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Special Section on CAPILLARY ZONE ELECTROPHORESIS AND RELATED TECHNIQUES

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

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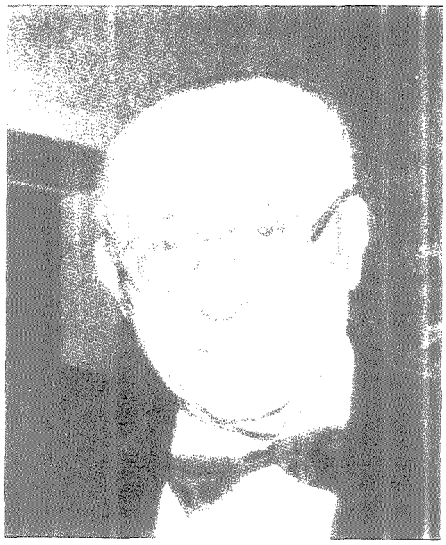
In Memoriam



Dr. Maurits Dekker
March 18, 1899 - January 16, 1995

Dr. Maurits Dekker (commonly known as Mau or Chairman Mau) was born in Amsterdam on March 18, 1899. He was the youngest of the four children of Elizabeth Pekel and Marcus Dekker. In Amsterdam, he attended elementary school and a three-year high school. His academic excellence allowed him to transfer to a more advanced five-year high school, from which he graduated in 1916. While in high school he demonstrated a gift for languages; for example, he read for pleasure the literature of Flaubert, Rabelais, and Balzac in the original French. He took great pride in this skill, fluently speaking Dutch, German, and English, as well as French.

In 1917 Mau began his university studies at the University of Amsterdam. He studied chemistry and microbiology and graduated in 1922 with a "Doctorandus, Drs." degree. During his studies he was supported by several organizations supervised by J. F. van Oss, a teacher and author of a textbook on "Warenkennis" (consumer goods). He helped van Oss in writing some of the chapters, reading and checking the manuscripts, and preparing the index. Dekker also did technical writing, which provided him with a small income. He wrote two or three times a week on the economics of the chemical industry. The columns were edited by J. F. van Oss and Dr. Gerrit Hondius Boldingh of the University of Amsterdam and published by D. B. Centen in two of its publications: *Chemisch Weekblad* and *Pharmaceutisch Weekblad*. These experiences were the forerunner of what was to come. Because of his experiences, Mau was aware of the importance and difficulty involved in editing technical material.

Instead, through his contact with *Chemisch Weekblad*, he developed an idea which was entrepreneurial in nature: he offered his services to D. B. Centen for six months free of charge in order to develop a direct mail book selling division for scientific and technical books. He was so successful that he became the paid managing director of this innovative division: "D. B. Centen's Wetenschappelijke Boekhandel."

Dekker continued to tutor a number of pupils after finishing his University studies. One of his students was Johann Gerhard Nordemann, who enlisted Dekker's help to teach him the principles of chemistry and physics. He planned to use this knowledge in his summer beer enterprise and winter anthracite business. During the lessons they often talked of other entrepreneurial possibilities. This led them to the idea in 1926 of Nordemann becoming the bookkeeper of D. B. Centen. In 1927, they decided to form a partnership called "Dekker and Nordemann's Wetenschappelijke Boekhandel." Since the owners of D. B. Centen wanted to retire, Dekker and Nordemann merged the two companies. Under the aegis of Dekker and Nordemann, the former owners received half the profits of the newly created company for several years.

The company grew very rapidly with its newfound strategy of selling Dutch books by mail, specializing in the fields of chemistry and pharmacy. After a period of time they decided to augment this activity by selling other scientific and technical works. They had the foresight to visualize that the English language would become the common international language for science and technology. To test their theory, they decided to purchase the translation rights to a few famous German texts. The most famous of these were the *Textbook of Organic Chemistry* by Paul Karrer and *Spot Test* by Fritz Feigl.

Mau learned that publishing was a different business from distributing. As a consequence, he came into contact with Johann Klautz, president of Elsevier Publishing Company, Amsterdam, who guided them through the production and printing processes of their publishing programs.

Since it was expected that the United States would in the future be a large, if not the largest, buyer of English language books, they decided in 1937 that a visit to the United States was necessary. They decided to establish the Nordemann Publishing Company, New York, owned by Dekker and Nordemann Wetenschappelijke Boekhandel, Amsterdam. Dr. Eric Proskauer was asked by Maurits Dekker to help to develop this publishing company in 1938.

In the spring of 1939 Elsevier Publishing, Amsterdam, asked Maurits Dekker to go to the United States for the primary purpose of founding their New York office. In June 1939, he and his family took care of all the necessary requirements for entry into the United States. These included physical examinations, passports, visas, and

appropriate bank papers. It was planned for Dekker to leave in the first week of September. His family—which included his wife, Rozetta Roos; his daughter, Elizabeth; and his two sons, Andrew and Marcel—were to follow within three months. However, when Germany declared war on England on September 3, Maurits changed his mind because the English and the Germans had mined the English Channel. Nevertheless, by the middle of October he finally decided to buy passage for himself only, but his wife insisted that the entire family accompany him. Within one week, possessions were sold or packed, and the entire family set sail on *The Zaandam* of the Holland American Line on October 26, 1939, arriving in New York on November 7, 1939. It was the next to last regularly scheduled liner to arrive successfully from Holland.

From December 1939 to May 1940, Maurits was salaried by Elsevier and managed the distribution of Elsevier books in the United States and Canada. At the same time, Dekker joined forces with Proskauer. When Germany invaded Holland in May 1940, contact with Europe ceased. As a consequence, all the assets were placed in an escrow account and a new publishing company was founded: Interscience Publishers, Inc., New York. Maurits became President and Eric S. Proskauer Vice President and subsequently Chairman of the Board.

With the help of such key people as Herman Mark for the field of polymers, I. M. Koltoff for analytical chemistry, A. Weissberger for organic chemistry, and Richard Courant for mathematics, Interscience grew rapidly and became internationally recognized as one of the leading scientific and technical publishers of its time. Mau was fond of telling stories of his weekly meetings in Brooklyn with Ray Kirk for the planning, developing, and publishing of the *Encyclopedia of Chemical Technology*. In the early days, this was Interscience's greatest success. It was also a prelude to Interscience's international acclaim because Mau traveled the world to sell the encyclopedia. He repeated these international visits periodically. He glowed when he stated, "I didn't go to sell; instead I traveled to smooth out our distribution." Even today, when I visit distributors throughout the world, they all ask for him and say there never has been a better publisher's ambassador who cared so much for the welfare of the book trade. Mau's education as a scientist and his activities as bookseller gained him the trust and admiration of colleagues in the book trade. Interscience became a premier publisher because of its ability to attract leading international authors, maintain standards of excellence, establish an unusually good market penetration, and maintain a publishing program at the forefront of scientific and technical developments. Examples of titles other than the *Encyclopedia of Chemical Technology* of which Mau was especially fond of remembering are: *The Treatise of Analytical Chemistry*, edited by I. M. Kolthoff and P. J. Elving; *Industrial Oil and Fat Products* by A. E. Bailey; *Medicinal Chemistry* by A. Burger; *Advanced Inorganic Chemistry* by Albert Cotton and G. Wilkinson; *Methods of Biochemical Analysis*, edited by David Glisk; *Fatty Acids* by S. Markley; *Industrial Hygiene and Toxicology*, edited by F. A. Patty; and the *Journal of Polymer Science*. All of these are now classics. Twenty-one years later, in 1961, Interscience merged with John Wiley & Sons, Inc. Mau remained with Wiley-Interscience until 1965.

Maurits and Ro Dekker's daughter, Elly, married Professor Dr. Hans Wynberg, a professor of organic chemistry, University of Groningen, the Netherlands. Their oldest son, Andrew, is a professor of pathology at Presbyterian Hospital, University of Pittsburgh. His son, Marcel, following in his father's footsteps in the publishing business, founded Marcel Dekker, Inc. in 1963. The *Journal of Liquid Chromatography*, in which this tribute is published, is one of Marcel Dekker, Inc.'s flagship publications.

Maurits joined Marcel Dekker, Inc. in 1966 with the responsibility for editorial acquisitions and was appointed Chairman of the Board. His interests ranged far and wide, but analytical chemistry was one discipline he revered, as exemplified by his association with such publications as *Advances in Chromatography* (now in 34 volumes), *Electroanalytical Chemistry* (in 32 volumes), the journal *Separation Science and Technology*, and the large program of titles in chromatography, spectroscopy, and a myriad of other books on analytical methods and techniques.

During Mau's 67-year career, he originated one successful bookselling business, founded two new publishing companies, and helped to develop a third. Most people would be proud to be the founder of one business establishment, never mind three. His motivation stemmed from the knowledge that he was advancing the building blocks of science and technology by publishing state-of-the-art works. As Herman Mark said in his letter of November 21, 1988 to Mau: "It was repeatedly said that you were a leading factor in the publication activities in this field of polymer science." The same can be said for other specialties and subspecialties, too many to mention here. His life and his career truly typify the spirit of a publishing pioneer.

His creative efforts were best captured in the last paragraph of the Honorary Doctoral Degree awarded to him in 1982 by the Polytechnic University:

> For your creative, preeminent, and continuing achievement as an international editor and publisher, and no less for your shining example as a human being, we confer on you the highest academic distinction that Polytechnic Institute of New York affords, the degree of Doctor of Science, honoris causa.

The world of publishing has been known as a sanctuary for the intellectual, dignified, discreet and refined. Maurits Dekker will be missed for all of those qualities and for the unifying force he represented in interfacing the scientific community with scientific and technical publishing.

Marcel Dekker

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IN MEMORIAM

"Chairman Mau"

I first became acquainted with Dr. Maurits Dekker about twenty years ago, when he sent me a letter which began... "Allow me to introduce myself to you. I am known to many as Chairman Mau." This was his inimitable way of "breaking the ice". And that's how innocently it all began! I didn't realize, then, the impact he would have on my life.

Mau had written to ask (no... to tell) me to become Editor of his Chromatographic Science Series of books. He assured me there would be "little, if any, work" on my part. He quickly pointed out the advantages of the notoriety this would bring to me and how it would immeasurably enhance my career. I refused... he followed up with another letter... then another... and a few phone calls and, finally, after a year of correspondence, he wore down my sales resistance and I accepted. Mau was certainly PERSISTENT and PERSUASIVE.

Needless to say, it did, in fact, involve work on my part, but it was the start of a business association which rapidly turned into a unique friendship I have always cherished and which has always resided in a special place in my heart.

If I have to pick the single most important lesson I learned from Mau it's **persistence**! He never tired and he never gave up. He never took **NO** for an answer. He never tired of asking me to accept yet another, and another assignment from him. He was persistent... and his persistence is the most important characteristic he imparted to me. And I have always been thankful for this. It has served me well.

No one has had the influence on my life that he has had. He was my greatest mentor; the principles and skills he taught me have served me well throughout my life, both in business and in social relationships.

To Mau, I say go with God... and let God occasionally have his way. It has been a pleasure and privilege to know you and to work with you. You are sorely missed.

Dr. Jack Cazes

CAPILLARY ZONE ELECTROPHORESIS AND RELATED TECHNIQUES

Edited by

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APPLICATIONS OF CAPILLARY ZONE ELECTROPHORESIS AND MICELLAR ELECTROKINETIC CHROMATOGRAPHY IN CANCER RESEARCH[1]

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ABSTRACT

The purpose of this communication is to illustrate the utility and advantages of capillary zone electrophoresis and micellar electrokinetic chromatography in cancer research. In our laboratory, both techniques have been used to explore many aspects of cancer research from the search for biological markers in urine, serum and tissue, to epidemiological studies, to the exploration of anticancer drugs in plant materials and marine organisms. Both CE and MEKC have proven to be useful in solving problems which faced us. We present a series of examples which illustrates the application of these techniques to problems faced by our laboratory.

INTRODUCTION

Analytical chemistry is an integral part of many aspects of cancer research, from testing of a chemical's purity and identity to pharmacokinetic, environmental, epidemiological, chemotherapeutic studies and the search for anticancer drugs and cancer markers. The rapid increase in the number and quantity of

[1]Presented in part at the 16th International Symposium on Capillary Chromatography, Riva del Garda, Italy, September 1994.

chemicals found in the environment, some of which pose a carcinogenic risk to animals as well as humans, require the continuous monitoring for their effect. The term chemical carcinogen refers to the response in experimental animals to a carcinogen which may be observed in several forms: a) as a significant increase in the frequency of one or several types of neoplasm, as compared with other than zero frequency in control animals; b) as the occurrence of neoplasma not observed in experimental animals; c) as a decreased latent period as compared with control animals; and d) as a combination of (a) and (c). Evidence that a particular chemical is carcinogenic in humans depends largely on epidemiological data. Analytical chemists (chromatographers, spectroscopists, etc.) have a role to play by developing methods for the extraction, separation, quantification, and structure elucidation of chemicals and their metabolites from environmental, biological, and clinical specimens.

Capillary zone electrophoresis (CZE) is a powerful micro-analytical technique which gives high resolution of large biomolecules as well as small ions. Micellar electrokinetic chromatography (MEKC) can be used to resolve mixtures of neutral as well as charged molecules. In our laboratory, CZE and MEKC have been used for the separation of varied groups of compounds of biomedical and clinical interest. In this review we will show the utility of CZE and MEKC as an important and useful analytical tool in cancer research. CZE and MEKC with UV-laser induced fluorescence (LIF), and photodiode array detection have been used for the separation and detection of proteins, purity determination of synthetic peptides and separation of amino acids in urine, and for the determination of cancer markers, anti-cancer drugs and other compounds and metabolites of interest to cancer researchers.

High performance liquid chromatography (HPLC) and gas chromatography (GC) are the main separation techniques used in solving analytical problems related to chemical carcinogen determination and quantification (1). Today, capillary electrophoresis (CE) is being used to solve many of the analytical problems facing cancer researchers. We present here a few examples which will illustrate the utility and effectiveness of CE and MEKC as an analytical tool in different aspects of cancer research.

Extraction of Anti-Cancer Natural Products:

Extraction and isolation of natural products from plant material and marine organism is an important aspect of drug discovery against cancer. The first example is that of the separation by MEKC of the anti-cancer drug taxol from the needles and bark of the Western Yew, taxus brevifolia (taxacaea). Taxol shows unique anti-tumor and anti-leukemic activities (2,3). It has been shown to produce responses in patients with different types of cancer, such as ovarian (4), breast (5), lung (6), head and neck region (7) and malignant melanoma (8). HPLC (9) and TLC (10) have been used, however, MEKC gave a baseline separation of taxol from other coeluting compounds. We were also able to resolve in the same experiment from needle and bark extracts taxol, baccatin III, cephalomannine and other closely related compounds which were not separated by HPLC (11). Figure 1 is a comparison of the separation of a needle extract by both HPLC and MEKC. Other natural products which were resolved by CZE (12) are bryostatin I and bryostatin II which are members of a group of closely related macrocyclic

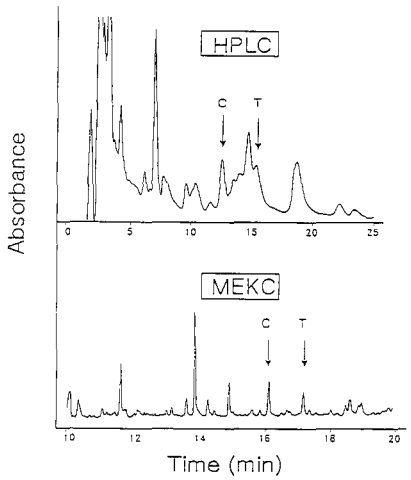


FIGURE 1. HPLC chromatogram and MEKC electropherograms of a crude needle extract from the taxus species. T = Taxol; C = depha-lomannine; B = baccatin III.

lactones which have been isolated from the marine Bryozoa Bugula neritina L. (Bugulidae). Bryostatins are activators of protein kinase C and show interesting antineoplastic and immunomoculating properties. These compounds were previously resolved by TLC (13) and HPLC (14). The advantages of MEKC over HPLC and TLC is the speed of analysis, the high resolution, and the negligible amount of organic solvent waste produced by MEKC.

Epidemeological Applications:

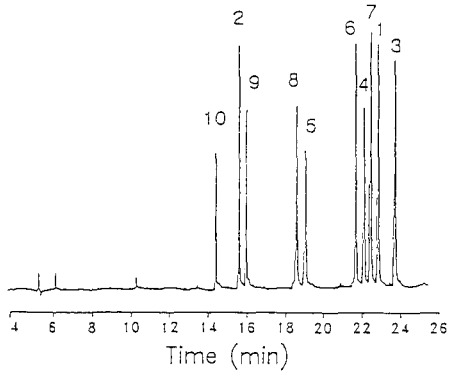
The modern epidemeologic study of hormone-dependent cancers, like breast, prostate and endometrial cancers, require the capability of highly accurate, specific and sensitive assay for steroid, peptide and protein hormones as well as growth factors. MEKC has been used in our laboratory for the separation of a group of endogenous and exogenous urinary estrogens which include estrone, estradiol, estriol, 2-hydroxyestrone, 2-hydroxyestradiol and others (15). Figure 2 shows the separation of 10 estrogens which have closely related structures.

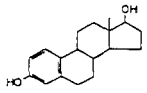
Retinoic Acids:

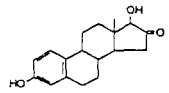
Retinoic acids (RAs) are metabolites or analogues of vitamin A that show important physiological functions. A variety of RAs have been shown to be capable of promoting growth and differentiation of epithelial cells and inhibiting tumorigenesis. A number of physiologically relevant RAs have been identified including all-trans-RA, 13-cis-RA, 4-oxo-RA and 13-cis-4-oxo-RA. Recently, the isomer 9-cis-RA has been identified as a high affinity ligand for the retinoid X receptor. Unfortunately, some RAs are also able to act as teratogens. Efficient separation among those isomers is needed for the assessment of their biological activities and for the most part high-performance liquid chromatography has been the method of choice. The separation of 5 retenoic acid isomers and internal standard from spiked rat urine by MEKC was achieved (16) using a micellar buffer made of 20 mM tris-borate (pH = 8.5), 25 mM SDS and 20% acetonitrile (figure 3).

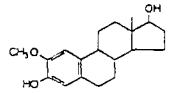
Amino Acids, Peptides and Proteins:

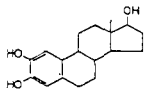
Amino acids, peptides and proteins play an important role in cancer research. Hydorxyproline (Hyp) is a secondary amino acid

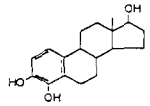


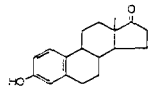

[1] 17β-Estradiol

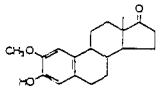

[2] 16-Keto-17β-estradiol

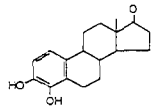

[3] 2-Methoxyestradiol

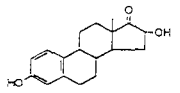

[4] 2-Hydroxyestradiol


[5] 4-Hydroxyestradiol


[6] Estrone


[7] 2-Methoxyestrone


[8] 4-Hydroxyestrone


[9] 16α-Hydroxyestrone

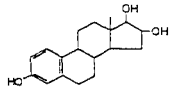

[10] Estriol

FIGURE 2. MEKC of ten estrogens. Buffers: 10 mM sodium phosphate (pH 7.0) containing 50 mM SDS and 20% methanol; capillary: 50 μm x 47 cm; voltage: 20 kV; pressure injection: 2S; detection: absorption at 200 nm.

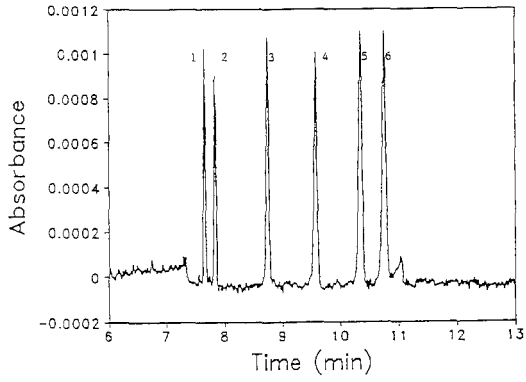


FIGURE 3. Separation of retinoic acid mixture from spiked rat plasma extract. Capillary: 75 μm x 47 cm; buffer: 20 mM Tris-borate (pH 8.5), 25 mM SDS, 20% acetonitrile; applied voltage: 13 kV; injection: 4S, pressure; peaks: 1 = 13-cis-4-oxo-RA, 2 = 4-oxo-RA, 3 = 13-cis-acitretin, 4 = 13-cis-RA; 5 = 9-cis-RA, 6 = all-trans-RA.

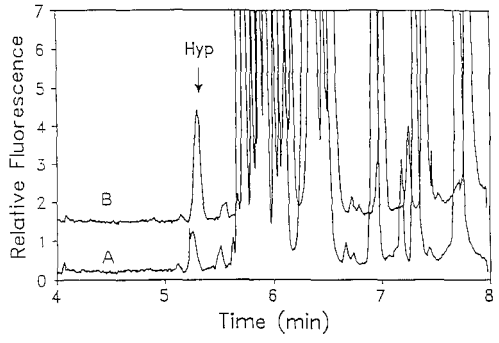


FIGURE 4. Electropherograms of a FMOC-derivatized serum (A), and a Hyp-spiked (~$6 \cdot 10^{-8}$ M) serum sample (B). The serum was diluted ca. 500-fold. Running conditions were: Capillary:60 cm x 50 μm I.D.; buffer: 20 mM sodium borate (pH 9.2) containing 25 mM SDS; applied voltage: 2 kV; injection: 15 s, gravity.

that reflects the metabolism of collagen. The determination of Hyp is important because it has been suggested as a marker for certain cancers and bone diseases. The detection of Hyp in biological samples is difficult due to its relatively low concentration compared to other amino acids and its lack of any strong spectroscopic property. Sensitive detection is possible by modifying the molecule using chemical derivatization. Micellar electrokinetic chromatography with UV laser-induced fluorescence (LIF) was used for the rapid and sensitive detection of Hyp in biological samples after pre-column derivatization with 9-fluorenylmethyl chloroformate (FMOC) (figure 4). Also, the application of the combined orthophthalic aldehyde (OPA)/FMOC derivatization in MEKC for the selective detection of secondary amino acids was achieved. The described MEKC-LIF method (17) allows rapid and sensitive detection of Hyp in serum and hydrolyzed urine that were derivatized with FMOC. The combined OPA/FMOC derivatization simplified the method (17) for the selective detection of proline (Pro) in serum and the simultaneous detection of Hyp, Pro, and sarcosine in hydrolyzed urine (figure 5). Also, the separation of a chiral mixture of amino acids was accomplished (18). Issaq and Chan (19) reviewed the separation and detection of amino acids and their enantiomers by capillary electrophoresis.

The separation of peptides (from dipeptides to 66 residue amino acids) have been accomplished using CZE (20-22). Purity determinations which could not be achieved by HPLC were easily done by CZE. In each case studied CZE was superior to HPLC in resolving the impurities in synthetic peptides (22). Figure 6 is a comparison of the separation by both HPLC and CZE of a 66 residue peptide. The results clearly show the superiority of the CZE separation. Proteins were also resolved by CZE and their native fluorescence was detected by LIF when they contained an aromatic amino acid (21).

Furthermore, CZE was used to study the binding of sequence-specific DNA to recombinant ETS1 oncoproteins (23). A time-dependent shift in the mobility of the P42-DNA complex was observed and the change in mobility was correlated with a net change in charge of the protein-DNA complex.

Catecholamines:

Capillary zone electrophoresis with untreated fused-silica capillaries and acetate buffer was evaluated for the separation and

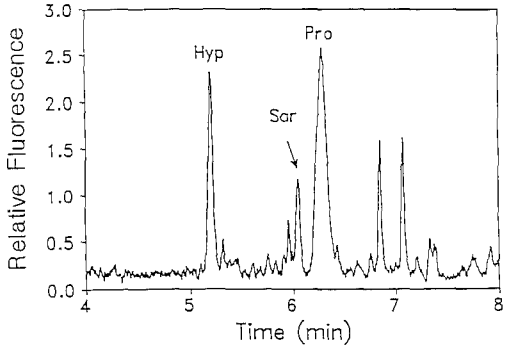


FIGURE 5. Electropherogram of a OPA/FMOC-derivatized urine. The urine sample was diluted ca. 5000-fold. Running conditions were the same as in Figure 4.

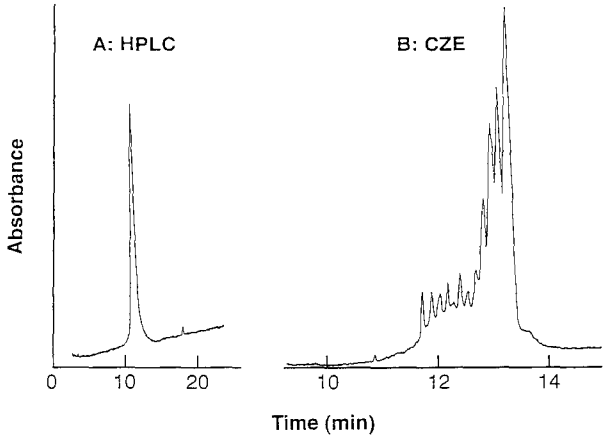


FIGURE 6. Analysis of the purity of a 66-residue peptide, LYQSNPPPNPEGTRQARRNRRRRWRERQRQIHSIZERILSTYLGRSAE-PVPLQLPPLERLTLDCNE-OH by (a) HPLC and (b) CZE. For HPLC the mobile phase program was from 40% (0.1 TFA in acetonitrile/water 4:1 and 60% 0.1 TFA in water to 100% 0.1 TFA in acetonitrile/water 4:1) in 40 min. For CZE the buffer was 50 mM phosphate at pH 3.0, the applied voltage 15 kV, and the current 55 μA. CE was performed with a Beckman Model P/ACE System 2000 fitted with a 57 cm x 75 μm i.d. polyacrylamide-coated fused silica column. Injection was by application of 0.5 psi pressure for 5 s. Detection was monitored at 214 nm.

analysis of catecholamine metabolites. Homovanillic acid and vanillylmandelic acid, which are excreted in abnormally elevated levels in the urine of patients with neuroblastoma, were separated from other possible catecholamine metabolites, using a 200 mM acetate buffer at pH = 4.10 (24). The high buffer concentration was necessary to minimize the peak tailing resulting from analyte-capillary wall interactions.

Nitrate and Nitrite Separation:

Capillary zone electrophoresis offers fast and efficient separation of inorganic ions. Nitrite, a meat preservative, reacts in vivo with amines and amides at an acidic pH to form carcinogenic nitrosamines. A limit of 10 mg nitrate per liter is imposed on drinking water to prevent methomoglobin in infants. Furthermore, the recent interest in the physiological importance of nitric oxide have heightened the need for sensitive methods for the determination of nitrite and nitrate in body fluids. Also, cancer patients receiving interluken II were found to have elevated levels of serum nitrate.

The problem with analyzing for nitrate and nitrite in biological samples by high performance ion chromatography is the presence of high concentration of chloride ions which interfere with the determination of nitrite due to lack of resolution and column saturation. The addition of silver reagents or silver-loaded cation exchange resins will react with the chloride and leads to its elimination as an interfering compound. However, it was reported that the silver chloride precipitation process causes a substantial reduction in the performance of ion exchange columns used for anions with insoluble silver salts (25,26). A capillary zone electrophoresis method for the separation and analysis of nitrate and nitrite in water and urine was developed (27). No interference in the electropherogram from other anions is observed by using a polyacrylamide-coated column with a modified phosphate buffer at pH 3 for the separation, and UV-absorption at λ = 214 nm for the detection (figure 7). The method does not require sample pretreatment or the use of organic solvents. The limit of detection for each analyte (S/N = 3), using a 75 μm i.d. capillary, is 0.5 μg/mL.

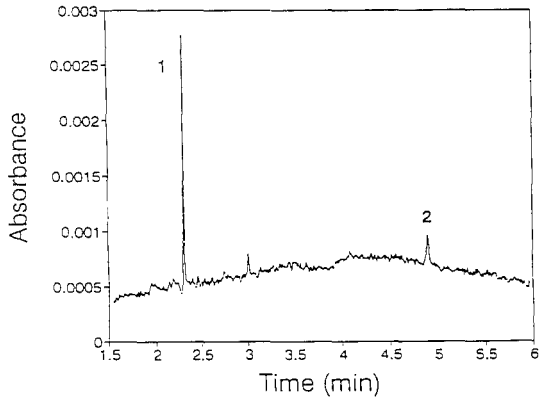


FIGURE 7. Electropherogram of a rat urine sample spiked with 50 μg/ml nitrite then diluted 40:1. Buffer: 25 mM phosphate containing 0.5% DMMAPS and 1.0% Brij-35; applied voltage: -15 kV; column: 10% T, polyacrylamide-coated fused silica [T = (g acrylamide + g N,N'-methylenebisacrylamide)/100 ml solution]; column dimensions: l_{total} = 57 cm, $L_{detection}$ = 50 cm, I.D. = 75 μm; instrument: beckman Model P/ACE System 2000. Detection: 214 nm. Solutes: 1 = nitrate; 2 = nitrite.

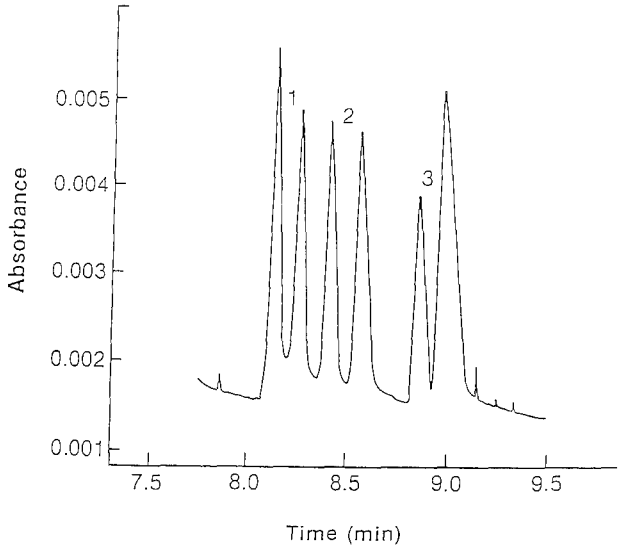
Nitrosoamino Acids:

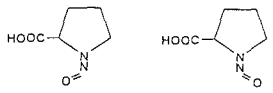
CE was also used for the separation of nitrosoamino acids and their syn and anti-conformers using a polyacrylamide-coated capillary and sub-ambient temperatures (28). The conformers of N-nitrosopro-line, N-nitroso-4-hydroxyproline and N-nitrosothiazolidine-4-carboxylic acid were resolved using CE at 5°C, figure 8.

Caffeine and Its Metabolites:

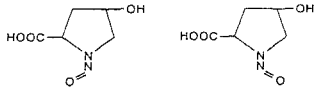
Caffeine (1,3,7-trimethylxanthine) is widely used in the human diet. It can be studied as a probe drug for the assessment of variability in biotransformation capacity. In the 1980's, examining caffeine metabolism to determine genetic acetylator phenotype and genotype of human population groups was common practice (29). Caffeine is particularly well suited as a test drug for many reasons such as availability, ease of administration and safety.

To date, high performance liquid chromatography is the method of choice for the determination of caffeine and its metabolites.

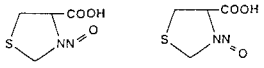




N-nitrosoproline



N-nitroso-4-hydroxyproline



N-nitrosothiazolidine-4-carboxylic acid

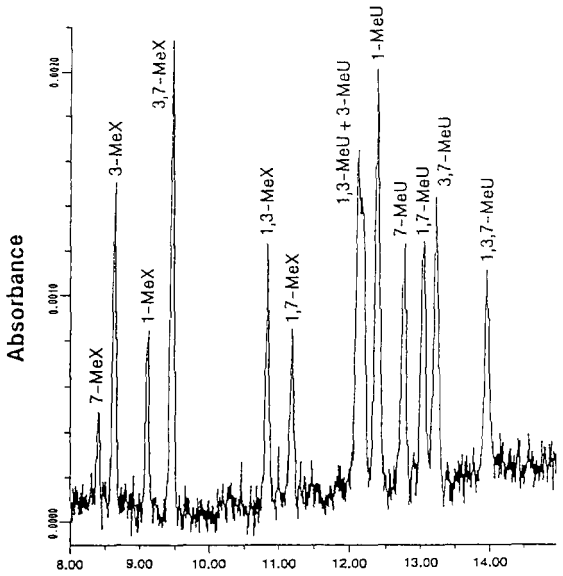
FIGURE 8. Electropherogram showing the separation of syn and anti conformers of selected nitrosoamino acids at 5°C. Solutes: 1 = N-nitrosothiazolidine-4-carboxylic acid, 2 = N=nitrosoproline, and 3 = N-nitroso-4-hydroxyproline; instrument: Beckman Model P/ACE System 5510; detection: 235 nm; column 10% T polyacrylamide-coated fused-silica; column dimensions: L_{total} = 57 cm, $L_{detector}$ = 50 cm, i.d. = 75 μm; buffer: 10 mM phosphate containing 2 mM DMMAPS, and 0.1% Tween 20; pH = 7.2; applied voltage: -25 kV; solute concentration: 2-5 μg/mL.

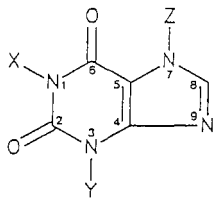
Grant et al. (30) developed a procedure for their extraction and subsequent HPLC separation and quantification. In our laboratory a method was developed for the separation of xanthines and uric acid derivatives which are normally present in human plasma and urine as metabolites of caffeine. The methyl-substituted uric acids were separated using in the CZE mode a 0.05 M sodium phosphate buffer (pH 7.0) at an applied voltage of 10 kV. In contrast, the separation of the methyl-substituted xanthines was only possible in the MEKC mode, where SDS (0.15 M) was added to the buffer system. When both types of compounds were present in the same sample, optimum resolution (12 peaks for 13 standard solutes) was realized in the MEKC mode with 0.15 M SDS added to the 0.05 M, pH 7 phosphate buffer at an applied voltage of 15 kV, figure 9 (31).

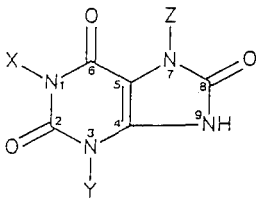
Nicotine and Its Metabolites:

The determination of nicotine and its principal metabolites in biological fluids provides information about exposure to cancer-causing cigarette smoke. Several methods have been published for the analysis of these compounds, of which HPLC is the most widely used (32). A CZE method was developed in our laboratory for the separation of nicotine, and three of its principle metabolites from a human urine matrix. Figure 10A shows the separation of a standard mixture using a polyacrylamide-coated column. Figure 10B shows the electropherogram resulting from a smoker's urine specimen, after sample clean-up according to the procedure of Zuccaro et al. (33) and reconstitution in the running buffer. The method offers: 1) orthogonality to HPLC in the separation mechanism; 2) selectivity, because only positively charged species migrate towards the negative electrode in the zero-electroosmotic flow environment provided by the polyacryl amide coated column; and 3) high resolution. The method is currently under evaluation for the analysis of biological samples.

The above examples illustrate the utility and advantages of both CZE and MEKC over other separation techniques in cancer research.







Solute	X	Y	Z
Xanthine (X)	H	H	H
1-MeX	Me	H	H
3-MeX	H	Me	H
7-MeX	H	H	Me
1,3-Mex	Me	Me	H
1,7-MeX	Me	H	Me
3,7-MeX	H	Me	Me
1,3,7-MeX	Me	Me	Me

Solute	X	Y	Z
Uric Acid (U)	H	H	H
1-MeU	Me	H	H
3-MeU	H	Me	H
7-MeU	H	H	Me
1,3-MeU	Me	Me	H
1,7-MeU	Me	H	Me
3,7-MeU	H	Me	Me

FIGURE 9. Electropherogram of a mixture of seven methyl-substituted xanthines and six methyl-substituted uric acids. Experimental conditions: 50 cm x 75 μm i.d.; buffer: 0.05 M Na_2HPO_4 + 0.15 M SDS; pH 7.0; pressure injection mode: 2 sec at 0.5 psi; temperature: 25°C; detector: UV at 280 nm; voltage: 15 kV. Solute symbols as given in Table I.

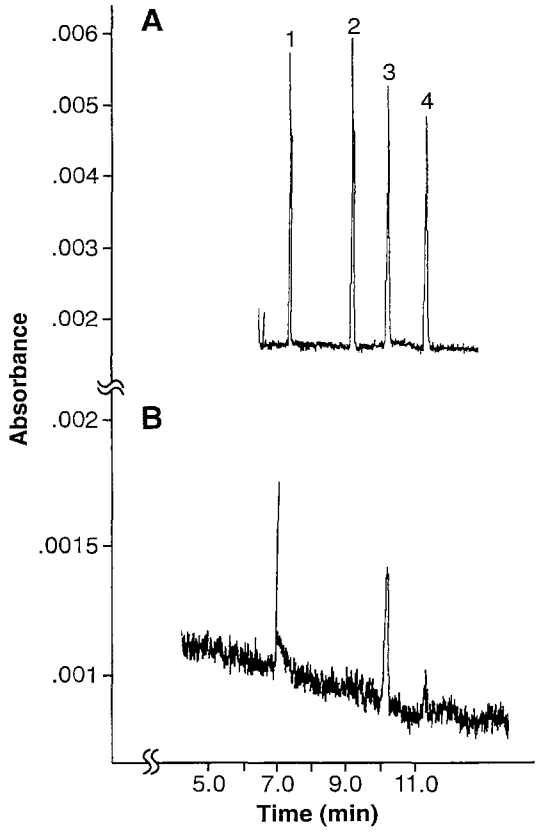


FIGURE 10. Separation of nicotine and three of its main metabolites. A: Standard mix and B: Smoker's urine sample. Buffer: 50 mM sodium acetate; pH: 4.7; voltage: 20 kV; pressure injection: 3S at 0.5 psi; detection: absorption of 260 nm; instrument: Beckman Model P/ACE System 5510; column: 10% T polyacrylamide-coated fused-silica; column dimensions: L_{total} = 47 cm, $L_{detector}$ = 40 cm; i.d. = 75 μm; solute concentration: 2-5 μg/mL in water; solutes: 1-nicotine, 2-demethylcotinine, 3-cotinine and 4-trans-3-hydroxycotinine.

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CAPILLARY ELECTROPHORETIC CHIRAL SEPARATIONS USING A SULFATED β-CYCLODEXTRIN-CONTAINING ELECTROLYTE

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ABSTRACT

Sulfated β-cyclodextrin (SO_3-β-CD) was utilized as a chiral additive for capillary zone electrophoresis (CZE). Chiral separations of several uhcharged enantiomers, such as phensuximide, indapamide, etc., which are difficult to separate using neutral CDs, were achieved. The effects of SO_3-β-CD concentration and the pH and ionic strength of the supporting electrolyte as well as the presence of an organic modifier, methanol, were discussed.

INTRODUCTION

In the last decade, capillary zone electrophoresis (CZE) has become an important analytical technique.[1] Like gas chromatography (GC) and high performance liquid chromatography (HPLC), CZE offers some conspicuous advantages over conventional electrophoresis, such as fast

separations, high efficiency, minute sample loading and ease of automation.[1]

CZE has been utilized to analyze a wide variety of charged and uncharged species, such as small ions,[2] simple organic molecules,[3] amino acids,[4,5] peptides,[6] and proteins.[7] One particularly challenging area of separations is that of enantiomeric separations. With identical physical and chemical properties in an achiral environment, enantiomers do not exhibit differences in electrophoretic mobility and have traditionally been considered to be the most difficult among all separations. Nevertheless, the unique high efficiency of CZE and the possibility of enantioselective interactions between the analyte and a chiral additive make CZE well-suited to enantiomeric separations. Separations are usually performed by adding a chiral selector to the buffer electrolyte and the separation is accomplished due to a preferred formation of a complex between the chiral additive and one of the enantiomers. The most commonly used chiral selectors in CE can be divided into three categories, in which different separation principles apply: host-guest complexation (e.g., native and derivatized cyclodextrins,[8,9,10] and crown ethers[11]); ligand exchange complexation[12] and optically active surfactants.[13,14]

Cyclodextrins (CDs) and their derivatives have been successfully used for GC, HPLC and TLC separations of a large number of chiral compounds including drugs and derivatized amino acids.[15,16,17] Although most of the GC and HPLC separations were achieved on immobilized CD chiral

stationary phases (CSPs), many different types of CSPs are required in order to cover a limited range of racemic compounds. The use of CDs as a mobile phase additive provides a flexible alternative for the separation of enantiomers, because separations can be performed on conventional columns which generally have higher efficiencies than CSPs.[18] Both native and derivatized CDs have been utilized as chiral mobile phase additives.[19,20,21]

Accordingly, the use of CDs in CE seems to be a logical extension of existing chiral separation techniques. Two approaches are generally used to achieve the chiral separation on CE. While CD may be immobilized within a polymeric gel,[4] most of the chiral separations in CZE with CDs have been achieved in free solution.

Chiral CZE separations have been achieved with all three native α-, β-, γ-CD and their mixtures.[22,23,24] The proposed separation mechanism seems to be dependent on a difference in stability of the inclusion complexes formed between each enantiomer and the CD coupled with differences in the mobility characteristics of the "host-guest" complexes.[25] Among the three native CDs, β-CD is by far the most widely used for CZE.[26] However, the low solubility of β-CD in water (1.85 g/100ml)[27] limits method optimization. Urea is commonly used as an additive to increase the solubility of the CD.[28,29] Alternatively, incorporation of different functional groups, such as methyl (e.g., dimethyl-β-CD[21,30] or trimethyl-β-CD[10,21]), glycosyl[23], and hydroxypropyl groups,[23,31] onto the CD result in different degrees of

enhanced solubility and enantioselectivity. However, all of these derivatized CDs and native CDs are neutral and therefore migrate with the same mobility as the electroosmotic flow. Hence, the separation "window" is limited to the time between the analyte migration time in the absence of CD and the migration time of the CD.

Nishi et al.[29] reported chiral resolution of electrically neutral compounds by micellar electrokinetic capillary chromatography (MECC), using a buffer containing CDs and negatively charged sodium dodecyl sulfate (SDS). Because the EOF flow was much stronger than the anodic electrophoretic migration of the micelle, the net migration of SDS was also in the direction of cathode, but with a slower velocity than the bulk solution or the CD. The proposed mechanism involved distribution of the solutes between three phases, i.e., the aqueous, the micelle and the CD phase. The retarded migration of the analytes as a result of partitioning into the micelle amplified the enantioselective interaction with the CD, thereby resulting in enhanced enantioselectivity.

Although most reports of CD-based electrokinetic chromatography use neutral CD, Terabe[32] has reported using an ionic derivatized CD, 2-O-carboxymethyl-β-CD to separate the structural isomers of cresols.

In this study, sulfated β-CD (SO_3-β-CD) was used as a chiral additive in the electrolyte. According to the manufacturer, the average degree of substitution is 7 to 11 sulfate/CD, with the substitution pattern unknown. The

presence of the negatively charged groups on the CD should not only slow the velocity of the SO_3-β-CD relative to the native CD, thereby increasing the chiral separation window, but also enhance the solubility of SO_3-β-CD relative to the native CD. It should be noted that derivatization of the CD no doubt changes the chiral recognition ability as well as hydrogen bonding capability of CD with analytes. Therefore, the SO_3-β-CD was anticipated to provide unique enantioselectivity. A variety of neutral racemic compounds were successfully resolved. The influence of experimental factors such as pH, the presence of organic modifier and the concentration of the chiral selector will be discussed subsequently. The contribution of electroosmotic flow may not be easily ascertained in the presence of ionic CD's because the neutral marker may complex with the CD. More importantly, however, the CD's contribute to the overall ionic strength of the buffer. Hence, the difference in the mobility or migration times and resolution of solutes is adopted to evaluate separations.

EXPERIMENTAL

Chemicals

SO_3-β-CD was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Racemic solutes were purchased from Aldrich or Sigma Chemical Company (St. Louis, MO) and dissolved in methanol (ca. 0.5 mg/ml). The methanol was HPLC grade. The buffer solutions were prepared from distilled, doubly deionized water.

Instrumentation

The experiments were carried out on a Water Quanta 4000 Capillary Zone Electrophoresis system equipped with a UV detector (214 nm) and a power supply delivering up to 30 kV. The CZE was operated in a conventional mode with the cathode at the detector end. The fused silica capillary column was 75 μm i.d., 60 cm total length and 52.4 cm capillary length to the detector window. Data acquisition was performed with a Shimadzu Chromatopac CR-501 data station.

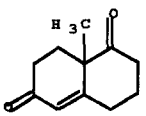
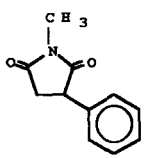
Run Conditions

Run buffers were prepared by dissolving disodium hydrogen phosphate (10-20 mM) and SO_3-β-CD (0-4%) in water, and adjusted with phosphoric acid to the appropriate pH values (5-8). All buffer solutions were filtered through a membrane filter of 0.45 μm pore size prior to use. In all experiments, a constant voltage was applied. The capillary was rinsed before each run with 0.1 M potassium hydroxide for 2 minutes and then buffer for 2 minutes. Samples were injected using the hydrostatic method (2 sec). Methanol was adopted as a marker for EOF.

RESULTS AND DISCUSSION

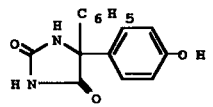
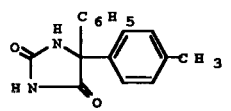
Table 1 lists the compounds used in this study along with results obtained under conditions optimized for each analyte to achieve baseline resolution of enantiomers within the shortest analysis time.

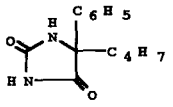
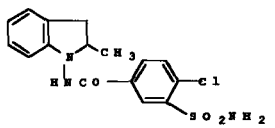
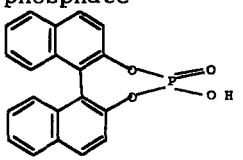
Table 1. Electrophoretic data for recemates resolved using SO-β-CD as CMA in CZE.

No	Compound	t	R_S	Electrophoretic condition	Applied Voltage
1	9-Methyl-$\Delta^{5(10)}$-1,6-dione 	17.88 18.46	2.94	10 mM Na_2HPO_4, 2% CD, pH 6.0	8 kV
2	Phensuximide 	19.32 20.10	1.94	10 mM Na_2HPO_4, 3% CD, pH 7.0	8 kV

(continued)

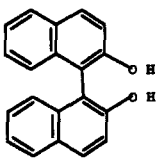
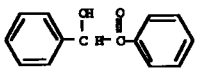
Table 1 (Continued).

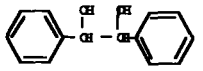
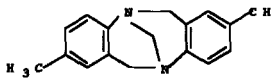
No	Compound	t	R_S	Electrophoretic condition	Applied Voltage
3	5-(4-Hydroxyphenyl) -5-phenyl hydantion 	15.61 16.72	2.94	10 mM Na_2HPO_4, 2% CD, pH 8.0	15 kV
4	5-(4-Methylphenyl) -5-phenyl hydantion 	15.20 16.06	2.96	10% MeOH / (10 mM Na_2HPO_4, 2% CD, pH 8.0)	15 kV

5	5-Cyclobutyl-5-phenyl hydantoin 	11.53 12.36	3.95	10% MeOH / (10 mM Na_2HPO_4, 2% CD, pH 8.0)	15 kV
6	Indapamide 	24.08 24.96	1.50	10 mM Na_2HPO_4, 4% CD, pH 7.0	8 kV
7	1,1'-Binaphthyl-2,2'-diyl hydrogen phosphate 	24.31 25.19	2.43	10 mM Na_2HPO_4, 2% CD, pH 8.0	8 kV

(continued)

Table 1 (Continued).

No	Compound	t	R_S	Electrophoretic condition	Applied Voltage
8	1,1'-Bi-2-naphthol 	9.35 10.17	2.72	10 mM Na_2HPO_4, 2% CD, pH 8.0	15 kV
9	Benzoin 	26.79 28.20	1.77	10 mM Na_2HPO_4, 2% CD, pH 7.0	8 kV

10	Hydrobenzoin 	8.63 10.12	4.40	10 mM Na_2HPO_4, 2% CD, pH 8.0	15 kV
11	Troger's base 	16.81 17.78	2.44	30% MeOH / (10 mM Na_2HPO_4, 2% CD, pH 8.0)	15 kV

From Table 1, it can be seen that most of the solutes resolved in this study either have a chiral center located on a ring (1-7) or have locked ring structures (7, 8, 11). As for many cases in HPLC, formation of an inclusion complex between the relative non-polar interior of the CD cavity and the hydrophobic moiety of the analyte seems to play an important role in chiral recognition with SO_3-β-CD.

Effect of SO_3-β-CD Concentration

The effect of SO_3-β-CD concentration on migration and selectivity was examined. For all compounds in this study, migration times increased as the concentration of SO_3-β-CD increased from 1% to 4%. Figure 1 shows the electrophoregrams for the resolution of 9-Methyl-$\Delta^{5(10)}$-1,6-dione into its enantiomers using different concentrations of SO_3-β-CD. Both the migration times and apparent mobility differences of the enantiomers increased as the concentration of SO_3-β-CD increased. Baseline separation (Rs > 1.5) was achieved when SO_3-β-CD increased to 3% (Figure 1). Although increasing the SO_3-β-CD concentration further improved the selectivity, the migration times also dramatically increased (Figure 1e).

Figure 2 further illustrates the effect of SO_3-β-CD concentration on the migration times and chiral recognition of some racemates in this study. The migration time difference between two enantiomers of each analyte increased as the concentration of SO_3-β-CD increased. It was found that complete enantiomeric resolution was achieved for

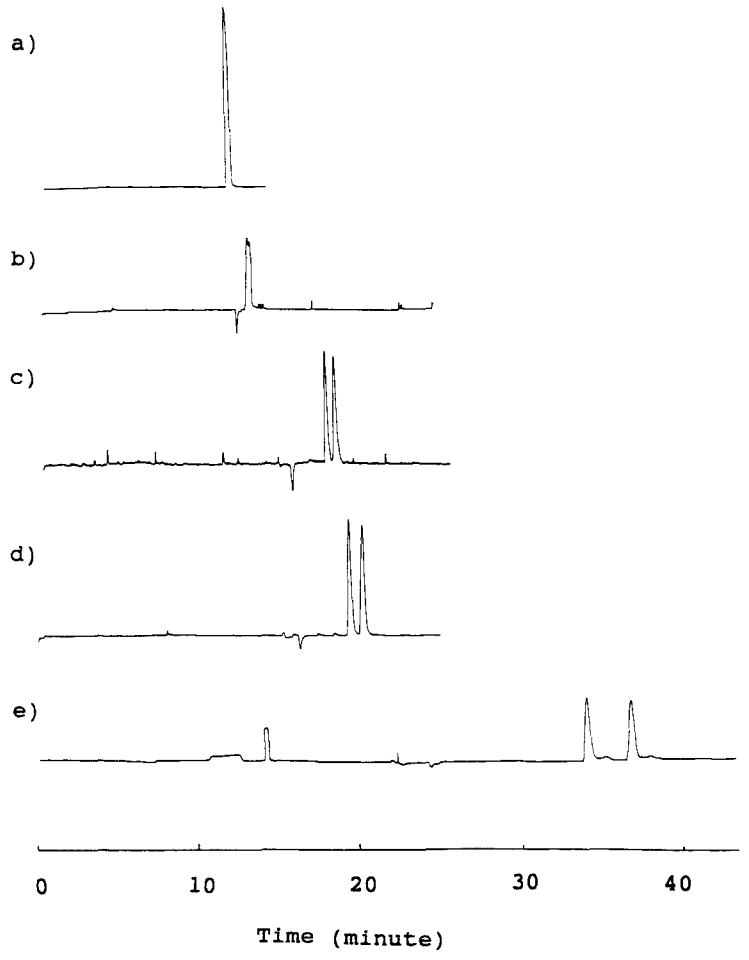


Figure 1 Electropherograms showing the effect of the concentration of SO-β-CD on chiral resolution of phensuximide. Conditions: electrolyte (10 mM Na_2HPO_4, pH 7.0); applied voltage, 8 kV; concentration of SO-β-CD:
a) 0%; b) 1%; c) 2%; d)3%; e)4%.

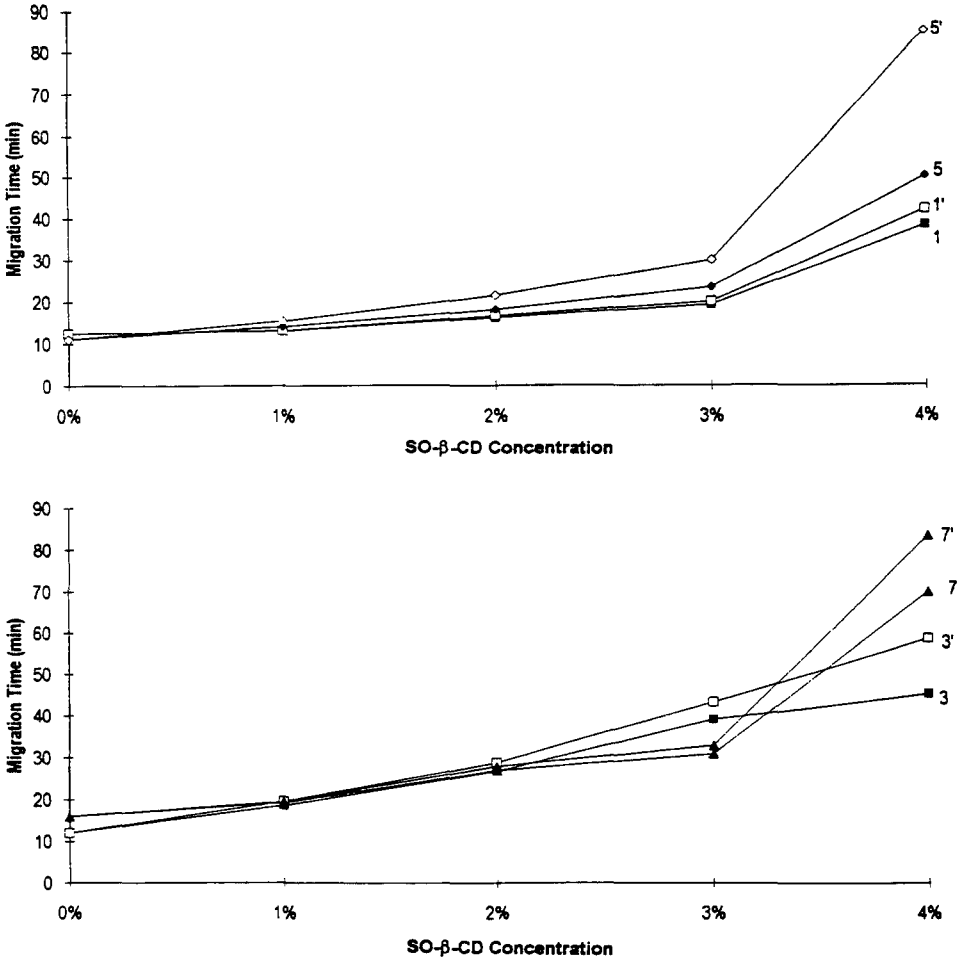


Figure 2 Effect of the concentration of SO-β-CD on the migration times of a) 1,1' = 9-Methyl-$\Delta^{5(10)}$-1,6-dione; 5,5' = 5-Cyclobutyl-5-phenyl hydantoin; b) 3,3' = 5-(4-Hydroxyphenyl)-5-phenyl hydantoin; 7,7' = 1,1'-Binaphthyl-2,2'-diyl hydrogen phosphate. Same conditions as in Figure 1.

analytes 3, 4, 5, 10 and 11 when 1% of SO_3-β-CD was added to the background electrolyte. To completely resolve compounds 2, 7, 8 and 9, 2% of SO_3-β-CD was required. To obtain baseline resolution of compound 1 and 6, it was necessary to add 3% and 4% of SO_3-β-CD to the run buffer, respectively. Although for most solutes, as SO_3-β-CD concentration increased, resolution increased as well (Figure 3), a maximum resolution was observed with compound 3 at 2% SO_3-β-CD.

In the absence of SO_3-β-CD, all analyte species moved toward the cathodic end with migration rates close to EOF. However in the presence of SO_3-β-CD, analytes complex with the SO_3-β-CD and were transported toward the negative electrode at slower velocity, presumably because the negatively charged sulfate groups on SO_3-β-CD electrophoretically attract it to the anodic end. Increasing the concentration of SO_3-β-CD provided more opportunity for analytes to complex with the SO_3-β-CD, therefore further slowing down the migration of analytes toward the detector end. It should also be noted that SO_3-β-CD contributes to the ionic strength and that increases in ionic strength suppresses the electroosmotic flow.

Effect of Electrolyte Ionic Strength

Unlike the native or methyl derivatized CDs, which are neutral in buffer solution, SO_3-β-CD is readily ionized in aqueous solution, and therefore contributes significantly to the ionic strength of background electrolyte. Thus,

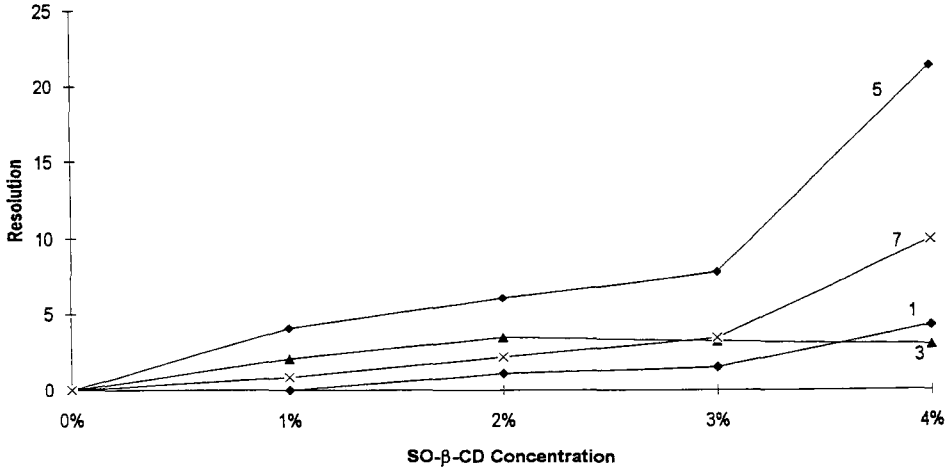


Figure 3 Effect of the concentration of SO-β-CD on R_S of: 1 = 9-Methyl-$\Delta^{5(10)}$-1,6-dione; 3 = 5-(4-Hydroxyphenyl)-5-phenyl hydantoin; 5 = 5-Cyclobutyl-5-phenyl hydantoin; 7 = 1,1'-Binaphthyl-2,2'-diyl hydrogen phosphate. Same conditions as in Figure 1.

increasing the concentration of SO_3-β-CD not only has a considerable impact on the chiral selectivity but on the ionic strength as well. Addition of 1% of SO_3-β-CD to the background electrolyte gave an average of almost 70% increase in current (e.g., current increased from 25.2 μA to 42 μA as SO_3-β-CD increased from 1% to 2%), thereby increasing the amount of Joule heating within the capillary.

To further evaluate the effect of electrolyte ionic strength on migration and chiral recognition, two series of

experiments were carried out at 2% of SO_3-β-CD with pH 8.0, containing 10 mM and 20 mM of phosphate buffer, respectively. The results of some test analytes are summarized in Table 2. It has been rationalized[33] that increased ion concentration reduces the thickness of the double layer on the capillary wall, leading to a decrease of EOF. Indeed, the migration times of most of the selected analytes increased when the phosphate concentration doubled, but the apparent mobility difference between two enantiomers as well as resolution did not show appreciable dependence on phosphate concentration for most analytes. Evidently, in most cases, the effect of phosphate buffer concentration on resolution was non-enantiospecific. Interestingly, the mobility and resolution of compounds 4 and 5 exhibit significant change. From Table 1, compounds 3, 4 and 5 bear similar structures. However, the polarity decreases from compound 3 to 5, which is due to the different substituents at the C5 position. The increase of migration time and resolution from compound 3 to compound 5 as phosphate concentration increased may provide evidence that hydrophobic complexation between the analyte and SO_3-β-CD cavity is important for chiral recognition. However, the overall contribution of phosphate buffer to the ionic strength of the background electrolyte was limited compared to SO_3-β-CD (e.g., current increased from 42 to 50 μA when phosphate concentration increased from 10 to 20 mM). Hence, SO_3-β-CD seemed to be the major contributor to the electrolyte conductivity in this experiment.

Table 2 Effect of phosphate concentration on the solute migration time and resolution. Electrolyte, 2% SO-β-CD, pH 8.0. Applied voltage, 8 kV.

	10 mM phosphate		20 mM phosphate	
Compound	t	Rs	t	Rs
2	15.87	1.17	15.86	1.14
	16.30		16.31	
3	31.78	3.99	32.16	3.46
	34.58		34.58	
4	26.43	3.05	33.64	3.69
	28.71		37.22	
5	16.88	6.21	21.04	11.18
	19.68		24.96	
6	14.95	1.06	16.74	0.74
	15.17		17.00	

Influence of pH

The effect of the run buffer pH was investigated in a pH range of 5 to 8. Generally, the migration times of the test solutes increased as pH decreased. Furthermore, when pH decreased to 5, no peaks were observed for any of the analytes within 120 minutes.

The increased retention at low pH may be attributed, in part, to the reduced EOF. Under low pH conditions, the

dissociation of silanol groups on the fused-silica capillary wall is suppressed. The reduced zeta potential thereby decreases EOF; consequently migration times increase. In aqueous buffer, the sulfate groups (pKa of H_2SO_4 -9) of SO_3-β-CD carry a negative charge. The migration of SO_3-β-CD is under the influence of both EOF and electrophoretic flow, which, in turn, is directly related to the pH of the electrolyte. The electrophoretic mobility of SO_3-β-CD is toward the anodic (injection) side while the EOF is toward the cathodic (detector) end. Presumably, at high pH, the EOF is stronger than the electrophoretic flow, thus the net migration of SO_3-β-CD may be in the direction of the cathode.[34] However, at low pH this may not be the case. Indeed, Stalcup and Agyei demonstrated that the net migration of another sulfated carbohydrate was toward the anode at pH 4.5.[34]

Figure 4 illustrates the effect of pH on the migration time and chiral recognition of compounds 1, 4, and 11. As the pH decreased from 8 to 6, the migration time of all three solutes increased. For compound 1, the increase of migration time for both enantiomers was almost parallel. In this case, the increase in retention seems to be related strictly to the decrease in EOF. This may be due to the fact that compound 1 is neutral and is not affected by the pH of the buffer. With compound 11, the migration time of the second eluting peak increased more than that of the first one at pH 6, resulting in an increase of the apparent mobility difference between the two enantiomers. Although

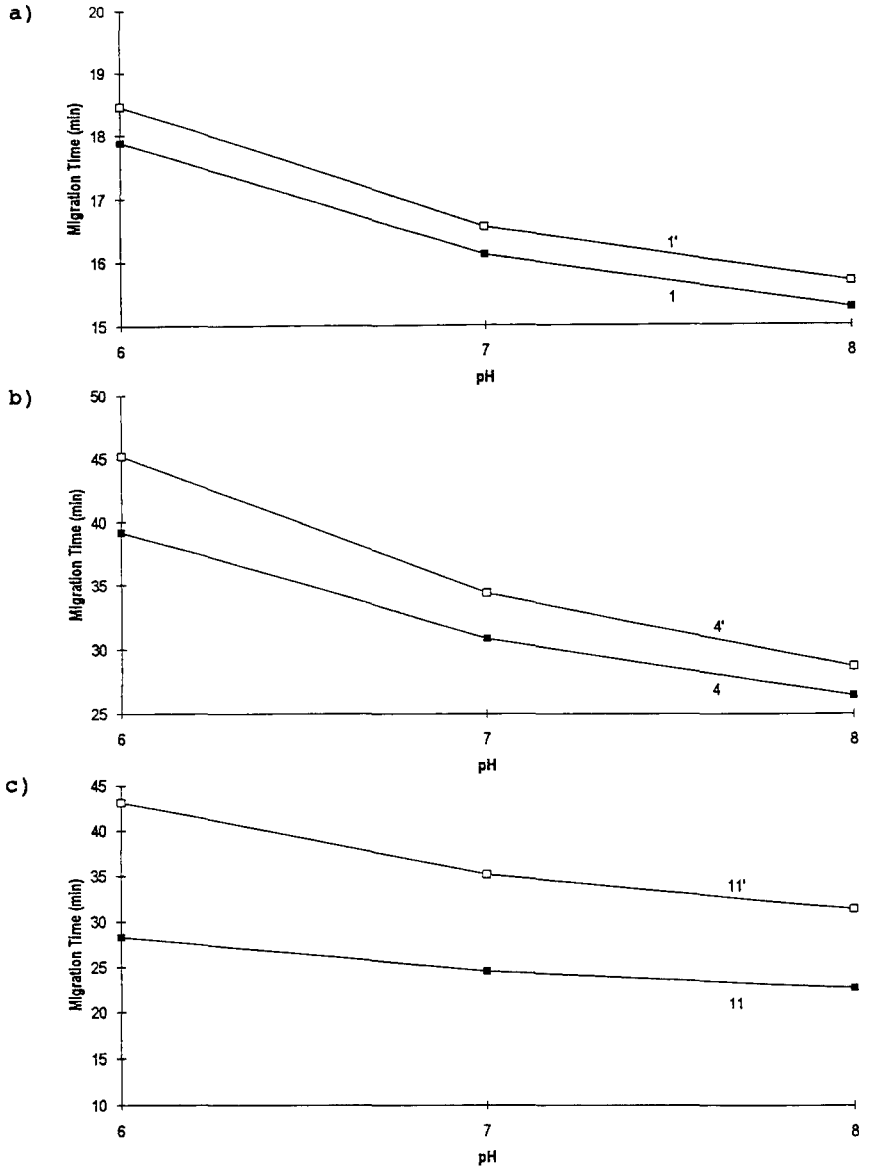


Figure 4 Effect of pH on the migration time of a) 1,1' = 9-Methyl-$\Delta^{5(10)}$-1,6-dione; b) 4,4' = 5-(4-Methylphenyl)-5-phenyl hydantoin; c) 11,11' = Troger's base. Conditions: electrolyte (Na_2HPO_4, 2% SO-β-CD); applied voltage, 8 kV.

apparently the same trend existed for compound 4, it should be noted that compound 11 was protonated under the experimental conditions ($pK_a \approx 10$ for tertiary alkylamine ammonium ion). In this case, at low pH or absense of EOF, the electrophoretic mobilities of the analyte and the additive are in the opposite directions thus further amplifies the differences in binding of the the enantiomers.

Effect of Organic Modifier

The effect of the addition of organic modifier to the run buffer on the performance parameters was also investigated. Methanol and acetonitrile are the two most frequently utilized water-miscible organic solvents.[35] In this study, methanol was added to the electrolyte in increasing proportion within a 0-30% (v/v) range. In all cases, the migration times of solutes increased as the amount of methanol in the electrolyte increased.

Addition of methanol resulted in a decrease of EOF and hence increased migration times of the solutes. It has been pointed out[36] that when methanol is added to the buffer the viscosity of the resulting solution increases while the dielectric constant decreases. Moreover, methanol is thought to interact with the capillary wall thereby masking the surface silanol groups, leading to an increase of the local viscosity in the double-layer.[36] The magnitude of the zeta potential which is generated on the wall of fused-silica capillary therefore decreases. According to equation[37]

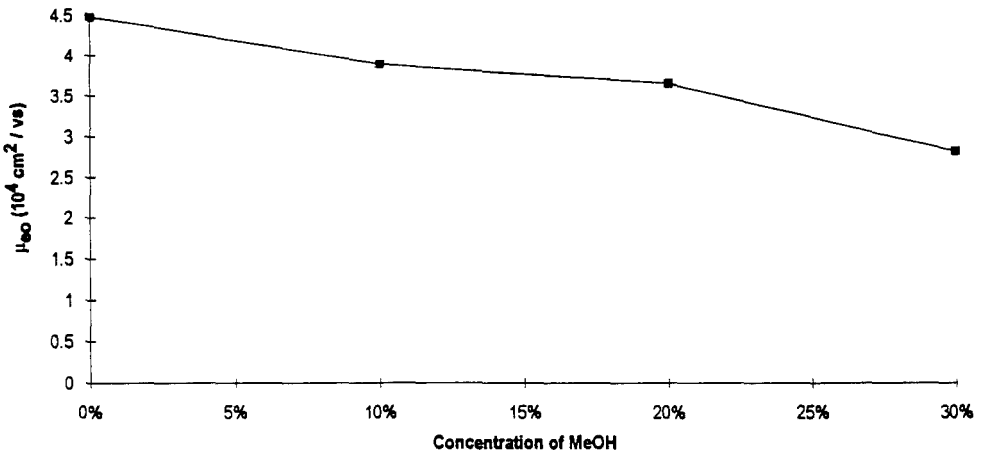


Figure 5 Effect of the concentration of methanol on electroosmotic flow. Electrolyte: 10 mM Na_2HPO_4, 2% SO-β-CD, pH 8.0; applied voltage, 15 kV.

$$v_{eo} = \frac{\varepsilon \xi}{4\pi\eta} E$$

where v_{eo} is the electroosmotic flow velocity; ε is the dielectric constant and η is the buffer viscosity, the EOF is a function of the buffer dielectric constant, viscosity and zeta potential. Figure 5 presents the effect of the addition of methanol on EOF.

Addition of methanol may also affect the CZE chiral separation in another way. Chiral recognition in SO_3-β-CD modified CZE is thought to be due to the host-guest inclusion complexation between the enantiomers and chiral selector. Addition of methanol in the run buffer may weaken the affinity of analytes for SO_3-β-CD, because methanol may

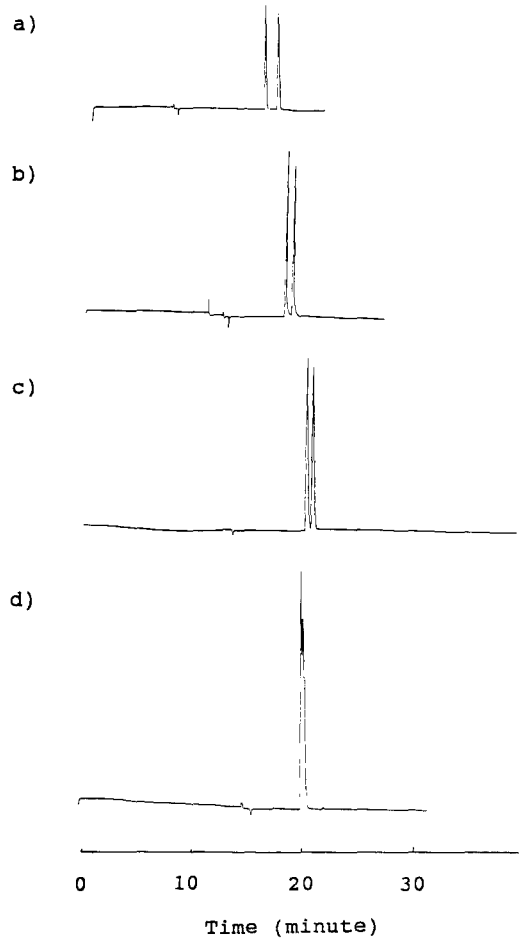


Figure 6 Electropherograms of 5-(4-Hydroxyphenyl)-5-phenyl hydantoin illustrating the effect of methanol concentration migration time and resolution. Same conditions as in Figure 4. Methanol concentration: a) 0%; b) 10%; c) 20%; d) 30%.

compete with solutes for the relatively hydrophobic cavity of SO_3-β-CD, therefore diminishing the chiral recognition. In addition, methanol may provide a more hospitable environment for nonpolar solutes.

It was observed that the resolution of the test enantiomeric compounds gradually deteriorated with the addition of methanol. Typical electropherograms of compound 3 are presented in Figure 6 to illustrate the change in resolution with changing methanol concentration. When the volume concentration of methanol increased to 30%, resolution of racemates 1, 2, 6, 7, 8 and 9 totally diminished and a single peak was observed. Partial resolution was obtained for compound 3, 4, 5 and 10, while base line separation of racemate 11 was still obtained. The case of compound 11 which is ionized under the experimental conditions, may lend support to the premise that the loss of chiral recognition for neutral species with the addition of methanol may be partially attributed to decreased electrolyte polarity.

With increasing volume concentration of methanol from 0% to 30% in solution, currents decreased from 85 μA to 30 μ A. This allowed operation of CZE with a higher electric field strength (15 kV).

CONCLUSIONS

The results demonstrate that SO_3-β-CD has sufficient solubility in an electrolyte to act as a good

enantioselective agent for the resolution of neutral enantiomers, which are difficult to achieve using neutral CDs. The concentration of SO_3-β-CD, pH and ionic strength of electrolyte, and the presence of organic modifier were all shown to exert a profound effect on solute migration and separation. However, the commercially available SO_3-β-CD used in this study is not well characterized. Future work may involve regiospecific sulfation of CD to maximize the separation and understand the separation mechanism. Unless separation and understand the separation mechanism. Unless a thorough understanding of separation mechanism is achieved, the optimization of electrophoretic separation will still be largely an empirical process.

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THREE-DIMENSIONAL ELECTROPHEROGRAM FOR THE SEPARATION OF OLIGODEOXY-NUCLEOTIDES AND DNA RESTRICTION FRAGMENTS USING CAPILLARY GEL ELECTROPHORESIS WITH A PHOTODIODE ARRAY DETECTOR

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ABSTRACT

A photodiode array detection system was applied to capillary gel electrophoresis and gave reliable spectra of single-stranded oligodeoxynucleotides and double-stranded DNA restriction fragments even in the presence of high UV background caused by polyacrylamide within the capillary. Photodiode array detection enabled accurate characterization of separated DNA fragments and was a powerful tool for the identification of oligodeoxynuelcotides.

INTRODUCTION

Slab gel electrophoresis has been widely used as a standard method for the separation of single-stranded DNA and DNA restriction fragments[1,2]. More recently, capillary gel electrophoresis (CGE) is developing rapidly as a powerful new analytical technique for the separation of single- and double-stranded DNA[3-7].

CGE is the automated technique offering the benefits of rapid separation and high resolution in comparison with time-consuming and labor-intensive slab gel electrophoresis.

CGE has some other advantages inherently, e.g., many valuable detectors developed for HPLC will be applicable to CGE. A photodiode array detector has been recognized as a powerful tool for HPLC[8] and capillary zone electrophoresis[9], but it has entered the CGE field more slowly. Due to high UV absorbance of polyacrylamide[10], the investigators may hesitate to apply the photodiode array detector to CGE.

In this paper, we demonstrates that a photodiode array detector is particularly useful for the analysis of spectra for DNA fragments in the presence of high UV background caused by polyacrylamide and the identification of oligodeoxynucleotides.

EXPERIMENTAL

The DNA restriction fragments of a ϕX174DNA/*Hae* III digest (0.24 μg/μL) was purchased from Toyobo (Osaka, Japan). The ϕX174DNA/*Hae* III digest contained 11 fragments of 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078, and 1357 base pairs (bp). The DNA samples were 10-fold diluted with Milli-Q water and stored at -18 °C until use. Oligodeoxynucleotide was purchased from Pharmacia (Uppsala, Sweden). The oligodeoxynucleotide sample was prepared by dissolution of 2.5 units sample into 100 μL Milli-Q water and stored at -18 °C until use. All other chemicals were of analytical-reagent or electrophoretic grade from Wako (Osaka, Japan).

Capillary gel electrophoretic separations were carried out by using an Otsuka CAPI-3000 (Otsuka Electronics, Osaka, Japan) and an HP ^{3D}CE (Yokokawa Analytical Systems, Tokyo, Japan) capillary electrophoresis instruments both equipped with a photodiode array detector. Polyimide coated fused silica capillaries (375 μm o.d., 100 μm i.d., GL Sciences, Tokyo, Japan) of 37.8 cm effective length and 50 cm total length were used. Gel-filled capillaries, in which crosslinked polyacrylamide gel (3 %T and 0.5 %C) or linear polyacrylamide (8 %T and 0 %C) is chemically bound to the capillary wall, were prepared

according to the literature[6,7]. A sample solution was introduced electrophoretically at negative polarity of 10 kV for 5 s into the gel-filled capillary and separated with a running buffer solution (50 mM tris-borate and 1 mM EDTA, pH 8.4) at negative polarity of 10 kV (200 V/cm, 8-15 μA) at 25 °C.

RESULTS AND DISCUSSION

Capillary gel electrophoresis (CGE) shows high resolving power in the single base resolution of single-stranded DNA[3-5] and separates completely a mixture of oligodeoxynucleotides of equal chain lengths but of different base composition[11,12]. In this case, each oligodeoxynucleotide is expected to be identified from its spectrum obtained by using a photodiode array detector, because the wavelength and the molar absorption coefficient for the UV absorption maxima of nucleotides in aqueous solutions are different from each other as follows: deoxyadenosine (260 nm, 1.52 x 10^4), deoxyguanosine (254 nm, 1.30 x 10^4), thymidine (267 nm, 9.65 x 10^3), and deoxycytidine (271 nm, 9.0 x 10^3), respectively[13]. The identification from the spectrum would be much easier and consume less amount of DNA sample than the conventional identification by spiking each peak with authentic sample. Although the high absorbance of polyacrylamide (3% T polyacrylamide gives 2.4-3.0 absorbance unit in the UV range of 190-260 nm and its spectrum is shown in Fig. 3 of ref. 10) within the capillary seems to interfere with reliable measurement for the spectrum of DNA fragment, the CAPI-3000 or the HP ^{3D}CE system, which measures a reference spectrum of polyacrylamide before electrophoresis, stores it on a hard disk, and corrects the background during electrophoresis, allows us to measure the spectrum accurately and obtain fine three-dimensional electropherogram. Other less-absorbing polymers such as dextran are easily employed instead of polyacrylamide.

Figure 1 shows the three-dimensional electropherogram (time-wavelength-absorbance) obtained by the photodiode array detector for the separation of a mixture of single-stranded oligodeoxynucleotides, using linear polyacrylamide (8 %T and 0 %C) filled capillary. We chose 20-mers of deoxyadenylate (dA_{20}) and thymidylate (dT_{20}) as model substrates. All spectrophotometric information over 200-400 nm of the DNA fragment migrating through a on-column cell of the gel-

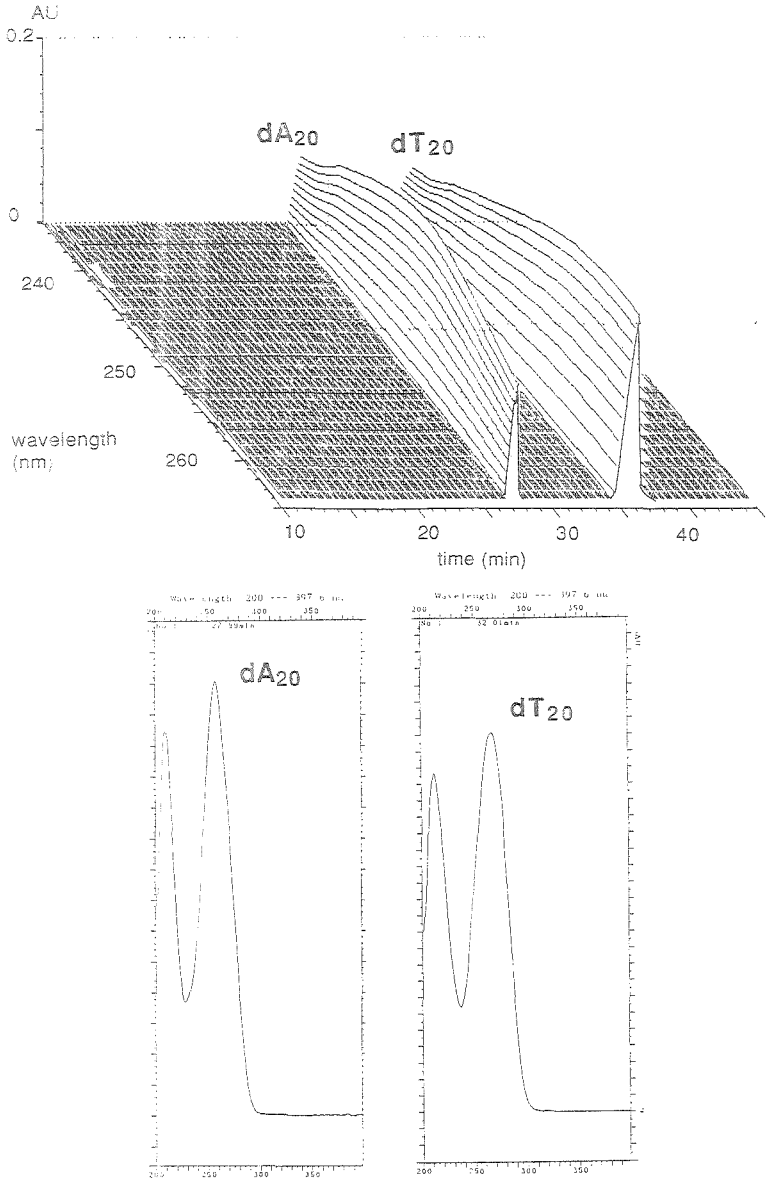

FIGURE. 1 Three-dimensional CGE profile for a mixture of oligodeoxynucleotides, dA_{20} and dT_{20}, obtained by a photodiode array detector, using linear polyacrylamide (8 %T and 0 %C) filled capillary. Conditions: Capillary, 100 μm i.d., 375 μm o.d., 50 cm length, 37.8 cm effective length; running buffer, 50 mM Tris-borate and 1 mM EDTA, pH 8.3; field, 200 V/cm; current, 10 μA; injection, 10 kV for 5 s; capillary temperature, 25 °C; recording, 0.1 AUFS, 230-280 nm.

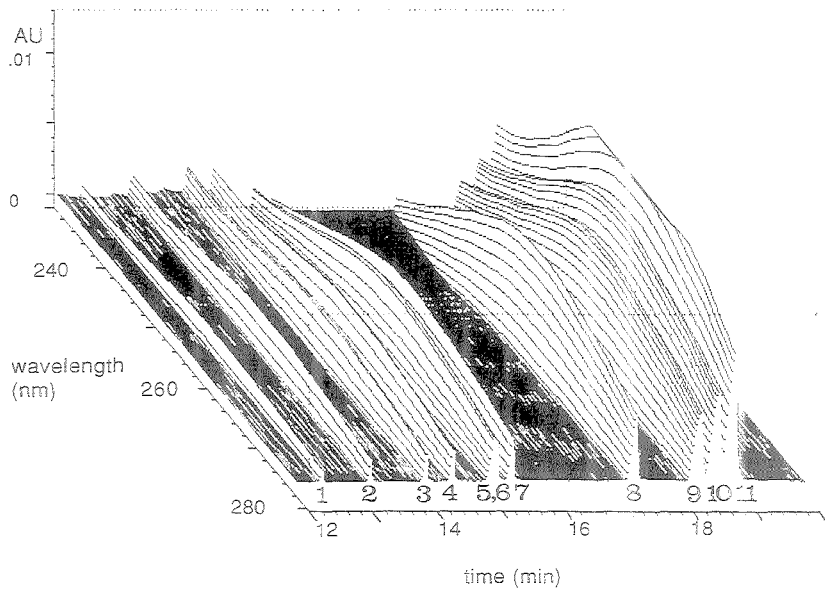

FIGURE. 2 Three-dimensional CGE profile for the *Hae* III restriction digest of φX174 DNA, using crosslinked polyacrylamide gel (3 %T and 0.5 %C) filled capillary. Other conditions as in Fig. 1. Peak assignment; 1=72, 2=118, 3=194, 4=234, 5=271, 6=281, 7=310, 8=603, 9=872, 10=1078, and 11=1357 bp.

filled capillary was computer-stored and a part of spectrum was reproduced three-dimensionally. Each well-resolved component is migrated at 27.99 min and 32.01 min, and gives the different absorption spectrum. The spectra of both components, superimposed in Fig. 1, show characteristic absorption maxima of 259 nm for the peak at 27.99 min and 267 nm for the peak at 32.01 min. These peaks, therefore, are assigned to dA_{20} for the former peak and dT_{20} for the latter peak from the spectrophotometric data obtained by the photodiode array detector and the assignments agreeing with those by spiking the authentic samples. These results show that the well-designed photodiode array detector is applicable to obtain the reliable spectrum for the identification of the sample solute migrated into the capillary filled with polyacrylamide having a very high UV absorbance.

We next examine the application of the photodiode array detector to gain three-dimensional electropherogram (Fig. 2) for the separation of a mixture of

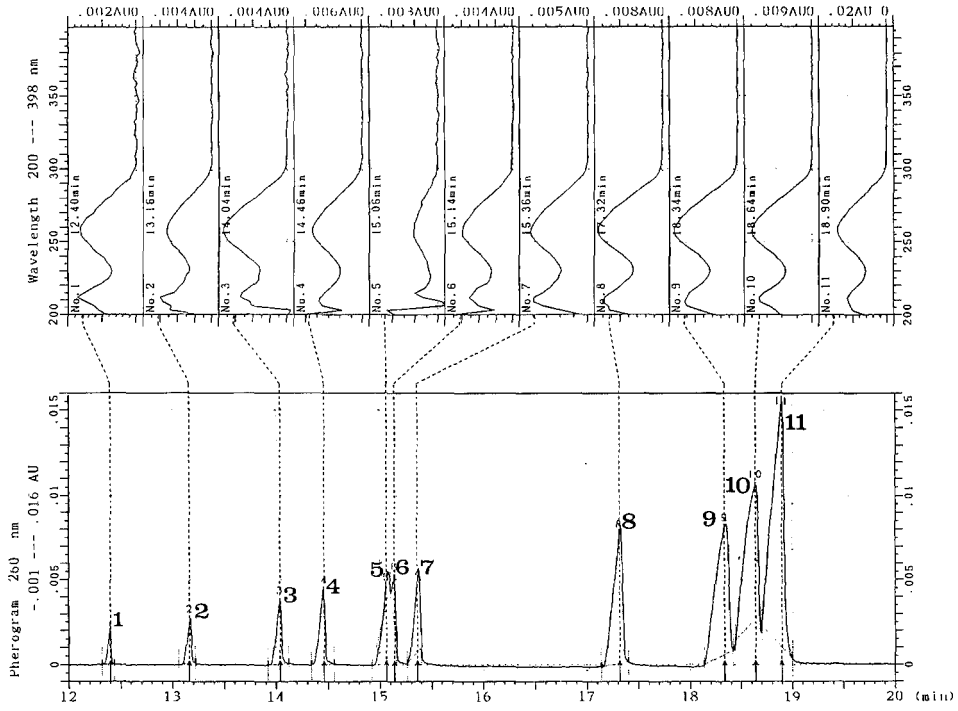

FIGURE. 3 Two-dimensional CGE profile at 260 nm for the *Hae* III restriction digest of ϕX174 DNA and the UV absorption spectrum of each peak. Other conditions as in Fig. 2. Peak assignment; 1=72, 2=118, 3=194, 4=234, 5=271, 6=281, 7=310, 8=603, 9=872, 10=1078, and 11=1357 bp.

double-stranded DNA restriction fragments (ϕX174 DNA/*Hae* III digest), using crosslinked polyacrylamide gel (3 %T and 0.5 %C) filled capillary. All fragments are well resolved and give the similar UV absorption spectrum, having a maximum around 255-260 nm. This illustrates that the photodiode array detector produces easily the three-dimensional electropherogram for the broader base pair range of DNA restriction fragments, ranging from 72 to 1353.

To investigate the feature of these spectra in more detail, two-dimensional electropherogram at specific wavelength (260 nm) and the spectrum of each component was reproduced from the spectrophotometric information stored on the

hard disk as shown in Fig. 3. The electropherogram indicates that the peaks are detected at the AU range from 0.0017 (72 bp) to 0.015 (1353 bp). The wavelength of the absorption maximum can be calculated from the spectrum accurately and it is noteworthy that each spectra of the individual fragments are reliably yielded even from the peak (72 bp) having very low absorbance unit of 0.0017. Each spectrum shows the specific absorption maxima in the range from 255 to 260 nm. These spectra indicate that the measurement of DNA restriction fragments at wavelength range from 250 to 265 nm is recommended for the sensitive detection of each DNA fragment, when a conventional variable-wavelength detector is used for routine analysis. The sensitivities relative to that at 260 nm are approximately 100 (260 nm), 90 (250 nm), and 90 (270 nm), respectively.

These results show that the photodiode array detector is effective for the check of PCR reaction through the identification of PCR product and reactants, including large DNA fragments (100-10,000 bp), oligodeoxynucleotide primer, and deoxynucleoside triphosphates (dNTP). The spectrum of large DNA fragments produced by PCR reaction is easily distinguishable from the spectra of oligodeoxynucleotides and dNTP, if migration time of PCR product is very similar to those of primers and dNTPs.

In this study, we demonstrate that the photodiode array detector is effective for the analysis of spectrum of single- and double-stranded DNA fragments even in the presence of high UV background caused by polyacrylamide within the capillary. The results in Figs. 1-3 clearly illustrate that the photodiode array detector shows very high detectability for the measurement of the absorption maximum from the spectrum of DNA fragments and will be applicable to identify oligodeoxynucleotides.

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DETERMINATION OF ORGANOLEAD AND ORGANOTIN COMPOUNDS IN WATER SAMPLES BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY

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Abstract

Micellar electrokinetic chromatography separation of organolead and organotin compounds was investigated. The effects of surfactant concentration and electrolyte buffer pH on the peak efficiencies and resolution were studied. Enrichment of the organolead and organotin species in water samples by liquid-liquid extraction and solid phase extraction using C_{18} membrane disk was performed. 1000-40,000 fold preconcentration for lead and tin species was obtained using solid phase extraction. The detection limits for organolead and organotin compounds were in the range of 1.1-5.2 parts per billion. The levels of these compounds in the collected water samples were determined.

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Introduction

Great attention has been paid to the speciation of organolead and organotin compounds since they permeate many aspects of human society and exert great effects on the economy and the environment. Both organolead and organotin have been widely applied in industrial, agricultural, forestrial and maritime fields. Tetraalkyllead (R_4Pb) compounds have been extensively used as petrol additives, although such use is diminishing, which have contributed significantly towards the problem of environmental pollution [1]. Various organotin compounds have been used as polyvinylchloride plastic stabilizer (dibutyltin or dioctyltin), antifouling agent in marine paint (tribulyltin), wood preservatives (triethyltin) and agricultural fungicide (triphenyltin). Mono- and tetra-organotin have little commercial use up to now, whereas di-and tri-organotin have been the main resources of environmental tin pollution [2-6].

In the past years, the successful determination and speciation of organolead and organotin species have been carried out by coupling separation techniques such as HPLC [7-16] and GC [17-28] with element-specific detectors, i.e. atomic absorption spectrometry (AAS), inductively coupled plasma-atomic emission spectrometry (ICPAES), microwave induced plasma-atomic emission spectrometry (MIPAES), and particularly the recent work of coupling supercritical fluid chromatography (SFC) and extraction [29-33] to inductively coupled plasma mass spectrometry [29] and ICPAES [32] for organotin speciation. For the determination of organolead and organotin compounds, GC methods are generally considered superior to HPLC because of the higher efficiency and the availability of more sensitive detectors. However, in most cases, it is necessary to convert the organolead and organotin into volatile forms by hydride-generation or Grignard reagent derivatization before GC separation can be performed because of the thermal instability of organolead and organotin compounds. These reactions sometimes maybe difficult and non-quantitative, and the by-products and the contaminants may cause interference in

subsequent determinations [31]. As for SFC, the poor solubility of organometallic compounds in common supercritical fluids prevent its wide application. Furthermore, customed-built interfaces for the on-line coupling of chromatography with spectrometry are not routinely available.

Capillary zone electrophoresis (CZE) and its modification in the form of micellar electrokinetic chromatography (MEKC) are newly developed separation methods, based on electrokinetic migration and chromatographic principles [34,35]. CZE and MEKC have demonstrated their enormous capacity and potential for separating ionic and neutral solutes due to the simplicity and high efficiency. CZE method has been recently used for aluminum [36] and arsenic [37] speciation, but there have been hardly any report dealing with the separation and speciation of organolead and organotin compounds by CZE and MEKC. It could be expected that, to separate organolead and organotin compounds, CE and MEKC would be preferred over HPLC due to the better efficiency, and over GC due to the lower temperature used, thus avoiding the derivatization steps.

It should be noted that the injection volume for CE is often several nanolitres, with a typical absolute detection limit of pg solute when conventional HPLC UV detector is used [34]. On the other hand, concentration in the peak is relatively high, e.g., more than 1 ppm for a common solute. This concentration detection ability is not sensitive enough for determining organolead and organotin in environmental samples, in which lead and tin exist at parts per trillion to low parts per billion level, unless preconcentration procedure is performed. Liquid-liquid extraction (LLE) and solid phase extraction (SPE) are the most commonly used methods for sample enrichment. LLE is a successful method but with recognized disadvantages: being laborious, time consuming, and also subject to problems such as the emulsification of the samples during extraction and the disposal of large volume of toxic solvents. In SPE extraction, water samples

are passed through a small cartridge packed with sorbent. Analytes are trapped by the sorbent and then are eluted with a small volume of suitable solvent. In many ways, SPE is superior to LLE methods by overcoming the problems in LLE and has been widely used in HPLC [38,39] and FIA [40,41] techniques. In the past few years, an alternative technique based on the principle of SPE but with a piece of sorbent membrane disk to replace the SPE cartridge has been reported [42]. This disk can be used as the filter membrane in a conventional HPLC filtration apparatus. Because the sorbent disk has larger contacting area than a SPE cartridge, larger flow rates are permitted, and therefore the extraction for large volume (1L or more) samples can be performed with much shorter times than with SPE cartridges. This disk extraction method has been used for the extraction of organic compounds in water samples [42-44]. Evans et al [46] also gave the first report on extracting tributyltin compounds in sea water using SPE disks.

The objectives of this investigation are (i) to develop a simple MEKC method for the separation of the most commonly encountered organo-lead and -tin compounds in environmental samples and (ii) to demonstrate the possibility of determining the organolead and organotin in real samples after disk SPE extraction.

Experimental

Chemicals and Materials

Trimethyllead chloride, triethyllead chloride, trimethyltin bromide, tributyltin bromide and dibutyltin dichloride were analytical grade and purchased from Aldrich Co. (Milwaukee, WI,

USA). *Ca.* 10 mg/ml stock solutions of the above organometallic compounds were prepared by dissolving accurately weighed amounts of the individual compounds in methanol [Caution: alkyllead and alkyltin compounds are highly toxic, and should be handled with great care]. The stock solutions were stored in refrigerator at 4 °C for one month without degradation. External standard solutions were prepared fresh daily. Sodium dodecylsulfate, sodium dihydrogen phosphate and disodium tetraborate from Merck (Germany) were used to prepare different electrolyte buffers, which were filtered with 0.45 μm membrane and degassed by ultrasonication before use. Sodium diethyldithiocarbamate, used as a complexing reagent, was obtained from Merck (Germany). All the other chemicals and solvents were analytical or better grade. Water purified with a Millipore-Q system was used throughout the experiments.

Instrumentation

The home-made electrophoretic system consisted of a Spellman model RHR30PN10/RVC high voltage D.C. power supply with a maximum voltage of 30 kV (Plainview, NY, USA), a piece of silica capillary separation column with dimension 550 or 600 mm length and 0.05 mm i.d. dipped in a pair of buffer reservoirs in which platinum electrodes were placed, and a model UVIS20 micro UV detector operated at 200 nm (Carlo Erba, Italy). Two capillary columns of 450 and 500 mm in effective length, i.e. from the anode to the detection window, were used to perform the separation of organolead and organotin. On-line detection was carried out through a window made by burning off 2 mm of the polyimide coating from the capillary. Samples were introduced into the anodic end of the column hydrodynamically by raising the sample reservoir to a height of 10 cm above the cathode end for 10 or 20 seconds. Electropherograms were recorded and processed with a Model DP-700 integrator (Carlo Erba, Italy). A standard 47 mm filtration apparatus purchased from Whatman (Maidstone, Kent, England) were used to perform membrane extraction. The C_{18} membrane disk with 47 mm diameter and 0.5 mm thickness,

containing 500 mg of 10 μm particle size and 60 Å pore size C_{18} bonded stationary phase, was manufactured by 3M (St. Paul, MN, USA) under the trademark Empore. A vacuum gauge was connected into the hose line between the vacuum pump and the filtration bottle to control the flow rates.

Water sampling and pretreatment

The rain water was collected during a heavy rain. The drainage water was taken from a drainage ditch besides a highway just after a rain. The water samples were passed through 0.45 μm filters, and high purity HNO_3 was added to the water sample to give a pH 1.5. These water samples were used for membrane extraction.

Procedure of C_{18} membrane extraction

1 litre water sample was added to 2.0 ml 5 M acetic acid, and the pH of the sample was adjusted to 8.2 with concentrated ammonia. 1.0 gram sodium diethyldithiocarbamate (NaDDC) was added to the sample and shaken until NaDDC dissolved completely. In some experiments, the samples were spiked with standard organolead and organotin compounds. The samples were then further shaken for another 10 minutes. The C_{18} membrane disk was placed on the disk holder in the Millipore filtration apparatus, the disk was conditioned using *ca.* 10 ml acetone, 10 ml hexane, and 10 ml acetone by applying a slight vacuum. This conditioning step was necessary to remove the accumulated contaminants due to the exposure to the environment and from the manufacturing process. After drawing air through the disk for several minutes, 10 ml methanol was added and drawn slowly through the disk until only a thin layer of methanol remained on the disk (the disk must not be allowed to become dry from then on until finishing the extraction). The disk was then washed with 100 ml Millipore water to remove any residual methanol. The water sample was added to the sample reservoir to start the membrane extraction.

During the extractions, the vacuum was adjusted to allow 1 litre sample to pass through the membrane disk in *ca.* 40 - 45 min. The disk was washed with 20 ml Millipore water, which was adjusted to pH 3.5 with high purity nitric acid, to remove water soluble matrix and complexing reagent trapped after the completion of the extraction. A full vacuum was applied to draw air through the disk for ten minutes. 4 × 2 ml methanol was used to elute the trapped organolead and organotin into an accurately calibrated tapered glass vial. In each elution, 2 ml methanol was added and a slight vacuum was applied to draw off *ca.* half of the methanol, the vacuum was interrupted at this point to allow the solvent to soak the disk for 2-3 minutes. The remaining portion was then drawn through. The eluted samples were kept in a fume cupboard overnight to evaporate the solvent to 0.5 to 1.0 ml. In some experiments, a small N_2 current was passed through the top of the sample vials to speed up the evaporation of the collecting solvent. The duration of evaporation was shortened to 3 to 4 hours by this method which allowed the determination to be carried out on the same day as extraction was done. Finally, the sample solutions were passed through a 0.45 μm filter prior to MEKC analysis.

Solvent extraction procedure given by Chakraborti *et al* [18] was modified as follows to extract organolead and organotin compounds as comparison. 200 ml water sample was transferred into a 250 ml separation funnel. 0.5 ml 5 M acetic acid was added to the sample and the pH was adjusted to 9.0 with 5 M ammonia. 0.25 gram NaDDC was then added and the sample was shaken until NaDDC dissolved completely. Next, 5 ml hexane was added and the sample was further shaken for another 15 minutes. After phase separation, the lower layer was run off. The organic phase was washed with 5 ml 1 M HCl by gently shaking the separation funnel for 5 minutes. The organic phase was transferred to a 5 ml tapered sample vial. The sample was dried by evaporating the solvent. Accurate 200 μl methanol was added to dissolve the sediment. The sample was passed through a 0.45 μm membrane for MEKC determination.

Results and Discussion

MEKC separation of organo-lead and -tin compounds

It has been demonstrated that MEKC has many advantages compared with CZE for separating ionic and neutral solutes [34]. In our preliminary experiments, no organolead and organotin peaks could be observed in the electropherogram obtained using CZE. This could be due to the decomposition of the organolead and organotin in the aqueous buffer. Another explanation was that the solutes were irreversibly adsorbed onto the column wall due to reaction with surface silanol groups. By adding the surfactant sodium dodecylsulfate (SDS) into the buffer, the MEKC separation of organolead and organotin was readily achieved. The effect of SDS concentration on the migration time of lead and tin species is shown in Figure 1. The migration times of the lead and tin species increased with increase of SDS concentrations, suggesting that interaction between organolead and organotin species and SDS micelles occurred. With the increase of SDS concentration, the solubilities of organolead and organotin in the micellar phase were increased, thus the migration times of all the lead and tin species increased. The change of the migration time for trimethyltin was not so remarkable. This could be explained by the weak interaction of trimethyltin with the micelles. Since trimethyltin has relatively small molecular size and large polarity, trimethyltin species are not so readily dissolved by the micelles and had the fastest migration. The migration order of lead and tin was dominated by the distribution ratios of the solutes in the micellar phase, i. e. the hydrophobicities of the solutes. For organolead and organotin species, the hydrophobicity were mainly attributed to their polarities which could be estimated from the ratio of central atom charges to the whole molecule size. Obviously, trimethyltin has the largest polarity and the fastest migration. In contrast, tributyltin has the same central atom but three large butyl groups. Therefore it has weak polarity and strong hydrophobicity. As a result, it has the slowest migration. The slower migration of trimethyllead than its tin counterpart could be explained by the fact that a central lead atom has a larger ion

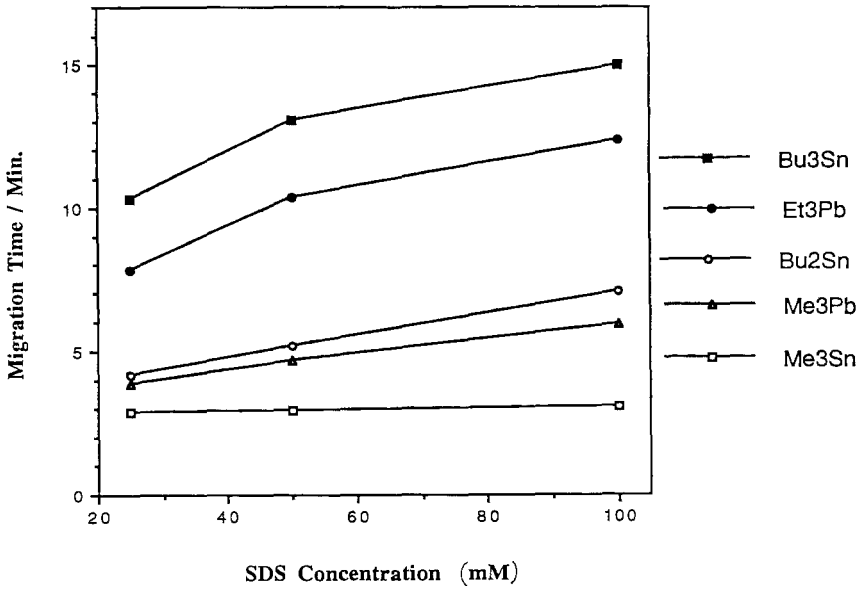

Fig. 1 Effect of the SDS concentration on the migration time of organolead and organotin compounds. MEKC conditions: micellar solution, different SDS concentrations in 0.025M $Na_2B_4O_7$ buffer (pH=9.30); separation column, 550 mm x 0.05 mm i. d. fused silica capillary tube; applied voltage, 20 KV.

radius than a tin atom does in the molecule. Thus trimethyllead had relatively lower polarity and stronger hydrophobicity [47], and its solubility in SDS micellar phase was relatively larger than trimethyltin.

Figure 2 shows the effects of applied voltage on migration of the organolead and organotin species. Generally, sharper peaks for lead and tin species were obtained when higher voltages were applied. The resolutions had also been improved due to the sharp peak shape. This observation was consistent with previous work [48]. The migration time of organolead and organotin compounds decreased with the increased applied voltages. This could be explained by

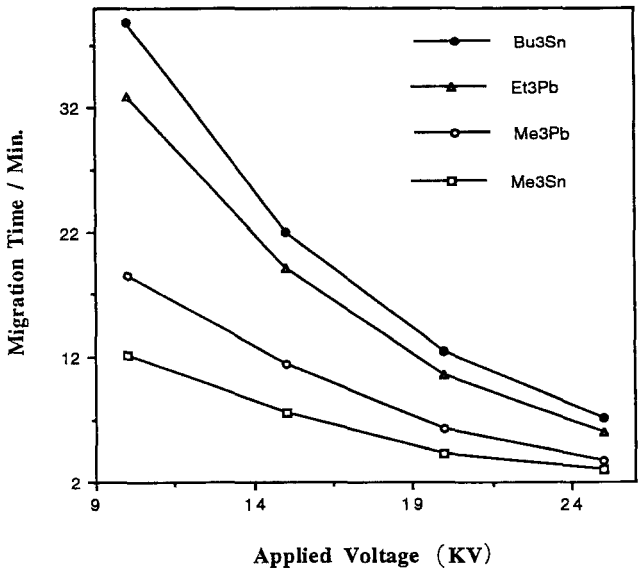

Fig.2 Effect of applied voltage on the migration time of the organolead and organotin compounds. MEKC conditions: micellar solution, 0.050 M SDS in 0.015 M $Na_2B_4O_7$-0.030 M NaH_2PO_4 (pH=7.65). Other conditions as in Fig. 1.

the fact that the organolead and organotin compounds had faster migration at higher electric field since the electroosmotic and electrophoretic flows are directly proportional to the applied voltage. The total separation time of *ca.* 40 min. for the chosen lead and tin species at 10 KV was shorten to 7.2 min. at 25 KV applied voltage. However, a high voltage resulted in a higher baseline noise level. Thus a poorer signal to noise ratio would be expected. It was also subjected to the danger of solutes decomposition at high voltages due to the Joule heat. In the present investigation, an optimum applied voltage of 20 KV was found to give satisfactory results.

The buffer pH affected the separation of organolead and organotin species significantly. The observation was attributed to the fact that these compounds could be partially ionized [18,32]

depending on the pH conditions. In addition, it was found that the buffer pH not only affected the resolution of organolead and organotin species by changing the electrokinetic migration of the solutes and micelles but also affected the detection sensitivities of organolead and organotin by changing the stability of lead and tin species. Figure 3 presents the electrokinetic chromatogram of the organolead and organotin species at different pH values. At pH 9.2 or higher trimethyltin had weak interaction with SDS. It was not separated from methanol completely, and therefore it would be impossible to determine trimethyltin quantitatively above pH 9.2 because of its overlap with the methanol peak. Dibutyltin had a narrow optimum pH range of 8.0 to 10.2 to give stable peaks. It partly overlapped with trimethyllead species at pH 9.2, but could be separated from trimethyllead at higher or lower pH values. Tributyltin and both organolead species gave good separation from other compounds at the pH range of 6.5 to 10.2, but with significant changes in migration times and sensitivities (Figure 3). When pH > 11.2, noisy baseline and unstable electropherogram were obtained, possibly due to the decomposition of organolead and organotin by hydrolysis. In most cases, the separations were carried out in the pH range of 7 to 9.

C_{18} membrane disc extraction of organo-lead and -tin in water samples

Kadokami et al [45] and Junk and Richard [49] reported the extraction of alkyltin species using cartridge extraction methods. Evans *et al* [46] described a method for the direct extraction of tribulytin from simulated water samples using C_{18} disks. These methods typically involve the use of acidified ethyl acetate with or without tropolone to elute alkyltin compounds from the cartridges or discs. Although different alkyltin species have been successfully extracted, it has been found that the extraction of alkyllead compounds exhibits low extraction efficiency without adding complexing reagent [6]. Our extraction procedure showed low extraction efficiencies for all the organo-lead and -tin compounds if no complexing reagent was added to the samples, but the extraction efficiencies were greatly improved when sodium diethyldithiocarbamate (NaDDC)

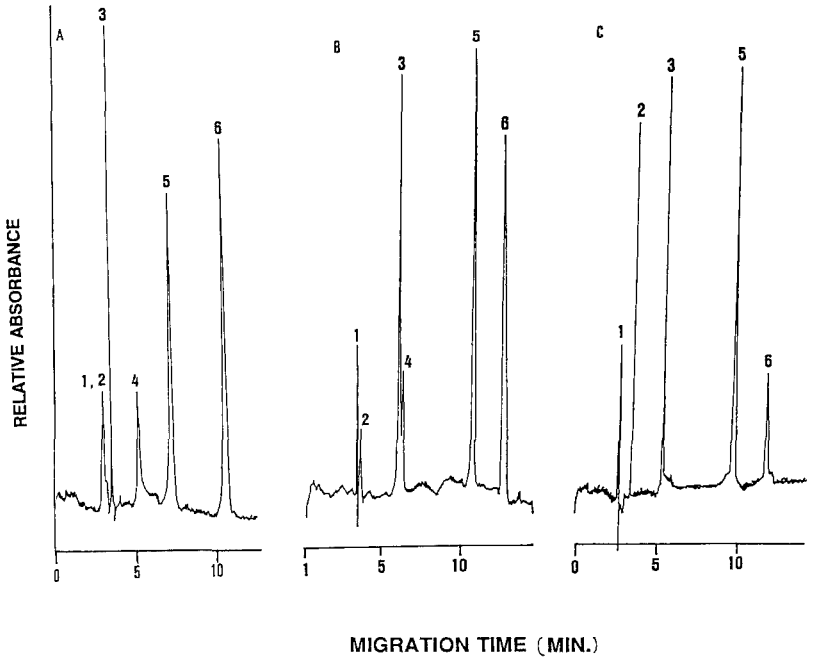

Fig. 3 MEKC separation of the organolead and organotin compounds at various buffer PH values. (A) pH = 10.2 (B) pH = 9.3 (C) pH = 7.65 Peak identification: 1 = methanol, 2 = trimethyltin, 3 = trimethyllead; 4 = dibutyltin, 5 = triethyllead, 6 = tributyltin. MEKC conditions: micellar solution, 0.05 M SDS in different ratios of 0.025M $Na_2B_4O_7$ - 0.075 M NaH_2PO_4 buffer. Other conditions as in Fig.1.

as complexing reagent was added to water samples. NaDDC has been widely used as complexing reagent for metal ion extraction using LLE and cartridge methods. Chau *et al* [6] demonstrated that alkyllead species could be extracted into organic phase using NaDDC as complexing reagent. Since organolead and organotin often exist in ionic forms in aqueous media [18, 32] , obviously, the extraction of alkyllead and organotin with addition of NaDDC was superior to the direct extraction method because of its applicability to both ionic and neutral forms of lead and tin species.

To achieve a high extraction efficiency, two key steps must be carried out effectively. The first is sample loading, which should allow the weak matrix compounds to pass through the membrane and only trapping the solutes of interest. The second step to consider is the elution of the organo-lead and -tin species from the disk. This step should be performed using a solvent with an appropriate polarity which is strong enough to elute the solutes while leaving strong matrix on the disk.

Different sample loading rates, i.e. 30, 60 and 120 min. per litre of sample, were used for the extraction of lead and tin species. It was found that the efficiencies were not affected by the loading rates. This was due to the fact that the membrane has a relatively large contacting area compared with SPE cartridges. Although the flow rates in disk extraction was larger than in the cartridge method, the linear velocity of solutes passing through the disk and the contacting time were comparable with those of the cartridge method. The problem often encountered using disc extraction was the plugging of the disk by the particles in real water samples, even after the water samples had been filtered with 0.45 μm membrane. Hagen *et al* [42] has also encountered this problem and suggested to acidify the samples to overcome this problem. However, this method was not suitable for the extraction of organolead and organotin. Therefore, the samples were passed through double 0.45 μm membranes before C_{18} disk extraction.

The pH dependence of extraction efficiency of alkyllead and alkyltin compounds using liquid-liquid extraction has been investigated by Chakraborti *et al* [18]. In the present study, extraction performed at pH 6.0 and 8.2 using C_{18} disks showed similar efficiencies. Since dithiocarbamate complexes are not stable in acidic medium, the extraction was carried out around pH 8. A high pH may result in C18 stationary phase deterioration and possible hydrolysis of alkyllead and alkyltin compounds.

As solvents to elute the trapped alkyllead and organotin from C_{18} disk, methanol, ethanol, acetone and hexane were compared. Acetone and hexane were strong enough to elute the organolead and organotin species from the disk, but large portion of matrix trapped was also eluted which formed precipitation after evaporization of the solvent. This precipitation made further sample handling (redissolving and filtration) difficult, and also caused potential interference for the following MEKC determination. Methanol and ethanol could elute organolead and tin with less companion matrix and therefore were considered as eluting solvents. Methanol was preferred over ethanol due to the low boiling point and fast evaporation after elution. To determine the volume of methanol needed to elute lead and tin species from C_{18} disks, the organolead and organotin eluted by several portions of 4 ml methanol were determined. It was found that 8 ml methanol could completely eluted organolead and organotin species. In practice, elution was performed using 4×2 ml or 3×3 ml methanol.

Figure 4 presents the electropherogram of the extracts from 1 litre of rain water samples. Since rain water often has a simple matrix, no interferences were encountered and the electropherogram was quite simple. Figure 5 is the electropherogram of the C_{18} disk extracts from 1 litre drainage water sample. This sample had more complicated matrix than rain water. A large matrix peak was detected at ca. 12 min, and tributyltin was masked by the matrix. Figure 6 is the electropherogram of extracts from the same water sample by liquid-liquid extraction method. An even larger matrix peak had been detected at the end of the electropherogram, in which triethyllead and tribulyltin had been masked. From the comparison of Figure 5 and Figure 6, it can be seen that C_{18} disk extraction had relatively lower interference than solvent extraction. The low interferences by C_{18} disk extraction could be explained by the selective separation of solutes from the matrix by the C_{18} disk. The weak matrix such as the main inorganic components, alkali and alkaline earth metals, in common water samples were not retained in the C_{18} stationary phase, whilst stronger main matrix

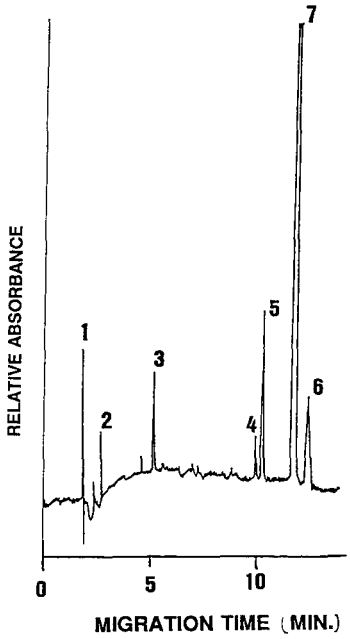

Fig. 4 MEKC electropherogram of C_{18} disc extracts from 1 litre rain water. Peak identification: 1 = methanol, 2 = trimethyltin (49.8 ng/ml), 3 = trimethyllead (18.4 ng/ml), 4 = unknown, 5 = triethyllead (50.0 ng/ml), 6 = tributyltin (28.8ng/ml), 7 = NaDDC + unknown. MEKC conditions: separation column, 600 mm x 0.05 mm i .d. fused silica capillary tube; applied voltage, 20 kV. Other conditions as in Fig. 2.

compounds could be left on the C_{18} disk by choosing a suitable solvent to elute only the solutes of interest with as little matrix as possible. Hagen *et al* [42] and Barcelo *et al* [44] have demonstrated that membrane extraction has lower interference than solid-phase cartridge extraction for pesticides in water samples with enrichment factors of 1000 to 10,000 folds.

Figure 7 shows the electropherogram of C_{18} membrane extracts from tap water with a large enrichment factor of 40,000 folds, i.e. 4 litre water sample was passed through the C_{18} disk and

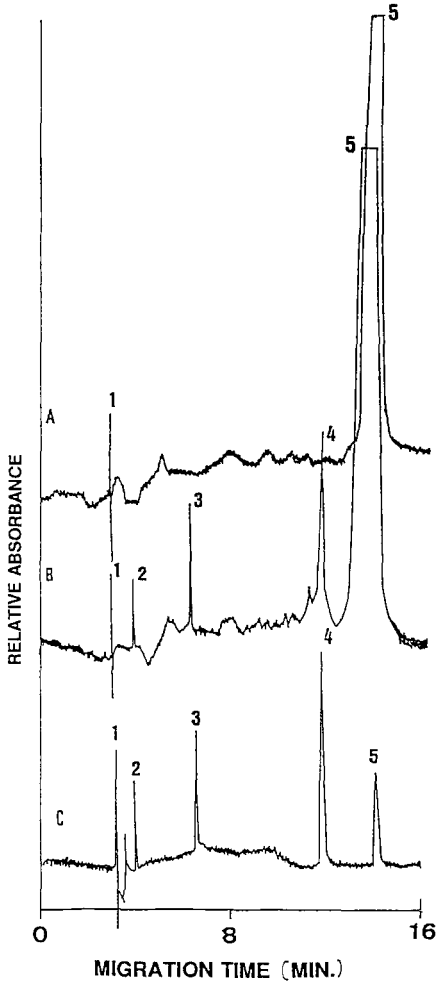

Fig. 5 Electropherogram of the extracts from 1 litre drainage water using C_{18} disc extraction. (A) drainage water sample.(B) same water sample spiked with organolead and organotin species. Peaks: 1 = methanol, 2 = trimethyltin (59.0 ng/ml), 3 = trimethyllead (18.4 ng/ml), 4 = triethyllead (49.3 ng/ml), 5 = tributyltin (14.4 ng/ml) + metrix. (C) direct injection of organolead and organotin species. Peaks: 1 = methanol, 2 = trimethyltin (49.8 μg/ml, 428 pg), 3 = trimethyllead (18.4 μg/ml, 158 pg), 4 = triethyllead (49.3 μg/ml, 423 pg), 5 = tributyltin (28.8 μg/ml, 247 pg). The injection volume for MEKC was 8.6 nl.

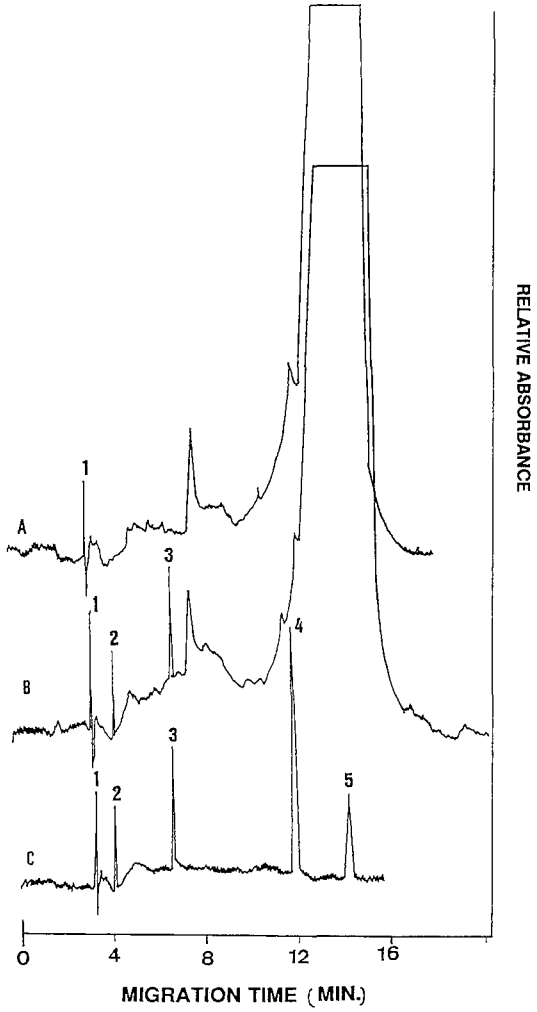

Fig. 6 Electropherogram of extract from drainage water using solvent extraction. Symbols, peaks and concentrations as in Fig. 6. MEKC conditions as in Fig. 5.

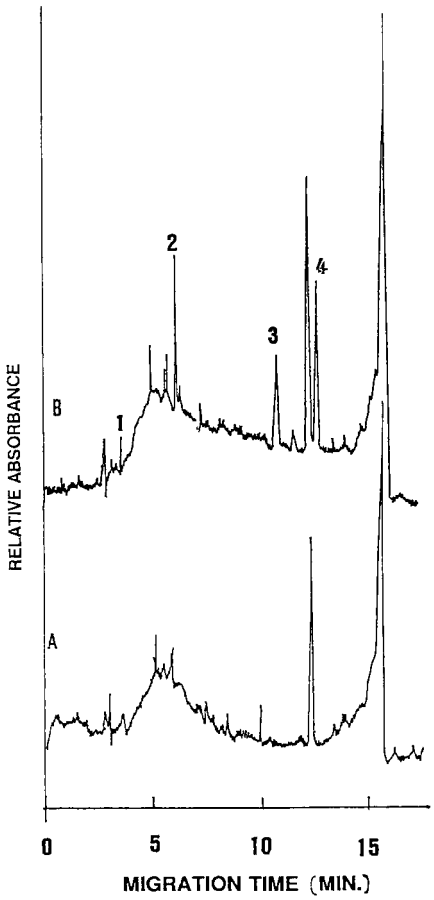

Fig. 7 Electropherogram of C_{18} disk extracts from 4 litres tap water samples. (A) Tap water extracts, (B) same tap water spiked with organolead and organotin species. Peaks: 1 = trimethyltin (2.4 ng/ml), 2 = trimethyllead (0.46 ng/ml), 3 = triethyllead (1.2 ng/ml), 4 = tributyltin (0.72 ng/ml). MEKC conditions as in Fig.5.

Table I. Recovery and precision for the determination of organolead and organotin compounds by CZE

		Me_3Sn	Bu_2Sn	Me_3Pb	Et_3Pb
Added (ppb)		49.8	28.8	18.4	49.3
Recovery (%)	(1)[a]	49.7	88.3	92.7	93.6
	(2)[b]	36.0	ND[c]	91.0	88.0
RSD (%)	(1)[a]	11.0	7.5	4.9	7.8
	(2)[b]	8.4	15.1	9.2	12.0

a: mean value of 5-6 extractions with C_{18} discs followed by MEKC determination.
b: mean value of 3-4 extraction with solvent extraction, followed by MEKC determination.
c: not determined.

a final volume of 100 μl sample was obtained. Although the matrix effects were stronger than the previous extraction with 1000 fold enrichment, the organolead and organotin peaks were still clearly identified. This indicated that the C_{18} disk extraction method may be more suitable than other preconcentration techniques such as solvent extraction and solid phase cartridge extraction for large volume and/or large enrichment factor extraction, provided that the samples had relatively weak matrix. In addition, MEKC method was found to be applicable to the analysis of organolead and organotin compounds due to its very small volume for sample injection.

Table I presents the recoveries of the organolead and tin species in water samples using C_{18} disk extraction. C_{18} disk extraction and solvent extraction gave similar recoveries for lead and tin species. For trimethyltin extraction, low recoveries were obtained. The reason for this is not yet known. A possible explanation is that the polarity of trimethyltin was relatively higher than the other lead and tin species, and the interaction with NaDDC was weak and hence a weak retention in C_{18} disk was expected. For organolead compounds, recoveries of close to 100 percent could be obtained. Table II shows the linear ranges and the detection limits for the

Table II. Linear range and detection limits of organolead and organotin compounds

	Concentration range (ppb)	Detection limits[a] (ppb)[b]	(pg)[c]
Me_3Sn	25.0-200	5.2	42.1
Bu_3Sn	8.2-100	1.1	15.5
Me_3Pb	9.2-100	1.9	22.4
Et_3Pb	14.0-200	1.5	17.2

a: S/N = 3
b: based on extraction from 1 litre of sample using C_{18} disc.
c: based on direct injection of 8.6 nl to MEKC.

determination of organolead and organotin compounds. The detection limits given in Table II were low enough for the determination of organolead and organotin compounds at parts per billion level concentration in environmental samples. For sub parts per billion level lead and tin species determination, larger factor enrichments must be performed, by using larger sample volume and longer extraction time.

Conclusions

MEKC was used to separate several organolead and organotin species most often encountered in environmental samples. MEKC has the advantages that the reactions between alkyltin and the stationary phase as observed in HPLC [23] can be eliminated and much better efficiencies than those of HPLC can be obtained. Unlike in most GC techniques, MEKC separations are carried out at low temperatures, thus avoiding the derivatization step which may introduce problems such as contamination, interference and difficulty in quantitation [24]. Membrane disk extraction was used to overcome the problem encountered in solvent extraction and solid-phase cartridge

extraction, such as emulsification, larger labour requirement and time consumption. The method developed in this work has the advantages of simplicity and rapidity. The potential applications of MEKC and membrane disk extraction for organo-lead and -tin in environmental analysis have been demonstrated.

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VALIDATION OF A MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY (MECC) METHOD FOR THE DETERMINATION OF p-TOLUENESULFONIC ACID IMPURITY IN A PHARMACEUTICAL INTERMEDIATE

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ABSTRACT

Micellar Electrokinetic capillary chromatography (MECC) has been employed for the quantitation of p-toluenesulphonic acid (pTSA) impurity levels in 5-fluoro-3-[3-(1-piperazinyl)-Propyl]-1H-indole (BMS 180317-01) a key intermediate used in the synthesis of a novel antidepressant drug candidate BMS 181101-02. The MECC method demonstrated good selectivity, precision,

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accuracy, linearity, limit of detection and quantitation. MECC of small ionic or polar molecules offers a useful alternative to ion chromatography, ion pair chromatography and RP-HPLC.

INTRODUCTION

In the past several years capillary electrophoresis (CE) has grown rapidly since the work of Jorgenson and Lukacs [1] in the early 1980's. This rapid growth has generated a variety of CE modes applicable to a broad range of charged and neutral species. Micellar electrokinetic capillary chromatography (MECC) was originally introduced in the mid 1980's by Terabe et. al (2) as a way to separate neutral molecules using CE. Terabe later classified the technique as a type of liquid-liquid chromatography without any solid support to hold the stationary liquid phase. Separation is achieved due to the selective partitioning of the ionic and nonionic solutes between the micelles and the surrounding aqueous phase. It was soon realized, that MECC could also be used for the separation of charged compounds [3,4]. In vast majority of all MECC studies sodium dodecylsulfate (SDS) is used as the micelle phase (5). This is primarily due to the fact that SDS is an extremely well behaved and well understood surfactant.

MECC has been shown to be of use for the separation of a range of ionic and nonionic drug classes including antibiotics [6,7] non-steroidal anti-inflammatories [8], steroids [9], analgesics [10] and water soluble vitamins [11].

When analyzing small ionic or polar molecules as impurities in pharmaceutical bulk materials by HPLC, early analyte elution (void volume) and the limited pH operating range of many silica columns are a commonly occurring problem. MECC was found well suited for the separation of the ionic impurity p-toluenesulphonic acid (pTSA) in our case. MECC was analogous to the conventional liquid-liquid partition chromatography producing separation comparable to the HPLC.

As shown in Figure 1, pTSA is a by-product generated in the synthesis of the key intermediate BMS 180317-01. Since the potential of pTSA and its related adduct in the final drug was of great concern, it was rather important to control the residual levels of pTSA at the intermediate step. A quick and reliable method was warranted for the quantitation of pTSA in BMS 180317-01. An HPLC method using a polymer based column and high pH mobile phase was developed with acceptable trace levels detection of pTSA. However, the use of high organic solvent content in the eluent resulted in frequent high back pressure and shorter column life. Establishing a

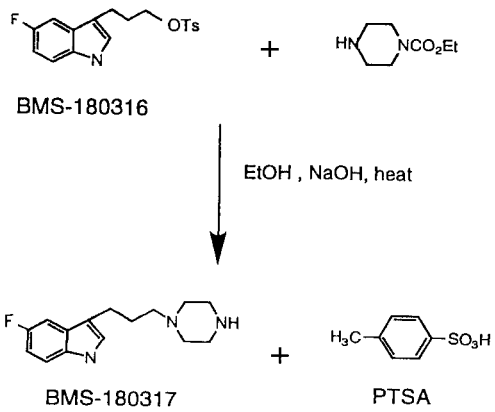

Figure 1 BMS 180317 synthesis scheme

rugged HPLC method may have been possible after extensive method development efforts. However, in this case, suitable MECC operating conditions were quickly identified and then validated for the separation and quantitation of residual pTSA levels in BMS 180317-01. Method validation included measurement of precision, accuracy, linearity, limit of detection and quantitation. The results obtained for several batches of BMS 180317-02 were comparable to those from an HPLC method.

EXPERIMENTAL

Materials and Chemicals

p-Toluenesulfonic acid, monohydrate (pTSA) was purchased from Aldrich chemicals (Milwaukee, WI, USA).

Sodium dodecyl sulfate, sodium borate and acetonitrile were purchased from Fisher Scientific (St. Louis, Mo, USA) and boric acid was purchased from Mallinckrodt (Paris, KY, USA). Water used in preparations of buffers and samples was obtained from a Millipore Milli-Q-System (Bedford, MA, USA). BMS 180317-01 was synthesized by the chemical process technology group at Bristol-Myers Squibb.

Instrumentation and Separation Conditions

A Biorad (Richmond,CA, USA) BioFocus 3000 capillary electrophoresis system was used in all measurements. The fused silica capillaries (50 μm I.D.) used in this study were purchased form Polymicro Technologies (Phoenix, AZ, USA). The separation conditions employed are summarized in Table I.

For optimal performance, the capillaries were preconditioned for 10 minutes at high pressure with 1.0 M NaOH, followed by 2 minutes water and 2 minutes run buffer before the first use. Between injections the capillaries were flushed with run buffer, water, and again run buffer for 20 seconds under high pressure.

Sample Preparation

The choice of an appropriate sample solvent is quite critical in CE since the sample solvent can improve separation efficiency and resolution. The pTSA working

TABLE I

SEPARATION CONDITIONS

System	Biorad Biofocus 3000 Capillary Electrophoresis System
Capillary	Uncoated, 25 cm effective X 50 μm I.D., Temperature 25 degrees
Run Buffer	100 mM boric acid/20 mM sodium borate/20 mM sodium dodecyl sulfate
Voltage	+5 kV, constant
Sample Introduction	8 psi*sec at 5 psi pressure
Detection	190 nm
Sample Concentration	500 ppm
External Standard	PTSA 2% w/w (10 ppm) in BMS 180317 (500 ppm)
Pre-injection Purge	20 seconds: run buffer/water/run buffer

standard (2% w/w) was prepared by spiking 10 ppm pTSA dissolved in 90:10 water:acetonitrile, into 500 ppm BMS 180317 reference standard solution. The stock solution of BMS 180317-01 samples were prepared by dissolving into 90:10 sodium borate (12mM) / boric acid buffer (12mM): acetonitrile to give 1.0 mg/ml concentration and then further diluted with water to 0.5 mg/ml.

RESULTS AND DISCUSSIONS

Optimum conditions for separating pTSA from BMS 180317-01 were obtained with a background electrolyte

containing 100mM boric acid/20mM sodium borate buffer containing 20mM SDS (pH 8.5). Under these conditions the anionic pTSA travels towards the anode; however, the net electroosmotic flow in the direction of the cathode sweeps it through the detector window. A typical separation achieved is given in Figure 2.

The validation undertaken for the MECC method follows the guidelines suggested for HPLC method validation [12].

Linearity

To fully assess the linear dynamic range of the UV detector response at 190 nm, a wide range of samples containing varying amounts of pTSA from 0.02% to 5.0%, relative to the BMS 180317-01 nominal concentration of 500 ppm, were analyzed. Linearity of the MECC method was demonstrated over this range and a correlation coefficient of 0.9999 was obtained for the normalized peak area of pTSA versus the %w/w concentartion of pTSA in BMS-180317. The higher pTSA response at 190nm was found to offset the less than optimum signal to noise ratio when compared with the response at the longer wavelength.

Precision of Peak Area and Migration Time

The precision results are summarized in Table II. A 2% w/w spike solution of pTSA (10 ppm) into BMS 180317-

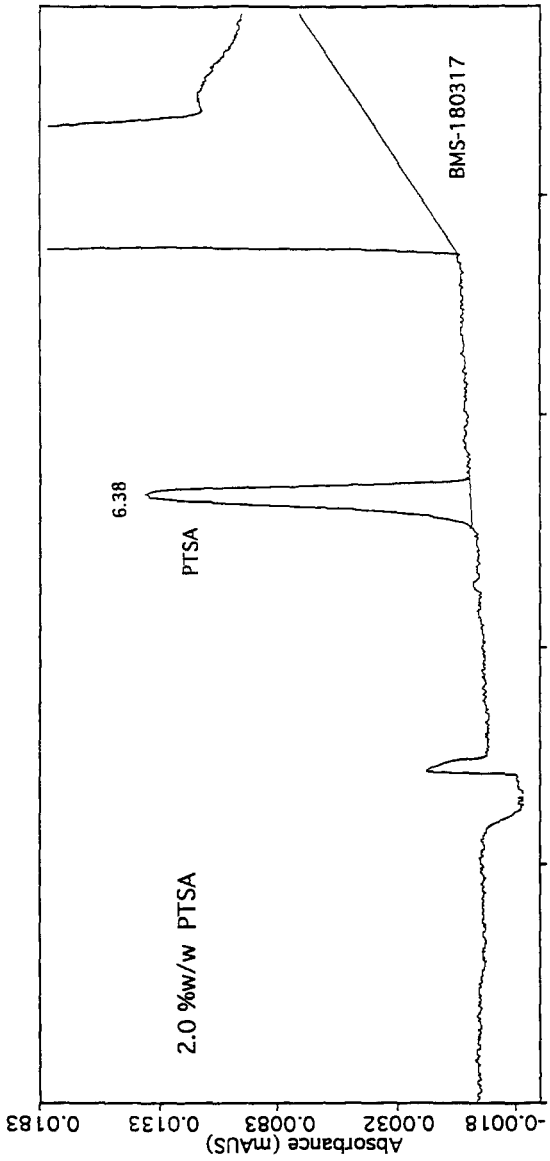

Figure 2 Electropherogram of a BMS 180317 spiked with 2% w/w pTSA. Separation conditions summarized in Table I.

TABLE II

PRECISION OF MIGRATION TIME AND PEAK AREA.

Injection Number	Migration Time (minutes)	Normalized Peak Area
1	6.44	308996
2	6.38	305166
3	6.38	300608
4	6.34	309332
5	6.33	291610
6	6.31	291154
7	6.39	283968
8	6.39	289758
Average	6.37	297574
RSD	0.6%	3%

01 (500 ppm) was injected 8 times and an RSD of 3% was obtained for the pTSA peak area. The RSD on the migration time of the pTSA peak was 0.6%. The area of the pTSA peak was normalized by its migration time in all the calculations to compensate for differences in the residence times in the detector [13].

The repeatability of the analysis for pTSA content was established by analyzing six individual preparations of BMS 180317-01 and calculating the levels of pTSA. The relative standard deviation of these repeat analysis for a sample containing 0.11% w/w pTSA was 7%.

Typical precision data of 0.5 - 2% RSD for peak area using an automated CE instruments have been reported in several publications [14,15]. Equipment manufacturers also typically quote that RSD's of less than 2% can be routinely obtained for peak areas. The less than optimum pricision of 3% for the peak area counts was primarily due to the difficulties associated with the integration and some what poor injection pricision of the Biorad system, precipitation or loss of the sample was not found to be the contributing factor. It is very important to emphasize that for impurities measurement at low levels it is rather difficult to maintain less than 2% RSD for peak areas. The reported values for precision of the method are adequate for its intended purpose of determining low levels of pTSA in BMS 180317-01 intermediate.

Accuracy

The accuracy of the MECC method was established by spike recovery approach. A reference standard of BMS 180317-01 containing no detectable levels of pTSA was spiked to get 0.05, 0.1, 0.2 and 2.0% pTSA relative to BMS 180317-01 (500 ppm). The average recovery was 99% when compared against a pTSA standard. For impurities measurement, this is considered acceptable. The data is summarized in Table III.

TABLE III

ACCURACY (RECOVERY) OF PTSA IN BMS 180317-01 SAMPLES

Spike Level	Recovery
0.05% w/w	104%
0.1% w/w	96%
0.2% w/w	96%
2.0% w/w	101%

Average Recovery 99.0%

Sensitivity

The sensitivity of the method in terms of limit of detection (LOD signal to noise ratio greater than 3) and minimum quantifiable level (MQL, signal to noise ratio greater than 10) was evaluated. The method was found to have an limit of detection of 0.02% (w/w) and a limit of quantitation of 0.08% (w/w). The LOD and MQL were determined by measuring the standard deviation of the peak-to-peak noise of the system using solvent blank. These low levels of LOD are comparable to levels reported by Altria for dimeric impurities present in salbutamol drug substance using low UV wavelength detection [16].

Comparison with HPLC

pTSA content determined by MECC for several batches of BMS 180317-01 was compared with the values obtained by

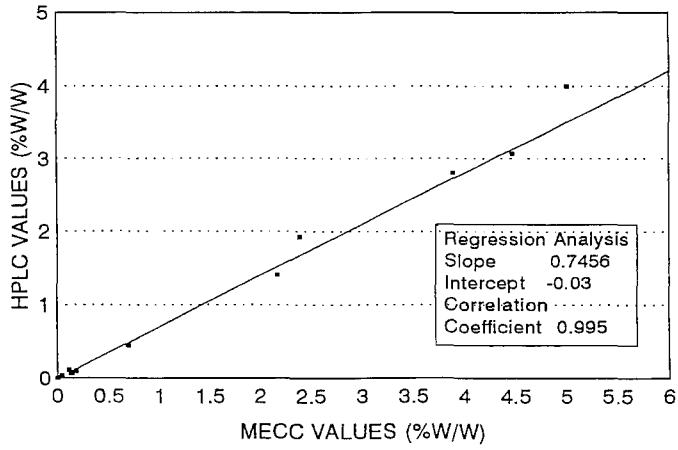

Figure 3 Comparative analysis by HPLC and MECC of pTSA levels in BMS 180317 samples.

a validated reverse phase HPLC method. Figure 3 shows the correlation between the two techniques. Although the HPLC method was found to give slightly higher results as indicative from the slope of the correlation line, the correlation coefficient of 0.995 was indicative of the overal agreement between the two methods.The small differences in results between the two methods at high levels could very well be due to the sample preparation and differences in the peak integration software packages used. Although the HPLC method was equally acceptable, MECC method was found to be faster, economical and rugged.

CONCLUSION

A MECC method was validated for the quantitative determination of p-toluenesulphonic acid impurity in BMS 180317-01, a pharmaceutical intermediate. Method validation demonstrated acceptable levels of performance in terms of precision, sensitivity, reproducibility and accuracy. The MECC method offered an useful alternative to the HPLC method and was demonstrated to give similar levels of method performance and comparable results. Although the MECC approach gave acceptable separation, the potential of free solution CE (FSCE) needs to be evaluated and it is possible that similar or better separation can very well be achieved with a simple FSCE method.

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DIRECT DETERMINATION OF EPHEDRINE ALKALOIDS AND EPINEPHRINE IN HUMAN URINE BY CAPILLARY ZONE ELECTROPHORESIS

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ABSTRACT

The complete separation of a mixture of six phenylamines and epinephrine in human urine was achieved by Capillary Zone Electrophoresis (CZE) in 15 min. For the CZE separation of the compounds, electrophoretic media with phosphate-borate and phosphate-acetonitrile buffer at different pH were used. A buffer solution that contained 40 mM phosphoric acid and 10 mM boric acid adjusted to pH 9.7 with NaOH 1 N, was found to be the most suitable electrolyte for epinephrine separation. The results successfully demonstrated the use of CZE with UV detection for screening and quantification of phenylamines and epinephrine in human urine without previous treatment, in concentration lower than 35.0 μg/ml, and quantification limit of 2.0±0.1 μg per millilitre of urine.

INTRODUCTION

Sympathomimetic or adrenergic drugs play important roles as neurotransmitter in the central and peripheral autonomic nervous systems and as hormones exerting endocrine and exocrine effects. It is not

surprising, therefore, that the lack of sufficient amounts of some of these drugs in the body can have a severe impact on both the quality and duration of life. These drugs have a stimulating effect on several systems of the body, mainly the central nervous, respiratory and the vasomotor systems. Consequently, the phenylamines are considered to be doping substances by the International Olympic Committee, and their maximum amount allowed per millilitre of urine is 5 μg/ml (for ephedrine).

These stimulants have been widely studied through different analytical techniques, and several methods have been reported for the determination of some of these compounds: for ephedrine, gas chromatography *(1-3)*, thin-layer chromatography *(4)*, spectrophotometry *(5)*, radioinmunoassays *(6)*, electrochemical methods with solid electrodes *(7,8)* and with selective liquid membrane electrodes *(9)*; for ephedrine and pseudoephedrine, ^{13}C-NMR-spectrometry *(10)*, and for different ephedrine alkaloids high performance liquid chromatography HPLC *(11-14)*, isotachophoresis *(15)* and the use of joint techniques such as HPLC-capillary electrophoresis *(16)*.

Capillary zone electrophoresis is a recently developed separation technique based on the differences in the mobility exhibited by different molecules in an electric field. It has many attractive features, including being a simple, fast and highly efficient technique applicable to a wide variety of analytes (for recent reviews, see refs. *(17,18)*). The development of effective methods of drugs separation and determination are important in pharmaceutical analysis as well as for the screening and quantification of drugs in biological fluids.

Several studies have shown that the application of CZE in real samples creates a great number of difficulties basically due to changes in the behaviour of substances *(19)*. They can be summarized as follows: (a) the

effect on the matrix on the charge of the analytes when performing the injection by electromigration, (b) the high concentration of salts presents in biological samples, (c) charges in the capillary walls, (d) adsorption of undesirable analytes on the capillary walls, and (e) overcharge in the capillary, caused by the total amount of components in the sample.

The analysis of several sympathomimetic drugs, such as the catecholamines, has been studied by CZE using electrochemical *(20)*, and spectrophotometric detection *(21-23)*. On the other hand, the simultaneous separation and quantification of this six phenylamines and epinephrine in human urine by CZE have not been reported. Only a recent method has been developed for the determination of ephedrine and pseudoephedrine in *Ephedra-Erba (24)*.

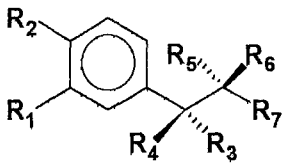
Following our previous paper *(25,26)*, we wish to report here a fast, simple and accurate method for the separation and direct determination of a mixture of six phenylamines and epinephrine in human urine by CZE. This method offers many advantages over some other methods described in literature, including that the urine samples can be directly injected without previous separation, so the procedure of analysis was greatly simplified.

EXPERIMENTAL SECTION

Chemicals

All chemicals were of analytical-reagent or research grade, Merck. The compounds investigated were obtained from Aldrich and Sigma. The formulae and abbreviations used in this paper are given in Table I. Stock

TABLE 1



ABBREVIATION	R_1	R_2	R_3	R_4	R_5	R_6	R_7	NAME	REFERENCE
(+)-E	H	H	OH	H	H	CH_3	$NH(CH_3)$	(+)-(1S,2R)-Ephedrine	Aldrich- 85-735-5
(+)-PE	H	H	OH	H	CH_3	H	$NH(CH_3)$	(+)-(1S,2S)-Pseudoephedrine	Aldrich- 29-461-6
(+)-NE	H	H	OH	H	H	CH_3	NH_2	(+)-(1S,2R)-Norephedrine	Aldrich- 19-362-3
(-)-NPE	H	H	H	OH	H	CH_3	NH_2	(-)-(1R,2R)-Norpseudoephedrine	Sigma- N-2758
(-)-ME	H	H	H	OH	CH_3	H	$N(CH_3)_2$	(-)-(1R,2S)-Methylephedrine	Aldrich- 25-521-0
(-)-MPE	H	H	H	OH	H	CH_3	$N(CH_3)_2$	(-)-(1R,2R)-Methylpseudoephedrine	Aldrich- 29-003-3
(-)-EPI	OH	OH	H	OH	H	H	$NH(CH_3)$	(-)-1R-Epinephrine	Aldrich- 21-930-4

solutions (0.01 M) of compounds were prepared in purified water (Milli-Q/Milli-RO, Millipore) and kept in the dark and under refrigeration.

Purified water was used to prepare all aqueous solutions, which were filtered through 0.45-μm filter Dynagard (Laguna Hills, CA, USA) before use. Samples of urine were similarly filtered before CZE analysis.

Electrophoretic Instrumentation and Running Conditions

All experiments have been performed in an EUROPHOR (Toulouse, France), equipped with a manual injector (Prime Vision I), high voltage source (Prime Vision V) and UV-VIS detector (Prime Vision II) adjusted to 210 nm. Supelco (Bellefonte, PA, USA; Cat. No. 77500) untreated silica (1 m x 75 μm I.D.; 363 μm O.D.) was used as capillary. The effective separation distance was 65 cm. Electropherograms were monitored with a Varian 4290 integrator. A constant voltage of 20 kV was applied. The cathode was on the detector side. Sample application occurred by hydrodynamic injection for a specified period of time (typically, 2 s).

Conditioning between runs was achieved by rinsing the capillary with 0.1 N NaOH for 2 min, purified water for 2 min and with buffer used in the analysis for another 2 min.

Procedure

Our own urine was employed as blank matrices. For the analysis of human urine, 2 ml of blank sample or one spiked with the seven drugs (see Table 1) was diluted to 10 ml with purified water.

The CZE data in this paper were obtained from three consecutive runs, and the run-to-run relative standard deviation (RSD) of the migration times was less than 2.3%.

RESULTS AND DISCUSSION

Optimum conditions for the separation were obtained by examining the effects of buffer pH, ionic strength and applied voltage. Of these, increasing the ionic strength of the buffer did not affect the migration time, but it did give a constant increment in the width of the peaks. Our studies showed that an ionic strength of 50 mM, produce the best separation of the phenylamines peaks. On the other hand, the buffer pH was found to be the most critical factor affecting the resolution.

The study of pH, was performed in urine samples spiked with the six phenylamines (E, PE, NE, NPE, ME, MPE) and epinephrine (EPI) using a buffer solution containing 50 mM phosphoric acid and adjusted the different pH with NaOH 1N.

Figure 1, shows the influence of pH on the migration times of 6 phenylamines and EPI. The electrophoretic peak of EPI was not visible for pH higher than 7.0 because this appears simultaneously on an endogenous peak of the urine.

It was found that at pH higher than 7.0 the separation between phenylamines was good. At pH lower than 7.0, only one electrophoretic peak was visible and the analysis time was increased two or three times. This is due to the fact that the amino groups of the stimulants are charged at this pH, making their separation impossible under these conditions. As the pH of the electrophoretic media increases, protonation of these amino groups would be inhibited, at a pH closer to the pK_a of the compounds the drugs were separated.

A pH of 9.7 was selected for the process because it produces the best resolution of compounds.

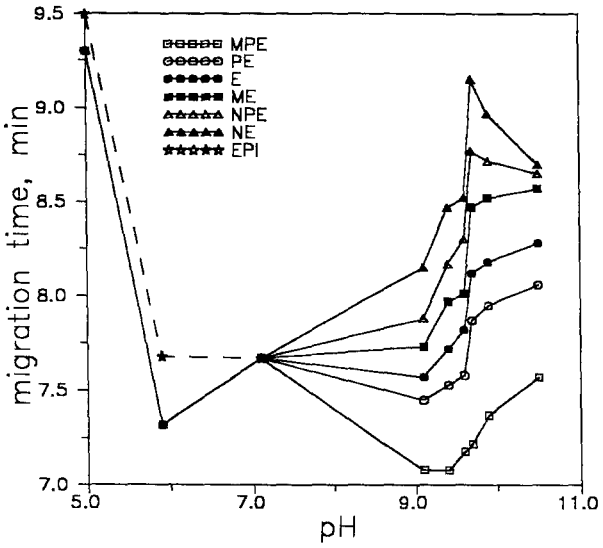


Figure 1.- **EFFECT OF pH ON THE MIGRATION TIME OF PHENYLAMINES AND EPINEPHRINE IN HUMAN URINE.**
Buffer: 50 mM phosphoric acid, UV detection: 210 nm, Hydrodynamic injection: 2 s., Running voltage: 20 kV, Urine sample five-fold diluted. Drug concentration $1.0x10^{-4}$ M of each one.

The effect of applied voltage on separation and resolution was investigated for the six phenylamines in phosphate buffer 50 mM at pH 9.7. By all accounts, experimentally determined resolution improves with increasing voltage up to a certain point (20 kV) beyond which the voltage is so high as to contribute to zone broadening by Joule's heating effect *(27)*. A voltage of 20 kV (current: 0.074 mA) was chosen, because it produces the shortest analysis time together with best efficiency (No. theoretical plates: $2.5x10^5$).

In a series of experiments the influence of organic solvent in the carrier electrolyte was investigated. Figure 2, shows the influence of acetonitrile on

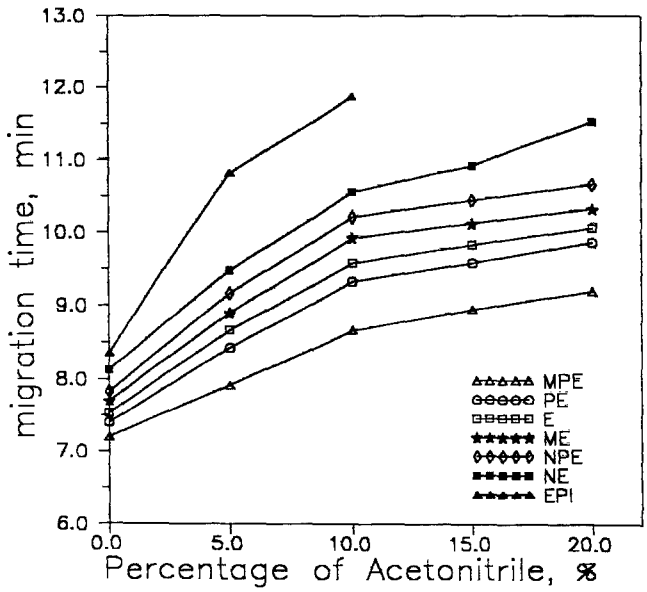


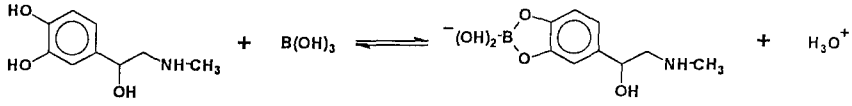
Figure 2.- **EFFECT OF ACETONITRILE ON THE MIGRATION TIME OF PHENYLAMINES AND EPINEPHRINE IN HUMAN URINE.**
Buffer: 50 mM phosphoric acid adjusted to pH 9.7. Other conditions as in figure 1.

the migrationtimes of phenylamines and EPI. Up to a 20% acetonitrile was used, from the results obtained, migration order of compounds in urine are not influenced by the content of organic solvent in the buffer. Furthermore, organic solvent caused a general increase in the migration times of all compounds, which can be attributed to two factors: on the first, the acetonitrile decreases the electrical conductivity, thereby decreasing the current (for a running voltage of 20 kV; buffer without acetonitrile, current: 0.074 mA; buffer with 20% acetonitrile, current: 0.047 mA); On the second, the acetonitrile possibly decreases the amount of electroosmotic flow modifier adsorbed onto the inner wall of the fused-silica capillary. Increasing amounts of acetonitrile produced a slight increase on the migration time of the compounds, improving the electrophoretic separations.

Thus, 10% of acetonitrile has been selected as modifier. Figure 3, shows typical electropherograms of the six phenylamines and epinephrine in human urine diluted 2:10 with purified water, using a 50 mM phosphate buffer at pH 9.7 with 10% acetonitrile. Under these conditions the electrophoretic peak of epinephrine was only visible for concentration higher than 10.0 µg/ml of this compound.

On the other hand, a matrix effect that slightly reduces the migration times of the all drugs and endogenous components of urine was observed when the samples were analyzed in this buffer. This effect disappeared with careful rinsing of the capillary and change of buffer vial in anode, with this the migration times returned to normal values in subsequent runs with the standard drug mixture.

In this paper, we have altered the migration behaviour of epinephrine through complexation with boric acid *(28,29)*. The complexation of boric acid with ortho-dihydroxy compounds such as catechols and certain carbohydrate has been studied extensively *(30,31)*. The complex forms via a reversible reaction with strongly pH dependent equilibrium indicated by the reaction scheme:



Of importance in this reaction is the negative charge on the boron atom which, when complexed with catechols, transforms cationic species into zwitterions and non ionic species into anions.

By adding boric acid to the running buffers, the retention time of a some solute is increased as they react with boric acid to form borate complex. To

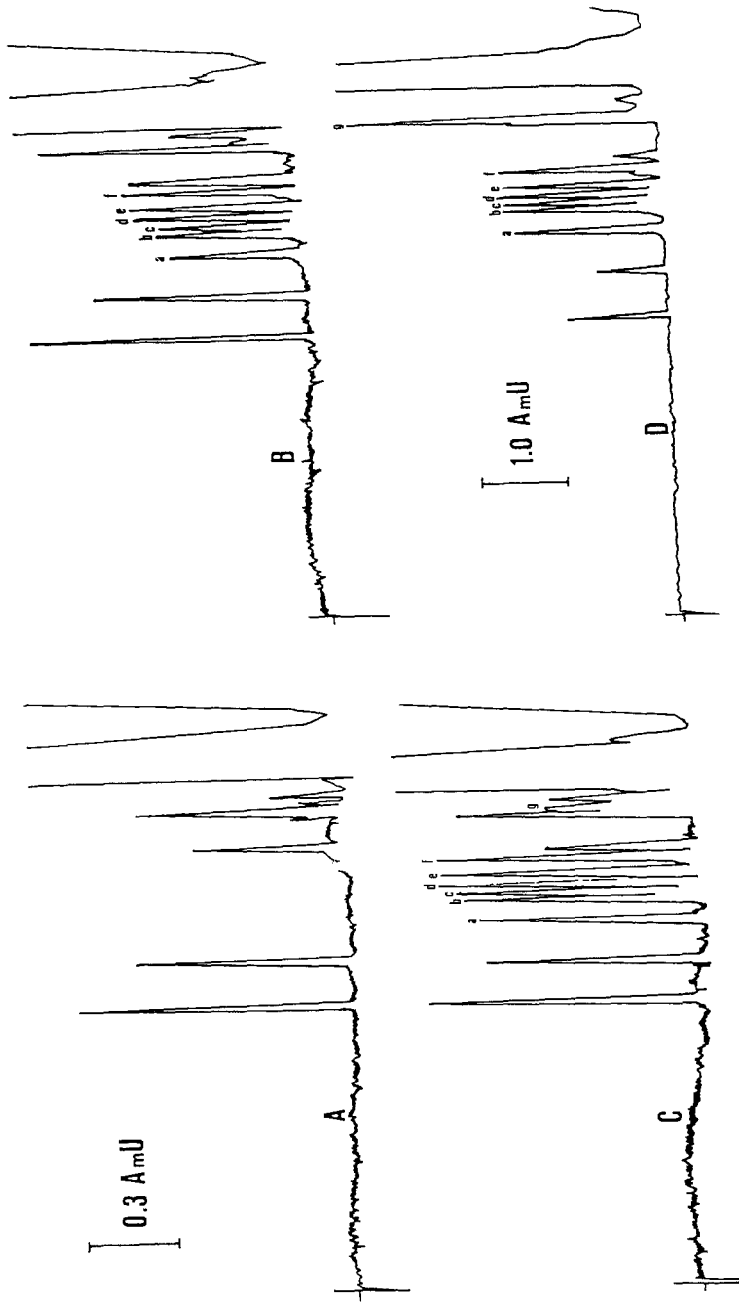


Figure 3.- **ELECTROPHEROGRAMS OF (A) FIVE-FOLD DILUTED BLANK URINE, URINE SPIKED WITH (B) 1.2×10^{-6} M, (C) 2.0×10^{-6} M, (D) 4.0×10^{-5} M OF EACH DRUGS.** Electrophoretic conditions: buffer, 50 mM phosphoric acid adjusted to pH 9.7 with 10% acetonitrile. Peak identification: a: MPE, b: PE, c: E, d: ME, e: NPE, f: NE, g: EPI. Other conditions as in figure 1.

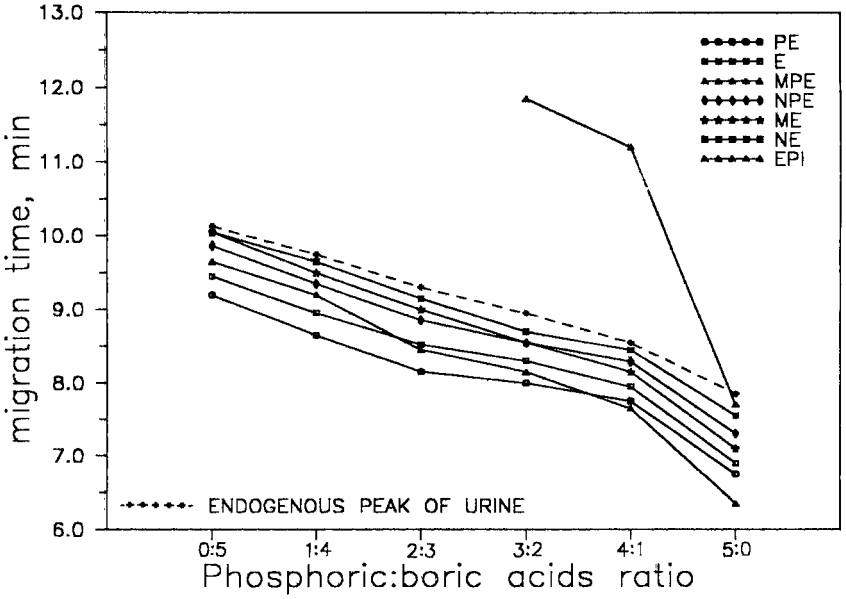


Figure 4.- **EFFECT OF DIFFERENT 50 mM PHOSPHORIC-BORIC ACIDS RATIOS ADJUSTED TO pH 9.7 ON THE MIGRATION TIME OF PHENYLAMINES, EPINEPHRINE AND ONE ENDOGENOUS PEAK OF URINE SAMPLE.** Drugs concentration: 1.0×10^{-5} M of each one. Other conditions as in figure 1.

control the best separation of EPI from the endogenous peak of human urine, different buffer solutions of concentration 50 mM with different ratios phosphoric acid-boric acid adjusted to pH 9.7 with NaOH 1 N were prepared.

Figure 4, shows the effect of different ratios phosphoric-boric acids (0:5; 1:4; 2:3; 3:2; 4:1; 5:0) on the migration times of seven drugs under study (phenylamines and EPI) and the endogenous component of urine. In all cases, increasing the concentration of phosphoric acid in the total composition of buffer, gave a reduction of the migration time of drugs. This effect is more pronounced for EPI, due to the complexation of boric acid

with ortho-dihydroxy groups is highly favorable. On the other hand, a large variation of migration times was observed for MPE and ME. It is very important to note that in a buffer 50 mM boric acid adjusted to pH 9.7, the effective mobilities of ME and NE are too close for them to be separated. Therefore, the resolution of this drugs can be improved by simply adding a little amount of phosphoric acid to the total buffer concentration.

When the buffer containing ratios of phosphoric-boric acids 3:2, 4:1 and 5:0, adjusted at pH 9.7 with NaOH 1 N, the electrophoretic peak of EPI was visible and separated from endogenous peak on the urine. When the buffer ratios containing more amounts of boric acid, ratios phosphoric-boric acids such as 2:3, 1:4 or 0:5, the electrophoretic peak of EPI was not observed, because the migration time increases due to the reaction with boric acid, (see figure 4).

Figure 5 shows the electropherograms obtained with different ratios of phosphoric-boric acids adjusted at pH 9.7 with NaOH 1 N.

In all the cases studied in 50 mM phosphoric acid with 10% acetonitrile adjusted to pH 9.7, electroosmotic mobility was 7.8×10^{-8} $m^2V^{-1}s^{-1}$, the resolution and efficiency of six phenylamines in human urine samples were very good, (No. theoretical plates: MPE, 3.4×10^5; PE, 2.1×10^5; E, 1.9×10^5; ME, 3.1×10^5; NPE, 2.6×10^5; NE, 2.0×10^5) but the resolution between EPI and endogenous peak of urine was poor, (No. theoretical plates: EPI, 1.0×10^4). However in 4:1 phosphoric-boric acids relation adjusted to pH 9.7, electroosmotic mobility was 6.9×10^{-8} $m^2V^{-1}s^{-1}$, resolution and efficiency between six phenylamines were slightly poor comparing with the previous buffer, but the resolution of EPI peak was good (No. theoretical plates: EPI, 1.6×10^5).

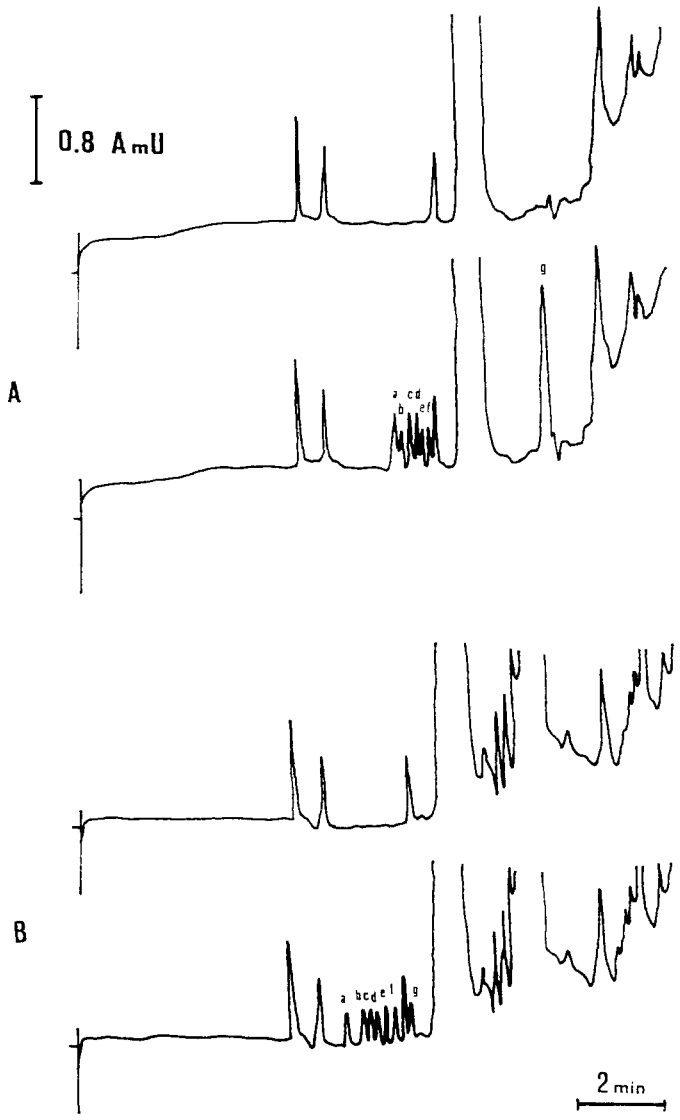


Figure 5.- ELECTROPHEROGRAMS OF BLANK URINE FIVE-FOLD DILUTED, AND URINE SPIKED WITH 1.0×10^{-5} M OF PHENYLAMINES AND EPINEPHRINE AT TWO DIFFERENT RUNNING BUFFERS ADJUSTED TO pH 9.7.
(A) 40 mM phosphoric acid + 10 mM boric acid, (B) 50 mM phosphoric acid. Other conditions as in figure 1.

Direct injection of urine provides complex electropherograms within the first half of the elution ranges, making unambiguous identification of zones and complete separation difficult *(32)*. However the last years different advantageous aspects of capillary electrophoresis for the determination of drugs in body fluids have been reported *(33)*.

Our studies have shown that the urine sample does not require any pretreatment, except dilution, before CZE process. For this reason, two millilitres of urine samples were diluted to ten millilitres with purified water, so the linearity was assessed in the concentration range 2.0 to 35.0 µg/ml, under next conditions: running voltage 20 kV, hydrodynamic injection 2 s, detection at 210 nm, buffers at pH 9.7 containing a) 40 mM phosphoric acid + 10 mM boric acid; b) 50 mM phosphoric acid + 10% acetonitrile. In these conditions, the migration order of drugs was: MPE, PE, E, ME, NPE, NE and EPI, and the analysis can be completed within 15 min in both of them.

The studies of influence of concentration were performed in the two previous buffers, with ten different concentrations, and each one of then was repeated three times. Quality parameters of proposed methods are shown in table II and III.

Determination of EPI in 50 mM phosphate buffer (pH 9.7, 10% acetonitrile) was only possible for concentrations between 13.0 to 40.0 µg/ml, for this conditions, the heigh of peak vs. concentration is linear with a sensitivity of 0.141±0.004 AmU.ml.μg^{-1}, r=0.998, with relative standard deviation lower than 8.9%, and a relative error no higher than 7.2%.

The determination of EPI in the electrophoretic media with a relation phosphoric-boric acid (4:1) adjusted to pH 9.7, was possible. The EPI peak increases linearly with the EPI concentration up to 10 µg/ml, with a sensitivity of 0.115±0.005 AmU.ml.μg^{-1}, r=0.992, with a relative standard

TABLE 2
Analytical Characteristics Obtained for the Determination of MPE, PE, E, ME, NPE, NE and EPI in Human Urine by Direct Injection. 50 mM phospate buffer (ph 9.7, 10% Acetonitrile)

COMPOUND	LINEAR RESPONSE (µg/mL)	SENSITIVITY[a] (Mean±RSD) (AmU.ml μg^{-1})	CORRELATION COEFFICIENT	DETECTION LIMIT[b] (Mean±RSD) (µg/ml)	MEAN RELATIVE ERROR %	RSD %
MPE	2.5 - 40.0	0.059±0.001	0.9993	0.8±0.1	4.2	3.8
PE	2.6 - 40.0	0.067±0.001	0.9996	0.8±0.1	4.3	3.5
E	2.3 - 40.0	0.065±0.001	0.9990	0.7±0.1	3.9	3.1
ME	2.3 - 40.0	0.058±0.001	0.9994	0.7±0.1	4.1	4.2
NPE	1.9 - 40.0	0.078±0.001	0.9995	0.6±0.1	4.5	4.2
NE	2.0 - 40.0	0.076±0.001	0.9990	0.6±0.1	4.6	4.2
EPI	13.0 - 40.0	0.141±0.004	0.998	3.9±0.2	7.2	8.9

[a] SLOPE OF THE CALIBRATION PLOT.
[b] SIGNAL-TO-NOISE RATIO= 3:1

TABLE 3
Analytical Characteristics Obtained for the Determination of MPE, PE, E, ME, NPE, NE and EPI in Human Urine by Direct Injection. 50 mM phosphoric-boric acids (4:1) adjusted to ph 9.7

COMPOUND	LINEAR RESPONSE (µg/mL)	SENSITIVITY[a] (Mean±RSD) ($AmU.ml.\mu g^{-1}$)	CORRELATION COEFFICIENT	DETECTION LIMIT[b] (Mean±RSD) (µg/ml)	MEAN RELATIVE ERROR %	RSD %
MPE	1.8 - 40.0	0.065±0.001	0.9993	0.6±0.1	1.9	2.1
PE	1.7 - 35.0	0.069±0.001	0.9992	0.5±0.1	2.3	2.4
E	1.4 - 35.0	0.088±0.001	0.998	0.4±0.1	2.4	2.4
ME	1.7 - 40.0	0.071±0.001	0.9991	0.5±0.1	2.3	2.6
NPE	1.4 - 35.0	0.085±0.001	0.9990	0.4±0.1	2.0	2.1
NE	1.3 - 35.0	0.092±0.001	0.9991	0.4±0.1	2.4	2.8
EPI	1.0 - 10.0	0.115±0.005	0.992	0.3±0.2	6.9	7.9

[a] SLOPE OF THE CALIBRATION PLOT.

[b] SIGNAL-TO-NOISE RATIO= 3:1

deviation no higher than 7.9% and a relative error lower than 6.9%. Comparison of the urine blank and urine spiked only with EPI reveals the presence of this drug in human urine.

The standard addition method used for urine samples spiked with drugs, gave similar results as the method using the calibration curves.

CONCLUSIONS

Because of its specific advantages, including automation, small sample size, easiness buffer change, direct injection of urine samples, when compared with chromatographic approaches, capillary zone electrophoresis appears to be very attractive for the monitoring of several drugs.

The separation of six phenylamines and epinephrine has been achieved using capillary zone electrophoresis. The addition of boric acid to the buffer system, containing phosphoric acid, has overcome the difficulty associated with the epinephrine peak in urine samples.

It can be concluded that the method developed is fast, simple and reliable, allowing simultaneous and direct determination of six phenylamines and epinephrine in human urine samples for concentration between 2.0 to 35.0 µg per millilitre of urine.

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GENERAL PAPERS

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HIGHLY SELECTIVE SIMULTANEOUS DETERMINATION OF EIGHT INORGANIC ANIONS IN DRINKING WATER BY SINGLE COLUMN HIGH PRESSURE ANION CHROMATOGRAPHY

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ABSTRACT

Ion Chromatography is a well-established analytical technique for the simultaneous determination of ions. This paper focuses on the analytical determination of eight inorganic anions: fluoride, hydrogen carbonate, chloride, nitrite, bromide, nitrate, hydrogen phosphate and sulphate, using conductometric detection.

The separation of the cited anions was achieved on a low capacity anion exchange column Hamilton PRP-X100 100 x 4.1 mm, 10 μm, with a mobile phase consisted of 2.5 mM p-hydroxybezoic acid and 2.0 mM sodium benzoate at a pH 9.0 adjusted with 1N NaOH and 8% CH_3OH as organic modifier, at a flow rate 0.7 ml/min.

For the quantitative determination bromide was used as internal standard at a concentration of 7.8 mg/l. A rectilinear relationship was observed up to 40 mg/l for all ions except for carbonate that was up to 30 mg/l. The detection limits (S/N =3) were 100 μg/l for carbonate and 50 μg/l for the rest of the cited anions, when 50 μl of the samples were injected onto the analytical column.

Recovery of anions in spiked samples ranged from 90.44% to 108.88% with the time of analysis being less than 10 min. The statistical evaluation of the method was examined performing intra-day (n=8) and inter-day calibration (n=10) and found to be satisfactory with high accuracy and precision results.

The applicability of the method was demonstrated on the analysis of drinking water: tap, table and mineral.

INTRODUCTION

The first reported method for separation and quantitative determination of inorganic ions by means of high-pressure liquid chromatography was developed by Small et al (1). This technique called ion chromatography used a combination of an analytical column and a suppressor column to decrease the conductivity of the mobile phase for conductometric detection. Since then significant drawbacks from the chromatographic point of view have been reported regarding this approach, most of which arise from the suppressor column itself(2):

(1) the number of injections is restricted by the capacity of the suppressor column,

(2) the suppressor column introduces extra band broadening, which results in lower resolution,

(3) special equipment is needed for IC,

(4) only those buffers can be applied which, after passage through the suppressor column, result in a low electrical background conductivity.

Solutions containing $NaHCO_3$ and Na_2CO_3 in low concentrations, 1-5 mM are used as eluents in dual column Ion Chromatography (suppressed conductivity).

In addition, packed suppressors require periodic regenaration to restore their ion-exchange capacity and the eluents applicable to suppressed ion chromatography are restricted to those which undergo suitable protonation or other reactions in the suppressor column. These deficiencies have provided an impetus for the development of chromatographic methods for inorganic anion analysis that do not require use of a suppressor column.

Solutions containing $NaHCO_3$ and Na_2CO_3 in low concentrations, 1-5 mM are used as eluents in dual column Ion Chromatography (suppressed conductivity).

Some investigations have been undertaken in an attempt to overcome these disadvantages. In particular Gjerde and co workers demonstrated that a suppressor column is not absolutely necessary for HPIC with electric conductivity detection. They used an anion exchanger having a low exchange capacity and an eluent having a very low conductivity. In this way the background conductivity is sufficiently low to allow the separated anions to be detected with a simple conductance detector(3-5).

A number of alternative methods have been reported in the literature, all of which use an analytical separator column (usually an ion-exchange column) without a suppressor column. These methods differ from each other chiefly in the mode of detection employed . Inorganic anions may be separated on a reversed-phase system either by prior formation of organic derivatives or by using ion-pair formation with subsequent direct detection of UV-absorbing ions at low UV wavelengths (210-220 nm). This same approach has been applied to an amino column (6,7), however these methods are not widely applicable since only a limited number of inorganic ions show UV absorbance. A more general method is the use of reversed-phase ion-pair HPLC with UV-absorbing pairing ions, where samples gave positive or negative peaks, depending on their charge and retention relative to the UV-absorbing pairing ion. Octylamine, cetrimide and alkylammonium salts e.g. tetrabutylammonium-acetate, phthalate or salicylate are most commonly used as eluents with this technique (8-20).

Various detection techniques reported in literature include conductivity, indirect UV photometry, amperometry, potentiometry and refractive index.

Among these techniques conductometric detection remains the mainstay of HPIC (21-23).

The detection of ions by conductance in non-suppressed Ion Chromatography (Single Column Ion Chromatography) is achieved due to the difference in ionic conductances of the sample and eluent ions.

When a large number of determinations are required the application of specific reactions for anions becomes time consuming and the possibility of the simultaneous separation, identification and quantitification of several anions is of interest High Pressure Ion Chromatography (HPIC) with suppressed conductivity detection is one of the most popular methods that satisfy these objectives.

Compared to non-chromatography techniques, Ion Chromatography methodology has the advantages of separation before detection increased detection, increased sensitivty, simple sample preparation and faster analysis time (24,25).

The United States Environmental Protection Agency (EPA) has established regulations and methodology for inorganic contaminants under the Safe Drinking Water Act. Fluoride, nitrite and nitrate are listed as primary pollutants since they can cause adverse health effects. Ion Chromatography has become a well established technique for the determination of nitrite and

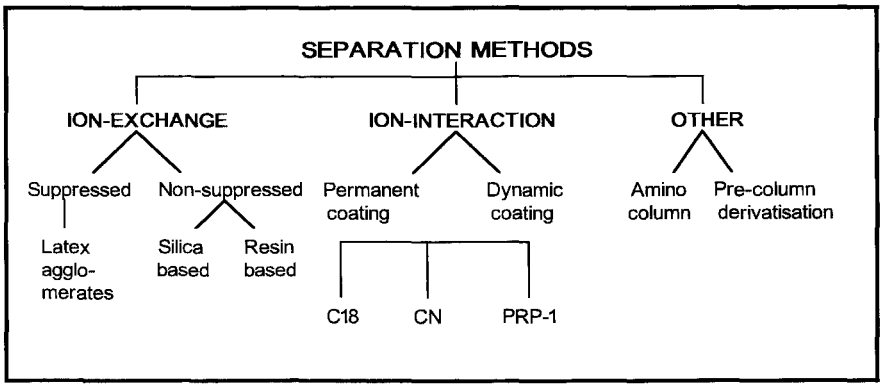

Figure 1. Schematic representation of separation methods used for inorganic anions.

nitrate in drinking water. Chloride and sulphate are listed as secondary contaminants because they are organoleptic (affect the smell, taste or appearance of water). They are not monitored by the EPA, hence, a laboratory can use any method (HPIC, ion selective electrode, flow injection analysis, etc.) to analyze for these analytes. The ability of HPIC to separate the analytes of interest from interferences provides a dinstinct advantage over other analytical techniques in terms of detection, sensitivity and is capable of multi-element analysis (26).

Several separation methods concerning the determination of inorganic anions are described in the literature. Figure 1 shows a schematic representation of these methods (27).

A summary of experimental conditions used in some non-suppressed ion-exchange methods using a variety of column types and detectors is given in table 1.

Detection limits in the range of 0.4-1600 ng are reported in the literature using different eluent and detection systems, while the time of analysis was in the most cases over ten minutes (16, 30).

In the present paper a non-suppressed chromatographic method with conductometric detection is described for the simultaneous determination of eight inorganic anions.

TABLE 1
SUMMARY OF EXPERIMENTAL CONDITIONS USED IN SOME ION-EXCHANGE METHODS USING A VARIETY OF COLUMN TYPES ELUENTS AND DETECTORS

ANIONS	COLUMN	ELUENT*	DETECTION	REF
$Br,Cl,NO_3,NO_2,PO_4,SO_4$	Nucleosil 5 or 10SB	0.03 M salicylate pH 4.0 0.075 M p-HBA pH 5.6 0.05 M KHP	Indirect Refractive Index	2
Br,Cl,F,PO_4,SO_4	Partisil SAX	0.05M $NaNO_3$ 0.04M KI , 0.05M KIO_3	Indirect UV Absorbance	27
Br,Cl,F,PO_4,SO_4,NO_3	Permutit ZeoKarb 225 Cd Form	0.0025 M $(CH_3COO)_2Cd$	Potentiometry	27
$Br,Cl,NO_3,NO_2,PO_4,SO_4,F,CO_3$	0.007 mequiv/g resin-based ion exchanger	6.5×10^{-4} M benzoate 5×10^{-4} M KHP	Conductivity	4,5
$Br,CO_3,NO_3,NO_2,PO_4,SO_4$	TSK GEL 620	1.3 mM gluconic acid in borate buffer	Conductivity	25
Cl,F,NO_3,NO_2,SO_4	TSK GEL 620 SA	2mM KOH	Conductivity	27
Br,Cl,NO_3,NO_2,SO_4	TSK GEL IEX 520	1mM citrate pH 5.2 1mM tartrate pH 3.2	Conductivity	28
F,NO_3,NO_2,PO_4,SO_4	TSK GEL IEX 520	0.1 M $NaNO_3$ or 0.05 M $NaNO_3$ in 0.05 M acetate buffer	Post-column reaction using $Fe(ClO_4)_3$	27
Cl,F,NO_3,NO_2,SO_4,PO_4	Surface agglomerated pellicular ion exchanger	1 mM phthalate pH 7.0 or 0.1 mM sulphobenzoate pH 8	Indirect UV absorbance	27
$Br,Cl,PO_4,NO_3,NO_2,SO_4$	Vydac 302 IC	5 mM KHP pH 4.0	Conductivity, Indirect UV	27
$F,Br,Cl,PO_4,NO_3,NO_2,SO_4$	Vydac 302 IC	0.5 mM KHP pH 4.6	Conductivity	27
$F,Br,Cl,PO_4,NO_3,NO_2,SO_4,CO_3$	Wescan 269-001	4 mM KHP pH 3.9	Conductivity	27
Br,Cl,PO_4,NO_3,SO_4	Vydac 302 IC	5 mM KHP pH 4.6	Conductivity, Indirect UV	9
Br,Cl,NO_3,NO_2	Wescan 269-001	10mM methanesulphonate pH5	UV 214 nm	29

*KHP=Potassium hydrogen phthalate
p-HBA= p-hydroxybenzoic acid

The purpose of this work was to characterize the method in terms of sensitivity, detection limits and working range in order to be applied to the routine analysis of drinking water.

The analysis of table water was also investigated and the validity of the method was established by inter-day and intra-day calibration studies.

EXPERIMENTAL

Instrumentation

The ion chromatograph used consisted of an SSI model 222D Pump (SSI, State College PA,USA) and a model Wescan 315 conductometric detector (Alltech, Deerfield, IL, USA) maintained at 35± 0.1 °C.

A Rheodyne model 9125 six-port high pressure switching injection valve (Rheodyne, Cotati, CA, USA) was assempled with a 50 µl injection loop.

The separation column Hamilton PRP-X100 100x 4.1 mm was packed with spherical 10 µm polystyrene-divinylbenzene trimethylammonium anion exchanger (Hamilton, Reno, NV, USA). The capacity of the column was 0.19±0.02 meq/g.

Data aquisition was performed using a Hewllett-Packard integrator, model HP 3396 II (Hewlett-Packard, Avondale, PA, USA).

Statistical evaluation of the experimental data was achieved by a VIP 312 IBM compatible PC.

Reagents

Stock standard solutions (1,000 mg/l) of fluoride, hydrogen carbonate, chloride, nitrite, bromide, nitrate, hydrogen phosphate and sulphate were prepared by dissolving appropriate amounts of analytical reagent grade sodium or potassium salts in de-ionised water. These solutions were subsequently diluted to give the multi-anion solutions required.

Analytical reagent grade compounds provided by Merck (Darmstadt, Germany) were used to prepare the eluent systems examined in the present study.

Stock solutions of 20 mM sodium benzoate and 20 mM p-hydroxy-benzoic acid were diluted daily to provide the working concentration of mobile phase 2.0 mM and 2.5 mM respectively.

The pH of the mobile phase was adjusted at 9.0 by adding 4 ml 1N sodium hydroxide solution per 1 liter of eluent. The sodium hydroxide solution was prepared by diluting properly the content of Fixanal ampoule (Riedel-deHaen, Hannover, Germany).

HPLC-grade methanol (Merck, Darmstadt, Germany) was added to the mobile phase at a concentration of 8%.

The mobile phase was filtered through 0.2 μm membrane filters (Schleicher-Schuell, Dassel, Germany).

All standard solutions and samples of drinking water were kept at 4 ^{o}C before and after analysis.

RESULTS AND DISCUSSION

Optimization of the chromatographic system

The chromatographic system for the separation of the eight, inorganic anions, was chosen among others, as shown in tables 2 and 3, in order to result in optimum separation regarding to selectivity and detection conditions and it was optimized in terms of its suitability for conductivity detection, considering its effectiveness on displacement of desired species with minimal conductivity.

pH of the mobile phase was sufficiently high in order to eliminate the system peak which is often encountered in SCIC (31). The system peak seems to be due to the elution of neutral molecules of the eluent on injection of a water sample. These molecules are believed to be retained on the chromatographic column by a reversed-phase mechanism, as about 85% of a low-capacity ion-exchange column is unfunctionalized (i.e. has no ionized group). The retention time of this peak depends on several parameters like: nature nad concentration of the eluent, pH of the mobile phase, presence of organic modifier in the eluent etc. Its existence can be a real problem if it overlaps with an analyte peak or it appears late in the chromatogram and it increases the time of analysis. To avoid this peak, the eluent must be completely ionized at the pH chosen.

The eluent anion must be retained by the anion-exchange resin sufficiently strongly that a very low concentration of the eluent salt will move anions to be separated down to the chromatographic column. At the same time the conductance of the eluent should be low so that the separated anions will give a detector signal well above that of the eluent background. The observed background conductivity of the eluent chosen was 12.25 $\mu S \cdot cm^{-1}$.

Keeping all the above practical considerations in mind the chromatographic system was optimized in terms of separation and detection.

The optimum conditions are reported in table 4.

TABLE 2
ELUENT SYSTEMS STUDIED FOR THEIR EFFECTIVENESS ON THE SEPARATION AND QUANTITATIVE DETERMINATION OF THE EIGHT INORGANIC ANIONS EXAMINED IN THE PRESENT STUDY

Eluent system	Compound	Concentration (mM)	pH	Background conductivity ($\mu S \cdot cm^{-1}$)	Flow rate (ml/min)	Pressure (psi)
A	Potassium hydrogen phthalate	2.0	6.0	6.8	1.3	700
B	p-hydroxy-benzoic acid (pHBA)	4.0	9.0	12.75	1.3	625
C	phthalic acid	5.0	9.0	11.7	1.3	875
D	Sodium benzoate	2.0	9.0	4.15	1.0	625
E	pHBA Na benzoate	2.0 2.0	9.0	14.15	1.0	675
F	pHBA Na benzoate	2.0 1.0	9.0	11.5	1.0	775
G	pHBA Na benzoate	3.0 2.0	9.0	19.6	1.0	1125
H	pHBA Na benzoate	2.5 1.5	9.0	14.25	1.0	1150
I	pHBA Na benzoate	2.0 1.5	9.0	9.75	1.0	1075
J	pHBA Na benzoate	1.5 1.5	9.0	15.0	1.0	900
K	pHBA Na benzoate	1.5 2.0	9.0	17.1	1.0	875
L	pHBA Na benzoate	2.5 2.0	9.0	12.25	0.7	675

TABLE 3
INORGANIC ANION SEPARATION WITH DIFFERENT ELUENT SYSTEMS AS DESCRIBED IN TABLE 2

Anions	Retention Time with Different Eluent Systems (min)											
	A	B	C	D	E	F	G	H	I	J	K	L
F^-	2.53	1.26	NS	4.53	1.79	1.63	1.66	2.31	2.49	3.54	1.52	1.71
HCO_3^-	-	1.79	-	-	-	-	5.76	11.9	4.05	8.99	7.91	2.35
Cl^-	2.53	2.15	2.91	6.61	2.71	3.10	2.62	3.18	3.85	3.61	3.51	2.66
NO_2^-	3.12	2.81	4.14	7.18	3.17	3.57	2.97	3.61	4.44	4.15	3.91	3.01
Br^-	4.28	4.41	3.91	9.36	4.01	4.66	3.79	4.71	6.04	5.73	5.41	4.21
NO_3^-	5.26	5.31	4.21	10.3	4.41	5.22	4.16	5.21	6.83	6.49	6.13	4.76
$HPO_4^=$	-	5.21	10.1	12.8	5.61	6.91	4.51	6.71	11.31	11.51	10.6	6.88
$SO_4^=$	7.8	7.41	13.6	-	7.01	9.12	5.81	8.91	16.5	12.2	11.21	9.39

TABLE 4
OPTIMUM CONDITIONS FOR THE CHROMATOGRAPHIC SEPARATION OF INORGANIC ANIONS.

Operation	Parameter	Value
Separation	p-hydroxybenzoic acid	2.5 mM
	sodium benzoate	2.0 mM
	pH	9.0
	methanol	8%
	Flow rate	0.7 ml/min
	Inlet pressure observed	675 psi
Detection	Conductometric	
	Time constant	0.2 s
	Sensitivity	0.5 $\mu S \cdot cm^{-1}FS$
	Temperature	35±0.1 ^oC

Figure 2 shows the chromatogram obtained during the separation of the eight inorganic anions by means of the chromatographic system developed at the present study.

The Rs values for the eight inorganic anions : fluoride, hydrogen carbonate, chloride, nitrite, bromide, nitrate, hydrogen phosphate and sulphate are 1.40, 0.25, 0.99, 2.15, 0.80, 2.24 and 1.99 respectively per couple of anions.

Performance characteristics of the proposed method.

The system described here was used for the simultaneous determination of eight inorganic anions: fluoride, hydrogen carbonate, chloride, nitrite, bromide, nitrate, hydrogen phosphate and sulphate.

Optimized chromatographic conditions were set and the following analytical characteristics were evaluated:

- Precision and accuracy.
-Working range and detectability.
-Analysis time.
-Calibration data.
-Real sample analysis.

Precision and Accuracy

In order to verify the reproducibility, replicate injections of standard solutions at low and high concentration levels were made and peak areas were measured in comparison to the peak area of the internal standard.

Statistical evaluation revealed relative standard deviations at different values for eight injections. The results are shown in table 5.

Long term stability was examined during routine operation of the system over a period of ten consecutive days. Results are presented in table 6. The inter-day reproducibility was usually better than 10% even when the instrument was shut down during working breaks.

Working range and detectability

For the analysis of a multi anion solution it is found that the maximum concentration or amount that can be injected is limited by saturation of the

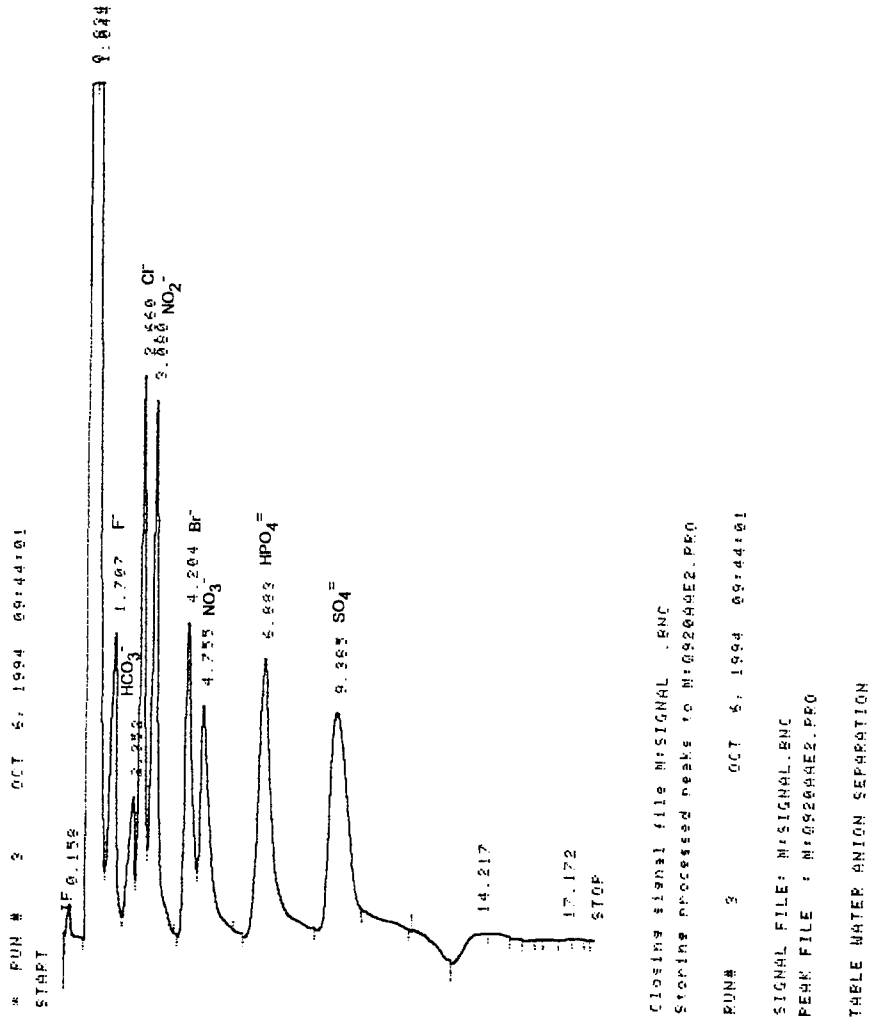

Figure 2. Chromatographic separation of the eight inorganic anions examined in the present study. Conditions are described in text.

TABLE 5
INTRA-DAY CALIBRATION RESULTS (n=8)

Anions	Added (ppm)	Found (ppm)	SD	RSD (%)	Recovery (%)
F^-	1.77	1.69	0.25	14.79	95.48
	2.66	2.59	0.12	4.63	97.37
	4.40	4.52	0.36	7.96	102.73
	6.20	6.70	0.52	7.76	108.06
HCO_3^-	1.94	1.97	0.14	7.11	101.55
	3.88	3.62	0.23	6.35	93.30
	5.81	5.66	0.28	4.95	97.42
	7.75	7.99	0.42	5.26	103.10
Cl^-	2.69	2.31	0.22	9.52	85.87
	4.04	3.93	0.16	4.07	97.29
	6.70	6.65	0.35	5.26	99.25
	9.43	9.06	0.59	6.51	96.08
NO_2^-	2.44	2.35	0.22	9.36	96.31
	3.66	3.52	0.37	10.51	96.17
	6.10	6.18	0.47	7.61	101.31
	8.53	8.48	0.68	8.02	99.46
NO_3^-	1.90	1.85	0.21	11.35	97.37
	3.79	3.79	0.33	8.71	100.00
	5.69	5.45	0.46	8.44	95.78
	9.50	9.66	0.56	5.80	101.68
HPO_4^{2-}	1.69	1.84	0.22	11.96	108.88
	3.38	3.48	0.29	8.33	102.96
	5.08	5.17	0.24	4.64	101.77
	8.50	8.42	0.53	6.29	99.06
SO_4^{2-}	1.99	1.93	0.18	9.33	96.98
	3.97	3.96	0.24	6.06	99.75
	5.96	5.39	0.51	9.46	90.44
	9.90	10.07	0.73	7.25	101.74

capacity of the column, resulting in poor peak shapes. The term working range is therefore more appropriate than linear range.

Table 7 provides the maximum amounts of multi-anion solution that can be injected onto the analytical column. It should be noted that the upper limit reported for each anion assumes the co-existing concentration of the other anions at a similar level. For this reason higher concentrations could also be injected in case of absence of other anions.

Detection limits were not evaluated by statistical treatment of the data because they depend on the injection volume and to a considerable extent

TABLE 6
PRECISION AND ACCURACY OVER A PERIOD OF 10 CONSECUTIVE DAYS.

Anions	Added (ppm)	Found (ppm)	SD	RSD (%)	Recovery (%)
F^-	1.77	1.73	0.17	9.83	98.30
	2.66	2.67	0.11	4.12	100.38
	4.40	4.08	0.11	2.70	92.73
	6.20	6.17	0.26	4.21	99.52
HCO_3^-	1.94	1.89	0.09	4.76	97.42
	3.88	3.95	0.31	7.85	101.80
	5.81	5.94	0.21	3.54	102.24
	7.75	7.82	0.39	4.99	100.90
Cl^-	2.69	2.50	0.21	8.40	92.94
	4.04	3.92	0.14	3.57	97.03
	6.70	6.67	0.15	2.20	99.55
	9.43	9.53	0.11	1.19	101.06
NO_2^-	2.44	2.32	0.09	3.88	95.08
	3.66	3.49	0.25	7.16	95.36
	6.10	6.00	0.15	2.50	98.36
	8.53	8.70	0.12	1.38	101.99
NO_3^-	1.90	2.05	0.26	12.68	107.89
	3.79	3.84	0.11	2.86	101.32
	5.69	5.52	0.14	2.46	97.01
	9.50	9.55	0.34	3.56	100.53
HPO_4^{2-}	1.69	1.69	0.07	4.14	100.00
	3.38	3.40	0.20	5.88	100.59
	5.08	5.04	0.41	8.13	99.21
	8.50	8.48	0.61	7.19	99.76
SO_4^{2-}	1.99	2.05	0.21	10.24	103.02
	3.97	4.00	0.32	7.92	100.76
	5.96	5.96	0.14	2.35	100.00
	9.90	9.87	0.29	2.94	99.70

TABLE 7
WORKING RANGE AND DETECTION LIMITS

Anions	Upper limit (µg)	Detection limit (ng)
F^-	2.0	2.5
HCO_3^-	1.5	5.0
Cl^-	2.0	2.5
NO_2^-	2.0	2.5
Br^-	2.0	2.5
NO_3^-	2.0	2.5
$HPO_4^=$	2.0	2.5
$SO_4^=$	2.0	2.5

on the noise level of the detectors employed. In conductivity detection noise is mainly created by temperature fluctuations. The proper thermostating of the measuring cell and suitable design of the flow cell allows the detection of very small conductivity differences.

Detection limits calculated as a three-fold signal-to-noise ratio at the baseline (S/N=3) are shown in table 7.

Analysis time

The analysis time in the proposed method is determined solely by the retention time of the most strongly retained ion in the chromatographic separation due to the lack of system peak. As is evident from figure 2 the sample analysis time can be less than 10 minutes, as sulphate the last eluting anion has 9.385 min. retention time.

Calibration data

Calibration of the method was done by injection of mixed ion standards covering the entire working range. The sensitivity setting of the conductivity detector was adjusted to give almost fullscale deflection for the highest standard concentration. Each sample was injected five times.

Linear correlation ($r>0.99148$) between concentration and peak area ratio was obtained for all ions using bromide as internal standard at a concentration of 7.8 mg/l. Bromide was chosen as internal standard for being absent in drinking water samples.

The results of the statistical treatment of calibration data for the seven anions are summarized in table 8.

REAL SAMPLE ANALYSIS

Analysis of drinking water

In order to examine the suitability of the method for the determination of inorganic anions, several drinking water samples: tap, mineral and table water provided by the water supply oraganization and local market respectively were analysed. Results are presented in table 9.

Examples of chromatograms obtained are illustrated in figures 3 and 4.

TABLE 8
CALIBRATION DATA FOR SIMULTANEOUS DETERMINATION OF INORGANIC ANIONS (PEAK AREA RATIO MEASUREMENTS WITH 7.8 MG/L BROMIDE AS INTERNAL STANDARD -RT=4.204 MIN).

Parameter	Value	F^-	HCO_3^-	Cl^-	NO_2^-	NO_3^-	$HPO_4^=$	$SO_4^=$
Concentration range	mg/l	0.5-40	0.5-30	0.5-40	0.5-40	0.5-40	0.5-40	0.5-40
Slope	$\mu S \cdot l \cdot cm^{-1} \cdot mg^{-1}$	0.06677	0.223272	0.19937	0.18422	0.07792	0.14698	0.33396
Intercept		0.45798	-0.002325	0.44741	0.24753	0.45555	0.42848	0.35135
Correlation coefficient		0.99148	0.99615	0.99459	0.99915	0.99684	0.99478	0.99966
Retention time	min	1.707	2.352	2.660	3.080	4.755	6.883	9.385

TABLE 9
SIMULTANEOUS DETERMINATION OF INORGANIC ANIONS IN DRINKING WATER SAMPLES.

Water sample	Anion	Label concentration (ppm)	Found[a] concentration (ppm)	RSD (%)
IVI	Cl^-	31.9	28.32±1.01	3.57
(Mineral Water)	NO_3^-	3.1	2.92±0.18	6.16
	SO_4^{2-}	5.8	5.74±0.26	4.52
	HCO_3^-	391.7	100.8±18.4	18.25
SOUROTI	Cl^-	69.1	67.70±3.62	5.34
(Mineral Water)	SO_4^{2-}	63.2	49.20±0.78	1.59
	F^-	0.3	ND	
	NO_3^-	0.4	ND	
	NO_2^-	0.005	ND	
	HCO_3^-	924.1	844.7±25.6	3.03
VIKOS	Cl^-	8.42	8.15±0.36	4.41
(Mineral Water)	NO_3^-	4.97	2.21±0.13	5.88
	NO_2^-	0.0	ND	
	SO_4^{2-}	2.40	2.01±0.09	4.47
	HCO_3^-	296.53	125.3±10.2	8.14
IRIS	Cl^-	28.4	20.03±1.83	9.13
(Mineral Water)	NO_3^-	-	ND	
	NO_2^-	-	ND	
	SO_4^{2-}	5.3	3.86±0.21	5.44
	HCO_3^-	388.1	94.9±12.6	13.28

a= Mean value n=6 ± SD
ND= Not detected.

TABLE 9 (CONT.)

NIGRITA (Table water)	Cl^-	17.10	14.41±0.62	4.30
	NO_3^-	5.70	4.35±0.15	3.45
	NO_2^-	-	ND	
	SO_4^{2-}	16.90	12.65±0.27	2.13
	PO_4^{3-}	0.08	ND	
	F^-	0.35	ND	
	HCO_3^-	398.5	136.2±21.4	15.71
AVRA (Table water)	Cl^-	10.2	8.56±1.31	12.84
	NO_3^-	28.6	28.0±2.9	10.36
	SO_4^{2-}	28.3	20.7±1.3	6.28
	HCO_3^-	146.4	68.5±9.4	13.72
SELI (Mineral Water)	Cl^-	3.5	3.06±0.32	10.46
	NO_3^-	0.6	0.2±0.01	5.00
	SO_4^{2-}	24.0	17.9±1.78	9.94
	HCO_3^-	231.9	127.2±21.2	16.67
ZAGORI (Mineral Water)	Cl^-	8.27	5.33±0.25	4.69
	NO_3^-	4.35	2.13±0.08	3.75
	SO_4^{2-}	7.85	4.12±0.48	11.65
	HCO_3^-	206.76	137.71±21.6	15.68
Municipal Water Supply Organisation	Cl^-	34-57	39.1±5.3	13.55
	NO_3^-	7-10	1.18±0.05	4.24
	SO_4^{2-}	14-23	12.2±0.5	4.10
	HCO_3^-		118.3±9.7	8.20
	F^-	0.13-0.16	ND	
	P_2O_5	0.032-0.055	ND	

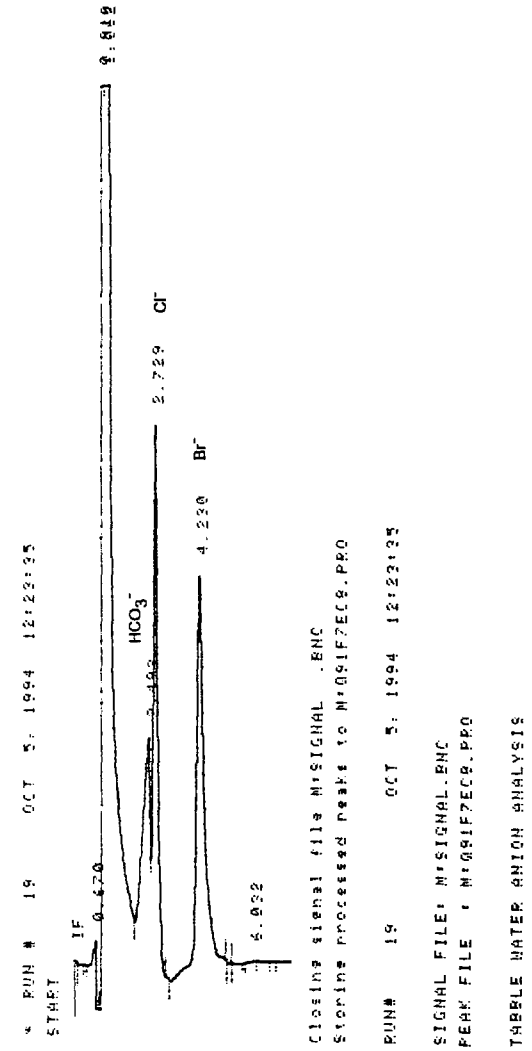

Figure 3. Simultaneous determination of inorganic anions in Mineral Water (IRIS). Conditions are described in text.

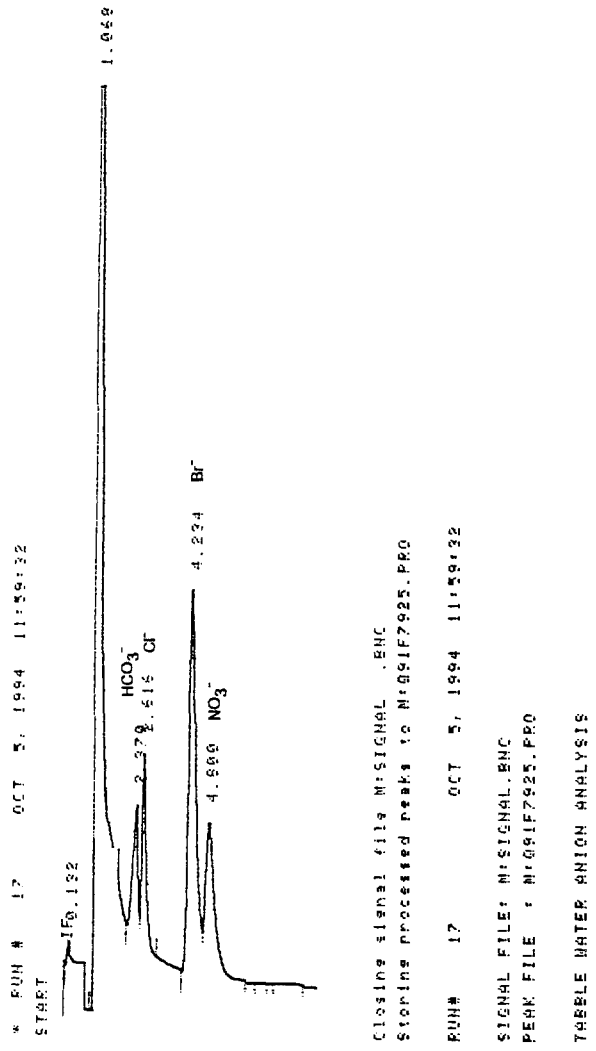

Figure 4. Simultaneous determination of inorganic anions in Table Water (AVRA). Conditions are described in text.

CONCLUSIONS

Eight inorganic anions: fluoride, hydrogen carbonate, chloride, nitrite, bromide, nitrate, hydrogen phosphate and sulphate were separated and analysed by means of Single Column Ion Chromatography (SCIC) technique with conductometric detection.

The combination of p-hydroxybenzoic acid and sodium benzoate at concentrations 2.5 mM and 2.0 mM respectively provides the required anions for the displacement of the desired species from the low-capacity polymer-based anion exchange column, as well as the sufficiently low background conductivity for enhanced sensitivity. The pH value of the mobile phase is high enough so that the system peak often encountered in SCIC does not occur.

The day-to-day precision was tested for the analysed anions over 10 consecutive days, while the repeatability was investigated by performing intra-day calibration (n=8).

The applicability of the method was demonstrated on the analysis of several drinking water samples.

The developed method is characterised by high selectivity, sensitivity, high accuracy and precision, and low cost of operation, providing multi-anion analysis within ten minutes.

No suppressor column is required so that peak dispersion is less, resulting in a better resolution. Moreover no regeneration step has to be included and no special equipment is needed.

The separation can be achieved on any liquid chromatograph equipped with a conductometric detector.

Detection limits are within 5.0 ng range for 50 µl injected sample volume.

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COMPUTER SIMULATION OF THE SEPARATION IN ONE- AND TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY BY ISOCRATIC AND STEPWISE GRADIENT DEVELOPMENT[1]

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ABSTRACT

A method is presented for the computer-assisted selection of the mode of development for the separation of ten phenolic components. The method is based on simulation of isocratic and gradient development. The different combinations of isocratic and gradient development in one (1D) and two (2D) dimensions are tested. The qualities of chromatograms are evaluated by application of criteria such as the distance function and multipeak criterion.

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INTRODUCTION

Thin-layer chromatography (TLC) has long been considered useful for the rapid and inexpensive qualitative analysis of simple mixtures. During the past 10 years however TLC has undergone a renaissance with the introduction of high performance TLC plates, precise spotting devices, sensitive spectrophotometric scanners (densitometers), full automated development process [1] and it has become accepted as a technique for precise and sensitive qualitative and quantitative analysis [2].

In planar chromatography some form of optimization is generally necessary if complete separation of all components in a sample is required and if the number of components is larger than a small fraction of the spot capacity of the system [3].

In recent years the study of the systematic strategies to optimize systems of HPTLC (TLC) has rapidly gained widespread acceptance combined with computer-assisted method development [4]. The optimization methods are in a very easy way transferred from HPLC to HPTLC, after consideration of differences between them.

The confident analysis from moderate to complex mixtures requires a very large peak capacity that is most readily obtained using multidimensional and multimodal separation techniques. The 2D (two-dimensional) TLC technique offers high capacity [4,5] and it is especially simple, since separations are normally carried out in a plane and

sequential development in orthogonal direction and this is all that is needed.

The advantages of 2D-TLC have been described by Guiochon and Nurok [4-6], who applied this technique to separation of complex mixtures. Their investigations were carried out by isocratic developments. The gradient development, especially the step-gradient TLC has become widely accepted and efficient method for the solution of the so-called general elution problem, i.e., the separation of compounds having greatly differing retentions. Another reasons are the enhancement of mutual displacement of the components to be separated and compression effect of the spots taking place during the gradient development. Considering above statements, it will be very interesting to investigate how the combination of 2D-TLC and the stepwise gradient mode work together and if it is possible to get better resolution of complex mixtures, with wide spectrum of polarity. In an earlier paper [7] an equation for the R_{FG} value of solute chromatographed under stepwise gradient conditions was derived, assuming a definite relationship between the k values and modifier concentration and in the subsequent papers computer programs were elaborated [7-12]. In the present paper equation derived determines the final position of solutes after 2D development, where 1D (one dimensional) developments can be carried out in different modes.

There are several established approaches [13,14] to optimizing separation quality using a suitable separation metric.

To judge the quality of an entire chromatogram, elementary criteria, used only for a pair of the peaks must be extended. A first possible combination is the summation of elementary criteria for successive compound pairs. A second class of criteria for the entire chromatogram multiplies the elementary criteria. The advantage of these product criteria is their sensivity, especially for the least resolved compound pairs.

THE THEORETICAL CONSIDERATION

One-dimensional development

The investigated sample is a multicomponent mixture, where components differ strongly in polarity. The development process is run in the ideal condition, in the isocratic as well in the gradient mode. For all components of the mixture a definite relationship between retention and the properties of mobile phase is known. This relationship can be described by the well known equation which follows from the Snyder-Soczewiński adsorption model [16,17] presented in the logarithmic form:

$$\log k_{AB} = \log k_o - m \log C_B \qquad (1)$$

Sometimes the relationship between the retention and the properties of mobile phase can be described by a polynomial form:

$$k_{(l)} = k_{(0)} + k_{(1)} C_{(l)} + k_{(2)} C_{(l)}^2 \qquad (2)$$

The isocratic development

When the chosen development mode is isocratic, then the final R_F values for solutes are determined by the following equation:

$$R_F = \frac{1}{1 + k} \tag{3}$$

The simple stepwise gradient development

If the applied mode is the stepwise gradient mode, then the final R_F values for solutes are determined by two equations:

- for solutes which migrated only in first zone (h = 1) of gradient program (for details see [7,8]) by equation:

$$R_{FG\,(j)} = \frac{1}{1 + k_{(j,l)}} \tag{4}$$

- for solutes which migrated through more than one zone (h ≥ 2) by equation:

$$R_{FG(j)} = \sum_{l=1}^{h-1} \frac{v_{(l)}}{k_{(j,l)}} + R_{F_{(j,h)}}\left[1 - \sum_{l=1}^{h-1} \frac{v_{(l)}}{1 - R_{F_{(j,l)}}}\right] \tag{5}$$

The unidimensional development

Again if the applied mode is unidimensial development (constant distance and the same mobile phase) then the final R_F value can be determined by equation [18]:

$$R_{FG_{(J)}^{m}} = 1 - (1 - R_{F_{(J,I)}})^{m} \tag{6}$$

The multiple gradient development

The last mode of development can be multiple development (increased elementary distance of development) where the development distance increase and the concentration of a modifier decreases for the consecutive steps. The details of the derived equations were presented in paper [12]:

$$R_{FG_{(J)}} = S_{(n-1,J)} + [Z_{(n)} - S_{(n-1,J)}] R_{F_{(n,J)}} \tag{7}$$

As mentioned earlier, to judge the quality of the chromatogram in one dimensional development the distance function is used:

$$D^{1D} = \sum_{I=1}^{n-1} \sum_{J=I+1}^{n} [(X_{(I)} - X_{(J)})^2] \tag{8}$$

where $X_{(i)}$, $Y_{(i)}$ are the values of R_F of solutes or distances of migration. Other function is MPC (multipeak criterium) [15] defined by equation:

$$MPC = \left[R_{F_{(MAX)}} - R_{F_{(n)}}\right]\left[R_{F_{(1)}} - R_{F_{(MIN)}}\right]\prod_{i=1}^{n-1}\frac{R_{F_{(i+1)}} - R_{F_{(i)}}}{\left[\frac{R_{F_{(MAX)}} - R_{F_{(MIN)}}}{n+1}\right]^{n+1}} \quad (9)$$

where:

$R_{F(MAX)}$ $R_{F(MIN)}$ extreme values within which all spots of compounds must lie

$R_{F(1)}$ - corresponds to the component of lowest R_F

$R_{F(n)}$ - corresponds to the component of highest R_F

n - number of components.

Two-dimensional development

The place of spotting the sample is considered as the origin of a coordinate system.

The process of development is carried out in two stages: the first in the direction of the x-axis on the distance L_x and the second (after evaporation of the first solvent) in the direction of y-axis on the distance L_y.

The positions of solutes after development in the x direction depend on the selectivity of the system; mobile phase/adsorbent for isocratic development and additionally on the gradient program if the latter technique is applied. Similarly, the migration distance in the y-direction depends on selectivity, the development distance and the applied gradient program.

After the development in the x-direction, the ordinates of all spots are zero. After the development in the y-direction, their abscissa values follow from their positions on the x axis after the first development.

The final positions of spots are thus determined by the coordinates $X_{(j)}$ and $Y_{(j)}$ which can be calculated from the equation:

$$R_{FXY_{(j)}} = [R_{FX_{(j)}}, R_{FY_{(j)}}] \tag{10}$$

The two-dimensional TLC separation is of no interest if the selection of the two different eluting systems and modes is not adequate. A good separation will be obtained when the surface area of plate over which the spots are spread is relatively large.

It is thus useful to calculate an estimate of the quality of a two-dimensional separation. In our case the distance function will be calculated [6].

In its simplest form the D^{2D} is:

$$D^{2D} = \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \left[(X_{(i)} - X_{(j)})^2 + (Y_{(i)} - Y_{(j)})^2 \right] \tag{11}$$

where: - $(X_{(i)} - X_{(j)})$ is the distance between an adjacent pair of spots in direction X

- $(Y_{(i)} - Y_{(j)})$ is the distance between an adjacent pair of spots in direction Y.

Equations (1 to 11) form the base to elaborate a computer program that simulates 2D-development in modes I-I, I-G, G-I, G-G, where I - means isocratic development, G - gradient development.

The flow diagram is shown in Fig. 8. The program allows not only for the calculation of the final R_F values of solutes in 1D and 2D development, but also the values of D^{1D}, D^{2D}, MPC for estimation of quality of chromatogram as well as the graphic representation of chromatogram. The program was written in Pascal (6.0).

EXPERIMENTAL

In this paper only the computer simulations were carried out. The group of phenolic compounds investigated by Lodi at all. [19] is a good example sample of which the components span a wide polarity range. From the plots (R_M vs. log φ) presented in paper [19], we estimated values of k_o and m for this group of solutes chromatographed on the diol-layer with the different mobile phases: acetonitrile + dichloromethane (the system I) and acetone + dichloromethane (the system II).

TABLE 1

Phenolic Compounds Used as Standards and Acetonitrile and Acetone as Modifier.

No.	Compound	Acetonitrile + dichloromethane		Acetone + dichloromethane	
		k_o	m	k_o	m
1	Rutin	6.45	3.00	0.330	4.33
2	Kaempferol-3-rutinoside	4.47	3.00	0.240	3.88
3	Quercetin-3-arabinoside	2.69	2.50	0.204	3.59
4	Quercetin-3-galactoside	1.91	2.85	0.120	3.30
5	Chlorogenic acid	1.02	1.80	0.072	2.88
6	Myricetin	0.99	1.38	0.051	2.49
7	Caffeic acid	0.50	1.30	0.056	1.77
8	Quercetin	0.50	1.03	0.054	1.59
9	Apigenin	0.27	0.97	0.052	1.42
10	Ferulic acid	0.24	0.45	0.035	1.30

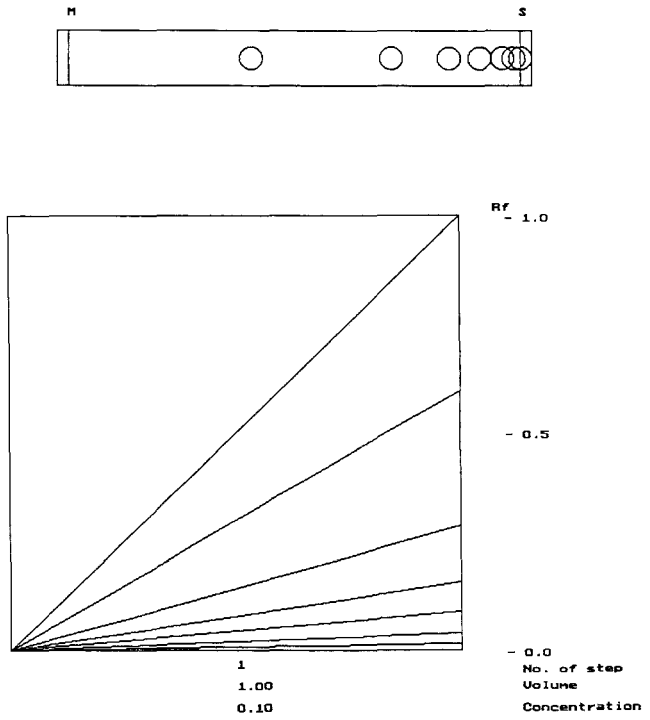

Fig.1. Computer simulated migration paths and chromatogram of ten phenolic components in system I obtained by isocratic development.

In both systems good selectivity is noticed relative to all pairs solutes.

Let us consider the isocratic mode of development for the system I. The optimal range of k values for most analysis by TLC is from k = 0.75 to k = 10 (this corresponds to R_F = 0.57 and R_F = 0.09). The solute of code 1 is the most strongly retained component of mixture. The

lowest concentration of modifier giving optimal range of k for the most strongly retained component is $\Phi = 0.46$ ($R_F = 0.10$). At this concentration the solutes of codes 5 to 10 are over the optimal range (Table 2). For the concentration of modifier higher than $\Phi = 0.46$ the components of codes 7, 8, 9, 10 have values k lower than 0.75 and will be accumulated up at the front of the mobile phase. When the concentration of modifier is lower than 0.46, the successive components of mixture have values of k higher than 10. From the above analysis it results that the isocratic mode of development is not satisfactory for the investigated components. Similar conclusion can be drawn from the analysis for system II. Table 2 presents the R_F values calculated for the minimal concentration of modifier, which gives optimal range for the most strongly retained components in system I and II. The quality of such chromatograms from the point of optimal range k values is not good considering all components.

The application of the stepwise gradient should improve the distribution of solutes on chromatogram.

For gradient application the main parameters having influence on the final values of R_F are: the initial concentration of modifier, the way of changing the concentration, the number of the steps and the volume of steps. The computer program which is able to predict the final values of R_F is very useful to simulate the gradients with many different values

TABLE 2

The R_F Values (>0.05) of Investigated Solutes for the Minimal Concentration of Modifier.

Code	System I	System II
	c_{mod} = 0.85	c_{mod} = 0.46
1	0.04	0.10
2	0.06	0.17
3	0.12	0.23
4	0.14	0.39
5	0.32	0.60
6	0.36	0.74
7	0.54	0.82
8	0.57	0.84
9	0.71	0.86
10	0.78	0.91

of parameters. The results of simulations of different gradients applied to system I and II are presented in Figures 2 and 3 and in Table 3.

At first, the simplest two-step gradient was considered and the system I was chosen. It was assumed that least retained solute should

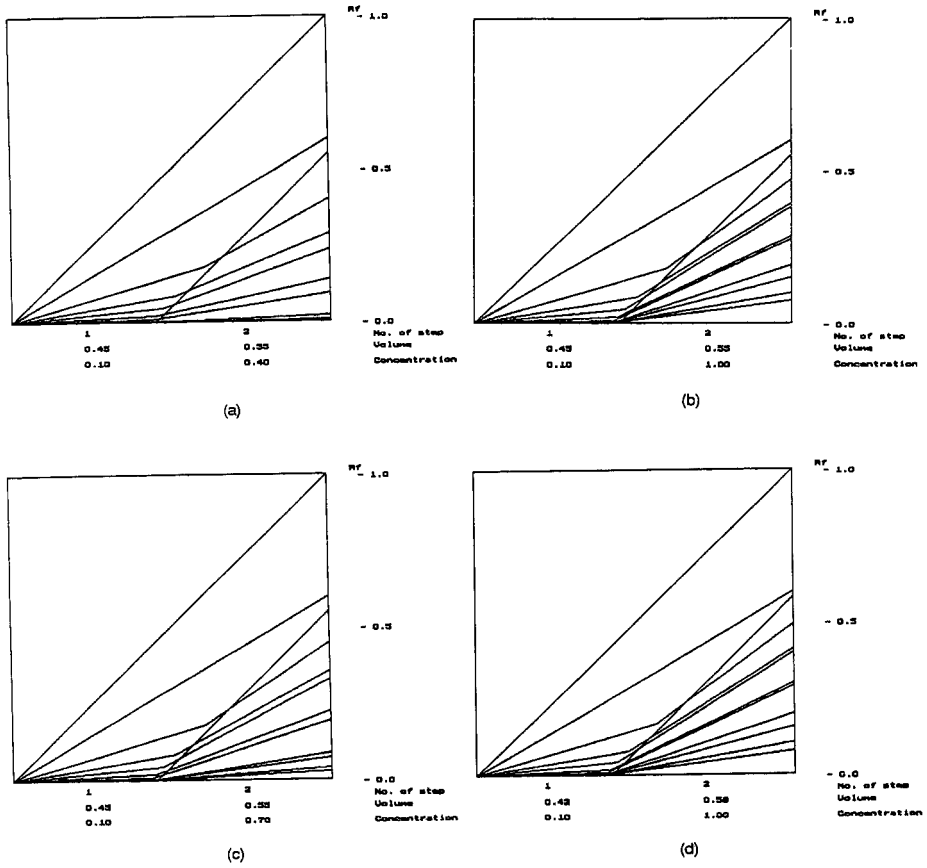

Fig.2. Computer simulated migration paths of ten phenolic components for different programs of gradient in system I.

have the R_F value equal to 0.6. The initial concentration of modifier can be calculated by equation (1) with the known values of k_o and m. It was Φ = 0.4 for system I. The simulation of the isocratic development with concentration 0.4 of modifier shows separation of solutes. Only the

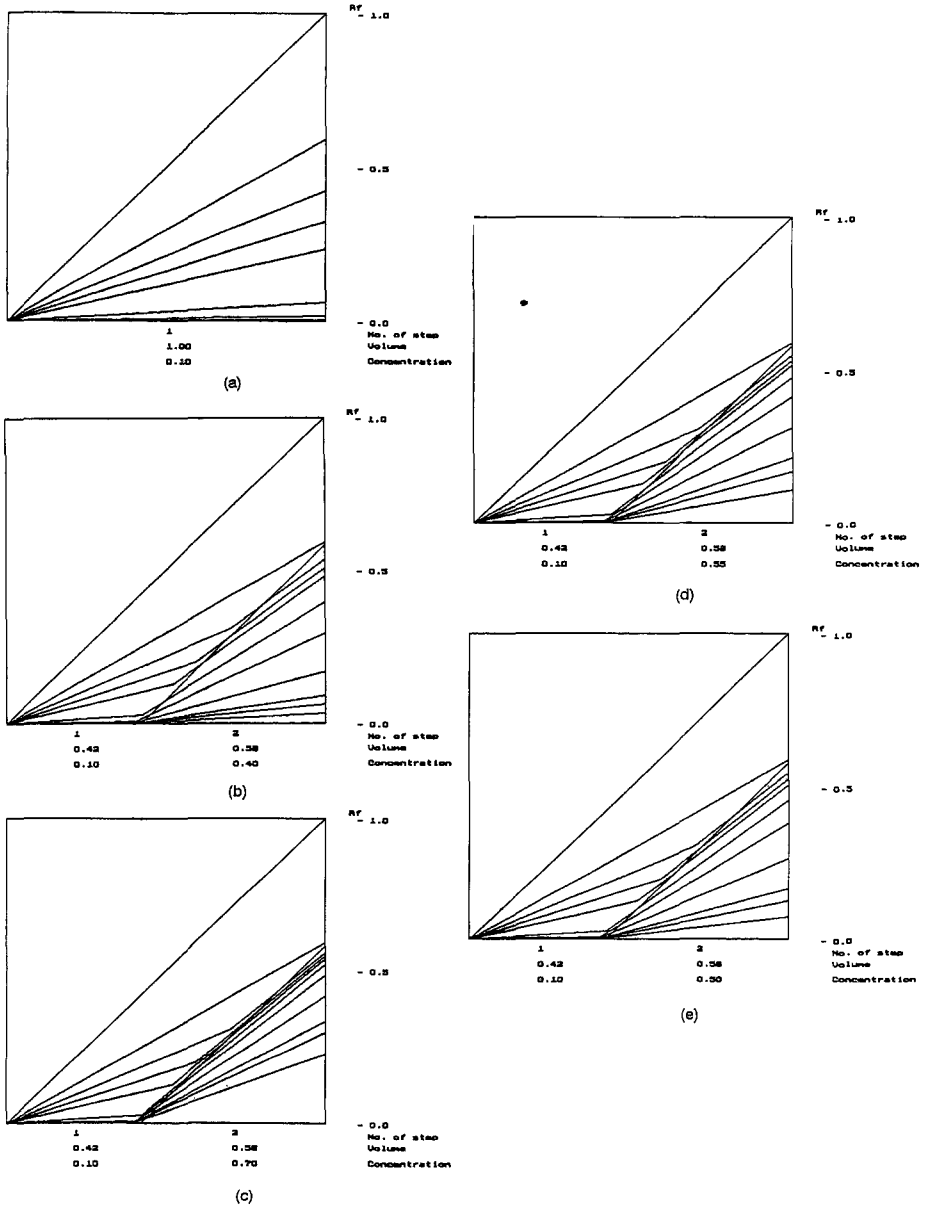

Fig.3. Computer simulated migration paths of ten phenolic components for isocratic and gradient developments in system II.

TABLE 3

The Characteristic of Simulated Separation in 1D Mode Obtained by the Isocratic and the Gradient Development.

No.	Figure	The range of R_F	Mode	Quality of chromatograms	
				D^{1D}_A	MPC [%]
1.	1	0.00 ÷ 0.60	ISO	3.3249	0.00
2.	2a	0.01 ÷ 0.60	GRAD	3.5916	0.00
	2b	0.03 ÷ 0.60	GRAD	3.3221	0.93
	2c	0.07 ÷ 0.60	GRAD	2.6076	3.97
	2d	0.08 ÷ 0.60	GRAD	2.6224	4.48
3.	3a	0.00 ÷ 0.59	ISO	4.1484	0.00
	3b	0.03 ÷ 0.59	GRAD	4.1724	8.07
	3c	0.23 ÷ 0.59	GRAD	1.4176	1.35
	3d	0.11 ÷ 0.59	GRAD	2.7669	10.76
	3e	0.08 ÷ 0.59	GRAD	3.2581	19.93

solutes of codes 7 to 10 have R_F values from optimal range and between solutes of codes 9 and 10 there is large gap (0.3 R_F unit).

From Fig. 2a, it follows that only solute of code 10 should be eluted in the first step. The volume of this step is 0.45. The next step is to choose the concentration of modifier for the next step and check if it is adequate and if all solutes are in the optimal range. If this condition is

fullfilled then the program is satisfactory. Next simulations can only refine the program. In other case, when two-step gradient is insufficient, the procedure is repeated until the suitable program is found.

Fig. 2a, b, c shows successive steps in choosing the gradient program. Fig. 2d presents refined separation. Similar simulation was done for the system II. The results are presented in Fig. 3a-e. In this system, the concentration of modifier in the second step $\Phi = 0.7$ was too high and in the next simulation, the values from the range $0.4 \div 0.7$ was chosen. It follows from above simulations that two-step gradient efficiently separates ten solutes in the optimal range of k.

The chromatograms obtained can be evaluated visually and with suitable quantitative criteria. In our case three criteria were used: optimal range of R_F values, the D criterion (the higher D value the better the separation) and the MPC criterion proposed by De Spiegeleer [15]. The results are presented in Table 3. The fulfillment of all three criteria in a satisfactory degree indicates a good result. In the case of system I the results represented in Fig. 2d may be considered satisfactory in spite of low value of MPC which is explained by low selectivity of the system relative to some pairs of components. It follows from Figs. 3 a-e and Table 3 that the use of system II for the separation of the investigated ten - component mixture gives considerably better results. The chromatograms obtained are characterized by considerably better values of criteria, both D as well as MPC. The chromatogram shown in Fig. 3e secures optimal

range of R_F values, moderately high D index and the highest MPC index which characterizes the regular distribution of spots in the required range of R_F values.

What are the advantages of combining isocratic and gradient development in the two - dimensional (2D)technique? Gradient elution permits the choice of such a programme that the investigated solutes of wide range of polarities are distributed along the whole distance of development. If the system chosen is highly selective relative to each pair of components, then in the second (orthogonal) distance a isocratic eluent selective to the most difficult pair (or several pairs) of components can be chosen. Another solution consists in consecutive steps corresponding to most selective systems for the individual difficult pairs. The simulations of 2D developments are presented in Fig.4-7 and Table 4. The latter lists the values of criterion of evaluation of chromatogram calculated according to eq.11 assuming equal development distances (10 cm) in direction X and Y.

It is known that the use of the same system for 2D development results in insignificant improvement of separation efficiency; $R_S^{2D} \approx R_S^{1D} \sqrt{2}$ which is illustrated in Fig. 4; for instance, for solutes 1 and 2 the separation distance is 2 mm after 1D and and 2.83 mm after 2D development. A similar insignificant increase of distance is observed for solutes 7 and 8. Since selectivity was the same for both systems, the spots are located after 2D development on a diagonal line.

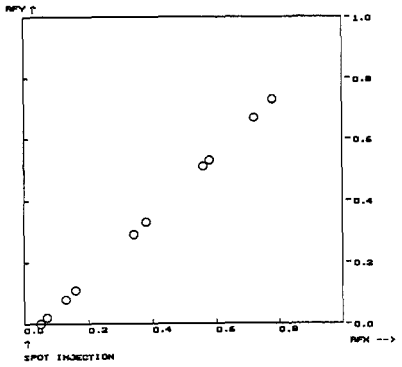

Fig. 4.

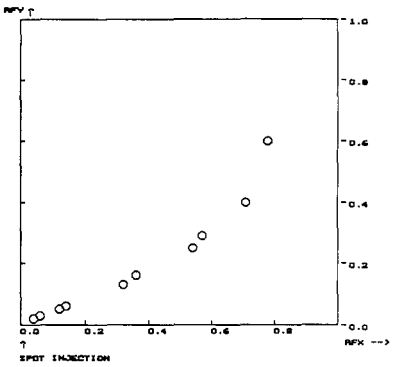

Fig. 5.

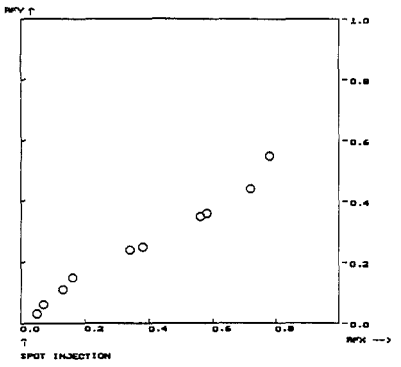

Fig. 6.

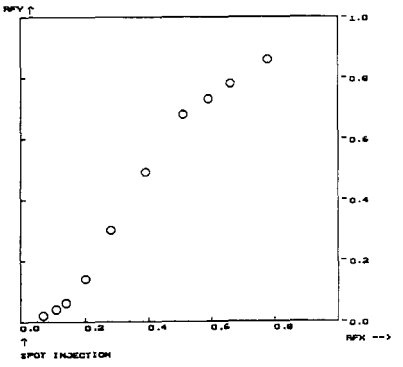

Fig. 7.

Fig.4 to Fig.7. Computer simulated chromatograms for different combinations of development mode.

Fig. 5 illustrates the use of combined isocratic and gradient development. Isocratic elution distributes the components along the whole distance of development; the shortest distances are observed for solutes 1, 2 (2 mm) and 7, 8 (2 mm). The use of gradient results in increased distance between solutes 1 and 2 (by 3 mm) but only by 1 mm

TABLE 4

The Characteristic of Simulated Separation in 2D Mode Obtained by Combination the Isocratic and Gradient Development.

No.	Figure	Mode 2D	D^{2D}	System
1.	4	ISO - ISO	133 082	I
2.	5	ISO - GRAD	92 765	I
3.	6	ISO - GRAD	99 573	I
4.	7	GRAD - ISO	158 561	II

between solutes 7 and 8. The final distances between solutes 1 and 2 are 3.61 mm and between 7 and 8 2.36 mm. On the other hand, distances between the remaining components are markedly increased.

Fig. 6 illustrates the case when the pair 7 + 8 is not separated but the components are eluted in the suitable range of R_F values. The choice of gradient programme in which the proper pair 7, 8 will be separated improved the overall separation too; the final distance between 7 and 8 is increased from 0 to 2 mm.

Further simulations in the system Iso-Grad permitted to get a still better final separation. Fig. 7 illustrates the result of Iso-Grad 2D development in which the distance between solutes 7 and 8 is increased to 5 mm.

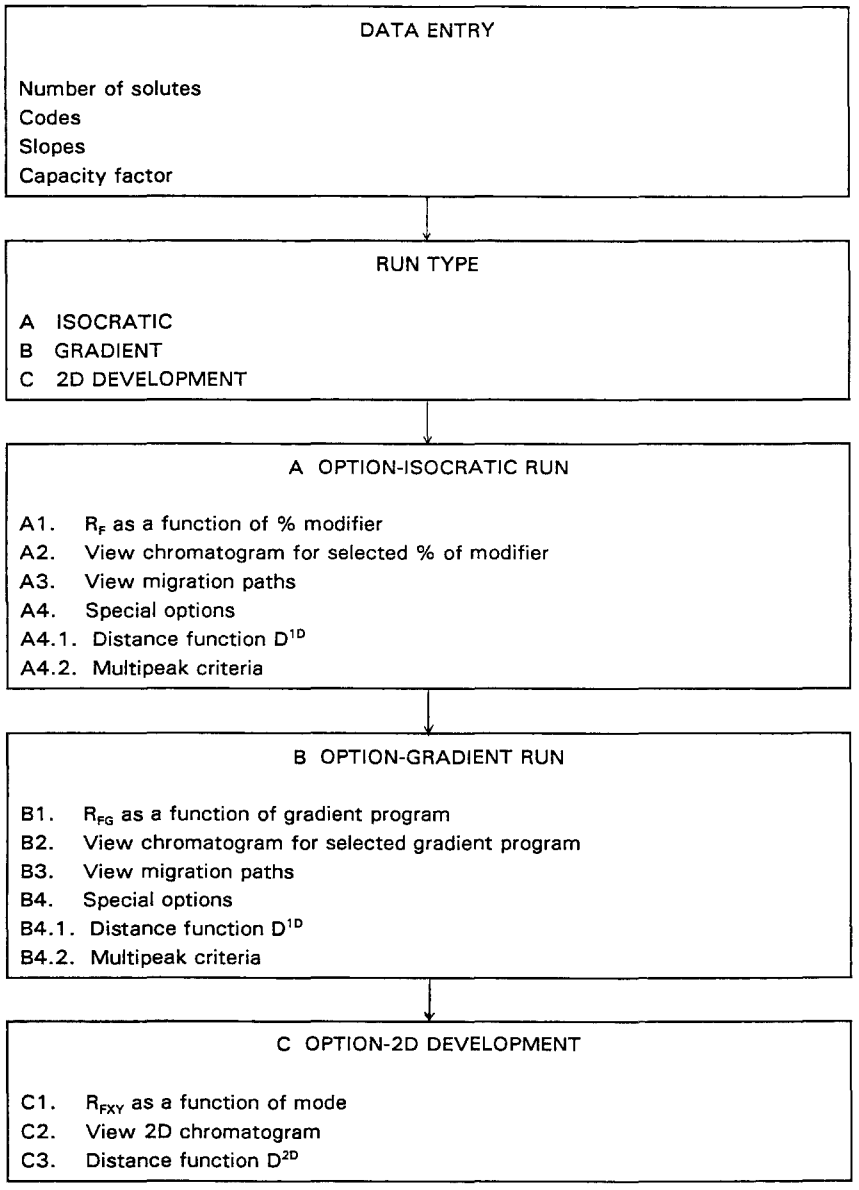

Fig.8. Flow diagram of computer program used to simulation of isocratic and gradient development in 1D and 2D.

Similar simulations were applied to system II where for 1D gradient elution was used, followed by isocratic elution for the second dimension. All pairs are well separated which is confirmed by the high value of D^{2D} = 158 000.

The programme elaborated for the isocratic and the gradient development in combination permits:

- the simulation of 2D development
- obtaining the diagram of 2D chromatograms
- calculation of values used for the estimation of chromatograms

The application of 2D simulation can be especially useful in the case of multicomponent mixtures of wide range of polarity to utilize the high capacity offered by 2D-TLC. It follows also from the above simulations that the investigated mixture of ten phenolics can be separated using a simple stepwise gradient or its combination with isocratic elution without necessity of use of time - consuming multiple development.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF NEW 5-ARYLOXY-METHYL-2-AMINO-2-OXAZOLINES: A COMPARATIVE STUDY OF THEIR LIPOPHILICITY

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ABSTRACT

The capacity factors (log k') for a series of bioactive 5-aryloxymethyl-2-amino-2-oxazolines were determined by a high-performance liquid chromatography method using methanol-water as a mobile phase and ODS column as stationary phase. The influence of mobile phase composition was examined, allowing the determination of log k'_w values through extrapolation to 100% water from capacity factors data. The influence of the apparent pH of the mobile phase was studied and discussed in terms of solute ionization. The partition coefficients (log $P_{o/w}$) and ionization constants (pK_a) of all the compounds were measured by classical methods permitting to correlate the different lipophilic indexes.

INTRODUCTION

The hydrophobic property of bioactive compounds, expressed by the 1-octanol/water partition coefficient ($P_{o/w}$), is one of the most important factors to be considered in quantitative structure-activity relationship

(QSAR) studies (1,2). For a number of years, chromatographic methods especially RP-HPLC have been proposed to determine the lipophilicity of drugs (3-6). An interrelationship between the partition coefficient P and the chromatographic column capacity factor k' in RP-HPLC has been established in a Collander-type equation (7).

We recently developed such a chromatographic approach of the lipophilicity applied to new series of 2-amino-2-oxazolines bearing different substituents on the heterocyclic ring (8,9).

The aim of the present study was to study the lipophilicity of new bioactive 5-aryloxymethyl-2-amino-2-oxazolines by mean of a RP-HPLC technique. The reliability of this methodology is checked by correlation of the k' data with the parameter P measured by the classical "shake-flask" method. The influence of the pH eluent on the capacity factor was studied by working at different pH values. The observed variations were related to the pK_a's values of 5-aryloxymethyl-2-amino-2-oxazolines determined by a potentiometric method.

MATERIALS AND METHODS

Apparatus and chromatographic conditions.

Chromatography was performed with a Waters Assoc. apparatus equipped with a Model 501 pump, a Model 455 ultraviolet detector operating at 254 nm and an U_6K manuel injector. The compounds were chromatographed on an Inertsil ODS2 column (250 mm x 4.6 mm, 5 µm particle size)(Interchim). The mobile phase composition ranged from 30 to 50 % (v/v) methanol with 0.06 M phosphate buffer at various pH (7.4 ; 8 ; 9). The flow rate was 1 ml/min. The detector output was recorded on a Model 746 data Module integrator

Standards and reagents

New 5-aryloxymethyl-2-amino-2-oxazolines were synthesized by condensation of monosodium cyanamide with the corresponding 1-aryloxymethyl-2,3-epoxypropanes (10). Their structures were supported by elemental analysis, IR, ^1H and ^{13}C NMR spectral data. Stock

solutions containing 1mg/ml of each drug were prepared in methanol and stored at -20°C.

HPLC-grade methanol (Prolabo) was used without further purification to prepare the mobile phase and water was glass-distilled deionized. To prepare the phosphate buffer solutions potassium dihydrogen phosphate and dipotassium hydrogen phosphate trihydrate (Merck) were used. Before use for HPLC, the mobile phase was filtered through a 0.45 μm membrane filter.

Measurement of log k'

The column dead-time of the system (t_0) was measured as the time from injection to the first distortion of the baseline after injection of pure water. Consequently, the stock solutions of tested compounds were diluted with water to the final injected concentrations of 50 μg/ml. According to their chromatographic behaviour, the retention time (t_r) of each compound was determined at six different methanol-phosphate buffer mixtures ranged from 30 to 50%. The compounds were injected separate from each other three times and the mean value of the retention time was retained. The log k'_w value for each compound was obtained by regression analysis of log k' data, expressed from the retention times t_r, through the formula : $k' = (t_r - t_0) / t_0$, and extrapolation to 0% methanol content.

The correlation/regression analysis were carried out with a statistical program on a Vectra computer (Hewlett Packard).

Measurement of pK_a

The pK_a's determinations were performed using a classical potentiometric method described elsewhere (11).

Determination of log P

The octanol-water partition coefficients ($P_{o/w}$) were determined by the classical "shake-flask" technique using a conventional methodology. Samples in a weight range of 5-10 mg were partitioned between 5 ml of n-octanol saturated with water and 50 ml of water saturated with n-octanol. The pH of the water phase was adjusted at 11, ensuring that all compounds were more than 99% unionized.

RESULTS AND DISCUSSION

The chemical formulae of the tested 5-aryloxymethyl-2-amino-2-oxazolines are given in Table 1.

Determination of log k'_w and S (slope of the regression analysis)

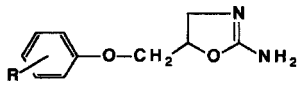
In this study we have chosen to measure the log k' value extrapolated to 0% of the organic modifier in the mobile phase (log k'_w). For some authors (3,12), this technique (polycratic method) allows more adequate evaluation of the hydrophobic nature of the solute and provides a scale of lipophilic parameters normalized to one set of conditions more closely related to log $P_{o/w}$. In these conditions, the test compounds were chromatographed under a variety of conditions in which the percentage of methanol varied from 30 to 50%. Experiments with lower percentages of methanol than 30% afforded experimental retention times too long to be measured, mainly at pH 9. For all compounds, linear relationships ($r > 0,98$) were proved to exist between the log k' values and methanol concentrations, allowing the calculation of log k'_w and S through extrapolation (Table 1). The slopes S for the equations were mostly constant. Statistically significant correlations were noted between log k'_w and S at each pH ($r = 0.919$, 0.877 and 0.917 - pH = 7.4, 8 and 9). These results may be related to the structural similarity of all tested compounds in regard to their partition behaviour in RP-HPLC.

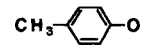
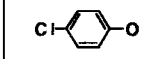
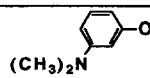
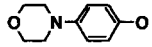
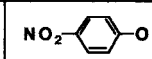
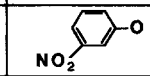
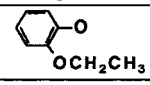
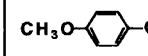
Effect of the pH eluent on log k'_w

The influence of solute ionization on RP-HPLC determination of capacity factors has been often discussed (4,5,13,14). In general, for the determination of hydrophobicity, the unionized form of solutes is taken as the reference state.

In order to study the variation of log k'_w in terms of ionization, the pK_a's determinations have been performed (Table 1). 5-aryloxymethyl-2-amino-2-oxazolines are basic molecules ($pK_a = 8.49 \pm 0.16$, variation coefficient 2.6%). These pK_a values are comparable with those of other 5-substituted-2-amino-2-oxazolines, indicating a slight influence of the

TABLE 1 : ANALYTICAL DATA OF 5-ARYLOXYMETHYL-2-AMINO-2-OXAZOLINES



N°	SUBSTITUENT	pH 7,4 logK'$_w$	Slope	pH 8 logK'$_w$	Slope	pH 9 logK'$_w$	Slope	log $P_{o/w}$	pK_a
1		1.804	-0.029	1.910	-0.026	1.998	-0.031	1.27	8.59
2		2.242	-0.033	2.115	-0.027	2.394	-0.034	1.79	8.52
3		1.977	-0.031	2.020	-0.028	2.512	-0.037	1.69	8.54
4		2.264	-0.032	2.503	-0.033	2.513	-0.035	2.00	8.71
5		2.037	-0.030	2.136	-0.030	2.374	-0.035	1.77	8.67
6		2.106	-0.031	2.318	-0.032	2.427	-0.038	1.40	8.49
7		1.537	-0.026	1.607	-0.027	1.612	-0.028	0.60	8.39
8		1.711	-0.028	1.700	-0.024	2.103	-0.034	1.17	8.35
9		1.695	-0.027	1.784	-0.027	2.030	-0.033	1.23	8.10
10		1.948	-0.030	1.966	-0.028	2.145	-0.033	1.24	8.52
11		1.739	-0.030	1.618	-0.024	1.881	-0.031	1.26	8.42
12		1.545	-0.027	1.587	-0.026	1.825	-0.031	0.85	8.56

nature of the 5-substituted moiety (7,8). For compounds 1-12, no particular influence due to the nature or the position of the substituent on the phenyl ring was observed.

For all compounds the ionization percentage is near 90% at pH 7.4, 75% at pH 8 and below 25% at pH 9. The variations of the log K'_w values with the apparent pH eluent (Table 1) are consistent with the ionization effects. At pH 9 the compounds were less ionized, they showed less hydrophilic properties and thus the capacity factor was maximum. On the contrary the capacity factor should be minimum at lowest pH. Nevertheless between pH 7.4 and 8 the increase of log k'_w was not always regular (i.e. compounds 2, 8, 10), suggesting particular ionization effects.

Correlation between lipophilic indexes

The log k'_w were correlated with log $P_{o/w}$, according to equations I, II and III for the experimental data listed in Table I

I pH 7.4 $\log k'_w = 0.561\ (\pm 0.082) \log P + 1.123\ (\pm 0.116)$
$(n = 12, r = 0.907, s = 0.110, F = 46.60, p \leq 0.0001)$

II pH 8 $\log k'_w = 0.606\ (\pm 0.129) \log P + 1.117\ (\pm 0.182)$
$(n = 12, r = 0.829, s = 0.173, F = 22.07, 0.0001 \leq p \leq 0.005)$

III pH 9 $\log k'_w = 0.668\ (\pm 0.093) \log P + 1.245\ (\pm 0.131)$
$(n = 12, r = 0.915, s = 0.168, F = 51.85, p \leq 0.0001)$

where n is the number of data, r is the correlation coefficient, s is the standard error of the estimate, F is a measure of the significance of the correlation and p is the probability level.

A definite correlation was established among the hydrophobic parameters ; it was better when log k'_w measured at pH 9 was used.

The apparent partition coefficient D has been calculated at the eluent pH, based on the knowledge of the acid dissociation constants :

$$D = \frac{P}{1 + [(H^+)/ Ka_1]}$$

In order to take into account the basic behaviour of the molecules, the correlations between log k'_w and log D were established. The substitution of log D instead of log P did not improve the correlation, especially at pH 7.4 and 8 (r = 0.828, 0.750 and 0.914 for pH 7.4, 8 and 9 respectively). This result could be related to the ionization effects already noticed during the experiment.

CONCLUSION

The influence of solute ionization on RP-HPLC determination of log P is obvious. In general, for the determination of hydrophobicity, the unionized form is taken as the reference state. This can be difficult to obtain retention data for acids and bases in their uncharged state due to the limited pH operating range of silica bonded phases.

In order to study the ionization effects of 5-aryloxymethyl-2-amino-2-oxazolines we determined the log k'_w at pH operating range near the ionization constant value (pK_a). We chose an ODS column with a stationary phase claimed to be stable up to pH 9 (with a phosphate buffer/methanol eluent).

The results indicated that the increase of log k'_w with the apparent pH eluent was consistent with the expected ionization effects for these basic compounds. However it should be noticed that the studied amines were all structurally related, and had nearly identical pK_a values.

From the octanol/water partition coefficients, satisfactory correlations among the hydrophobic parameters have been established. In particular the correlation coefficients calculated for the experiments at pH 7.4 and pH 9 were not greatly different ; thus it should be tempting to use the log k'_w values measured at physiologic pH for our further QSAR studies.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF MONO- AND OLIGOSACCHARIDES DERIVATIZED WITH *P*-AMINOBENZOIC ETHYL ESTER ON A C_{18}-BONDED SILICA COLUMN

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ABSTRACT

This report examines a behavior of mono- and oligosaccharide-ABEE derivatives in RP-HPLC. Sugar-ABEE derivatives are saparated by reverse-phase partition chromatography rather than normal-phase partition chromatography. Reducing sugars are derivatized with *p*-aminobenzoic ethyl ester(ABEE) for the UV detection at 254 nm. C18-bonded silica column is used for the separation of sugar-ABEE derivatives in an isocratic mode. RP-HPLC is performed by using ternary mixture as a mobile phase and column temperature is maintained at 45°C. Sugar-ABEE derivatives are separated well within a short run time(*ca.* 25 min) by reverse-phase partition chromatographic mode, and C18-bonded silica column is very stable during the analysis. The (1→6)-linkage type of dihexose-ABEE derivatives has shorter retention time than (1→4)-linkage type. β-Anomeric configuration of [Glc-β-(1→4)Glc]-oligomer has shorter retention time than α-anomer in RP-HPLC. Column

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temperature and ionic strength slightly affect the elution behavior of sugar-ABEE derivatives.

INTRODUCTION

Saccharides are very abundant in nature and important in biology, medicine, pharmacology, and food industry. Traditionally, paper[1] and thin-layer chromatography[2] have been used to separate the saccharides, but these techniques have poor resolution and are not always quantitative[3]. Although gel filtration[4] and ion-exchange column chromatography[5] have been used to obtain large amount of sugars, they are not adequate for microanalysis and require very long analysis time. Gas-liquid chromatography with flame ionization detector or mass spectrometer has been used for the analysis of saccharides. This methodology is very powerful for the structural analysis, but it needs very tedious derivatization steps before the analysis and is not applicable to the purification of the sample because of the destructive nature of the method [6].

Nowadays, high-performance liquid chromatography has been used as a powerful technique to separate the saccharides. For the separation, various stationary phase have been used include silica gel[7], amine-bonded silica[3,8-11], polyfunctional amine modified silica[12-14], polystyrene-based anion-[15] and cation-exchange[16] resin, cyano-bonded silica[17], and C18-bonded silica [18-24]. Although the breakthrough has been achieved with the introduction of amine-bonded phase for the seaparation of saccharides, there are many disadvantages; their short life time because of the formation of Shiff's bases between the sugars and the amine-bonded phase[13,19,21], the limited saccharides solubility in mobile phase due to higher organic solvent contents demand as an eluent [13,22], and their low efficiencies. Ion-exchange resin is very useful for the separation of saccharides, but its throughput is low and it gives poor resolution for the higher oligosaccharides[22].

There is another problem in saccharides separation with high-performance liquid chromatography because saccharides have no chromophores or fluorophores. Although underivatized saccharides have been detected by refractive index detector[6,11,14], it is insensitive, requires pulseless pump for low noise levels and can not be used for gradient mode[6]. Many researchers have derivatized the saccharides by using pre- or post-column derivatization for more sensitive detection[18,23-27].

In this report, mono- and oligosaccharides are derivatized with ABEE in the presence of sodium cyanoborohydride for the detection and separated by C18-bonded silica column[18]. The seperation behavior of mono- and oligosaccharide-ABEE derivatives is studied by varying mobile-phase composition, column temperature, and ionic strength in RP-HPLC. All sugar-ABEE derivatives are saparated well by reverse-phase partition chromatographic mode rather than normal-phase partition chromatographic mode.

MATERIALS AND METHODS

Materials

Mono- and oligosaccharide standards, ABEE, sodium cyanoborohydride, sodium chloride and sodium acetate trihydrates were obtained from Sigma Chemical Co. (St. Louis, MO). HPLC-grade acetonitrile and methanol were purchased from Baxter Healthcare Corp. (Muskegon, MI). HPLC-grade distilled, deionized water (Millipore, Bedford, MA) was used. All other chemicals and solvents were of analytical reagent or HPLC grade.

Derivatization of mono- and oligosaccharides with p-aminobenzoic ethyl ester

The procedure employed for derivatization of mono- and oligosaccharides at their reducing end with ABEE was done by the method of Wang *et al* . [27]. The sample solution was made by dissolving 100 μmole sugars in 10 ml water. The ABEE reagent was made by mixing 165mg ABEE, 35 mg sodium cyanoborohydride, 41 μl glacial acetic acid, and 0.35 ml warm methanol. 40 μl of the ABEE-reagent was added to a 6 x 50 mm glass tube (Corning 9820, Corning, NY) containing 10 μl of sample solution and reacted at 80°C. After 1 h, the reaction mixture was cooled to room temperature. The distilled water (0.2 ml) and equal volume (0.2 ml) of chloroform were added to the reaction mixture. After vigorous vortexing, it was centrifuged for 1 min. The upper aqueous layer was subjected to HPLC analysis.

Reverse-phase high-performance liquid chromatography of sugar-ABEE derivatives

A Hewlett-Packard HP 1050 Series system (Hewlett-Packard, Avondale, PA) was used. Detection was performed with a Hewlett-Packard HP 1050 variable wave length detector at 254 nm. The Rheodyne injector was used with

a 10 μl sample loop and the data was collected by using a Hewlett-Packard HPLC ChemStation system. A 3.9 x 150 mm Pico · Tag column (Waters, Milford, MA) was used.

Samples were injected onto a Pico · Tag column (3.9 x 150 mm, Waters) at 45°C, if not specified otherwise. Elution (1.2 ml/min) was performed isocratically with Solvent A(Sol A) (50 mM sodium acetate buffer, pH 4.5 with glacier acetic acid) and Solvent B(Sol B) (50 mM Sodium acetate buffer, pH 4.5 with glacial acetic acid / acetonitrile / methanol = 40 / 40 / 20) in a ratio of Sol A / Sol B = 80 / 20 (%,v/v), if not specified otherwise.

RESULTS AND DISCUSSION

High-performance liquid chromatography has been used as a powerful technique to separate the saccharides. In this report we have described a behavior of mono- and oligosaccharide-ABEE derivatives in RP-HPLC. We have derivatized the sugars with ABEE in the presence of sodium cyanoborohydride for the UV detection [18,27]. This procedure is simple and only chloroform extraction is required for removal of excess ABEE. Although underivatized sugars can be detected by refractive index detector, sugar-ABEE derivatives are detected more sensitively and separated with high resolution [18].

To study the behavior of sugar-ABEE derivatives on a C18-bonded silica column, ternary mixture of aqueous buffer, methanol, and acetonitrile was used as a mobile phase. When we used the optimized condition for resolving of sugar-ABEE derivatives, many sugars were separated very well within a short run time (*ca.* 25 min) with high reproducibility (Figure 1). As shown in Figure 1A, the blank chromatogram had a very good baseline with few side products formed during the derivatization procedure. Many amino- and neutral saccharide-ABEE derivatives were separated in a single chromatography (Figure 1B and 1C) and Gal, Glu, and Man were separated each other when Sol A / Sol B = 90 / 10 (%,v/v) was used as a mobile phase [18]. The shape of peaks was very sharp and was not a doublet which was usually found in the separation of underivatized sugars [8,21]. There were many advantages in derivatization of saccharides with ABEE in the presence sodium cyanoborohydride as shown in Figure 1; few side products in background(Figure 1A), higher sensitivity(*ca.* 50 pmol) [18], and elimination of the possible doublet, which could be formed by

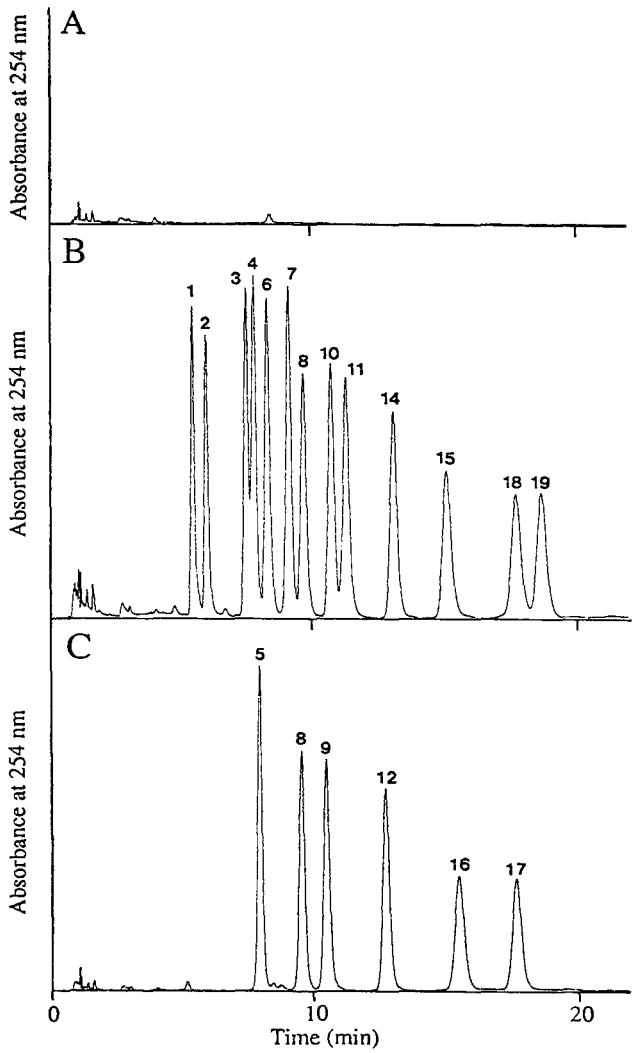

FIGURE 1. Separation of mono- and disaccharide-ABEE derivatives by RP-HPLC. Conditions for RP-HPLC are described under Experimental. (A) Chromatogram of blank reaction. Derivatization step was performed without sugar component. (B) and (C) Chromatogram of mono- and disaccharide-ABEE derivatives. The identity of sugars was as follows; 1. glucosamine(GlcN); 2. galactosamine(GalN); 3. meliobiose(Mel); 4. lactose(Lac); 5.isomaltose(Iso); 6. gentiobiose(Gen); 7. cellobiose(Cel); 8. maltose(Mal); 9. galactose(Gal); 10. glucose(Glc); 11. mannose(Man); 12. arabinose(Ara); 13. ribose(Rib); 14. xylose (Xyl); 15. *N*-acetylglucosamine(GlcNAc); 16. *N,N'*-diacetylchitobiose(Chi); 17. *N*-acetylgalactos amine; 18. fucose(Fuc); 19. rhamnose(Rha); 20. 2-deoxyglucose(DeGlc). The amount of each sugar was 5.0 nmole.

TABLE I
RETENTION TIME OF MONO- AND DISACCHARIDE-ABEE DERIVATIVES

ABEE derivatives	Structure	Retention time (min)	
		SolA/SolB=80/20	SolA/SolB=75/25
Monoaminohexose-ABEE			
1. Glucosamine	GlcN	5.3	3.7
2. Galactosamine	GalN	5.8	4.0
Dihexose-ABEE			
3. Meliobiose	Galα1-6Glc	7.4	4.7
4. Lactose	Galβ1-4Glc	7.8	4.9
5. Isomaltose	Glcα1-6Glc	7.9	5.0
6. Gentiobiose	Glcβ1-6Glc	8.2	5.1
7. Cellobiose	Glcβ1-4Glc	9.0	5.0
8. Maltose	Glcα1-4Glc	9.7	6.0
Monohexose-ABEE			
9. Galactose	Gal	10.7	6.8
10. Glucose	Glc	10.8	6.8
11. Mannose	Man	11.4	7.2
Monopentose-ABEE			
12. Arabinose	Ara	12.8	8.1
13. Ribose	Rib	13.0	8.1
14. Xylose	Xyl	13.2	8.2
Mono- and Diacetylatedamino hexose-ABEE			
15. *N*-acetylglucosamine	GlcNAc	15.0	8.8
16. *N,N'*-diacetylchitobiose	GlcNAcβ1-4GlcNAc	15.6	8.2
17. *N*-acetylgalactosamine	GalNAc	17.9	10.2
Deoxyhexose-ABEE			
18. Fucose	Fuc	18.0	10.8
19. Rhamnose	Rha	18.6	11.3
20. 2-deoxyglusose	2DeGlc	22.0	13.0

mutarotation of free reducing end, by using the reductive amination of reducing end with ABEE.

To study the behavior of sugar-ABEE derivatives in RP-HPLC, mobile phase composition was changed (Table I). Although many amine- and C18-bonded silica columns were used for sugar separation, most of them were performed by normal-phase partition chromatographic mode[4,11-14,24-26]. As shown in Table I, all sugar-ABEE derivatives were eluted more rapidly as increasing organic solvent (Sol B). These behaviors mean that the separation of sugar-ABEE derivatives was performed by reverse-phase partition chromatography[18,24,25] rather than normal-phase partition chromatography.

Figure 2 showed the RP-HPLC elution profiles of mono- and oligo saccharide-ABEE derivatives separated at different column temperature. The retention time of sugar-ABEE derivatives was decreased as increasing column temperature. As shown in Figure 2, the retention times of sugar-ABEE derivatives were decreased as increasing the column temperature, but the elution profile of sugar-ABEE derivatives was scarcely affected by varying the column temperature in RP-HPLC. Although the elution time of derivatives was decreased as increasing the column temperature, many sugar-ABEE derivatives were separated well because the column efficiency was improved by increasing the rate of mass transfer of sugar-ABEE derivatives into C18-bonded stationary phase.

The retention times of mono- and disaccharide-ABEE derivatives separated with two mobile phase were summarized in Table I. When we examined the elution profile of sugar-ABEE derivatives, we found out the elution patterns of sugar-ABEE derivatives according to the basic structure of sugar moieties. Monoaminohexose-ABEE (GlcN and GalN) derivatives generally exhibited shorter retention times than dihexose-ABEE (Mel, Lac, Iso, and Gen) derivatives and were eluted in following monohexose-ABEE (Gal, Glc, and Man), monopentose-ABEE (Ara, Rib, and Xyl), *N*-acetylamionohexose-ABEE (GlcNAc, Chi, and GalNAc), and deoxyhexose-ABEE (Fuc, Rha, and DeGlc) derivatives. When we examined the elution profiles of dihexose-ABEE derivatives, we could find that (1→6)-linkage in the structure had shorter retention time than (1→4)-linkage and these results were comparable to the oligosaccharide separation profile on an amine-bonded silica column[8-11]. When amine-bonded silica column was used for separation, the (1→6)-linkage in structure had longer retention time than the (1→4)-linkage because sugar-ABEE derivatives[9]. When we examined the elution profile of

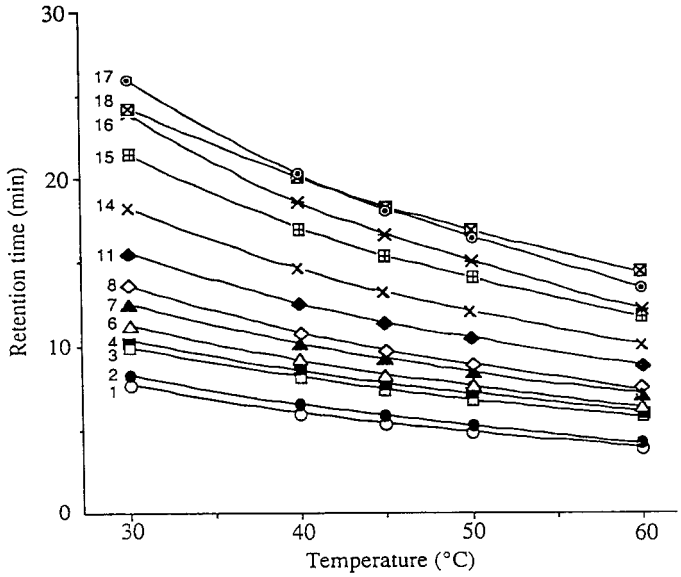

FIGURE 2. Effect of column temperature on retention time. Conditions for RP-HPLC are described under Experimental. The identities of the sugars are the same as those of FIGURE 1.

dihexose-ABEE derivatives, the effect of an anomeric configuration of the linkage was not clear. To obtain the information about the effect of anomeric configuration on separation of sugar-ABEE derivatives, we used malto oligosaccharide([Glc-α-(1→4)Glc])-ABEE and cellooligosaccharide([Glc-β-(1→4)Glc])-ABEE derivatives as a sample (Figure 3). The Glc-ABEE (DP=1) was eluted at the same retention time but cellooligosaccharide-ABEE (DP≥2) derivatives were eluted more rapidly than maltooligosaccharide-ABEE derivatives. In the ABEE derivatives of glucooligomers polymerized through (1→4)-linkage, the β-anomeric configuration of [Glc-β-(1→4)Glc]-oligomer was eluted more rapidly than α-anomer. These results confirmed that the linkage type and the anomeric configuration of the oligosaccharides were very important factors on separation [3, 9, 20].

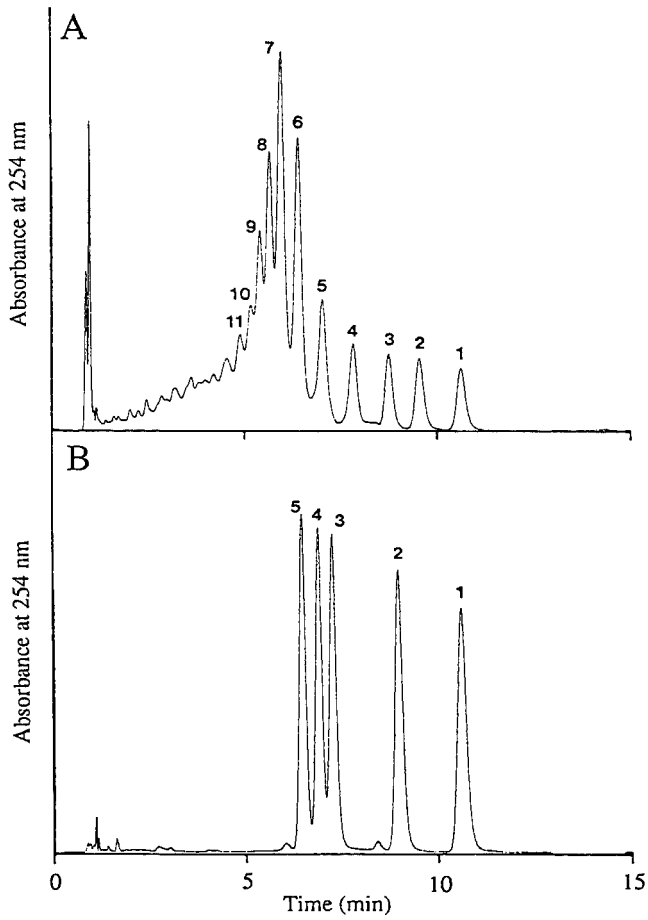

FIGURE 3. Separation of oligosaccharide-ABEE derivatives by RP-HPLC. Conditions for RP-HPLC are described under Experimental. (A) Maltooligosaccharide-ABEE derivatives. (B) Cellooligosaccharide-ABEE derivatives. The number on chromatogram indicate degree of polymerization (DP).

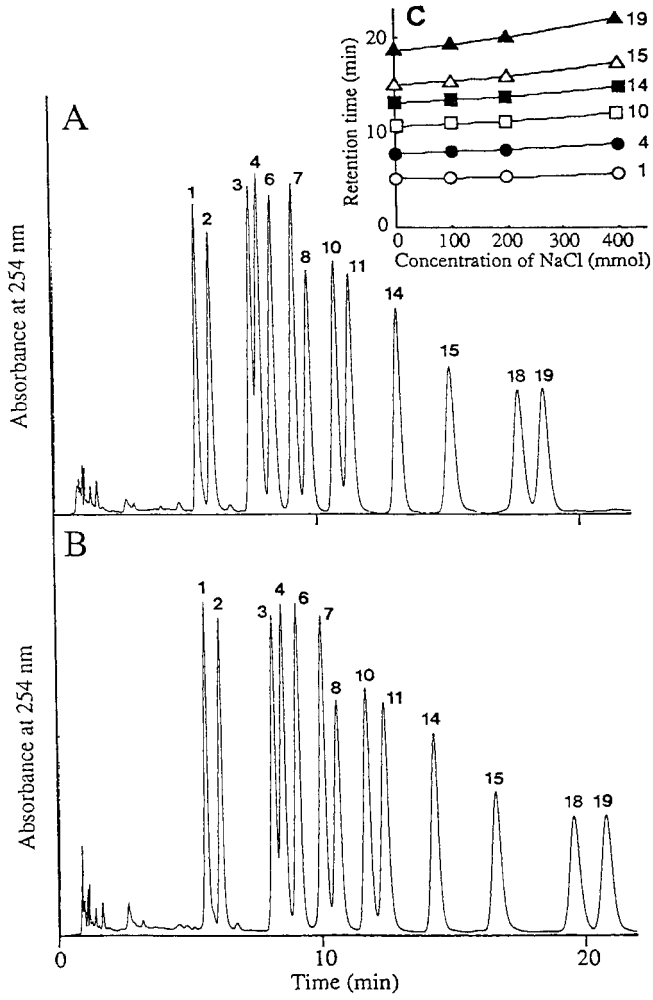

FIGURE 4. Effect of salt concentration on retention time. Conditions for RP-HPLC are described under Experimental. (A) 0 mmol NaCl in mobile phase. (B) 200 mmol NaCl in mobile phase. (C) Retention time profile of sugar-ABEE derivatives in proportion to increasing salt concentration. The identities of the sugars are the same as those of FIGURE 1.

C18-bonded silica column has the advantage of being pressure stable and readily available in suitable particle size and properties. Although there were lots of success of silica based packings for chromatography, there has been a number of occasions on which they were noted to fail dismally in achieving a separation of basic amino group containing compounds and all the problems of chromatography were assigned to the unbonded silanol groups. We examined the silanol group effect on separation by varying ionic strength because sugar-ABEE derivatives have a positive charges at pH=4.5, but the effect of ionic strength on retention time and peak shapes was not critical (Figure 4A and 4B). Although the pK_a of silanol groups is around 7.1[28], unbonded silanol groups are partilally ionized at pH=4.5[29]. If there were unexpected ionic interactions between unbonded silanol groups and positively charged sugar-ABEE derivatives, the retention time of positively charged molecules should be decreased, and then increased as increasing the salt concentration[29-31]. Figure 4C showed that the retention times of sugar-ABEE derivatives were only slightly increased because the solubility of sugar-ABEE in mobile phase was decreased due to the salting out effect[30], and unexpected silanol group effects on separation had to be eliminated by using sodium acetate buffer as a mobile phase component because sodium cations in mobile phase were powerful suppressors of free silanol groups[31].

CONCLUSIONS

This report has demonstrated behaviors of mono- and oligosaccharide-ABEE derivatives on a C18-bonded silica column. All sugar-ABEE derivatives were separated by reverse-phase partition mode rather than normal-phase partition condition. Sugar-ABEE derivatives were resolved well with a simple isocratic mode within a short run time(*ca.* 25 min). C18-bonded silica column is very stable for analysis of saccharides in contrast to widely used amine-bonded silica column[13, 19, 21]. The linkage type and the anomeric configuration of oligomers were important factors on separation of oligosaccharide-ABEE derivatives in RP-HPLC. The (1→6)-linkage type of dihexose-ABEE derivatives has shorter retention time than (1→4)-linkage type. β-Anomeric configuration of [Glc-β-(1→4)Glc]-oligomer has shorter retention time than α-anomer. Column temperature and ionic strength slightly affected the elution behavior of sugar-ABEE derivatives in RP-HPLC.

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AN IMPROVED ASSAY FOR ACADESINE (AICA-RIBOSIDE) IN HUMAN PLASMA USING LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC ELECTROCHEMICAL DETECTION

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ABSTRACT

A LC method using amperometric electrochemical detection has been developed for the quantitation of the adenosine regulating agent acadesine (AICA-riboside) in human plasma. Ultrafiltrates of plasma were chromatographed under isocratic conditions on a reverse-phase (C18) LC column using a mobile phase of 1.5% methanol in phosphate buffer at pH 6.3. The column eluant was monitored with a working electrode potential of +750 mV vs Ag/AgCl. Using a 250 μL sample of plasma for analysis the method has a validated limit of quantitation (LOQ) for acadesine of 6.25 ng/mL with a run-time of 12 minutes/sample. This new method provides a 20-fold decrease in the detection limit for acadesine compared to a previous procedure which employed UV detection.

INTRODUCTION

Acadesine (AICA-riboside; 5-amino-4-imidazole carboxamide ribonucleoside, Figure 1A) is a purine nucleoside analog with anti-ischemic properties that has been tested for the prevention of adverse cardiovascular outcomes in patients undergoing coronary artery bypass graft (CABG) surgery (1). Since acadesine has been viewed as a novel site and event-specific adenosine-

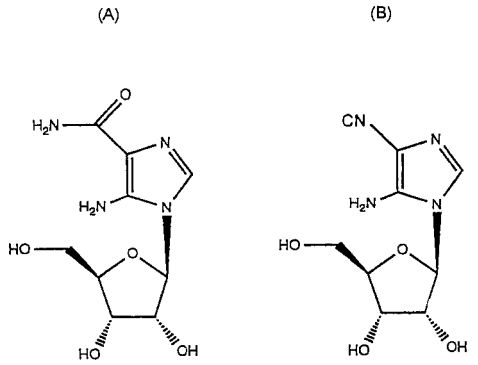

FIGURE 1. A: Structure of Acadesine (AICA-riboside)
B: Structure of compound A used as internal standard

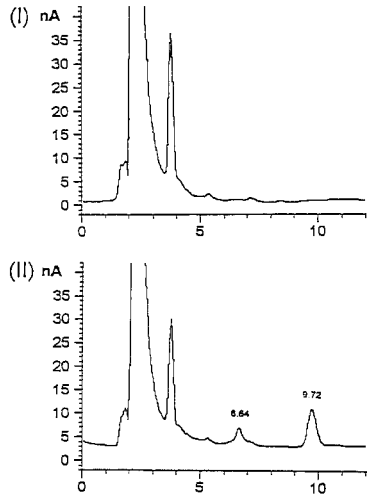

FIGURE 2. Chromatograms showing the retention time for acadesine and compound A (internal standard) in human plasma (I: Control; II: 50 ng/mL of acadesine Rt=6.64; compound A Rt=9.72)

regulating agent (ARA) that may be used for the treatment of certain cardiovascular disorders (2,3), the development of a sensitive method for the detection of acadesine in plasma is important.

A number of procedures have been described for the measurement of acadesine in biological fluids, including a high performance liquid chromatography method with spectrophotometric detection (4,5) and a colorimetric procedure (6). The limit of quantitation (LOQ) of both these assays is 125 ng/ml. In the present study we analyzed ultrafiltrates of plasma sample (4) in combination with amperometric electrochemical detection.

MATERIALS AND METHOD

All chemicals used were either HPLC or reagent grade. Methanol, water, and sodium hydroxide were obtained from Fisher (Fairlawn, NJ, USA). Phosphoric acid (85%, w/v) was purchased from Curtin Matheson Scientific (Houston, TX, USA). Acadesine (AICA-riboside) and GP115 (Figure 2) were obtained from Gensia, Inc.. (San Diego, CA).

The LC system consisted of a pump and autosampler (Hewlett-Packard HP1090, Wallbronn, Germany) and an amperometric electrochemical detector (HP1049A, Wallbronn, Germany). The working electrode potential was set at 750 mV vs Ag/AgCl (3 M KCl). Samples were chromatographed on a Beckman Ultrasphere C-18, 4.6 x 250 mm, 5 μ column (Beckman, Fullerton, CA, USA) with a Brownlee precolumn, 3.2 x 15 mm, 7 μ (Applied Biosystems, Foster City, CA, USA) and eluted with a mobile phase consisting of 0.01 N phosphoric acid and 1.5% v/v methanol adjusted to pH 6.3 with 10% w/v solution of sodium hydroxide. The mobile phase was filtered by vacuum through an HV-filter (Millipore, Bedford, MA, USA) and was continuously degassed with helium during sample injection.

The standard solutions were prepared by successive 1:1 dilutions of 1600 ng/mL of acadesine in human plasma down to 6.25 ng/mL. Low, medium and high *in vitro* quality control samples were prepared at 6.4, 128 and 1280 ng/mL. These quality controls were stored in aliquots at -20^{o}C and used in the daily method validation (7). Since acadesine has negligible (~1%) plasma protein binding, approximately 250 μL of each plasma sample or

TABLE 1

Intra-day precision (n=4) of acadesine in human plasma.

Amount acadesine added (ng/mL)	Amount acadesine found (ng/mL)	RSD %	Relative error %
1600	1740	3.41	+8.75
800	900	3.27	+12.50
400	430	3.04	+7.50
200	211	4.11	+5.50
100	96	2.55	-4.00
50	43.3	4.77	-13.40
25	20.7	3.03	-17.20
12.5	11.50	4.76	-8.00
6.25	6.87	2.74	+9.92

TABLE 2

Inter-day precision (n=5) of acadesine in human plasma.

Amount acadesine added (ng/mL)	Amount acadesine found (ng/mL)	RSD %	Relative error %
1600	1700	4.40	+6.25
800	850	3.93	+6.25
400	421	5.21	+5.25
200	204	2.71	+2.00
100	97	4.53	-3.00
50	46.1	7.68	-7.80
25	22.5	6.24	+10.00
12.5	11.86	12.81	-5.12
6.25	6.62	6.70	+5.92

TABLE 3

Standard curve statistics (mean ± SD, n=5) for acadesine in human plasma.

Date	Slope	Y-intercept	Correlation coefficient
05/04/94	4.200	-0.001	0.9998
05/05/94	3.866	-0.006	0.9995
05/06/94	3.617	0.004	0.9995
05/07/94	4.320	-0.001	0.9999
05/08/94	3.981	-0.002	0.9999
mean	3.997	-0.001	0.9997
SD	0.277	0.003	0.0002
RSD %	6.94	-329.35	0.02

standard were filtered through an Amicon Centrifree Micropartition System (Amicon, Beverly, MA, USA) by centrifugation (Beckman, Model T-6 Centrifuge, Fullerton, CA, USA) at 2000 x g for 30 minutes at room temperature. An internal standard of compound A (Figure 1B) (25 μL of a 10 μg/mL stock solution) was added to 100 μL of ultrafiltrate. After vortexing for 5 sec, a 25 μL injection volume was injected into the LC. The data were collected and analyzed using ChemStation based software (HP, Kennet Square, PA, USA). Variance stabilized transformation regression analysis was used to fit the standard curve (8).

RESULTS

Acadesine was eluted at 6.64 min while the internal standard GP115 eluted at 9.72 min (Figure 2). The intra- (n=4) and inter-day (n=5) precision were <13% (RSD %) (Tables 1 and 2). The standard curves (n=5) were linear throughout the range tested (r>0.999) (Table 3). The relative standard deviation of the slope during the 5-day analysis was 6.94% (Table 3). The accuracy using *in vitro* quality control samples in high, medium, and low concentrations was between 94 and 105% (Table 4). The method was used to measure acadesine in human plasma with concentrations ranging from 1600 to 6.25 ng/mL.

TABLE 4

Accuracy for the determination of acadesine in quality control plasma samples (n=10).

Amount acadesine added (ng/mL)	Amount acadesine found (ng/mL)	RSD %	Accuracy %
1280	1340	4.51	104.77
128	121	4.97	94.14
6.40	6.47	14.62	101.14

CONCLUSIONS

We have developed a highly sensitive HPLC-amperometric electrochemical detection assay for the quantitation of acadesine in human plasma. The detection limit of the assay (LOQ = 6.25 ng/ml) is twenty times lower than previously reported methods (4,5,6). Another benefit is that volumes of plasma as low as 100 µl can be analyzed. In summary, the validated assay described has the advantage of being simple and exhibiting good precision, linearity, accuracy, and lower detection limit (9).

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DETERMINATION OF RETINOL AND RETINOIC ACID IN CAPILLARY BLOOD BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simplified method for quantitation of retinol in capillary blood by high-performance liquid chromatography (HPLC) is described. Serum (10-20 μl) obtained by centrifugation of capillary blood is extracted with a solvent mixture of isopropanol-dichloroethane, and the extract is directly injected into a HPLC system to quantitate retinol. The lower limit of quantitation is 0.25 ng retinol, thus permitting quantitation of serum or plasma level of 8 μg or more retinol/L. Although endogenous retinoic acid cannot be analyzed, retinoic acid, following an oral dose, can be extracted along with retinol in presence of acetic acid and quantitated. The lower limit of quantitation of retinoic acid is 0.5 ng, thus permitting quantitation of 50 ng or more of retinoic acid/ml serum or plasma. The small sample size required, their simple preparation, and rapid analysis make this method well suited for clinical studies. The method should be very useful for monitoring retinol level in blood of patients suspected of vitamin A deficiency or of retinol and retinoic acid levels in patients undergoing retinoic acid therapy for skin disorders or acute promyelocytic leukemia.

INTRODUCTION

Vitamin A is an essential micronutrient for several life processes. Vitamin A is required for growth, reproduction, vision, differentiation, hematopoiesis, bone development, and pattern formation [1]. Vitamin A deficiency is a major public health problem in many developing countries [2]. Deficiency of vitamin A results in defective vision, including blindness [2]. It is a major cause of morbidity and mortality among children in developing countries [2-4]. Retinol (ROL) and retinoic acid (RA) are naturally occurring forms of vitamin A that are continuosly circulating in human blood. Although blood ROL level does not reflect vitamin A status, blood levels of vitamin A continue to be the most commonly used biochemical measure of the vitamin A status of individuals and populations, even with all the limitations in their interpretation [2]. Measurement of plasma or serum retinol level is an essential part in the dose-response tests that have proved very useful for indirectly assessing inadequate liver stores of vitamin A [5,6]. All-trans-RA and 13-cis-RA are currently being prescribed for the treatment of various skin disorders [7] and acute promyelocytic leukemia [8]. Monitoring RA levels in the blood of such patients is often necessary and useful. There are a number of methods available for the analysis of ROL only or of ROL, carotenoids, and tocopherols [9]. Because the concentration of endogenous RA is negligible when compared with endogenous ROL in the blood, simultaneous analysis of RA and ROL in blood poses a problem. Using as much as 0.5 ml or more of serum, simultaneous quantitation of ROL with endogenous [10] or exogenous [11] RA has recently been reported. In 1993, we reported simplified procedures for the extraction and HPLC analysis of ROL, carotenoids, and tocopherols in human

serum [12]. Although endogenous RA could not be detected in the small volumes of sera used, we demonstrated the possibility of using RA as an internal standard in this assay [12]. In this paper, we demonstrate that modifications of the extraction and HPLC procedures have allowed analysis of ROL singly or simultaneously with RA in very small volumes of blood.

MATERIALS AND METHODS

All experiments were carried out under yellow light in laboratories illuminated with Gold fluorescent lights (F40GO made by Philips, USA). Retinoids and their solutions were kept under argon at -20°C.

Chemicals and Reagents

All-trans RA, all-trans ROL, and all-trans retinyl acetate (RAC) were purchased from Sigma Chemical Co., St. Louis, MO. The purity of retinoids was checked by high performance liquid chromatography (HPLC). Whenever necessary, retinoids were purified by HPLC according to the procedure described [13]. HPLC grade methanol and acetonitrile and reagent grade dichloroethane and glacial acetic acid were purchased from Fisher Scientific Co., Fair Lawn, NJ.

Preparation of Standards

Concentrated solutions of retinoids were first prepared by dissolving about 1-2 mg of each pure retinoid in methanol. The solutions were diluted until an absorbace reading of 0.3-0.5 was reached at the wave length of maximum absorption. These solutions served as stock solutions. At the time of extraction of retinoids

in serum, the stock solutions were diluted 10-fold with a mixture of isopropanol/dichloroethane (2:1, v/v). These solutions, therefore, contained 0.2-0.5 ng retinoid/μl of the solution. The following E (1%, 1 cm) values were used: 1850 at 325 nm for ROL, 1500 at 350 nm for RA, and 1500 at 325 nm for RAC [9].

HPLC System

The HPLC system consisted of: a model 510 pump (Waters Associates, Milford, MA), a model V^4 absorbance detector (ISCO, Lincoln, NE), a dual-channel CR-4A integrator (Shimadju, Columbia, MD), and a Rheodyne injector (Cotati, CA).

For analysis of ROL only, a Microsorb-MV C_{18} 3-μm column (100 x 4.6 mm; Rainin, Woburn, MA) was used. For simultaneous analysis of ROL and RA, a Ultracarb 5 ODS30 (150 x 4.6 mm) column (Phenomenex, Rancho Palos Verdes, CA). A guard column of C_{18} material (Upchurch Scientific, Omaha, NE) preceded the main column. The mobile phase consisted of acetonitrile-dichloromethane-methanol (85:12:3) containing 0.1% ammonium acetate. Ammonium acetate must first be dissolved in methanol; then acetonitrile and dichloromethane were added. Dichloromethane can be substituted by the less volatile dichloroethane without affecting the analysis. The flow rate was 0.5 ml/min, and the detection wavelength was 325 nm when the Rainin column was used; the flow rate was 1 ml/min, and the detection wavelength was 335 nm when the Phenomenex column was used for simultaneous analysis of ROL and RA.

Collection of Blood and Preparation of Serum

Capillary blood was drawn from fingers by means of an Autolet II (Fisher Scientific, Fair Lawn, NJ) and 100-

μl micro pipets (DADE, American Hospital Supply Corp., Miami, FL). The tubes were sealed at the bottom, and centrifuged for 5 min at 2000 rpm at 4°C. The serum was pipetted out by means of a 50 μl Hamilton syringe (Cobert Associates, St. Louis, MO), and 10-20 μl of portions of serum were transferred into clear glass autosampler inserts (100 μl; Kimble/Kontes, Skokie, IL). The serum was used immediately, or stored at -20°C.

Venous blood was drawn by medical staff at the University Health Center.

Extraction of Retinol:

A solution containing retinyl acetate in isopropanol/dichloroethane (0.2-0.5 ng RAC/μl; 30 μl) was added to each 10 μl of serum in the insert. The insert was closed by means of a Parafilm (American National Can, Greenwich, CT). The mixture was vortexed for 30 sec, then centrifuged for 1 min, and 10-20 μl of the supernatant solution was injected into the HPLC system (Rainin column).

Extraction of Retinol and Retinoic Acid:

Retinoic acid was not extracted efficiently by the above procedure [12]. Hence, 5 μl of glacial acetic acid was added per 30 μl of isopropanol/dichloroethane in the above extraction procedure. After vortexing and centrifugation, 10-20 μl of the supernatant was injected into the HPLC system (Phenomenex column).

Recovery, Quantification and Calibration

ROL was first quantitated in five replicate serum samples. Next, measured quantities of ROL, RA, and RAC

were added to the serum sample and quantitated again to determine the percentage of recovery.

Standard curves of peak area ratios (compound of interest to internal standard RAC) versus concentration ratios were calculated for ROL and RA. Calibration mixtures contained differing amounts of ROL or RA with a constant amount of internal standard. Concentration of ROL and RA was selected to reflect expected concentrations in samples studied.

RESULTS

Chromatographic Separation

The separation of ROL present in 10 μl of extract prepared from 10 μl of serum with 30 μl of the solvent mixture of isopropanol and dichloroethane containing RAC as the internal standard is shown in Fig. 1. HPLC was performed on the Rainin 3μ column. There was baseline separation of ROL from RAC, and the analysis time was less than 5 min. Precipitation of proteins followed by vortexing and centrifugation resulted in a total volume of the final extract at 25-30 μl. Injection of 10 μl of this extract, therefore, was equivalent to about 3-4 μl of serum.

Unless extracts from large volumes of serum were injected, the endogenous level of RA present in 10-20 μl of serum extract could not be detected by our procedure (Fig. 2A). However, analysis of 10-20 μl of extract of serum obtained 0.5-3 h after an oral dose of 50-mg RA, could be detected and analyzed. The separation of RA from an unidentified compound present in serum was not satisfactory on the Rainin column. Therefore, for simultaneous analysis of ROL and RA in serum, the Phenomenex column was used. A chromatogram of 20 μl of

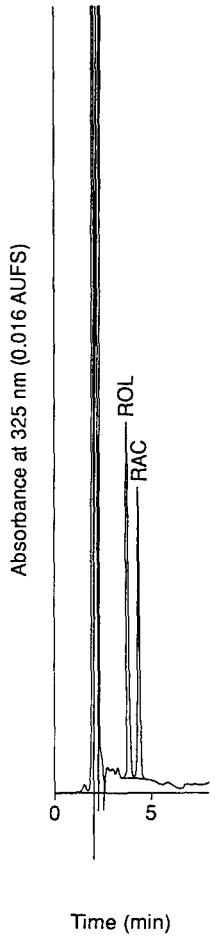

FIGURE 1. Chromatogram of HPLC analysis of retinol (ROL) in human serum: 10 μl of serum was extracted with 30 μl of the solvent mixture containing retinyl acetate (RAC) as internal standard, and 10 μl of the extract was injected directly.

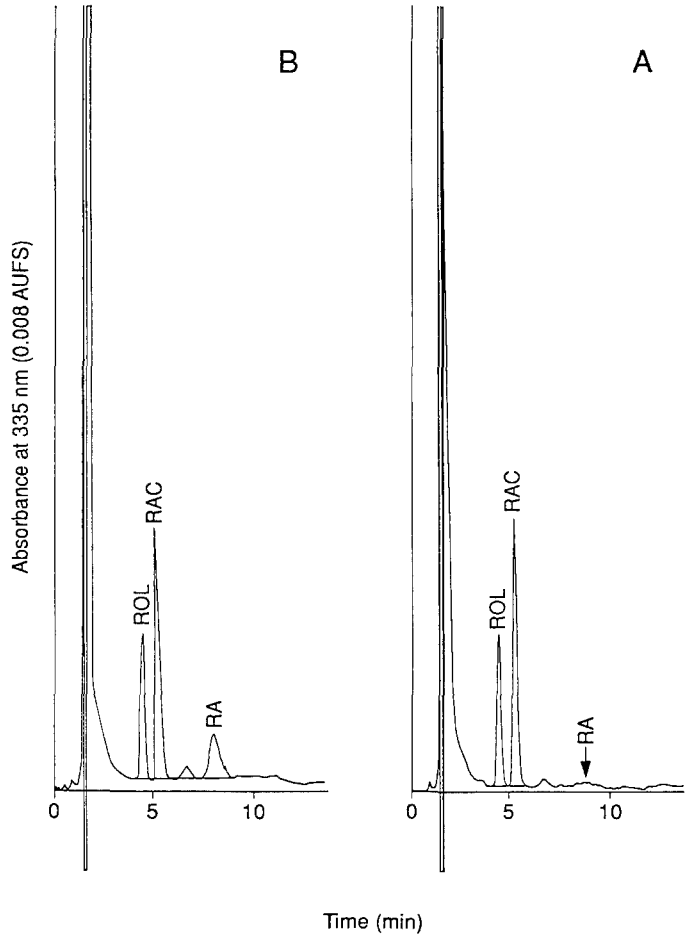

FIGURE 2. Chromatogram of HPLC analysis of retinol (ROL) and retinoic acid (RA) in human serum: 20 μl of serum was extracted with 60 μl of the solvent mixture containing acetic acid and RAC (internal standard), and 20 μl of the extract was injected directly. A, before; and B, 1 h after an oral dose of 50-mg RA.

the extract from 20 μl of serum (processed with 60 μl of the solvent mixture) obtained 1 h after a 50-mg dose of RA is shown in Fig. 2B.

Limit of Quantitation and Detection

Retinol: With both the Rainin and the Phenomenex column, there was a linear relationship between peak areas obtained and the concentration over the range of 0.25 ng to >10 ng of ROL. The satisfactory lower limit of quantitation on the Rainin column was 0.25 ng of ROL, although about 0.1 ng of ROL could be detected. The Phenomenex column was not as sensitive as the Rainin column, and the limit of quantitation on this column was 0.5 ng of ROL.

Retinoic acid: The lower limit of satisfactory quantitation of RA on the Phenomenex column was found to be 0.5 ng RA, equivalent to 50 ng/ml of serum. The lower limit of detection of RA, however, was found to be 0.1 ng of RA.

Recovery

The recovery of added ROL in five replicate serum samples was 101 ± 3%. The recovery of added RA in presence of acetic acid was similarly determined in five replicate analysis, and was found to be 98 ± 5%. The recovery of the internal standard RAC was found to be 99 ± 2%.

Precision of The Extraction Procedure

The within-run and between-run imprecision of the extraction procedure for ROL was estimated by repeated

TABLE I

Comparison of Retinol Level in Sera Obtained from Venous and Capillary Blood

Capillary blood	Venous blood
μg/dl Serum	
38.65 ± 3.8	38.46 ± 2.5

analysis of three serum samples over a period of 2 wk. The within-run and between-run results were found to be 31.4 ± 0.8 μg/dl and 34.2 ± 1 μg/dl, respectively.

Comparison of analysis of Venous and Capillary Blood

Sera (10 μl) each obtained from capillary blood and venous blood were extracted under identical conditions and analyzed on the Rainin column by HPLC. The results are shown in Table I. There was no significant difference in the values obtained from the two blood samples.

DISCUSSION

We reported recently a simplified extraction procedure for the simultaneous analysis of retinol, tocopherols, and carotenoids in serum by vortexing and centrifuging 50-100 μl of serum with a mixture of isopropanol/dichloromethane and injecting 25-100 μl aliquots of the extract [12]. The extraction procedure eliminated the need for repeated solvent extraction, evaporation of solvent, and reconstitution of the residue. This resulted, not only in reduction of analysis time, but also in chances of loss of analytes and

isomerization and decomposition of the otherwise labile retinoids. Although the procedure described in this research for extraction of ROL is similar to the published procedure, a modification was made for the simultaneous extraction of RA with ROL. We found that acetic acid needs to be added to the serum for efficient extraction and recovery of retinoic acid. In our previous method [12], we demonstrated that, whereas the recovery of ROL was 101 ± 4.7%, the recovery of RA was only 67.4 ± 1.5%. In this study, we found that, in the presence of acetic acid, the recovery of RA was 98 ± 5%. Besides the modification in the extraction procedure, we have made other modifications of the assay procedure. It is now possible to quantitate ROL in very small volumes of blood that can be obtained from capillaries. We found no difference in serum ROL content of capillary and venous blood (Table 1). Because larger volumes of blood are often difficult to obtain in large-scale population study, especially amongst infants, the present methodology should be very valuable in such studies. Moreover, retinyl acetate, which is readily available and which separates very well from retinol with the modified solvent composition, can be used as an internal standard.

We also found that retinoic acid can be quantitated simultaneously in small volumes of serum obtained from volunteers taking a single 50-mg oral dose of RA. The method should find usefulness in analyzing ROL and RA in the blood of patients undergoing RA therapy. Such patients usually take daily 1 mg/kg body weight of RA for the treatment of acne [7] or 45 mg/m^2 body for the treatment of acute promyelocytic leukemia [8]. Because ROL and RA can be simultaneously quantitated, the method should be useful for monitoring serum ROL level of patients undergoing RA therapy when there is a danger of defective vision.

Analysis of ROL on the Rainin 3μ-column is very sensitive and rapid. Because the flow rate is only 0.5 ml/min and the analysis time is 5 min, less solvent is required. The column is also relatively inexpensive. The method should, therefore, be useful for the analysis of retinol in large-scale epidemiological studies in developing countries where cost often limits studies.

ACKNOWLEDGEMENTS

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LANTHANIDE LUMINESCENCE DETECTION OF BLEOMYCINS AND NALIDIXIC ACID

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ABSTRACT

Bleomycins and nalidixic acid transfer energy to Tb(III). Subsequent emission from Tb(III) provides for a selective and sensitive detection method. Temperature studies indicate that energy transfer for both compounds involves an intramolecular process. The efficiency of reversed-phase separations of bleomycins is improved by adding Tb(III) to the mobile phase. Tb(III) luminescence detection is compatible with a wide range of mobile phases suitable for the separation of nalidixic acid. Detection limits for bleomycin A_2 and nalidixic acid are 3×10^{-6} M and 4×10^{-7} M respectively.

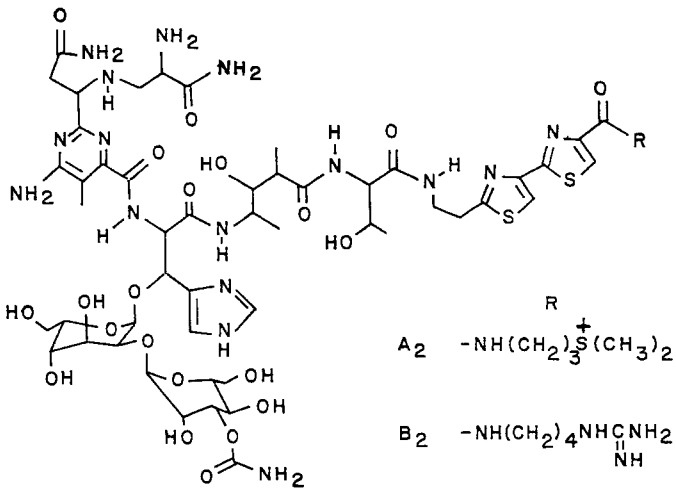
INTRODUCTION

Luminescent methods of detection are important in liquid chromatography because of their sensitivity and selectivity. Since many compounds are not inherently luminescent it is common to use either a pre- or post-column derivatization scheme with a suitable luminescent chromophore. We have previously described the use of the luminescent lanthanide ions europium(III) and

terbium(III) as detection chromophores in liquid chromatography[1-3].

These ions do not exhibit intense luminescence when directly excited. Instead, the lanthanide ions are excited indirectly by an energy transfer from the first excited triplet state of certain organic compounds. In particular, compounds with a benzoyl moiety often have triplet states close to that of the excited state of terbium and europium. Certain nitrogen heterocycles such as observed in nucleic acids also have triplet energies well matched with those of the lanthanides. The energy transfer can occur by an intermolecular or intramolecular process depending on whether the transferring compound has a strong binding group. Compounds such as dimethoxybenzophenone and naphthaldehyde transfer energy by an intermolecular process[1], whereas tetracycline[2,4], orotate[5], and single-stranded nucleic acids[2] transfer by an intramolecular process. Intramolecular transfer is generally preferred since it provides for more sensitive detection, and is less susceptible to variables such as the anion of the lanthanide salt and quenching by water or oxygen[1,2]. Non-transferring compounds such as aliphatic thiols have been detected by lanthanide luminescence by derivatizing with a transferring moiety such as 4-maleimidylsalicylic acid[6,7]. Compounds that quench lanthanide luminescence either by accepting energy from excited state lanthanide ions[1,8] or by ligand exchange with a transferring moiety[9] can also be detected.

This report investigates the liquid chromatographic detection of bleomycins(I) and nalidixic acid(II) by lanthanide luminescence. These compounds contain moieties that should have triplet state energies close to those of the lanthanides. They also have the potential to form chelate bonds with lanthanide ions.



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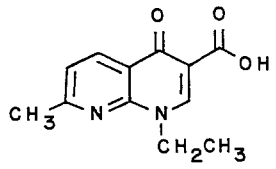

II

EXPERIMENTAL

Reagents:

All reagents were used without further purification. Terbium was purchased as its oxide in 99.9% purity from Alfa Products (Danvers, MA, USA) and converted to its chloride salt using Ultrex hydrochloric acid (J,T. Baker, Phillipsburg, NJ, U.S.A.) according to published procedures[1]. Bleomycins were kindly provided by Mead Johnson Oncology Products (Evansville, IN, U.S.A.). Nalidixic acid was obtained from Aldrich Chemical Co.(Milwaukee, WI, U.S.A.). Solvents used in preparing mobile phases were HPLC grade except water, which was

purified by passage through a Milli Q water purification device. All mobile phases were prepared as specified in the literature.

Apparatus:

Chromatographic analysis was performed using a Beckman 110B pump and Beckman-Altex 210A injector with 20 uL sample loop. A Beckman 163 variable wavelength absorbance monitor was used for ultraviolet detection. A Beckman ODS column (15 cm x 4.6 mm, 5 um) was used for reversed-phase separations and an Alltech Anion R column (25 cm x 4.1 mm, 10 um) with Wescan Guard Cartridge for anion exchange separations. Fluorescence measurements were carried out on a Perkin-Elmer LS-5 spectrofluorometer fitted with either a cuvette holder or high pressure flow cell. Excitation wavelengths were 318 nm for bleomycins and 310 nm for nalidixic acid. The emission wavelength was 544 nm when Tb(III) was the emitting species. Excitation and emission slit widths were 5 nm. Response time was set at 1. For time-resolved measurements, the instrument was switched to the phosphorescence mode and appropriate delay and gate times were set.

The effect of temperature on the intensity of lanthanide luminescence was studied by flow injection analysis. A 25 ft. stainless-steel coil (0.01 in I.D.) was placed between the injector and spectrofluorometer and immersed in a constant temperature water bath.

Evaluation of Mobile Phases:

Preliminary evaluations of the compatibility of mobile phases with lanthanide luminescence detection were carried out by cuvette studies. Solutions were prepared by adding 30 uL of a 1 x 10^{-3} M solution of nalidixic acid (pH 6 by adding 1 M potassium hydroxide) to 3 mL of the appropriate mobile phase containing 0.01 M of Tb(III)

either as its chloride salt or EDTA complex. The emission spectrum was then recorded.

RESULTS AND DISCUSSION

Bleomycins:

A comparison of emission from solutions of bleomycin with and without terbium(III) upon excitation of the bleomycin at 318 nm is shown in Figure 1. The two peaks in Figure 1b are characteristic of terbium emission. The greater intensity of the peak at 545 nm is typical. The pyrimidine ring of bleomycin is expected to be the moiety responsible for energy transfer to Tb(III). Although terbium is known to bond to bleomycin with an association constant of approximately 2.5 x 10^4, NMR experiments indicate that the site of binding cannot be determined with certainty[10]. Presumably the bleomycin associates with Tb(III) in a multidentate manner.

The efficiency of intramolecular transfer depends on the proximity of the lanthanide to the transferring moiety[2,4]. Given the relative concentration of terbium and bleomycin employed in liquid chromatographic detection, and the likely possibility that Tb(III) does not bind directly at the pyrimidine ring site, it is possible that energy transfer from bleomycin to Tb(III) can involve both intra- and intermolecular processes. Previous work on systems involving intermolecular transfer has shown that lanthanide luminescence increases as a function of temperature[1,11,12]. In these cases the increase in energy transfer more than offsets the increase in collisional deactivation, thermal quenching from low lying excited state levels in Eu(III)[13], or lanthanide to organic energy transfer[14]. The latter process can occur if the triplet energy of the organic is only slightly higher than the first excited state of the lanthanide.

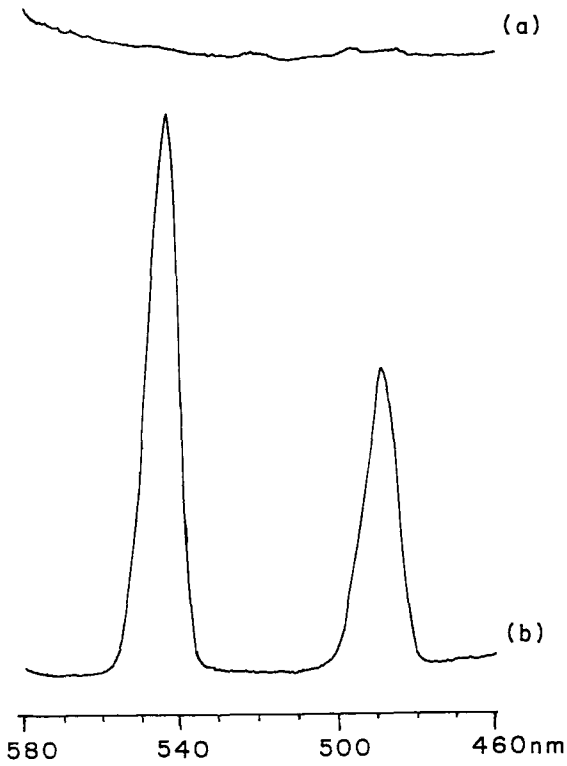

FIGURE 1. Emission spectra of a) Tb(III)chloride (10 mM) in MOPS (pH 6.8) and b) a 1:1 mixture of Tb(III)chloride (1 mM) in MOPS (pH 6.8) and bleomycin sulfate (0.1 mM) in PIPES (pH 6.8).

The intensity of terbium luminescence in a mixture of terbium and bleomycin decreases with increasing temperature (Figure 2a). This is similar to the temperature effect of europium with tetracycline (Figure 2b), a system known to involve intramolecular transfer[2,4]. Energy transfer between bleomycin and Tb(III) is therefore dominated by intramolecular exchange.

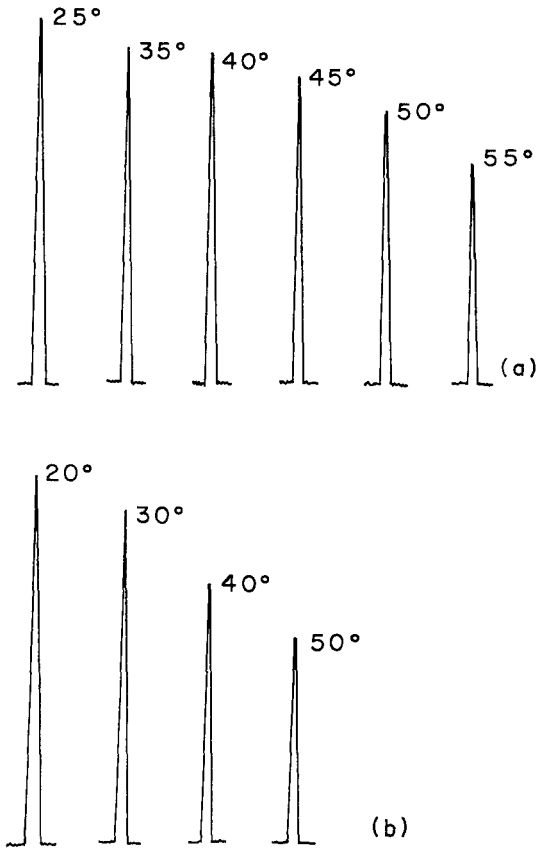

FIGURE 2. Effect of temperature on the intensity of lanthanide luminescence. Sample in a) was bleomycin (0.1 mM) and Tb(III)chloride (10 mM) in water (5 mM heptanesulfonic acid, 0.5 % glacial acetic acid, pH 6.8)-methanol, 55:45 v/v. Sample in b) was tetracycline (0.1 mM), Na[EuEDTA)]·$5H_2O$ (0.1 mM), ammonium chloride (0.2 M) (pH 9) in water. Excitation wavelength, 392 nm; emission wavelength, 616 nm.

The intensity of terbium luminescence in terbium-bleomycin mixtures increases at higher pH. Deprotonation of the bleomycins, which have pKa values of 2.9, 4.7, and 7.3 for B_2[15] and 2.9, 5.0, and 7.7 for A_2[16] must either strengthen the terbium-bleomycin interaction or facilitate binding of the terbium closer to the pyrimidine ring. Terbium(III) chloride or nitrate form insoluble hydroxides at basic pH, and cannot be used above pH 7. Formation of EDTA complexes with lanthanide ions prevents such precipitates from forming. In previous work with tetracycline, efficient energy transfer was observed with Na[Eu(EDTA)] at basic pH[2]. In the case of bleomycin, however, the energy transfer to Na[Tb(EDTA)] was poor. Since the bleomycins are assumed to bind in a multidentate manner, the strong complexation of EDTA ($\log K_F \approx 17$)[17] must block bleomycin association with the terbium. Terbium(III)chloride was therefore used at a pH of 6.8 for detection.

The chromatographic efficiency in reversed-phase separations of bleomycins improves significantly when copper(II) is added to the mobile phase[18]. Complexation with copper would reduce the number of sites on the bleomycin capable of simultaneously adsorbing to the stationary phase. Such multi-site adsorption has been proposed as a reason for poor chromatographic efficiency in the reversed-phase separation of proteins[19]. Addition of a one hundred-fold excess of terbium(III) to a solution containing a one-to-one copper-bleomycin complex did not show significant enhancement of terbium luminescence. Terbium(III) forms a much weaker complex with bleomycin than copper(II) ($\log K_F = 18.1$)[20], and does not effectively compete for the binding site.

Terbium(III) can be added to the mobile phase in place of copper(II), and a similar improvement in chromatographic efficiency is observed. Because of the

relatively weak complexation, a ten to one hundred-fold excess of terbium is needed to achieve good efficiency. Both bleomycin A_2 and B_2 are detected using Tb(III) luminescence, although the sensitivity for the A_2 congener is better than that of B_2 under the conditions employed(Figure 3). Both the A_2 and B_2 congeners transfer energy to Tb(III)[10]. Other congeners of bleomycin that do not involve modification at or near the pyrimidine moiety are expected to transfer energy as well.

By using a pulsed source and incorporating a delay between the excitation and detection, it is possible to discriminate the long-lived lanthanide luminescence from scatter and short-lived fluorescence from impurities[2,21]. For bleomycins, an approximately five-fold increase in sensitivity was realized using time-resolved methods (Figure 4). At the optimum conditions of delay time of 0.01 msec and gate time of 3.5 msec, the limit of detection, defined as three times the signal-to-noise was measured to be 3×10^{-6} M for bleomycin A_2, or approximately 80 ng of injected sample. Linear response was observed over two orders of magnitude. This detection limit is not as low as what was measured with ultraviolet detection (2×10^{-7} M), but the excellent selectivity of lanthanide luminescence detection should facilitate the analysis of bleomycins in complex mixtures.

Nalidixic Acid:

Nalidixic acid transfers energy to Tb(III). Binding is expected to occur at the carboxylate group. A pH above six ensures deprotonation of the acid moiety and maximizes transfer to Tb(III). The effect of temperature on the intensity of Tb(III) luminescence (Figure 5) indicates that intramolecular transfer dominates the process. The signal decreased from 30°C to 50°C at

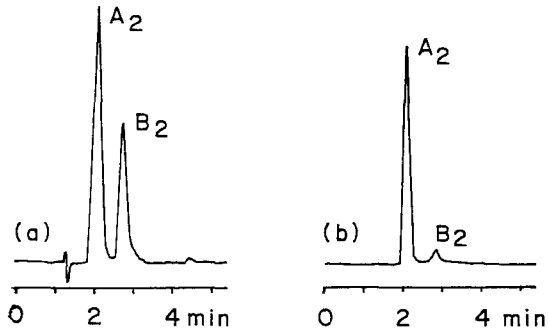

FIGURE 3. Chromatograms of bleomycins (50 uM) using a) ultraviolet detection (318 nm) and b) fluorescence detection. Mobile phase: Tb(III)chloride (10 mM) in water (5 mM heptanesulfonic acid, 0.5 % glacial acetic acid, pH 6.8)-methanol, 55:45 v/v. Flow rate, 1 mL/min.

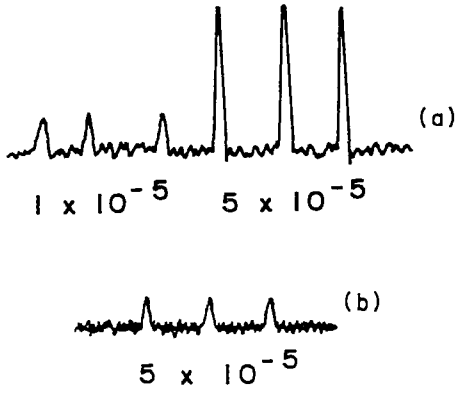

FIGURE 4. Comparison of the sensitivity of a) time-resolved and b) normal measurements on the detection of bleomycin. Sample was bleomycin (concentration shown in figure) and Tb(III)chloride (10 mM) in water (5 mM heptanesulfonic acid, 0.5 % glacial acetic acid, pH 6.8)-methanol, 55:45 v/v. Excitation wavelength, 318 nm; emission wavelength, 544 nm.

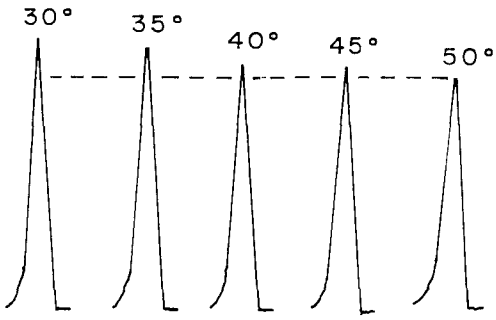

FIGURE 5. Effect of temperature on the intensity of Tb(III) luminescence. Sample was nalidixic acid (10 uM) and Tb(III)chloride (1 mM) in water (10 mM sodium acetate).

Tb(III)-nalidixic acid ratios of 20:1, 100:1 and 200:1. The use of time-resolved methods did not enhance the signal-to-noise ratio or detection limits for this system.

A number of separations of nalidixic acid and similar compounds have been reported[22-34]. The compatibility of each of these phases with lanthanide luminescence detection was assessed. Insoluble complexes formed when terbium was mixed directly with phases containing phosphate, citrate, or oxalate ions. In most cases the insoluble complexes could be eliminated by adding EDTA to the mixture. Transfer from nalidixic acid to Tb(III) was observed after addition of the EDTA. Lanthanide luminescence detection is therefore compatible with a range of available mobile phases.

Two reversed-phase separations using sodium dihydrogen phosphate (5 mM), Na[Tb(EDTA)] (10 mM)-acetonitrile (60:40)[29] and oxalic acid (5 mM), Na[Tb(EDTA)] (10 mM)-acetonitrile (55:45)[34] as mobile phases and one anion exchange separation using sodium

tetraborate (10 mM), sodium sulfate (3 mM), and Na[Tb(EDTA)] (10 mM) at pH 9.2[23] as the mobile phase were attempted. In all three cases we were unable to reproduce the literature results. Either no elution or extremely inefficient separations with severely tailed peaks were observed. The detection limits and linearity was therefore assessed in a flow-injection mode. The measured detection limits were 7×10^{-7} M for the two reversed-phase conditions and 4×10^{-7} M in the anion exchange phase. Linearity was observed over more than two orders of magnitude. Work is continuing in an attempt to find efficient chromatographic conditions for the separation of nalidixic acid.

ACKNOWLEDGEMENTS

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LIQUID CHROMATOGRAPHY CALENDAR

1995

MAY 21: Techniques for Polymer Analysis and Characterization, a short course, Sanibel Island, Florida. Contact: ISPAC Registration, 815 Don Gaspar, Santa Fe, NM 87501, USA.

MAY 22 - 24: 8th International Symposium on Polymer Analysis and Characterization, Sanibel Island, Florida. Contact: ISPAC Registration, 815 Don Gaspar, Santa Fe, NM 87501, USA.

APRIL 25 - 28: Biochemische Analytik '95, Leipzig. Contact: Prof. Dr. H. feldmann, Inst. fur Physiologische Chemie der Universitat, Goethestrasse 33, D-80336 Munchen, Germany.

MAY 23: Miniaturization in Liquid Chromatography versus Capillary Electrophoresis, Pharmaceutical Institute, University of Ghent, Ghent, Belgium. Contact: Dr. W. R. G. Baeyens, Univ of Ghent, Pharmaceutical Inst, Harelbekestraat 72, B-9000 Ghent, Belgium.

MAY 28 - JUNE 2: HPLC'95, 19th International Symposium on Column Liquid Chromatography, Convention Center, Innsbruck, Austria. Contact: HPLC'95 Secretariat, Tyrol Congress, Marktgraben 2, A-6020 Innsbruck, Austria.

MAY 31 - JUNE 2: 27th Central Regional Meeting, ACS, Akron Section. Contact: J. Visintainer, Goodyear Research, D415A, 142 Goodyear Blvd, Akron, OH 44236, USA.

JUNE 5 - 8: 5th Symposium on Our Environment / 1st Asia-Pacific Workshop on Pesticides, Singapore. Contact: The Secretariat, 5th Symp on our Environment, Chem Dept, National University of Singapore, Kent Ridge, Republic of Singapore 0511.

JUNE 6 - 8: 28th Great Lakes Regional ACS Meeting, LaCrosse-Winona Section. Contact: M. Collins, Chem. Dept., Viterbo College, La Crosse, WI 54601, USA.

JUNE 11 - 14: 1995 International Symposium and Exhibit on Preparative Chromatography, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JUNE 13 - 16: Capillary Electrophoresis, Routine Method for the Quality Control of Drugs: Practical Approach (in English); L'Electrophorese Capillaire, Methode de Routine pour le Controle de Qualite des Medicaments: Approche Pratique (in French), Monpellier, France. Contact: Prof. H. Fabre, Lab. de Chimie Analytique, Inst. Europeen des Sciences Pharmaceutiques Industrielles de Montpellier, Ave. Charles Flahault, 34060 Montpellier Cedex 1, France.

JUNE 14 - 16: 50th Northwest/12th Rocky Mountain Regional Meeting, ACS, Park City, Utah. Contact: J. Boerio-Goates, Chem Dept, 139C-ESC, Brigham Young Univ, Provo, UT 84602, USA.

JULY 9 - 15: SAC'95, The University of Hull, UK, sponsored by the Analytical Division, The Royal Society of Chemistry. Contact: The Royal Society of Chemistry, Burlington House, Picadilly, London W1V 0BN, UK.

JULY 7 - 8: FFF Workshop, University of Utah, Salt Lake City, UT. Contact: Ms. Julie Westwood, FFF Research Center, Dept. of Chem., University of Utah, Salt Lake City, UT 84112, USA.

JULY 10 - 12: FFF'95, Fifth International Symposium on Field-Flow Fractionation, Park City, Utah. Contact: Ms. Julie Westwood, FFF Researcvh Center, Dept. of Chem. Univ. of Utah, Salt Lake City, UT 84112, USA.

JULY 23 - 28: 35th Rocky Mountain Conference on Analytical Chemistry, Hyatt Regency, Denver, Colorado. Contact: Patricia Sulik, Rocky Mt. Instrum. Labs, 456 S. Link Lane, Ft. Collins, CO 80524, USA.

Juny 25 - 28: Method Development in HPLC, Virginia Tech, Blacksburg, Virginia. Contact: Dr. H. McNair, Chem Dept, Virginia Tech, Blacksburg, VA 24061-0212, USA

AUGUST 13 - 17: ICFIA'95: International Conference on Flow Injection Analysis, Seattle, Washington. Contact: Prof. G. D. Christian, Dept of Chemistry BG-10, University of Washington, Seattle, WA 98195, USA.

AUGUST 14 - 19: 35th IUPAC Congress, Istanbul, Turkey. Contact: Prof. A. R. Berkem, 35th IUPAC Congress, Halaskargazi Cad. No. 53, D.8, 80230 Istanbul, Turkey.

AUGUST 20 - 25: 210th ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 20 - 25: ACS sponsored Symposium on Saponins: Chemistry and Biological Activity, Chicago, Illinois. Contact: G. R. waller, Oklahoma State University, Dept of Chem & Molecular Biology, Stillwater, OK 74078, USA.

SEPTEMBER 4 - 7: 13th International Symposium on Biomedical Applications of Chromatography and Electrophoresis and International Symposium on the Applications of HPLC in Enzyme Chemistry, Prague, Czech Republic. Contact: Prof. Z. Deyl, Institute of Physiology, Videnska 1083, CZ-14220 Prague 4, Czech Republic.

SEPTEMBER 12 - 15: 5th International Symposium on Drug Analysis, Leuven, Belgium. Contact: Prof. J. Hoogmartens, Inst. of Pharmaceutical Sciences, Van Evenstraat 4, B-3000 Leuven, Belgium.

OCTOBER 18 - 21: 31st Western Regional Meeting, ACS, San Diego, Calif. Contact: S Blackburn, General Dynamics, P. O. Box 179094, San Diego, CA 92177-2094, USA.

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

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

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

NOVEMBER 5 - 7: 30th Midwestern Regional Meeting, ACS, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr, Miami, OK 74354, USA.

NOVEMBER 29 - DECEMBER 1: Joint 51st Southwestern/47th Southeastern Regional Meeting, ACS, Peabody Hotel, Memphis, Tenn. Contact: P.K. Bridson, Chem Dept, Memphis State Univ, Memphis, TN 38152, USA.

DECEMBER 17 - 22: 1995 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1996

FEBRUARY 26 - MARCH 1: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

MARCH 24 - 29: 211th ACS National Meeting, New Orleans, LA. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

MARCH 31 - APRIL 4: 7th International Symposium on Supercritical Fluid Chromatography and Extraction, Indianapolis, Indiana. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

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MAY 7 - 9: VIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Monte Carlo, Monaco.. Contact: Prof. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

AUGUST 18 - 23: 212th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 1 - 6: 11th Symposium on Quantitative Structure-Activity Relationships: Computer-Assisted Lead Finding and Optimization," Lausanne, Switzerland. Contact: Dr. Han van de Waterbeemd, F. Hoffmann-La Roche Ltd., Dept PRPC 65/314, CH-4002 Basle, Switzerland.

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1997

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1998

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AUGUST 23 - 28: 216th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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2000

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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

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

AUGUST 19 - 24: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2002

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Book:

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

2. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in HPLC of Biological Macromolecules, K. M.

Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

8. Each page of manuscript should be numbered lightly, with a light blue pencil, at the bottom of the page.

9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used.

10. Material that cannot be typed, such as Greek symbols, script letters and structural formulae, should be drawn carefully with dark black India ink. Do not use any other color ink.

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