (Germany). All the other reagents were of analytical grade.

The water needed in the experiments was double distilled. The buffer was prepared by adding phosphoric acid to 0.067 M potassium dihydrogen phosphate to obtain a final pH of 2.4.

Apparatus

The HPLC system consisted of a Model 302 solvent delivery pump from Techma-Robot Warsaw (Poland) and a Model LCD-2040 variable wavelength UV detector from Laboratorni Pristroje Praha (Czech Republic). Chromatograms were recorded with a Model TZ-4620 recorder from Laboratorni Pristroje Praha. A Model 327 reciprocating shaker from Premed (Poland) was applied.

METHODS

Chromatographic Conditions

Chromatography was carried out on LiChrosorb RP-18 column (250×4 mm) with particle size of 10 μm. The mobile phase consisted of phosphate buffer pH 2.4 and acetonitrile (7:3, v/v). The flow rate was 1 mL/min. The column effluent was monitored at 211 nm using a detector range of 0.08 AUFS and a chart speed of 0.06 cm/min. Sample volumes of 20 μL were injected into the analytical column with a manual HPLC injector fitted with a 20 μL loop (from Laboratorni Pristroje Praha). All assays were performed at ambient temperature.

Solutions

The stock solutions of benazepril-HCl (1 mg/mL), cilazapril (1 mg/mL) and of internal standard-enalapril maleate (4 mg/mL) were prepared by dissolving appropriate amounts of the substances in methanol. These solutions were stable for at least two months if stored at 4°C.

Calibration for Benazepril Assay

0.1, 0.2, 0.3, 0.4, 0.5 mL volumes of the stock solution of benazepril-HCl were pipetted into 10 mL volumetric flasks. Then, 0.2 mL volume of the internal standard solution was added to each sample and made with methanol up to the mark. 20 μl volume of each sample was then injected into the column. All measurements were repeated three times for each concentration.

The peak heights were measured and the peak height ratios of analyte to internal standard were then plotted against the respective concentration of benazepril-HCl.

Calibration for Cilazapril Assay

0.4, 0.8, 1.2, 1.6, 2.0 mL volumes of the stock solution of cilazapril and 0.5 mL volumes of the stock solution of internal standard were mixed in volumetric flasks and completed to 10 mL with methanol. A 20 μL volume of each sample was then injected into the column. All measurements were repeated three times. The calibration curve was constructed by plotting the peak height ratios of cilazapril to the internal standard versus the respective drug concentration.

Tablets of Benazepril: Extraction and Quantification

The weighed tablets of Lotensin® were ground to a fine powder. The amounts equivalent to 25 mg of the compound were extracted with methanol in 25 mL volumetric flasks. 0.1, 0.3 and 0.5 mL volumes of the filtered extracts were transferred into 10 mL flasks; 0.2 mL volumes of the internal standard solution were added and made up with methanol. Then, 20 μl volume of each sample was injected into the column.

Tablets of Cilazapril: Extraction and Quantification

Tablets of Inhibace® were weighed and pulverized. The amounts equivalent to 25 mg of the compound were extracted with methanol in 25 mL volumetric flasks. 0.4, 1.2, 2.0 mL volumes of the filtered extracts and 0.5 mL volumes of the internal standard solution were pipetted into 10 mL flasks and completed with methanol. 20 μl volume of each sample was then injected into the column.

RESULTS AND DISCUSSION

A reversed phase HPLC procedure was proposed as a suitable method for the analysis of benazepril-HCl and cilazapril in the dosage forms. The chromatographic conditions were adjusted in order to provide a versatile HPLC procedure capable of separating benazepril or cilazapril and the internal standard. A mixture of phosphate buffer, pH 2.4 - acetonitrile (7:3, v/v) at a flow rate of 1 mL/min, was found to be an appropriate mobile phase allowing adequate separation of active substances and the internal standard. As shown in Figure 2, the substances were eluted, forming well shaped, symmetrical single peaks, well separated from the solvent front.

The relationship between the peak height ratios of benazepril-HCl to the internal standard and the concentration of drug, was linear over the concentration 10-50 μg/mL. The detection limit for the analysis of benazepril was 5 μg/mL with 20 μl injection. The regression equation was y=1.46x-0.004 (standard error of slope=0.0191485, standard error of intercept=0.0127017), where y=peak height ratio of drug to that of internal standard and x=concentration of drug in μg/20 μl. The correlation coefficient for the regression line was 0.9997.

For the quantitative determination of cilazapril, the linear calibration curve was obtained in the range of 40-200 μg/mL, with the detection limit 10 μg/mL. The regression equation was y=0.31375x-0.003 (standard error of slope=0.00314576, standard error of intercept=0.0083466), where y=peak height ratio of cilazapril to that of the internal standard and x=concentration of drug in μg per 20 μl; the correlation coefficient was 0.9998.

Methanol was chosen as the extraction organic solvent because of solubility properties of the examined drugs and a reversed phase mode of chromatography. The recoveries, after extraction from the tablets, were found

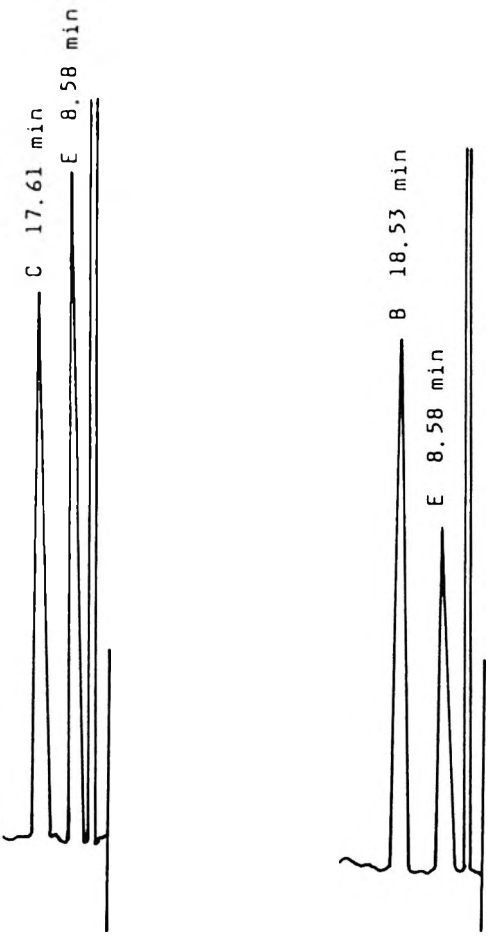


Figure 2. Typical chromatograms of benazepril (B), cilazapril (C) and internal standard-enalapril (E).

to be 99.99 ± 1.96% for benazepril-HCl and 106.04 ± 1.78% for cilazapril(mean ± standard deviation). The precision of the chromatographic analysis in tablets was determined at three concentrations of both drugs. The coefficients of variation were obtained by repeating the procedure five times for each sample (Table 1).

Table 1

Results of the Determination of Benazepril and Cilazapril in Tablets (n = 5 for each Sample)

No	Amount Expected μg/20 μL	Amount Found (Mean ± SD)	Coefficient of Variation (%)
	Benazepril		
1	0.20	0.200 ± 0.0089	4.45
2	0.60	0.584 ± 0.0102	1.75
3	1.00	1.002 ± 0.0103	1.02
	Cilazapril		
1	0.80	0.806 ± 0.0282	3.50
2	2.40	2.526 ± 0.0102	0.40
3	4.00	3.975 ± 0.0157	0.39

SD = Standard Deviation

The described method is simple and fairly reliable for the pharmaceutical analysis. As mentioned above, the literature relating to benazepril and cilazapril determinations is rather scarce. Therefore, it should facilitate the analytical investigation of both drugs.

ACKNOWLEDGMENTS

We wish to thank Ciba-Geigy, Hoffmann-La Roche, and Polpharma companies for supplying benazepril hydrochloride, cilazapril and enalapril maleate pure substances.

REFERENCES

1. A. Sioufi, F. Pommier, G. Kaiser, J .P. Dubois, J. Chromatogr., **434**, 239-46 (1988).

2. B. M. Michniewicz, J. C. Hodges, B. G. England, T. Chang, C. J. Blankley, G. D. Nordblom, J. Clin. Immunoassay **10**, 111-15 (1987).

3. H. Tanaka, Y. Yoneyama, M. Sugawara, I. Umeda, Y. Ohta, J. Pharm. Sci. **76**, 224-7 (1987).

4. F. Barbato, P. Morrica, F. Quaglia, Farmaco, **49**, 457-60 (1994).

Received September 15, 1996
Accepted December 13, 1996
Manuscript 4289

J. LIQ. CHROM. & REL. TECHNOL., 20(13), 2143 (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 GPC, and has taught at Rutgers University. He is currently Visiting Scholar at Florida Atlantic University.

Details may be obtained from Dr. Jack Cazes, P. O. Box 970210, Coconut Creek, FL 33097. Tel.: (954) 570-9446; E-Mail: jcazes@icanect.net.

J. LIQ. CHROM. & REL. TECHNOL., 20(13), 2145–2154 (1997)

LIQUID CHROMATOGRAPHY CALENDAR

1997

AUGUST 2 - 9: 39th Rocky Mountain Conference on Analytical Chemistry, Hyatt Regency Denver, Denver, Colorado. Contact: Patricia Sulik, Rocky Mountain Instrumental Labs, 456 S. Link Lane, Ft. Colleins, CO, USA. (303) 530-1169; Email: gebrown@usgs.gov.

AUGUST 11 - 13: 10th International Symposium on Polymer Analysis and Characterization (ISPAC-10), University of Toronto, Canada. Contact: Prof. S. T. Balke, Dept. of Chem. Engg & Appl. Chem., Univ. of Toronto, Toronto, Ont., Canada, M5S 1A4. Tel/FAX: (416) 978-7495; Email: balke@ecf.toronto.edu.

SEPTEMBER 2 - 5: 12th International Bioanalytical Forum, Univ. of Surrey, Guildford, UK, sponsored by the Chromatographic Society (U.K.). Contact: Dr. E. Reid, 72 The Chase, Guildford GU2 5UL, U.K. Tel/FAX: (0) 1483-565324; Email: D.Stevenson@surrey.ac.uk.

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

SEPTEMBER 8 - 10: 1997 PrepTech Conference, Hyatt Regency Hotel, Orlando International Airport, Florida. Contact: S. Galla, ISC Technical Conferences, Inc., 30 Controls Dr., Box 559, Shelton, CT 06484-0559, USA. Tel: (203) 926-9300; FAX: (203) 926-9722.

SEPTEMBER 14 - 17: International Ion Chromatography Symposium, Westin Hotel, Santa Clara, California. Contact: Janet Strimaitis, Century International, P. O. Box 493, Medfield, MA 02052-0493, USA. Tel: (508) 359-8777; FAX: (508) 359-8778; Email: century@ixl.net.

SEPTEMBER 14 - 19: ACS Int'l Symposium on Systems Approach to Service Life Prediction of Organic Coatings, Breckenridge, Colorado. Contact: J. Martin, NIST, Bldg. 226, Rm B-350, Gaithersburg, MD 20899, USA. Email: jmartin@nist.gov.

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.

SEPTEMBER 22 - 25: AIChE Conference on Safety in Ammonia Plants & Related Facilities, Fairmont Hotel, San Francisco, California. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

SEPTEMBER 30 - OCTOBER 2: 53rd Southwest ACS Regional Meeting, Tulsa, Oklahoma. Contact: F. B. Growcock, Amoco Corp., E&PT, P. O. Box 3385, Tulsa, OK 74012, USA. Tel: (918) 660-4224; Email: fgrowcock@amoco.com.

OCTOBER 5 - 8: Conference on Formulations & Drug Delivery, La Jolla, California, sponsored by the ACS Div. of Biochem. Technol. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036. Tel: (202) 872-6286; FAX: (202) 872- 6013; Email: miscmtgs@acs.org.

OCTOBER 6 - 10: Validation d'une Procedure d'Analyse, Qulification de l'Appareillage; Application a la Chromatographie Liquide, Montpellier, France. (Training course given in French) Contact: Prof. H. Fabre, Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15; Email: hfabre@pharma.univ-montpl.fr

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 - 23: Sensors Expo: Conference on Exposition of Sensors, Detroit, Michigan. Contact: Expocon Mgmt. Assoc., 3363 Reef Rd, P. O. Box 915, Fairfield, CT 06430-0915, USA. Tel: (203) 256-4700; Email: sensors@expo.com.

OCTOBER 21 - 23: Biotechnica Hannover '97: Int'l. Trade Fair for Biotechnology, Hannover, Germany. Contact: D. Hyland, Hannover Fairs USA, Inc., 103 Carnegie Center, Princeton, NJ 08540, USA.

OCTOBER 21 - 24: 152nd Fall Technical Meeting & Rubber Expo'97, Cleveland, Ohio, sponsored by ACS Div. of Rubber Chem. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036. Tel: (202) 872-6286.

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 25 - 30: 24th Annual Conference of the Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Providence, Rhode Island. Contact: ACS Div. of Anal. Chem., Tel: (301) 846-4797; FAX: (301) 694-6860; Internet: http://FACSS.org/info.html.

OCTOBER 26 - 29: ISPPP'97 – 17th International Symposium on the Separation of Proteins, Peptides, and Polynucleotides, Boubletree Hotel, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. FAX: (301) 898-5596.

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 2 - 6: AAPS Annual Meeting & Expo, Boston, Mass. Contact: AAPS, 1650 King Street, Alexandria, VA 22314-2747, USA. Tel: (703) 548-3000.

NOVEMBER 2 - 6: 11th International Forum on Electrolysis in the Chemical Industry, Clearwater Beach, Florida. Contact: P. Klyczynski, Electrosysthesis Co., 72 Ward Rd., Lancaster, NY 14086, USA.

NOVEMBER 6 - 8: 2nd North American Research Conference on Emulsion Polymers/Polymer Colloids, Hilton Head Is., SC. Contact: A. V. Patsis, SUNY New Paltz, Inst. of Mater. Sci., New Paltz, NY 12561, USA.

NOVEMBER 7 - 8: 6th Conference on Current Trends in Computational Chemistry, Jackson, Miss. Contact: J. Leszcynski, Jackson State Univ., Chem. Dept., 1400 J. R. Lynch St., Jackson, MS 39217, USA. Tel: 601) 973-3482; Email: jersy@iris5.jusms.edu.

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: AIChE Annual Meeting, Westin Bonaventure/Omni Los Angeles, Los Angeles, California. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

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.

NOVEMBER 20 - 21: New Horizons in Chemistry Symposium, Los Angeles. Contact: J. May, Tel: (213) 740-5962; Email: jessy@methyl.usc.edu.

1998

FEBRUARY 28 - MARCH 1: 28th Annual Spring Prog. in Polymers: Degradation & Stabilization of Polymers, Hilton Head Is., South Carolina. Contact: A. V. Patsis, SUNY New Paltz, Inst. of Mater. Sci., New Paltz, NY 12561. Email: ims@mhv.net.

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 8 - 12: AIChE Spring National Meeting, Sheraton, New Orleans. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA.

MARCH 15 - 18: 14th Rocky Mountain Regional Meeting, Tucson, Arizona. Contact: R. Pott, Univ of Arizona, Tucson, AZ 85721.

MARCH 23 - 25: 5th Meeting on Supercritical Fluids: Materials and Natural Products Processing, Nice, France. Contact: F. Brionne, Secretariat, ISASF, E.N.S.I.C., 1 rue Grandville, B.P. 451, F-54001 Nancy Cedex, France. Email: brionne@ensic.u-nancy.fr.

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036, USA.

MAY 3 - 8: HPLC'98 – 22nd International Symposium on High Performance Liquid Phase Separations and Related Techniques, Regal Waterfront Hotel, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596; EMail: Janetbarr@aol.com.

MAY 20 - 22: 32nd Middle Atlantic ACS Regional Meeting, Wilmington, Delaware. Contact: G. Trainor, DuPont Merck, P. O. Box 80353, Wilmington, DE 19880-0353. Email: reglmtgs@acs.org.

MAY 26 - 29: VIIIth International Symposium on Luminescence Spectrometry in Biomedical and Environmental Analysis: Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Las Palmas de Gran Canaria (Canary Islands), Spain. Contact: Dr. Jose Juan Dantana Rodriguez, University of Las Palmas of G. C., Chem. Dept., Faculty of Marine Sci., 35017 Las Palmas de G. C., Spain. Tel: 34 (9) 28 45.29.15 / 45.29.00; FAX: 34 (9) 28 45. 29.22; Email: josejuan.santana@quimica.ulpgc.es.

MAY 27 - 29: 30th Central Regional ACS Meeting, Ann Arbor, Michigan. Contact: D. W. Ewing, J. Carroll Univ., Chem. Dept., Cleveland, OH 44118. Tel: (216) 397-4241. Email: ewing@jevaxa.jeu.edu.

JUNE 1 - 3: 31st Great Lakes Regional ACS Meeting, Milwaukee, Wisconsin. Contact: A. Hill, Univ. of Wisc., Chem. Dept., Milwaukee, WI 53201, USA. Tel: (414) 229-4256; Email: reglmtgs@acs.org.

JUNE 2 - 5: Third International Symposium on Hormone and Veterinary Drug Residue Anbalysis, Congres Centre Oud Sint-Jan, Bruges, Belgium. Contact: C. Van Peteghem, Dept. of Food Analysis, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel: (32) 9/264.81.34;

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.

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.

JULY 12 - 16: SFC/SFE'98: 8th International Symposium & Exhibit on Supercritical Fluid Chromatography & Extraction, Adams Mark Hotel, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596; Email: Janetbarr@aol.com.

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.

AUGUST 31 - SEPTEMBER 3: AIChE Conference on Safety in Ammonia Plants & Related Facilities, Charleston Place, Charleston, South Carolina. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

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 13 - 18: 22nd International Symposium on Chromatography, ISC'98, Rome, Italy. Contact: F. Dondi, Chem. Dept., Universita di Ferrara, Via L. Borsari, 46, I-44100 Ferrara, Italy. Tel: 39 (532) 291154; FAX: 39 (532) 240707; Email: mo5@dns.Unife.it.

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, Res. 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.

NOVEMBER 15 - 20: AIChE Annual Meeting, Fontainbleu/Eden Roc, Miami Beach, Florida. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

1999

MARCH 7 - 12: PittCon '99, Orlando, Florida. Contact: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA

MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings. 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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

JUNE 25 - 30: HPLC'2000 – 24th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Seattle, Washington. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville. MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596.

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

DECEMBER 14 - 19: 2000 Int'l Chemical Congress of the Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th St., NW, Washington, DC 20036, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: pacific@acs.org.

2001

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

AUGUST 25 - 31: 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 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2003

MARCH 23 - 28: 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 - 12: 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 2: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC.

AUGUST 22 - 27: 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 - 18: 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 2: 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.

2006

MARCH 26 - 31: 231st ACS National Meeting, Atlanta, GA. 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 10 - 15: 232nd ACS National Meeting, San Francisco, 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.

2007

MARCH 25 - 30: 233rd 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.

AUGUST 19 - 24: 234th ACS National Meeting, Boston, Massachusetts. 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.**

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, all 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, tables, and figure captions, should be saved in a single file on the diskette; tables and figure captions should be placed at the end of the text. Label the diskette with the corresponding author's last name, the title of the manuscript and our 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.

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 New 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

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 address(es). 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
EXPERIMENTAL
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 in boldface, 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 third of an 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, except the abstract, should be **typed 1½ or double-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 separate from the text, at the end of thye manuscript. **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 printed or professionally drawn with 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. All labels and legends in figures should be large enough to remain legible when figures are reduced to fit the journal's pages. They should 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. Figure numbers, name of senior author and an arrow indicating "top" should be written in light blue pencil on the back of each 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.

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

Take advantage of the latest theoretical and applied research developments in the field with...

Advances in Chromatography

VOLUME 37

edited by

PHYLLIS R. BROWN, *University of Rhode Island, Kingston*
ELI GRUSHKA, *Hebrew University of Jerusalem, Israel*

December, 1996 / 544 pages, illustrated / $195.00

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 37** examines timely subjects such as

- single and multichannel techniques for the assessment of peak purity
- technological and methodological innovations in steady-state and dynamic-state fluorescence detection
- structure, performance, retention mechanisms, and applications of carbon-based packing materials for liquid chromatography
- instrumentation and applications of supercritical fluid extraction/gas chromatography
- the use of conventional solid-phase extraction and solid-phase microextraction for gas chromatography sample preparation
- analytical techniques and clinical applications in the capillary electrophoresis of proteins
- chiral separations in capillary electrophoresis with chiral micelle polymers
- peptide analysis techniques employing both high-performance liquid chromatography and capillary electrophoresis
- and 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; biotechnologists; biochemists and biological chemists; agrochemists; chemical, materials, pollution, and quality control engineers; toxicologists; pharmacologists; pharmacists; physiologists; zoologists; botanists; food, cosmetic, polymer, pharmaceutical, and environmental scientists; microbiologists; virologists; oceanographers; research and quality control scientists in industry; scientists in government hospitals and industrial plants; and upper-level undergraduate and graduate students in all scientific disciplines.

Contents

ISBN: 0—8247—9804—X

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

Understand the most recent developments in the field of. . .

Ion Exchange and Solvent Extraction

VOLUME 13

edited by

JACOB A. MARINSKY
State University of New York at Buffalo

YIZHAK MARCUS
Hebrew University of Jerusalem, Israel

February, 1997
416 pages, illustrated
$195.00

Volume 13 of this important series continues in the tradition of its widely received predecessors, presenting **current** advances and results in solvent extraction.

Containing nearly **800** helpful drawings, tables, equations, and bibliographic citations, ***Volume 13***

- shows how mixed extractants can advantageously extract divalent metal salts from aqueous media
- discusses the extraction of acids by the acid-base-coupled extractants
- explores the potential use of supramolecular chemistry for the extraction of organic compounds involving host-guest associates
- examines aqueous biphasic extraction systems for several important applications, including the removal of technetium from active nuclear waste
- demonstrates methods of polymeric resin impregnation and provides a physicochemical characterization of solvent-impregnated resins
- treats the selective extraction of the alkali metal ions emphasizing cesium which features prominently in nuclear waste
- and more!

The ***Ion Exchange and Solvent Extraction Series*** treats ion exchange and solvent extraction both as discrete topics and as a unified, multidisciplinary study—presenting **new insights** for researchers in many chemical and related fields. The volumes in this now classic series are of major importance to analytical, coordination, process, separation, surface, organic, inorganic, physical, and environmental chemists; geochemists; electrochemists; radiochemists; biochemists; biophysicists; hydrometallurgists; membrane researchers; and chemical engineers.

Contents

ISBN: 0—8247—9825—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

Simplify and improve techniques for analyzing samples while meeting regulatory requirements with...

Contains section summaries and a glossary of the latest validation terminology—facilitating a deeper understanding of the subject as well as eminent negotiations between scientists and regulators!

Analytical Method Development and Validation

MICHAEL E. SWARTZ
Waters Corporation, Milford, Massachusetts

IRA S. KRULL
Northeastern University, Boston, Massachusetts

May, 1997 / 96 pages, illustrated / $35.00

This concise guide delineates specific approaches to the successful development, optimization, and validation of high-quality HPLC-based analytical methods—demonstrating how these steps can be integrated into a formal process reflecting present United States Pharmacopeia (USP) regulations and International Conference on Harmonization (ICH) contributions.

Analytical Method Development and Validation furnishes

- step-by-step procedures for methods development and optimization—including guidelines for the *future* development of analytical techniques *as well as* discussing instrument qualification and system suitability
- validation protocols for *new* and *improved* analytical methods—aiding in the creation of validated procedures *and* revealing which steps can be employed today
- regulatory agency guidelines—assessing methods validation criteria stipulated by the USP and the FDA and undergoing final approval by the ICH
- examples of complete methods development and validation—illustrating what a final validated analytical method contains and how it is presented
- pathways by which acceptable, validated methods of analysis are built—providing directions for the development of assays
- and more!

Supplemented with an up-to-date bibliography, tables, and drawings, this convenient handbook is useful for analytical chemists and biochemists, pharmaceutical development chemists, pharmacologists, pharmacokineticists, laboratory and validation managers, and graduate-level students in these disciplines.

CONTENTS

ISBN: 0-8247-0115-1

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 • 061-261-8482
Visit our Web Site at http://www.dekker.com
(Register in our Web Site Guestbook and receive a discount on any future purchase of Marcel Dekker, Inc. products.)

JOURNAL OF LIQUID CHROMATOGRAPHY
& RELATED TECHNOLOGIES

Volume 20, Number 13, 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

J. LIQ. CHROM. & REL. TECHNOL., 20(13), (1997)

Contents Continued