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ISOLATION, SEPARATION, AND DETECTION OF ENKEPHALINS: A REVIEW OF METHODS FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ELECTROPHORESIS

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ABSTRACT

High Performance Liquid Chromatography(HPLC) and Capillary Electrophoresis (CE) are valuable analytical techniques for the determination of neuropeptides in biological fluids. A crucial aspect of HPLC and CE analyses is the preparation of samples. This review outlines sample preparation protocols for HPLC and CE methods that have been used for this determination, with a focus on enkephalins. Sample preparation protocols have ranged from simple techniques of extraction and centrifugation to solid phase extraction (SPE) and ultrafiltration. Additionally, this review also discusses the final determinant steps of HPLC and CE using various modes of detection . Detection modes for the HPLC methods included ultraviolet (UV), fluorometry, electrochemical and mass spectrometry (MS). Modes of detection when using CE for this determination have been more limited, focusing on UV and MS detection modes.

Introduction

Since the discovery of the pentapeptides, methionine enkephalin (ME) and leucine enkephalin (LE) by Hughes in 1975 (1) there has been a great deal of interest focused not only on the function of these compounds and other members of the opioid class but also on selective and sensitive methods to allow for their measurement in biological systems (2). A search of the

literature conducted with a time frame from January 1990 to January 1995 indicated more than 1500 citations on enkephalins with a substantial number of these reports concerned with analysis. Much of the activity in the determination of these compounds has centered on the use of radioimmunoassys (RIA) which, for some time, was the only technique that afforded sufficient sensitivity to be of use to researchers. In RIA, antiserum generated to peptide and protein conjugates is generally directed towards the C-terminus. Attack at the C-terminus of the molecule appears to afford better selectivity when compared to attack at the N-terminus of the molecule. However, since many of the enkephalins in questions are structurally similar, a certain amount of cross-reactivity between related peptides would be expected. (3).

In recent years, developments in high performance liquid chromatography (HPLC) and more recently, capillary electrophoresis (CE) have allowed for their use in neuropeptide analysis. This review will concentrate on HPLC and CE methods for the determination of ME and LE. It will outline methods of sample preparation for this class of compounds and also provide selected HPLC and CE methods that have been used for the determination of these substances. Methods of sample preparation and analysis outlined in this review range from very direct protocols that may be appropriate for some samples to extremely intricate methods that have been used for other sample types. Comprehension of this information should permit a scientist to make informed decisions regarding the assessment of neuropeptides in biological samples.

Sample Preparation

Before any analysis can proceed, some form of sample preparation must be performed. With some compounds and samples this can be as simple as dilution and filtration prior to analysis. Alternatively, it can be a multi-step sample preparation protocol. In the case of neuropeptides, initial efforts utilized the unit operations of solubilization, centrifugation, and

filtration in a variety of combinations. Loeber (4) extracted rat pituitary glands with a mixture of 0.2 M HCl and cold acetone. After the samples were homogenized, they were centrifuged at 30,000 g and the supernatant removed. The supernatant was lyophilized and the sample dissolved in ammonium acetate at a pH of 4.15 prior to HPLC analysis. Mousa and Couri (5) analyzed serum for enkephalins and neuropeptides using a sample preparation technique which entailed centrifugation at 2400 g prior to analysis. A third method (6) reported for the extraction of enkephalins in tissue samples was the use of a mixture of acetic acid, HCl and beta-mercaptoethanol which contained pepstatin and phenylmethyl sulfonyl chloride. After extraction, the samples were centrifuged at 26,000 g followed by open column chromatography on Sephadex G-10 or G-25, or precipitation with TCA. Samples treated with TCA were centrifuged at 26,000 g and the compounds of interest extracted with ether prior to HPLC analysis.

Laatikainen and his coauthors (7) reported a method for blood samples where they were collected in polyethylene tubes containing heparin and aprotinin. Samples were then chilled and centrifuged at 1000 g for 10 minutes at O degrees C. After addition of an internal standard, plasma proteins were precipitated by treatment with 0.1 M acetic acid and acetonitrile. The samples were centrifuged at 4500 g and diluted with distilled water before analysis with cation exchange HPLC.

A second type of sample preparation that has been used in this assay is ultrafiltration (UF), a method used in the HPLC analysis of ME in the retina of the developing rat (8). In this technique, samples were prepared for analysis by mixing in a phosphate buffer and ultrafiltration through a 10,000 MW cutoff membrane. UF also has been employed extensively in the analysis of numerous pharmaceutical compounds and for the study of protein bound and unbound drugs. (9,10) . The sample preparation can be performed with either a centrifuge or a syringe (11, 12).

Solid phase extraction (SPE) is a third method of sample preparation and has replaced many of the earlier protocols . There is substantial literature pertaining to the basics of this technique (13), and many vendors of these columns have developed data bases for applications (14,15). The SPE technique for neuropeptides has been pioneered by Desiderio (16). As an example of this SPE protocol , tissue was homogenized with 1N acetic acid and the proteins precipitated with a mixture of acetone/ 0.1N HCl (8/2). The resulting solution was centrifuged at 27,000 g for 20 minutes. The supernatant was withdrawn, dried under a stream of nitrogen, resuspended in 1% trifluoroacetic acid (TFA), and applied to a previously conditioned C18 solid phase extraction column. The salts were removed by a TFA wash and the peptide rich fraction eluted with a mixture of acetonitrile/0.05% TFA (8/2) for HPLC analysis (17). Figure 1 provides an outline of the overall scheme that was employed.

SPE was applied by Fleming and Reynolds for the determination of ME and LE in the rat brain (18) . Since the final analysis used electrochemical detection for the HPLC system and was operated at high applied potentials it was necessary to develop highly effective sample preparation protocols. Fleming and Reynolds utilized a technique called chromatographic mode sequencing (CMS) which resulted in decreased amounts of electroactive interfering substances. In this method, samples were initially extracted with acetic acid , lyophilized and frozen at -70 degrees C. The lyophilized samples were resuspended in 10% TCA containing 0.1% sodium metabisulfite, and centrifuged. The samples were extracted with two volumes of diethyl ether and vortexed. The ether layer was removed and nitrogen blown over the remaining aqueous layer to eliminate any residual ether. The pH of this layer was adjusted to approximately 2 with 1.0 M NaOH and phosphate buffer (pH 2.3) for a final phosphate concentration of 40 mM. An aliquot of tissue no larger than 300 mg was applied to a 400 mg C-8 SPE column. The column was washed with 3.0 ml of 50 mM potassium dihydrogen phosphate (pH 2.3), 2.0 ml of water/methanol (83/17 v/v), 2.0 ml of water /acetonitrile (92/8 v/v) and 2.0 ml of water/acetic

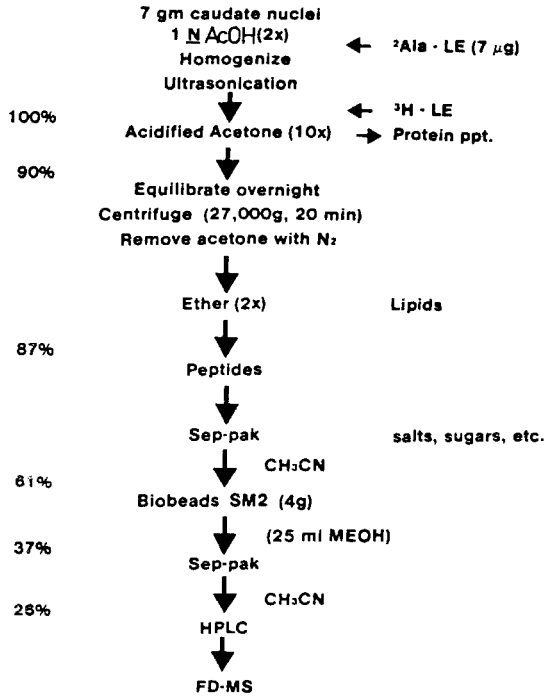

Figure 1

Solid Phase Extraction Protocol (59)

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acid (85/15 v/v) with a 3.0 ml water wash used between each organic wash step. The enkephalin fraction was eluted with 1.0 ml of triethylamine phosphate/acetonitrile (50/50) . The extract was dried under a stream of nitrogen and redissolved in water. It was applied to a 180 mg activated Type W column. This column was washed with 3.0 ml of water, 2.0 ml of the previous water/methanol mixture, 2.0 ml of the water/acetonitrile mixture and 2.0 ml of water/acetic acid (88/12 v/v). A water wash was used between each organic wash. The enkephalins were eluted with acetonitrile/water (56/44 v/v), evaporated to dryness and then redissolved in an appropriate

Table 1

Additional Uses of SPE for the Preparation of ME and LE

Compounds Analyzed	Reference
ME, LE	21, 26
Neuropeptides including ME	22, 23, 24, 25, 27

amount of the 50 mM phosphate buffer for analysis. While this was an extremely detailed protocol, the authors indicated that no spurious peaks appeared, and that after injecting these tissue extracts for a week, it was only necessary to wash the column with 100% methanol before additional analyses.

In the determination of beta-endorphin fragments in human plasma (19), an acidified sample was applied to an C18 SPE column. which had been conditioned with methanol, 6M urea and water. The acidified plasma was applied to the column and washed with water and 4% acetic acid. The retained peptides were eluted with a mixture of 1-propanol/acetic acid (96/4). While this method focused on endorphins, alteration of the elution solvent would make it appropriate for ME and LE.

Additional uses of SPE cartridges have allowed for the development of rapid isolation protocols for enkephalins and endorphins. In this method (20), C-18 SPE columns were conditioned with methanol, water and 0.5 M formic acid-pyridine at pH 3 or pH 4. Enkephalins were isolated using the pH 3 system while endorphins were isolated with the pH 4 solvent system. A number of other SPE protocols have been reported for enkephalins and are summarized in Table 1.

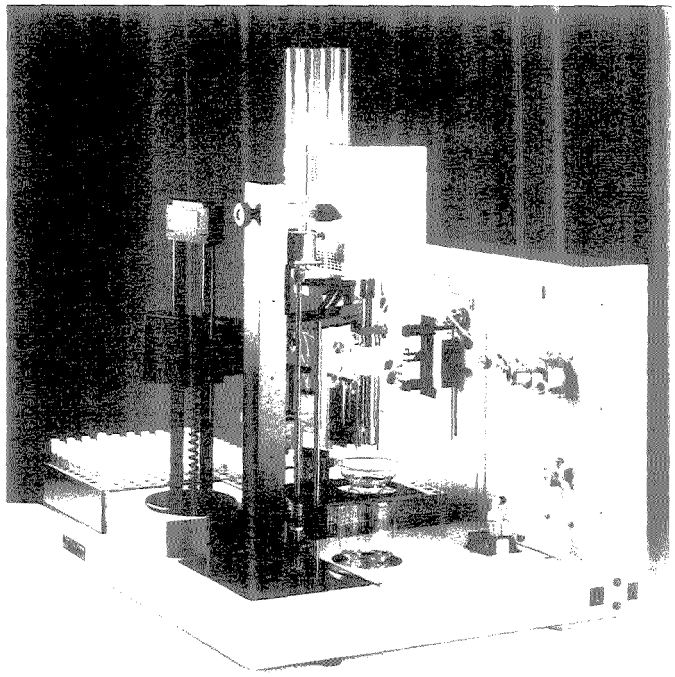

Figure 2

Automated Sample Preparation Workstation

An exciting development in the use of SPE for sample preparation of ME and LE is the commercialization of a number of automated systems for solid phase extraction (28). These systems range from a full fledged laboratory robot to a robotic workstation. An example of one of these instruments is seen in Figure 2. While no one has yet reported the use of these systems for these isolations, one could expect that the uses of automated systems will increase in the clinical laboratory as prices decrease and the demand for determinations continues to rise.

Another mode of sample preparation for enkephalins is microdialysis. Microdialysis is a very attractive technique since it can be used on freely moving conscious

animals (29). Microdialysis can introduce and remove molecules from the brain and the sample will contain no blood cells or macromolecules. Samples obtained by this technique can often be used without the need for additional sample refinement. One of the concerns about samples prepared in this fashion is that they must contain sufficient amounts of the compound of interest to detect. However, salts which dialyze with the compound of interest may need to be removed prior to analysis (30). This technique has been applied to the determination of neuropeptides in median eminence of the ewe by Advis and Guzman (31).

HPLC Analysis

HPLC is widely used in analytical and neuroscience laboratories in two distinct ways. In some laboratories it is used as a supplement to established RIA protocols and in others it is employed as a primary method for enkephalin analysis. There are numerous books about HPLC (32, 33, 34) that deal with subjects ranging from the basics of HPLC to advanced topics. There also are a number of volumes dealing with detection in HPLC (35, 36).

Five modes of detection have been used in the HPLC analysis of the enkephalins; spectrophotometric, fluorescence, electrochemical, radioimmunoassay (RIA) and mass spectrometry (MS). This review will briefly discuss all of these modes of detection except RIA.

The first type of HPLC detection system for enkephalins is ultraviolet spectroscopy (UV). Most peptides exhibit no characteristic chromophore, so detection in the 200 nm region is used. Figures 3 and 4 provide illustrations of this mode of detection showing the separation of ME and LE on a reversed phase HPLC with a mobile phase of acetonitrile/water and TFA with detection at 200 nm. Since many solvents associated with HPLC adsorb in this region some compromise is seen between sensitivity and wavelength. While this region can be used for the detection of these neurochemicals, a substantial number of other compounds also absorb in this region. ME and LE contain tyrosine residues which absorb in the 254 and 280 nm

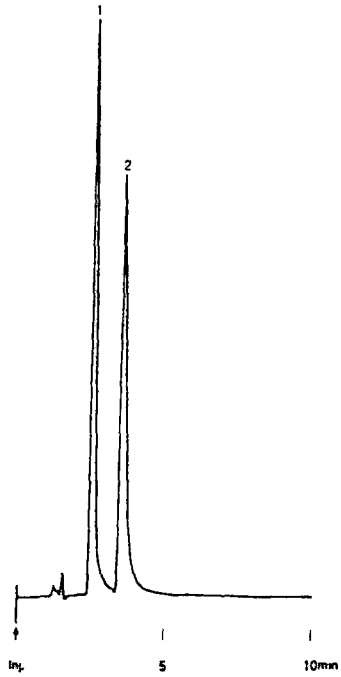

Figure 3

Reversed Phase Separation of ME and LE (59)

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region, so this wavelength can be used for these compounds but at a loss of sensitivity compared to detection at 200 nm (37). Initially, HPLC detectors had fixed wavelengths of either 254 or 280 nm, so these wavelengths were the obvious choice. As detector technology evolved, variable wavelength detectors were developed that allowed the analyst to monitor the 200 nm region of the spectra with good signal to noise (S/N) ratios. As detectors continued their evolution, there have been two developments in the technology: scanning and photodiode array (PDA) detectors. The scanning detectors scan peaks of interest as they elute from the HPLC column while the PDA detector affords additional capabilities such as absorbance ratioing for peak purity, searching of

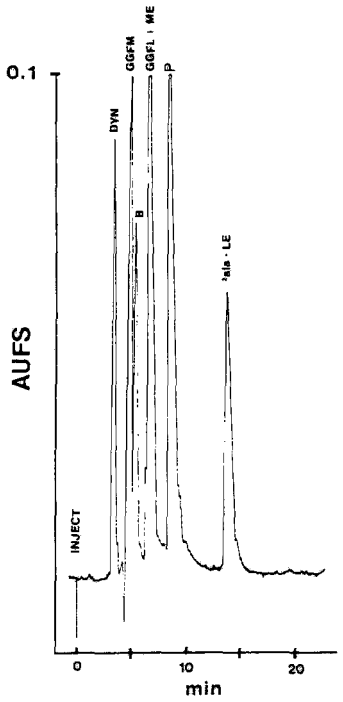

Figure 4

Reversed Phase Separation of Neuropeptides

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spectral libraries, and the calculation of derivative spectra. Since many similar compounds have similar UV spectra, the ability to calculate and display derivative spectra is an important component of a detection system. PDA detection systems provide an excellent complement to MS detection.

Fluorescent and other detection mechanisms has also been applied to the analysis of enkephalins. A paper by Zhang (38) reported the determination of ME, LE and substance P using fluorescence detection of the 9-fluorenylmethyl chloroformate derivative (FMOC). The

derivatized compounds are separated in a two step gradient in approximately 18 minutes with lower limits in the range of 300-500 fmoles. After treatment of the FMOC-neuropeptide derivative with mild acid, the intact neuropeptide could be recovered. Jencen (39) reported on the formation of napthalene-2,3-dicarboxyaldehyde/cyanide (NDA/CN) derivatives of ME, LE and D-[Ala^5] -ME using conventional fluorescence detection with column switching for increased chromatographic selectivity. This work was expanded to allow for chemiluminescence detection. Electrochemical detection also has been used for the determination of neurochemicals with numerous references appearing in the literature (40). While other modes of detection for HPLC are valuable for the analysis of neuropeptides, none has the broad applicability or provides the amount of information that is available from mass spectrometry (MS). Research in liquid chromatography- mass spectrometry (LC-MS) has been active since the 1960's. There has been an expansive evolution of the technology in the 1980's, with this technology expanded to the analysis of neuropeptides. An overview of LC-MS has been provided by Tomer and Parker (41), while the recent article by Heath and Giordani (42) provides a comparison of UV, fluorescence and electrospray for the detection of selected peptides.

In 1982, Yamada and Desiderio (43) published a method for the analysis of endogenous LE using a combination of HPLC and Field Desorption MS (FD-MS) . In this application samples were prepared off-line for analysis. Kenyon and coworkers reported the sequencing of underivatized peptides by Direct Liquid Inlet (DLI) LC-MS using a reversed phase column and a buffered mobile phase consisting of triethylammonium acetate and acetonitrile (44) . Positive and negative ion spectra were obtained and M+1 and M-1 ion were seen for the compounds of interest. The use of thermospray (TSP) LC-MS in neurochemistry was reported in 1987 by Artigas and Gelpi (45) who provided data of the MS of 19 indolic compounds in positive and negative ion modes. In the past few years, flow fast atom bombardment (FAB) MS has been used extensively for the identification of selected neuropeptides. FAB allows for the production of

M+H ions from peptides up to 10,000 daltons for the determination of underivatized peptides (46) . An article by Hill (47) stated that the two developments in bioanalytical mass spectrometry that allowed it to develop at an accelerated pace were tandem MS (MS-MS) and spray ionization. MS-MS enhanced the signal to noise ratio and allowed for better sensitivity and spray ionization allowed MS to be applied to molecules with a high mass and low volatility such as proteins and peptides. An extreme amount of activity in the use of electrospray ionization (ESI) and it's variants has been reported. Electrospray is another soft ionization technique (48) like thermospray, but unlike thermospray allows for the production of multiply charged molecular ions. It has been successfully applied to assays by a number of researchers. Dass and colleagues (49) reported the use of ESI-MS for the analysis of opioid peptides and the quantification of endogenous ME and beta-endorphin. ME and LE produced only [M + H] + ions while the other peptides produced a series of multiply charged ions. Samples of pituitary extract were prepared using a SPE protocol and ME was quantified with an internal standard. The ME content in the extracts ranged from 4.7 to 9.1 pmol/mg protein. Dimond (50) has provided a technical review of mass spectrometry for biomolecular analysis and states that ESI-MS produces molecular weight data with an accuracy of 0.01% for proteins and peptides in the range of 100-100,000 daltons and can be performed on 1 pmol of material. Figure 5 gives an example of the spectra recorded for 6 neuropeptides . In this application, a fused silica column 50 um ID x 8 cm was packed with 10 um C-18 material. A gradient elution was performed using a mobile phase of 2-50% methanol in 0.25% acetic acid at a flow rate of 0.82 ul/min. Nanoscale packed column capillary LC coupled to continuous flow FAB has been applied to a number of peptides including synthetic mixtures of bioactive peptides (51) . The column consisted of fused silica with ID of 50-74 um divided into 2.5 m lengths and packed with 780 um C-18 material. The mobile phases consisted of linear gradients of acetonitrile/water or methanol/water containing 0.1% TFA. Figure 5 provides a sample separation of seven neuropeptides using this technique. Woolfit (52) reported the LC/MS

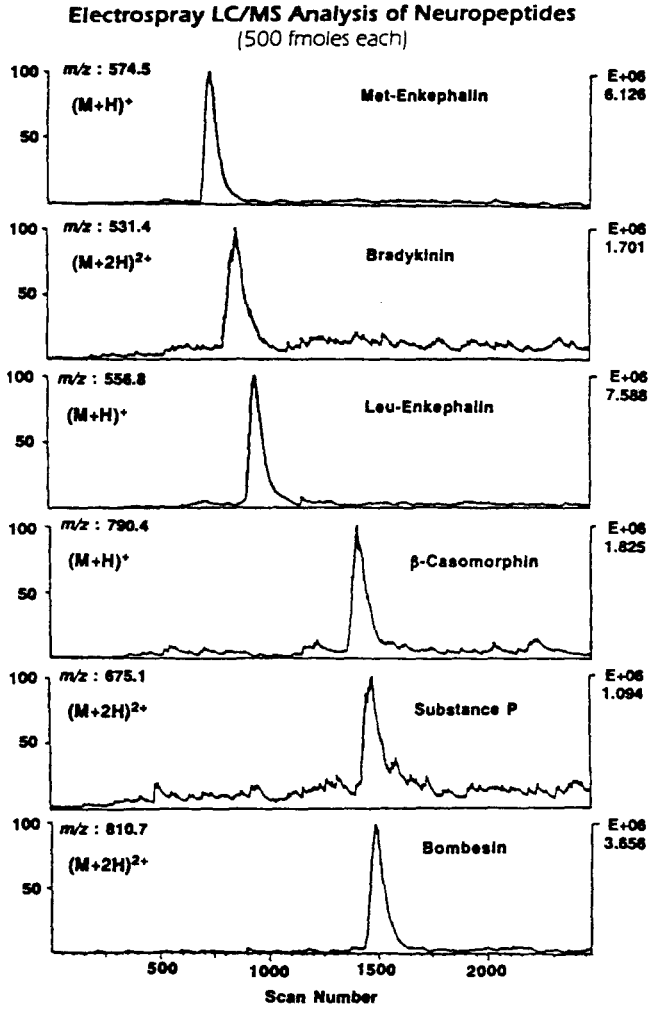

Figure 5

Series of Electrospray SIC of a Mixture of Neuropeptides (50)

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analysis of an enkephalin mixture using a liquid secondary ion MS interface and Lovelace (53) has described the determination of ME in human pituitary tissue by multidimensional reversed phase HPLC, radioreceptor assays, FAB-MS and MS-MS. A paper by Kusimerz (54) outlined a FAB-MS method for the quantitation of picomole levels of ME in human pituitary extracts using a deuterated internal standard followed by multidimensional LC. The application of pattern recognition techniques to MS data for sequencing C-terminal peptide residues was described by Degoda and Pulfer (55) . In this application, pattern recognition techniques were investigated on sequence information obtained from FAB and MS-MS data and applied to amino acid sequences up to pentapeptides. Recently, Emmett and Caproli reported a micro-electrospray technique for the high sensitivity analysis of peptides and proteins (56). Figure 6 shows injections of ME desorbed from a C-18 packed spray needle with (a) 10 ul of 5 fmol/ul in Ringers solution and (b) 10 ul of 100 amol/ul in water. Table 2 provides additional references on the analysis of enkephalins and other selected neuropeptides by HPLC.

Reversed phase HPLC columns are utilized for peptide determinations since they have excellent resolution and are able to resolve similar polypeptides which might differ by only one amino acid residue. Other developments in reversed phase HPLC variants involve the application of a larger pore size particle which allows larger peptides better access to the interior of the silica and also the development of a C-4, butyl phase. The physical dimensions of HPLC columns have been altered with narrow bore columns having a diameter of 2.1 mm that allow for increased sensitivity and flow rates of about 20% of standard 4.0 mm diameter columns. Minibore columns with 1.0 mm diameter offer a five fold improvement in sensitivity and flow rates in the 25-50 ul /minute flow range. Finally, there have been a resurgence in capillary chromatography where flow rates are in the 1-5 ul/minute flow range. Review articles and several other books dedicated to the determination of proteins and peptides by HPLC should be consulted for further information (62-64).

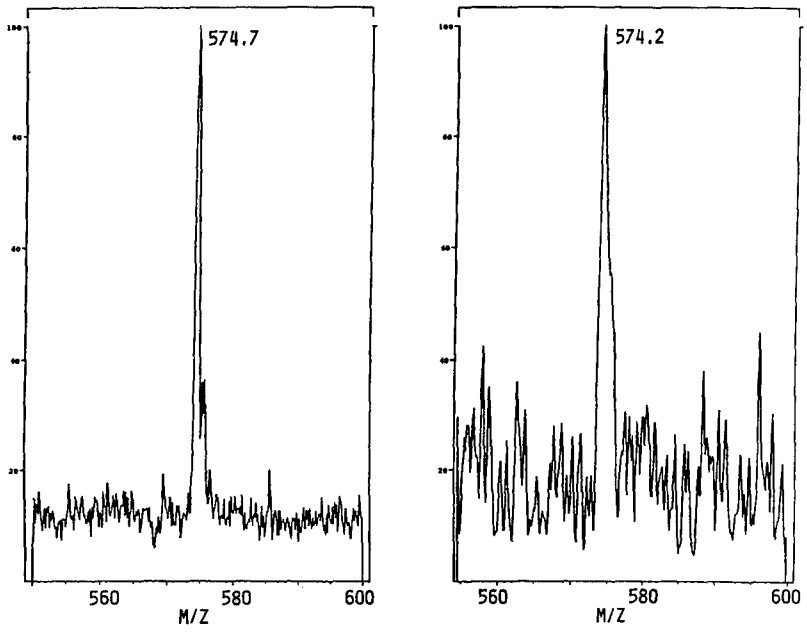

Figure 6

Micro-ES Spectrum of ME Desorbed from C-18 Packed Spray Needle (56)

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Table 2

Additional HPLC Methods for the Determination of ME and LE

Column	Detection	Reference
μBondapak C18	UV/RIA	57
C18	UV	58
μBondapak C18	RIA	59
C18	Electrochemical	60
μBondapak C18	UV	61

Capillary Electrophoresis

HPLC, with it's various modes of separation and detection, has provided the neuroanalyst with excellent capabilities. The introduction of CE in the 1980's, however, has provided exciting capabilities for the analysis of neuropeptides. CE offered rapid analysis times, high column efficiencies, high resolution, and automation in both methods development and analytical phases of the determination (65). It is not within the scope of this manuscript to provide an extensive review of this technology in it's various forms, and the reader is referred to other reference materials including some recent reviews and books on the subject (66 -69).

HPLC offers a wide variety of modes of detection for the analysis of ME and LE. Presently CE has limited detection options. The most recent generation of commercially available instruments come equipped with a UV detector. These detectors vary greatly with some vendors providing a variable wavelength detector while others offer variable wavelength detectors with scanning capability. Additionally, CE instruments equipped with photodiode array (PDA) detectors are becoming common. Even though CE uses very small volumes of sample and buffer, it cannot be called a trace technique, so a number of protocols and instrumental variations have evolved to allow for the enhancement of sensitivity (70). These embellishments have occurred essentially in two areas: sample concentration and cell geometry. One sample concentration strategy has been stacking which is based on the electrophoretic concentration of the peptide of interest and has been said lower the detection limits of compound 5-fold (71). One vendor now markets a special capillary that contains a small amount of reversed phase HPLC packing at the injection site, thereby allowing for enhanced sensitivity. In the area of cell geometry, there have been developments of bubble cells and Z-shaped detector optics (72). One vendor has introduced a high sensitivity option to allow for enhanced UV detection (73). Fluorescence detection is also becoming commercialized with vendors offering laser induced fluorescence (LIF) and a filter based instrument fluorescence detector. In the examination of peptides, UV detection at 200 or

280 nm has been used for the same reasons that it is employed in HPLC determinations. In CE, the capillary column becomes the detector window and on-column detection of approximately 100 pg has been reported (74). Moreover, with LIF, attomole detection levels of selected compounds have been published (75). In addition to these two modes of detection, indirect UV and amperometry have also been employed in CE (76, 77).

While the most widely used detection mode in CE is UV, considerable activity has been associated with the coupling of CE with MS. A review of CE-MS was recently published by Niessen and coworkers (78). Mosley and others (79) reported the use of CE-MS with continuous flow FAB interface for the determination of bioactive peptides. ESI has also been applied to this determination. CE-ESI-MS has also been applied to the analysis of small proteins up to 30,000 daltons. Muck and Henion (80) reported the determination of ME and LE in equine CSF by microbore HPLC and CE-MS. The CE separation was performed on a 90-100 cm x 100 um I.D. untreated fused silica capillary using a 50/50 mixture of acetonitrile and 20-30 mM ammonium acetate buffer @ pH 6.8 with MS detection.

At the 1994 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Sweedler (81) presented a paper on the analysis of neuropeptides using multichannel detection in CE. While it was not applied to the enkephalins, laser-induced fluorescence and radiochemical detection was applied to the determination of eight neuropeptides from the giant marine snail with the aim of measuring these compounds within the processes of the neurons and following the release of the peptides from different presynaptic release sites. The detection system was an imaging spectrograph and a slow-scan CCD detector. The system was able to detect zeptomole (10^{-21}) amounts of fluorescent labeled peptides from a nanoliter sample.

The applications base on protein and peptide analysis continues to grow at a rapid rate as can be seen in Table 3, and the use of specialty columns for capillary gel electrophoresis and other modes of separation will increase. There are now kits available from vendors that will

Table 3

Selected Applications of CE for the Determination of Proteins and Peptides

Protein	Reference
Human Growth Hormone	82
Anticoagulant Peptide	83
Growth Hormone Releasing Peptide	84
Motilin Fragments	85
Tryptic Peptides from Myoglobin	86

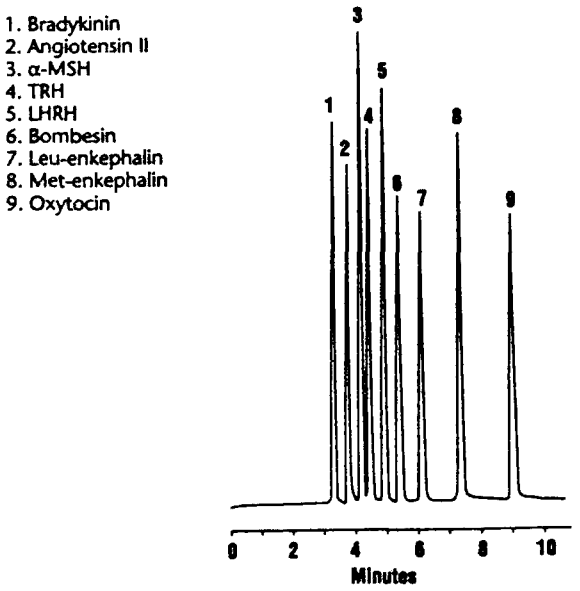

Figure 7

Sample Electropherogram of Peptides Using SEC Separation

(Compliments of Bio-Rad)

Table 4

Selected HPLC and CE Techniques for Amino Acid Composition

Derivative	Reference
PITC	88
OPA	89
FMOC	90
Dabsyl	92

allow many of these separations to move from research environments to routine usage. Figure 7 illustrates the separation of a peptide standard mix using one of these kits. With the extreme interest in the assay discussed in this review, one would expect the applications base for neurochemicals and neuropeptides to continue evolving at a rapid pace.

Confirmatory Techniques

MS, in one of its many forms, is the most direct method to confirm the identity since it can provide not only extremely precise information about the molecular weight of a substance but also provide information about it's composition due to the fragmentation pattern of the material of interest.

Should one not have access to an MS, a series of studies using amino acid analysis could be performed to further identify a substance. Amino acid analysis with HPLC or CE using any one of the accepted techniques listed in Table 4 would provide information regarding the amino acid composition of the substance. In the case of ME and LE, the resulting information would prove that each was a pentapeptide but would not address the amino acid sequence . To determine the sequence of the amino acids, an Edman degradation

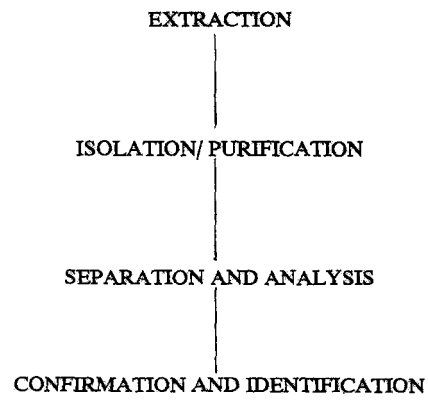

Figure 8

General Protocol for the Isolation and Identification of Neuropeptides

(87) would be performed. The Edman degradation is a classical method that can be accomplished in either a manual or automated fashion. These complimentary techniques could be used to provide more information about the peptide in question.

The determination of the enkephalins ME and LE in biological fluids is a multi-step effort requiring extraction, isolation, separation and final identification. Figure 8 provides an overview of the general protocol needed to successfully isolate and identify ME and LE.

Conclusion

This review has discussed previous and current applications on the isolation, separation and analysis of enkephalins. It also provided the scientist with a bibliography to seek further information since reseach efforts indicate that neuropeptides are not only neuromodulators and neurotransmitters, but also growth factors, and methods of analysis of these compounds are becoming increasingly important.

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A NEW SEQUENTIAL PROCEDURE FOR THE EFFICIENT AND AUTOMATED LOCATION OF OPTIMUM CONDITIONS IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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ABSTRACT

A new sequential Optimization Procedure by Search Point (OPSP) based on Hooke-Jeeves algorithm is developed. The procedure is an automated multifactor optimization of conditions for an HPLC separation. Its usefulness in computer-assisted method development is shown by the experimental mobile phase optimization of an isocratic reverse phase liquid chromatography separation of a mixture of six selected pesticides. The relative composition of a ternary mobile phase (Acetonitrile, Methanol, Water) was varied during the optimization process. An objective function (OF) which was used as the criterion of quality of the chromatographic

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separation is described. The perfomance of this new chromatographic method is evaluated either by plotting the map of the separation quality using a Grid Search method or by comparing the results with the ones obtained by the application of a Modified Simplex method, in both cases over the same triangular (Acetonitrile, Methanol, Water) parameter space. The optimum solvent composition for the satisfactory separation of pesticides by application of the OPSP was determined from only eleven chromatographic experiments while by applying the Modified Simplex or the Grid Search procedure the number of experiments were 15 and 36 respectively.

INTRODUCTION

The use of the computer as an aid in selecting adequate or optimum conditions in order to perform a given analytical separation within an acceptable analysis time has played an important role in the development of HPLC methods of analysis. This general topic continues to attract great interest as it has been attested by several recent books [1-5] and by the availability of the new commercial software offered by several HPLC manufacturers [2,6,7].

Optimization of the operating conditions neccesary to accomplish adequate separations by HPLC generally requires a formal experimental design coupled with the use of an optimization strategy. Most optimization techniques fall into two categories, viz., sequential experimental procedures and simultaneous experimental procedures [8,9]. Both approaches have been used to optimize the operating conditions of HPLC methods of analysis. The advantages and disadvantages of both approaches have been extensively described [1,3,4].

Quality criteria

Sequential procedures depend basically on two factors, the power of the directing algorithm (search algorithm) and the effectiveness of the quality criterion that is being used. The quality of a chromatographic separation must be expressed as a single numerical value or experimental response. The selection of a suitable criteria to achieve this may vary considerably from one example to another according to the different goals to be met in the optimization process. Often a compromise between conflicting goals has to be found. Optimization of chromatographic separation may therefore be considered as a multicriterion problem [10]. To solve such a problem it is necessary to translate such different analytical goals into objective functions [11] (OF) or desirability functions [12] (DF). These are complex functions incorporating a measure of the amount of separation by sum criteria (ΣRs, ΣP, etc.), product criteria (ΠRs, ΠP, etc.), etc. and other factors such as number of peaks, time requirements, etc. (by separate terms, penalty functions, etc.).

Search algorithm

The Basic Simplex method [13] (BSM) or Modified Simplex method [14] (MSM) unequivocally is the sequential method of choice to optimize HPLC separations. Nevertheless, the Simplex method has serious limitations mainly derived from its limited searching capability [15,16]. This fact leads to the development of different modifications such as Super Modified Simplex [17] (SMS), Weighted Centroid method [18] (WCM). Each of these search methods includes symmetry criteria [19] by which the search algorithm may continue to decide the next search direction after each step in the search process. These limitations are especially important to select mobile phase composition for HPLC due to the limitations in the experimental modification of solvent percentages. Therefore, the existence of

such problems or limitations of this search algorithm means that its implementation for the optimization of a particular analytical method may be very difficult in the hands of one who is not expert in these chemometrics methods.

In this paper a new sequential Optimization Procedure by Search Point (OPSP) based on Hooke-Jeeves algorithm [20] is described, and it is applied to the optimization of the composition of a ternary mobile phase for the HPLC separation of six selected pesticides. The result of the separation is compared with those obtained by application of a Modified Simplex and by application of a Grid Search design over the same parameter space. An objective function (OF) based on resolution (Rs) and time requirements is described and used as quality criterion. The major advantages of this multi-factor procedure are its high simplicity, efficiency, great search capacity, and the suitability for automation of the optimization process.

THEORY

The OPSP method is guided by calculations and decisions that are rigorously specified but trivially simple. The procedure is a hill-climbing method in which the direction and increment of advance is dependent only on the experimental responses and not on any particular values on an absolute scale. Only one experiment is needed for each step of the optimization process.

The algorithm

The objective of this sequential procedure is to force a point inside the selected factor space, named "search point", to move away from regions of poor response towards the region of optimum experimental response. In the present discussion a point at a given location within the factor space

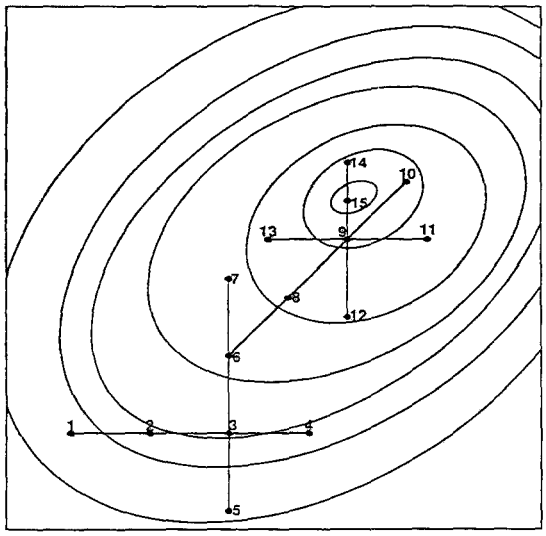

FIGURE 1. Illustration of a two-dimensional OPSP optimization. Concentric lines are contour lines.

corresponds to a specific ternary mobile phase composition. Movement implies a change in the composition of the ternary mobile phase and, thus, a point at a different location within the factor space. In Figure 1, a simple experiment beginning at point 1 is carried out to determine the experimental response. Subsequent movements or steps in the process are made according to a set of "rules". Figure 2 summarizes the rules of the OPSP procedure.

Rule 1.- After each experiment a movement is made according to any of the orthogonal or bisecants directions toward the limits of the factor space chosen (for non orthogonal experimental design, e.g. triangle, it will be considered as orthogonal the two firstly selected variables to locate the movements) by applying Rule 2 (orthogonal movement) or Rule 4 (bisecant movement). Figure 1 shows the progress of the "search point" in a two

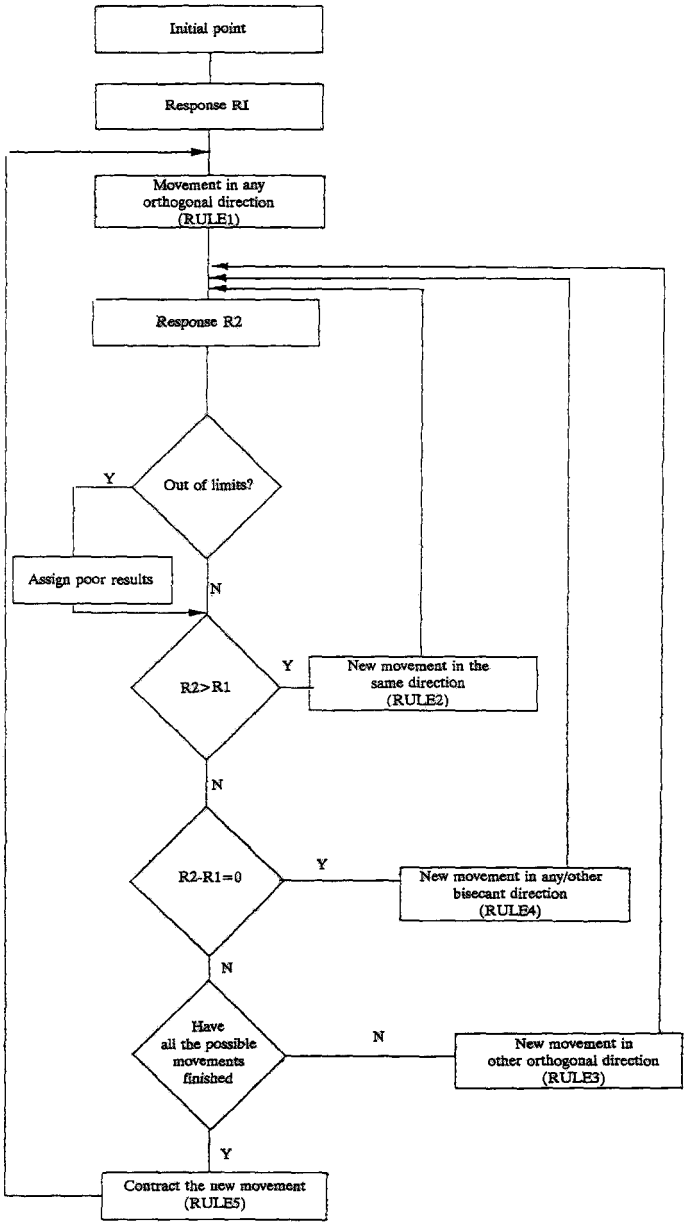

FIGURE 2. Flow chart of OPSP procedure.

variable factor space. Having carried out an experiment at point 1 at the beginning of the procedure, the experimental response is calculated by an adequate objective function and a new point, 2, is then calculated by application of a vector.

Rule 2.- Direction, sense and magnitude of the movement remain constant if the experimental response obtained is classified as better. For example, as shown in Figure 1 the movement of point 2 gives a new point, 3, and the movement of point 3 leads to point 4. This procedure is repeated until the optimum is located or until a violation of Rule 3, 4 or 5 occurs.

Rule 3.- If the new point has a worse response, do not apply Rule 2 but instead change the direction 90 degrees from the previous movement. As it can be seen in Figure 1, point 3 is followed by two sequential attempts before a better response, 6, is obtained.

Rule 4.- If the response at a new point differs very little or not at all from the previous point (R_2-$R_1 \approx 0$ or minor than a target value previously fixed from 0.01 to 1.00) do not apply Rule 2 but instead change the direction 45 degrees of the previous movement. As it can be seen in Figure 1, point 7 has an equal response than 6 and then it is followed by point 8.

Rule 5.- If the response at a new point is worse than the experimental responses in all the possible displacements from the previous point, then re-apply Rule 1 with a contracted increment. Referring to Figure 1, the movement of point 9 gives a new point, 15, as a result of a contracted vector displacement.

Increment of movement: The choice of the step sizes for Rule 2 and Rule 5 are arbitrary but usually a large increment (e.g. 1/6-1/8 of the range of a selected variable for Rule 2) is an advantage for two reasons. First,

to ensure some change in response and second a large step can more rapidly approach the optimum. In the OPSP program discussed here the increment can be fixed arbitrarily or generated automatically following the criterion mentioned above.

Location of the initial search point: Locating the initial search point is closely related to the size of the movement selected, but we considered a good practice to choose a central point over the domain of factor space to force the search point to back itself. In the OPSP program described here, location of the initial point can be fixed arbitrarily or generated automatically by application of a single mathematical relation among the limits of the search area selected.

End of Search criterion: One can choose among various criteria, such as the level of response, the number of experiments carried out or whether the different subsequent responses approaches the random noise and error.

EXPERIMENTAL

Instrumentation

High perfomance liquid chromatography system used includes a(n):

- 600E pump (Waters, Mildford Massachusetts),
- Rheodyne six-port injection valve with 20 µl loop,
- 990 photodiode array detector (Waters),
- 990 printer/plotter (Waters) and
- Olivetti microcomputer using a 991 software (Waters) implemented with the OPSP program developed by the authors.

TABLE 1

Pesticides studied in this work.

No	Pesticide studied	Aqueous solubility ($mg.\ l^{-1}$)	Use in Europe[21]
1	Metomyl	58×10^3	local
2	Dimethoate	25×10^3	major
3	Aldicarb	6×10^3	major
4	Dichlorvos	10×10^3	local
5	Carbofuran	700	local
6	Atrazine	30	major

HPLC separations were conducted using a Hypersil (Green Env.) 3x150 mm (5 μm particle size) C18 column (Shandon).

Chemicals and solvents

HPLC-grade solvents (Merck) were used in this work. Mobile phases were degassed with helium prior to use. Distilled water was obtained from a Millipore water purification Milli-Q system.

The pesticide standards (pestanal quality) listed in Table 1 were obtained from Riedel-de Haën (Seelze, German). Stock solutions were prepared by dissolving 20 mg of each purity certified pesticides in 100 ml of acetonitrile. A standard sample solution was prepared by mixing 200 μl of each of the stock solutions and making up to 10 ml with acetonitrile. All solvents and samples were filtered through a Millipore membrane filters (0.45 μm) before injection onto the column.

Chromatographic conditions

The following initial chromatographic conditions were used: the ternary mobile phase consisted of solvent A acetonitrile (AcN), solvent B methanol

(MeOH), and solvent C (water); flow-rate was 1 ml. min^{-1}; the UV detector was set at 210 nm; volume injected was 20 μl and column temperature was 30°C. The isocratic composition of the mobile phase in the reversed phase elution was optimized to obtain the best separation of pesticides. The void time was obtained by using methanol as the unretained component for all mobile phases. All chromatographic runs were duplicated. Reproducibility of retention times was ±0.5% or better.

Quality criteria

Objective Function. The first step in the optimization procedure was to select a suitable criteria to achieve an optimum judgement according to the different goals that have to be met in optimization procedure. The selection of an objective function (OF) based on penalty functions -p- type "infinite wall" was realized on the criteria given by Deming [11]. This function is defined as

$$OF = \Sigma (R_{i,j} \cdot P) \quad (I)$$

$$P = 1 \text{ for } Y > Y_{it};\ Y < Y_{ft} \quad (II)$$

$$P = 0 \text{ for } Y < Y_{it};\ Y > Y_{ft} \quad (III)$$

where $R_{i,j}$ is the resolution of each pair of peaks i and j. The maximum value assigned to $R_{i,j}$ is fixed in 1.5 in order to avoid that $\Sigma R_{i,j}$ is determined largely by the largest values of $R_{i,j}$.

P is the value of the penalty, y is the value of the retention time and Yt is the target retention time (in all the cases i is referred to the initial peak and f the last peak considered).

We assign a target value for Y_{it} =2 minutes and the second target retention time is Y_{ft} = 15 minutes as maximum acceptable retention time of the last peak considered. This objective function was used in the different optimization procedures applied in this work.

RESULTS AND DISCUSSION

Variable space selected

A water, acetonitrile, methanol triangular variable space is selected. Relying on literature data and previous experience, we observe that domains near the vertexes are of no interest because they have lower values of Y_{it} than 2 min or higher values of Y_{ft} than 15 min. So, in order to restrict the search area we select as esperimental boundary conditions: %AcN 20-70, %MeOH 0-65 and %Water 20-60. Therefore the variable space is restricted as shown in Figure 3 where the search region is darkened.

Over this search domain we apply the procedure proposed (OPSP computer program elaborated by the authors) to locate the coordinates of the optimum mobile phase (highest criterion value OF) to separate the standard mixture selected.

OPSP optimization

Inital point. The first point was automatically defined by the program using the equation,:

$$Vi = Vmin + Range\ (100 - \Sigma Vmin_{A,B,C}) / \Sigma Vmax_{A,B,C} - \Sigma Vmin_{A,B,C} \qquad (IV)$$

where Vi is the initial volume percentage selected of each variable (A, acetonitrile; B, methanol; and C, water), Range is the range of each

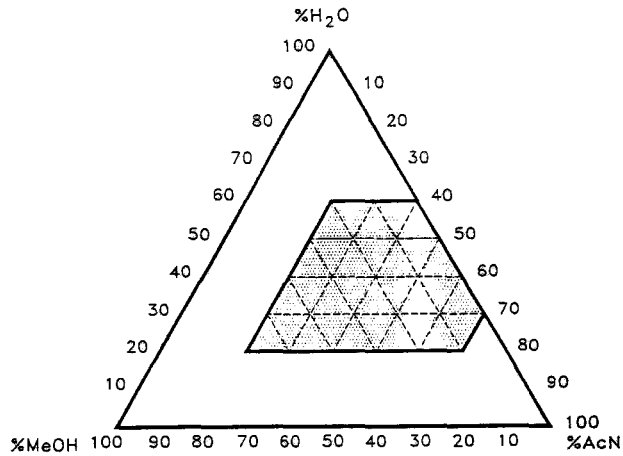

FIGURE 3. Graphical representation of search area (darkened) in the OPSP optimization.

variable (A,B,C), $\Sigma Vmin_{A,B,C}$ is the sum of minimum values of volume percentages of each variable and $\Sigma Vmax_{A,B,C}$ is the sum of maximum values of volume percentages of each variable. The initial value selected consists of the smallest possible value plus a factor proportional to the range of each variable. The initial experimental conditions are 39% AcN, 25% MeOH, 36% Water. The chromatogram of this initial experiment are shown in Figure 4.

Size of movements. The size of movements were automatically generated with sizes of 1/8 of the range of each variable considered for Rule 2 and Rule 4 and half ratios for Rule 5. The only two variables as criteria for the size and direction of orthogonal movements were the two first variables selected; %AcN and %MeOH.

OPSP movements. A set of experiments (Figure 5A) was therefore carried out guided by the search algorithm. A satisfactory separation (OF =

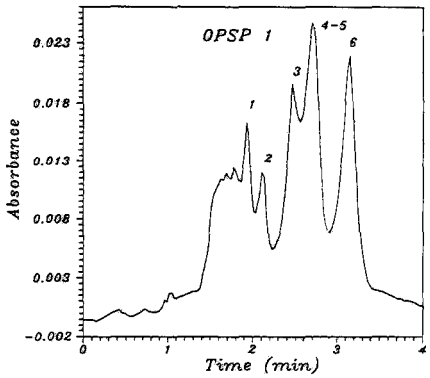

FIGURE 4. LC chromatogram of standard mixture of pesticides using a mobile phase corresponding to the initial point selected in the OPSP procedure. Chromatographic conditions are described in text. For peak identification see Table 1.

7.5) was achieved when eleven experimental chromatographic runs had been performed. This value OF =7.5 was the maximum possible, so we stop the movements at this point (see Table 2). The optimum conditions are 27% AcN, 17% MeOH and 56% Water. Figure 6A shows the chromatogram of the selected pesticides using these conditions.

Modified Simplex optimization

Initial Simplex. The location of the initial Simplex was (close to initial point of the OPSP procedure): vertex 1, 30% AcN: 30% MeOH: 40% Water; vertex 2, 40% AcN: 32% MeOH: 28% Water; and vertex 3, 32% AcN: 40% MeOH: 28% Water.

Size of movements. The size of the movements was calculated using a step size [22] of 10% (close to the sizes of the OPSP procedure) and a α value of 1. For constractions were used alpha values of 0.8, 0.6 and 0.5

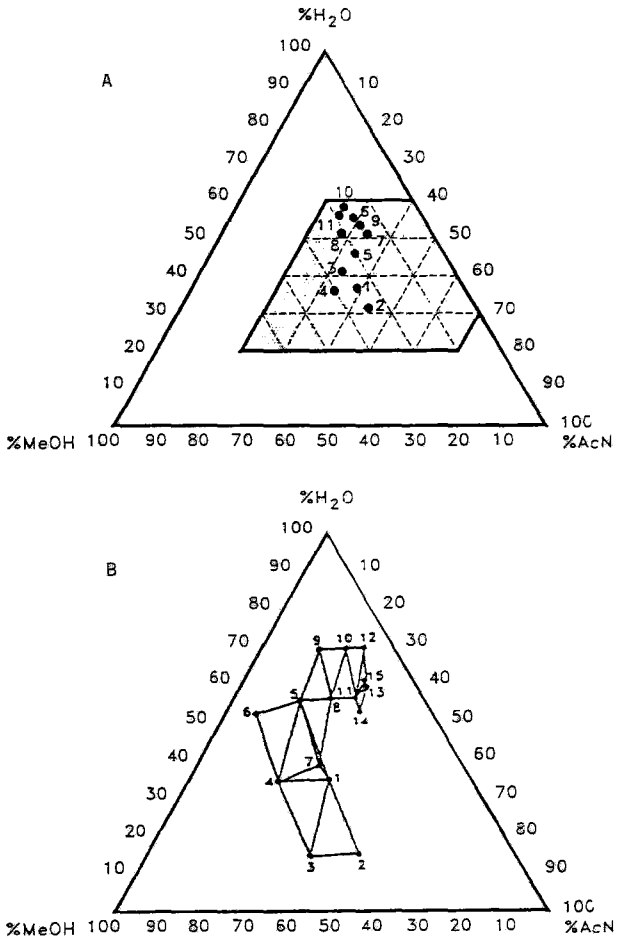

FIGURE 5. (A) Movements of the OPSP procedure across the variable space selected; (B) movements of the Modified Simplex procedure across the variable space selected; (C) Overview of the mobile phases compositions requiered in the Grid Search used.

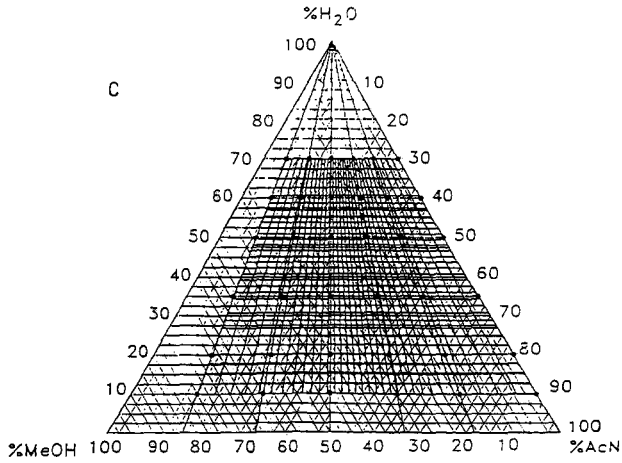

FIGURE 5 (continued)

TABLE 2
Experimental runs and results for the OPSP optimization.

Experience	%AcN:%MeOH:%H_2O	FO
1	39:25:36	3.18
2	44:25:31	3.02
3	34:25:41	3.42
4	34:30:36	2.88
5	34:20:46	3.84
6	29:15:56	5.04
7	34:15:51	3.67
8	29:20:51	5.74
9	31:15:54	4.99
10	27:15:58	6.36
11	27:17:56	7.50

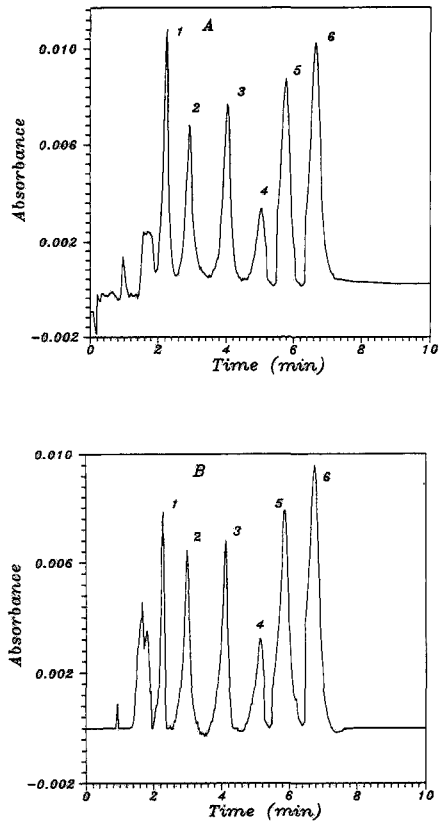

FIGURE 6. LC chromatograms of standard mixture of pesticides under optimum mobile phase obtained by: (A) the OPSP procedure; (B) the Modified Simplex procedure and (C) the Grid Search procedure. Chromatographic conditions are described in text. For peak identification see Table 1.

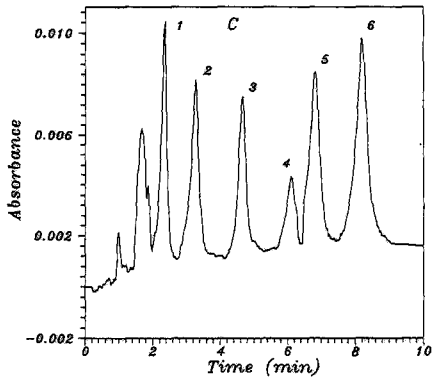

FIGURE 6 (continued)

TABLE 3
Experimental runs and results for the Modified Simplex optimization.

Initial vertexes	%AcN:%MeOH:%H_2O	α	FO
1	30:30:40	—	2.93
2	40:32:28	—	1.62
3	32:40:28	—	1.80
Movement N° (Vertex N°)	**%AcN:%MeOH:%H_2O**	**α**	**FO**
1 (4)	23:37:40	1.0	5.20
2 (5)	20:28:52	1.0	5.25
3 (6)	15:34:51	0.8	5.16
4 (7)	27:31:42	0.8	5.22
5 (8)	24:23:53	0.8	5.86
6 (9)	19:20:61	0.8	5.36
7 (10)	22:17:61	0.8	6.09
8 (11)	27:19:54	0.8	7.20
9 (12)	25:14:61	0.8	6.80
10 (13)	28:17:55	0.6	7.06
11 (14)	29:20:51	0.6	7.05
12 (15)	27:17:56	0.5	7.50

(see Table 3). These size values are close to the sizes used in the OPSP procedure.

Simplex movements. The movements of the simplexes are listed in Table 3 and them can be seen in Figure 5B. The best value of OF is obtained in the movement 12. So, 15 experiences have been necessary. Figure 6B shows the chromatogram of the selected pesticides using the optimum conditions.

Grid Search optimization

To check the results obtained by the OPSP procedure an additional set of 36 experiments was performed to draw the map of the separation quality using a grid of small increments over the same water-acetonitrile-methanol solvent triangle as shown in Figure 5C and with the mobile phases indicated in Table 4. The different OF values obtained let the graphic representation of the response surface as shown in Figure 7. The set experimental conditions corresponding to the top of the tallest "mountain" gives the best possible separation. Figure 7 predicts the global optimum in a narrow region near %AcN = 20-40, %MeOH = 10-20 and %Water = 45-65 which are in very close agreement with that obtained by the proposed procedure. Figure 6C shows the chromatogram of the selected pesticides using the best conditions.

Comparison of optimization methods applied

The results of the application of the OPSP, Grid Search and Simplex optimization are listed in Table 5 and optimum chromatograms obtained in each procedure are shown in Figure 6. It can be noted lower experiences number for OPSP procedure and an optimum separation in this case.

TABLE 4

Objective function (OF) values corresponding to the Grid Search chosen.

Experience	% AcN:% MeOH:%H_2O	FO	Experience	% AcN:% MeOH:%H_2O	FO
A1	5:25:70	3.00	D1	11:54:35	2.56
A2	10:20:70	3.00	D2	22:43:35	3.03
A3	15:15:70	3.00	D3	33:32:35	3.12
A4	20:10:70	4.50	D4	43:22:35	3.07
A5	25:5:70	7.00	D5	53:12:35	1.62
A6	30:0:70	6.74	D6	65:0:35	2.16
B1	7:33:60	5.09	E1	13:67:20	1.92
B2	13:27:60	4.00	E2	27:53:20	1.17
B3	20:20:60	6.68	E3	40:40:20	0.00
B4	27:13:60	7.28	E4	53:27:20	0.77
B5	33:7:60	6.36	E5	66:14:20	0.00
B6	40:0:60	6.45	E6	80:0:20	0.00
C1	8:42:50	5.14	F1	15:75:10	0.00
C2	17:33:50	6.09	F2	30:60:10	0.00
C3	25:25:50	5.70	F3	45:45:10	0.00
C4	33:17:50	5.15	F4	60:30:10	0.00
C5	41:9:50	4.56	F5	73:17:10	0.00
C6	50:0:50	4.89	F6	90:0:10	0.00

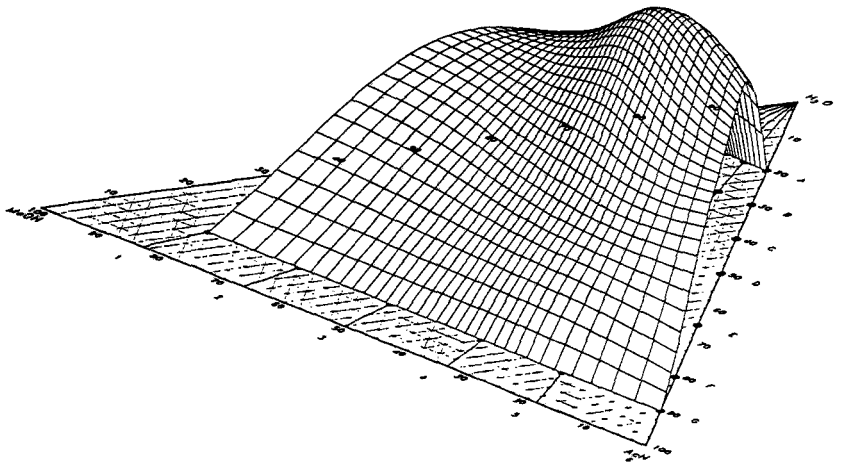

FIGURE 7. Pseudo-three dimensional response surface obtained by the Grid Search method.

TABLE 5

Movile phase composition optimum, experiences number and objective function (FO) for the three methods of optimization studied.

OPTIMIZATION METHOD	MOVILE PHASE COMPOSITION (AcN:MeOH:H_2O)	EXPERIENCES No	FO
GRID SEARCH	28:15:57	44	7.29
OPSP	27:17:56	11	7.50
MODIFIED SIMPLEX	27:17:56	15	7.50

CONCLUSIONS

A new sequential procedure (OPSP) has been developed and applied to the optimization of the chromatographic selectivity in the separation of selected pesticides by HPLC using isocratic reverse phase elution. The above results demonstrate that the OPSP procedure is an useful technique to find eluent compositions that give optimal separation. Althought the present study has involved only three factors and isocratic mode, the concepts are entirely general and can be extended to higher dimensional factor spaces, to other LC modes and to other operating parameters. The OPSP program can be requested from the authors.

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5,6-DIMETHOXY-2-(4'-HYDRAZINOCARBONYL-PHENYL)BENZOTHIAZOLE AS A HIGHLY SENSITIVE AND STABLE FLUORESCENCE DERIVATIZATION REAGENT FOR CARBOXYLIC ACIDS IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

5,6-Dimethoxy-2-(4'-hydrazinocarbonylphenyl)benzothiazole (BHBT-hydrazide) was found to be a highly sensitive and stable fluorescence derivatization reagent for carboxylic acids in liquid chromatography. Its reactivity was investigated for various $C_{18:0}$ - $C_{22:6}$ saturated and unsaturated fatty acids. The reagent readily reacted with the fatty acids in aqueous solution in the presence of pyridine and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at 37℃ to produce the corresponding fluorescent derivatives. The derivatives were separated on a reversed-phase column, TSK-gel ODS 120T, with gradient elution using 40 - 100 % (v/v) aqueous acetonitrile, and were detected spectrofluorimetrically at 447 nm with excitation of 365 nm. Calibration curves were linear over the range 10 fmol - 5.0 pmol per 20-μl injection (r=0.992 - 0.999). The relative standard deviations for ten replicated determinations did not exceed 2.0 % for any of the fatty acids (1.0 nmol/ml). The detection limits (signal-to-noise ratio=3) for the acids were 1 - 2 fmol

for an injection volumn of 20 μl. Further, the BHBT derivative of stearic acid was synthesized to examine the fluorescence properties.

INTRODUCTION

Various biogenic carboxylic acids occur in extremely small amounts in biological samples, and play very important roles. In addition, many drugs with carboxylic acids, which have strong efficacy against diseases and are administered at extremely small doses, have been developed. Therefore, a sensitive and selective high-performance liquid chromatographic (HPLC) method is required for the determination of the biogenic carboxylic acids and drugs.

For the purpose, numerous fluorescence derivatization reagents have been developed for the determination of carboxylic acids by HPLC: *e.g.* 4-bromomethyl-7-methoxy- (1-3), 4-hydroxymethyl-7-methoxy- (4), 4-diazomethyl-7-methoxy- (5), 4-bromomethyl-7-acethoxy- (6), 4-bromomethyl-6,7-dimethoxycoumarins (7), *N,N'*-dicyclohexyl-*O*-(7-methoxycoumarin-4-yl)methylisourea (8), 8-(chloromethyl)- (9), 9-(hydroxymethyl)- (10) and 9-aminophenanthrenes (11), 9-anthryldiazomethane (12,13), 9,10-diaminophenanthrene (14), *p*-(9-anthroyl)phenacyl bromide (15), 1-aminoethyl-4-dimethylaminonaphthalene (16), 1-bromoacetylpyrene (17), monodansyl cadaverine (18,19), 2-(2,3-naphthalimino)ethyl trifluoromethanesulphonate (20), 4-substituted 7-aminoalkylamino-2,1,3-benzoxadiazoles (21,22), 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one (23), 9-bromomethylacridine (24), *N*-(9-acridinyl)bromoacetamide (25), 1-pyrenyldiazomethane (26) and 3-bromomethyl-6,7-dimethoxy- (27) and 3-bromomethyl-6,7-methylenedioxy-1-methyl-2(1*H*)quinoxalinones (28).
However, these reagents generally require almost dried aprotic solvents, higher temperatures and/or prolonged heating in the derivatization reaction.

Most of biogenic substances and drugs having carboxylic acids are polor molecules, and occasionally labile against heat and light. Accordingly, a stable and highly sensitive reagent, which reacts with carboxylic acids in aqueous solution under mild reaction conditions, is required for the determination of their substances. Recently, in order to overcome the problems, some sensitve and selective fluorescence derivatization reagents having a hydrazinocarbonyl group as the reactive

group towards carboxylic acids have been reported; *viz* 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-propionylcarboxylic acid hydrazide (DMEQ-hydrazide) (29), 4-(5,6-dimethoxy-2-benzimidazoyl)benzohydrazide (30) and 2-(4-hydrazinocarbonylphenyl)-4,5-diphenylimidazole (31). The reagents react with carboxylic acids in aqueous solution in the presence of pyridine and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide under moderate derivatization conditions. Of these reagents, DMEQ-hydrazide has been already successfully applied for the direct determination of plasma free fatty acids (32) and urinary steroid glucuronides (33).

In previous research, we reported that 5,6-dimethoxy-2-benzothiazole, which is produced by the reaction between benzaldehyde and 2,2'-dithiobis-(1-amino-4,5-dimethoxybenzene), gives a more intense fluorescence than DMEQ and is excellent as a fluorophore (34). Hence, we newly synthesized 5,6-dimethoxy-2-(4'-hydrazinocarbonylphenyl)benzothiazole (BHBT-hydrazide), which has a novel 5,6-dimethoxy-2-benzothiazole moiety, as a fluorescence derivatization reagent for carboxylic acids. The reagent was demonstrated to be very useful for the highly sensitive determination of linear saturated and unsaturated fatty acids in the fluorimetric HPLC.

Moreover, the BHBT derivative of stearic acid was isolated to examine the fluorescence properties.

EXPERIMENTAL

Apparatus

Uncorrected fluorescence spectra and intensities were measured with a Hitachi (Tokyo, Japan) Model 650-60 spectrofluorimeter in 10 x 10 mm quartz cells; the spectral bandwidths of 10 nm were used for both the excitation and emission monochromators. Electron impact (EI) and fast atom bombardment (FAB) mass spectra (MS) were recorded with a Jeol (Tokyo, Japan) DX-300 spectrometer. ^{1}H-nuclear magnetic resonance (NMR) spectra were obtained with a Jeol JNM-GX400 spectrometer at 400 MHz using a ca. 1 % (w/v) solution of chloroform-d_1 or dimethyl-d_6 sulphoxide containing tetramethylsilane as an internal standard (abbreviations used : s, singlet; d, doublet; t, triplet; m, multiplet). Uncorrected melting temperatures were measured on a Yanaco (Tokyo, Japan) micro melting-point apparatus.

Chemicals and solutions

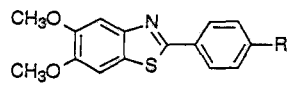
All chemicals were of analytical-reagent grade, unless stated otherwise. Deionized distilled water was used. Caprylic ($C_{8:0}$), capric ($C_{10:0}$), lauric ($C_{12:0}$), myristic ($C_{14:0}$), myristoleic ($C_{14:1}$), palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$), margaric ($C_{17:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), linolenic ($C_{18:3}$), arachidic ($C_{20:0}$), arachidonic ($C_{20:4}$) and docosahexaenoic ($C_{22:6}$) acids were purchased from Sigma (St. Louis, MO, USA). Stock solutions of $C_{8:0}$ - $C_{22:6}$ acids (1×10^{-4} M) were prepared in *N,N*-dimethylformamide and diluted further with water to give the required concentrations. 2,2'-Dithiobis(1-amino-4,5-dimethoxybenzene) (DTAD) was prepared as described previously (34).

Synthesis of BHBT-hydrazide

BHBT was synthesized via compounds I-II from DTAD in satisfactory yields by the following method (Fig. 1).

Compounds I and II

To a solution of 4-carboxylbenzaldehyde [1.2 g (8 mmol)/20 ml methanol] was added DTAD solution [3.7g (10 mmol)/40 ml methanol containing 1.2 g of tri-*n*-buthylphosphine and 0.8 M disodium hydrogen phosphite]. The mixture was allowed to stand at 37℃ for 2 h with stirring. The precipitates were filtered, washed with methanol - water (7:3, v/v) and dried under reduced pressure. The resulting crude product (compound I) dissolved in absolute methanol (50 ml) was treated with ethereal diazomethane solution prepared by the established method (35). The reaction mixture was evaporated to dryness under reduced pressure. The residue was subjected to chromatography on a silica gel 60 (ca. 130 g. 70-230 mesh; Japan Merck, Tokyo) column (25 x 3.5 cm i.d.) with *n*-hexane - ethylacetate - chloroform (2:1:1, v/v) as eluent. The main fraction was collected and the solvent was removed under reduced pressure to give compound II (800 mg, 30 %) as pale yellow needles, m.p. 223 - 224℃. ^{1}H NMR spectra (chloroform-d_1); δ 3.96, 3.98, 3.99 (s each, 3H each, OCH_3 each); 7.31, 7.56 (s each. 1H each, aromatic proton in beozothiazole moiety]; 8.08, 8.12 ppm [d (J=8 Hz) each, 2H each, aromatic proton in phenyl moiety]. Elemental analysis, calculated for $C_{17}H_{15}NO_4S$, C 61.99, H 4.59, N 4.25; found, C 62.11, H 4.66, N 4.41 %. EI-MS, *m/z*=329 (M^+, base peak), 314 ($M^+ - CH_3$), 286 ($M^+ - CO - CH_3$).



	R
Compound I	COOH
Compound II	$COOCH_3$
BHBT-hydrazide	$CONHNH_2$
BHBT-ST	$CONHNHCO(CH_2)_{16}CH_3$

Fig. 1. Compound, reagent and product structures.

BHBT-hydrazide

To a solution of compound II (500 mg, 15 mmol) in ethanol (50 ml) was added 100 ml of aqueous 45 % hydrazine hydrate, and the mixture was refluxed for ca. 60 min. The resulting precipitate was collected and recrystallized from ethanol to give BHBT-hydrazide (400 mg, 80 %) as colorless needles, m.p. 252 - 254℃. ^{1}H NMR (dimethyl-d_6 sulphoxide), δ 3.87, 3.89 (s each, 3H each, OCH_3 each), 4.55 (s, 2H, NH_2), 7.62, 7.71 (s each, 1H each, aromatic proton in benzothiazole moiety), 7.98, 8.08 [d (J=8 Hz) each, 2H each, aromatic proton in phenyl moiety], 9.90 ppm (s, 1H, NH). Elemental analysis: calculated for $C_{16}H_{15}N_3O_3S$, C 58.35, H 4.59, N 12.76; found, C 58.03, H 4.63, N 12.69 %. El-MS, *m/z*=329 (M^+, base peak), 314 ($M^+ - CH_3$), 286 ($M^+ - CO - CH_3$).

BHBT-hydrazide was stable in the crystalline state for a year or longer even in daylight. The reagent dissolved in *N,N*-dimethylformamide could be used for at least 2 weeks.

Isolation of the reaction product of stearic acid and BHBT-hydrazide

BHBT-hydrazide (200 mg), stearic acid (180 mg) and EDC (95 mg) were dissolved in pyidine - ethanol (2:8, v/v) (50 ml) and the mixture was allowed to stand at 37℃ for 1 h. The mixture was evaporated to dryness under reduced pressure. The residue in a small amount of ethylacetate, was chromatographed on a silica gel 60 colunm (25 x 2.5 cm i.d., 75 g, 70-230 mesh) with the same solvent. The main fraction was evaporated to dryness and the residue was recrystallized from ethanol to give 160 mg (44 %) of BHBT-ST (Fig. 1) as colorless needles, m.p. 230

- 232℃. ^{1}H NMR (dimethyl-d_6 sulphoxide), δ 0.85 [t (J=7 Hz), 3H, CH_3], 1.24 - 3.54 (m, 32H, $C_{16}H_{32}$), 3.88, 3.89 (s each, 3H each, OCH_3 each), 5.6 (broad, 1H, NH), 7.63, 7.73 (s each, 1H each, aromatic proton in benzothiazole moiety), 8.02, 8.03 [d (J=8 Hz) each, 2H each, aromatic protons in phenyl moiety], 9.03 ppm (broad, 1H, NH). Elemental analysis: calculated for $C_{34}H_{49}N_3O_4S$, C 68.32, H 8.41, N 6.91; found, C 68.54, H 8.29, N 7.05 %. FAB-MS, *m/z*=596 (MH^+), 298 (MH^+ - $C_{18}H_{36}N_2O_2$).

HPLC apparatus and conditions

A Hitachi 655A liquid chromatograph equipped with a Rheodyne 7125 syringe-loading sample injection valve (20-μl loop) and a Shimadzu (Kyoto, Japan) RF-535 fluorescence spectromonitor fitted with a 12-μl flow-cell was used. The fluorescence spectrometer was operated at an excitation wavelength of 365 nm and an emission wavelength of 447 nm. A TSK gel ODS 120T (Tosoh, Tokyo, Japan) column (250 x 4.6 mm i.d.; particle size, 5 μm) was used. The column temperature was ambient (20 - 27℃). For the separation of the BHBT derivatives of fatty acids, a gradient elution with aqueous 40-100 % (v/v) acetonitrile was carried out by using a Hitachi 833A solvent gradient device. The flow-rate was 1.0 ml/min.

Derivatization procedure

To 100 μl of a test solution of fatty acids in water (or 20-times water diluted serum) were added 100 μl each of 1 M EDC and 20 % (v/v) pyridine (both in methanol) and 100 μl of 15 mM reagent in *N,N*-dimethylformamide. The mixture was allowed to stand at 37℃ for 20 min, and then a portion (20 μl) was injected into the chromatograph. To prepare the reagent blank, 100 μl of methanol in place of 100 μl of the test solution were subjected to the same procedure.

RESULTS AND DISCUSSION

Product of the reaction of stearic acid with BHBT-hydrazide and its fluorescence properties

The fluorescent reaction products obtained in the determination of carboxylic acids were examined by using stearic acid. As carbonylhydrazino groups react with carboxylic acid compounds in the presence of EDC to give acid hydrazides (29-31),

the reaction product from stearic acid should be the corresponding BHBT-carboxylic acid hydrazide derivative. The derivative was confirmed as BHBT-ST (in Fig. 1) by the elemental analysis data and by the FAB-MS and ^{1}H NMR spectral data.

The fluorescence properties of BHBT-ST in methanol, acetonitrile, water and their mixtures, which have been widely used as mobile phase in reversed-phase chromatography, were examined to find a suitable mobile phase for the HPLC separation of the BHBT derivatives of fatty acids ($C_{8:0}$ - $C_{22:6}$).

The fluorescence excitation (maximum, 365 nm) and emission (maximum, 447 nm) spectra of BHBT-ST in methanol were almost identical with those in water and acetonitrile. The maxima in aqueous methanol and acetonitrile were independent of the concentration of water. The maximum and constant intensity was obtained at wide ranges in aqueous acetonitrile [0-50 % (v/v)] in comparison with aqueous methanol [0-10 % (v/v)] (Fig. 2). These results suggest that aqueous acetonitrile is suitable as a mobile phase in reversed-phase chromatography of BHBT derivatives of fatty acids with gradient elution.

Derivatization conditions

The conditions were examined using a mixture of the fatty acids (1.0 nmol/ml each).

BHBT-hydrazide was found to dissolve easily in *N,N*-dimethylformamide and dimethylsulphoxide and slightly in acetonitrile, methanol, ethanol, 2-methoxyethanol and tetrahydrofurane, but not in water and acetone. The former seven solvents affected the reactivity between BHBT-hydrazide and fatty acids. Since *N,N*-dimethylformamide gave the most intense peak heights (Table 1), the reagent solution was therefore prepared in *N,N*-dimethylformamide.

The most intense peaks were obtained at concentrations greater than ca. 10 mM of the reagent solution for all the fatty acids; 15 mM was used in the procedure.

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and pyridine were used to facilitate the derivatization of fatty acids with BHBT-hydrazide. Maximum and constant peak heights could be attained at EDC concentrations in the solution in the range of 0.8 - 1.3 M; 1.0 M was selected as optimum. The peak heights for the acids were maximum and constant at concentrations of pyridine higher than 10 % (v/v); 20 % was employed.

The derivatization reaction of fatty acids with BHBT-hydrazide apparently occurred even at 0℃; higher temperature allowed the fluorescence to develop more

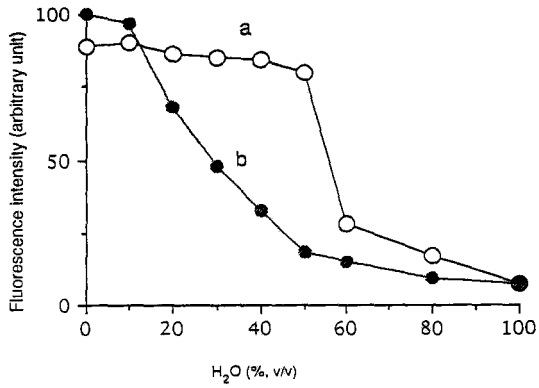


Fig. 2. Effect of water concentration in (a) aqueous acetonitrile and (b) aqueous methanol on the fluorescence intensity of BHBT-ST (1.0 nmol/ml). The fluorescence intensity was measured at the excitation and emission maxima.

TABLE 1

Effect of reaction solvent on the peak height

Solvent	Relative peak height[a)]
N,*N*-Dimethylformamide	100.0
Dimethylsulphoxide	94.5
Acetonitrile	76.4
Methanol	54.2
Ethanol	53.1
2-Methoxyethanol	60.4
Tetrahydrofurane	15.3

a) The peak height obtained with *N*,*N*-dimethylformamide was taken as 100.0.

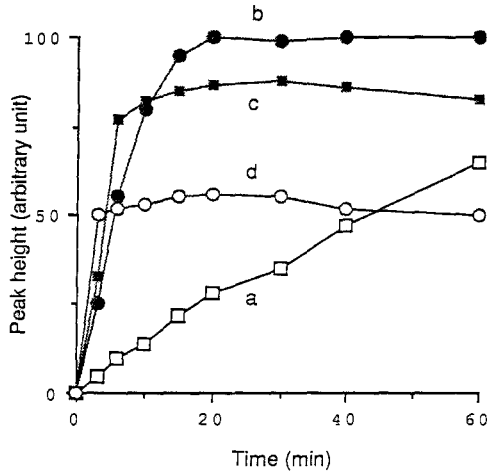


Fig. 3. Effect of reaction time and temperature on the peak height. A portion (100 μl) of a standard solution of $C_{16:0}$ acid (1.0 nmol/ml) was treated according to the described procedure. Temperature : a=0℃; b=37℃; c=50℃; d=80℃.

rapidly (Fig. 3). However, temperatures higher than 50℃ caused reduction of the fluorescence, probably because of decomposition of the produced derivatives. At 37 ℃, the peak heights for all the fatty acids were almost maximum after standing for 20 min. Hence, the solution was allowed to stand at 37℃ for 20 min in the procedure. The BHBT derivatives in the final mixture were stable for at least 24 h in the daylight at room temperature. The yield of the fluorescent derivative from stearic acid under the conditions employed was found to be 74.2 % by comparing the value of the peak height for stearic acid with that of BHBT-ST.

The similar optimal derivatization conditions were obtained when 2.0 pmol - 1.0 nmol/ml solutions of fatty acids were used.

Separation of BHBT derivatives of fatty acids

The good separation of BHBT derivatives of fifteen ($C_{8:0}$ - $C_{22:6}$) fatty acids was achieved on a reversed-phase column, TSK gel 120T, by gradient elution with acetonitrile between 40 and 100 % (v/v) in the mobile phase. However, the peaks for $C_{18:2}$ and $C_{20:4}$ acids could not be resolved successfully under any HPLC

conditions tested. The peak for $C_{20:1}$ acid (t_R=91.5 min) was slightly separated from the peak for $C_{18:0}$ acid (t_R=90.1 min). A typical chromatogram obtained with a standard solution of the fatty acids is shown in Fig. 4. The individual fatty acids each gave a single peak in the chromatogram. The change in the acetonitrile concentration had no effect on the fluorescence excitation and emission maximum wavelengths of the BHBT derivatives of all the fatty acids; the spectra were virtually identical with those of BHBT-ST.

It appears from Fig. 4 that different fatty acids have different peak responses. This might be due to the differences in the yields of the fluorescence derivatives from fatty acids and/or the quantum yields of the derivatives.

Precision, calibration graph and detection limit

The precision was established by repeated determinations using a standard mixture of fatty acids (1.0 nmol/ml each). The relative standard deviations did not exceed 2.0 % for any of the fatty acids examined (n=10 each instance).

The relationships between the peak heights and the amounts of the individual fatty acids were linear from 10 fmol to at least 5.0 pmol per injection volume (20 μl). The linear correlation coefficients were 0.992 or better for all the fatty acids.

The detection limits for all the fatty acids were 1 - 2 fmol on-column (signal-to-noise ratio=3). The method with BHBT-hydrazide was more sensitive 3 - 4 times than that with DMEQ-hydrazide (30), which is one of the most sensitive HPLC method.

Reaction of BHBT-hydrazide with compounds other than linear saturated and unsaturated $C_{8:0}$ - $C_{22:6}$ fatty acids

Hydroxycarboxylic acids (lactic and malic), dicarboxylic acids (oxalic, malonic, succinic and adipic) and aromatic carboxylic acids (benzoic, salicylic and cinnamic) reacted with BHBT-hydrazide under the derivatization conditions described to produce fluorescent derivatives. However, these compounds did not interfere with either the detection or the separation of the peaks for all the fatty acids , because BHBT derivatives of the compounds were eluted at 5 - 20 min under the recommended HPLC conditions. The metabolites of arachidonic acid (prostaglandin B_2, thromboxane B_2 and leukotriene B_4) also reacted with BHBT-hydrazide to yield the corresponding fluorescent derivatives and their retention times were 35 min, 15 min and 5 min, respectively, under the HPLC conditions. α-Keto acids (pyruvic,

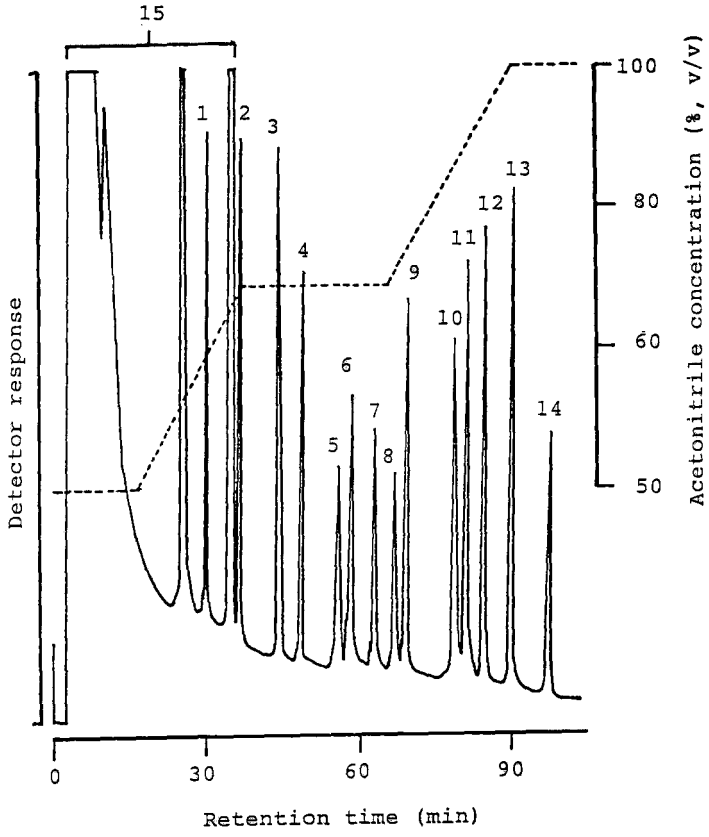


Fig. 4. Chromatogram of BHBT derivatives of fatty acids. A portion (100 µl) of a standard solution of the acids (1.0 nmol/ml each) was treated according to the described procedure. Peaks: 1=$C_{8:0}$; 2=$C_{10:0}$; 3=$C_{12:0}$; 4=$C_{14:1}$; 5=$C_{18:3}$; 6=$C_{14:0}$; 7=$C_{16:1}$; 8=$C_{22:6}$; 9=$C_{20:4}$ and $C_{18:2}$; 10=$C_{16:0}$; 11=$C_{18:1}$; 12=$C_{17:0}$; 13=$C_{18:0}$; 14=$C_{20:0}$; 15, others=reagent blank components. Mobile phase, gradient elution with aqueous acetonitrile (0-15 min, 40 %; 15-35 min, 40-70 %; 35-65 min, 70 %; 65-90 min, 70-100 %; 90-110 min, 100 %).

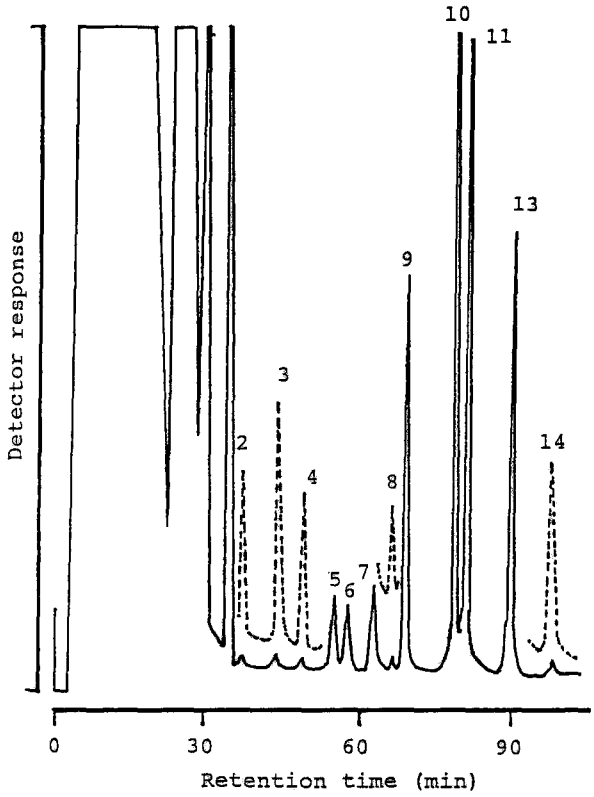


Fig. 5. Chromatogram obtained with human serum. Free fatty acid concentrations (nmol/ml serum):$C_{10:0}$=1.98; $C_{12:0}$=2.65; $C_{14:1}$=1.76; $C_{18:3}$=10.56; $C_{14:0}$=10.22; $C_{16:1}$=12.21; $C_{22:6}$=2.05; $C_{16:0}$=100.75; $C_{18:1}$=120.64; $C_{18:0}$=45.87; $C_{20:0}$=4.82.

α-ketoglutaric and phenylpyruvic) and 17 different α-amino acids did not fluoresce under the derivatization conditions employed. These observations suggested that the reagent is selective for carboxylic acids.

Application to serum free fatty acid assay

The proposed method was applied to the determination of free fatty acids in human serum.

Figure 5 shows a typical chromatogram obtained with serum. The serum levels of the acids except for $C_{8:0}$ were successfully quantified using an extremely small amount of serum (5 μl) by means of the standard addition method; $C_{8:0}$ acid was overlapped with endogenous substances in serum. The serum levels of (shown in Fig. 5) were in reasonable agreement with those by the HPLC (27,32) and GC (36) methods previously reported. The recoveries of the fatty acids (0.2 nmol per 5 μl each) added to a pooled normal serum were 95.5 - 98.3 %. This result indicates that BHBT-hydrazide is useful for the determination of carboxylic acids in biological materials.

CONCLUSION

BHBT-hydrazide is easy to synthesize and fairly stable in air and daylight, and even in the reagent solution. The reagent permits the derivatization of carboxylic acids in aqueous solution under the moderate conditions and is more sensitive (ca. 3 times) than DMEQ-hydrazide, which is one of the most sensitive fluorescence derivatization reagent. Therefore, the reagent should be useful for the detection of thermally labile carboxylic acids of biological importance at femtomole levels by HPLC.

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MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY OF METHYLATED FLAVONE AGLYCONES

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Abstract

Thirty four flavone aglycones isolated from herbs and spices were analysed by MECC on fused silica capillaries with sodium borate buffers and SDS micelles. Addition of organic solvents was necessary to improve the separation. However, when methanol was used, either as a sample solvent or as a constituent of the buffer, the most hydrophobic flavones appeared as double or triple peaks in the electropherograms. These double peaks disappeared when acetonitrile was used instead of methanol. The best separation was obtained with buffers containing acetonitrile when samples were dissolved in the same running buffer. In the separation of these flavones, in addition to the hydrophobic interaction with micelles, the ionization of phenolic hydroxyls and the borate complexation had some effect in the migration order of the molecules. A correlation between the migration order in MECC and the elution order previously reported for reversed-phase HPLC analyses was generally observed.

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INTRODUCTION

Analysis of plant flavonoids by Capillary Electrophoresis has been developed in the last few years since the first work published by Pietta and coworkers in 1991 [1]. Capillary Zone Electrophoresis (CZE) has been used in the analysis of flavonol O-glycosides [2-4], flavone O-glycosides [5] and flavonoid C-glycosides [6]. In addition, Micellar Electrokinetic Capillary Chromatography (MECC) has been used in the analysis of flavone O-glycosides [5] and flavonol O-glycosides [1,7,8]. However, analysis of flavonoid aglycones by these techniques has not been studied to the same extent, and only a few works on the MECC analysis of polyhydroxylated flavonol aglycones [7,9], and on the flavonoid aglycones (flavonols, flavones and flavanones) from honey [10,11] have been published. To date no analysis of lipophilic methylated flavones by Capillary Electrophoresis has been reported. These flavonoids are constitutive of many herbs and spices, and are partly responsible for their food antioxidative properties [12]. In addition, these lipophilic flavonoids have pharmacological [13] and ecological effects [14]. The aim of the present work is to establish conditions for the analysis of these flavonoid aglycones and to study the effect of the structures on their electrophoretic mobility.

EXPERIMENTAL

Materials

The flavonoid aglycones listed in table I were used as standards. All these compounds had previously been isolated and identified from Labiatae herbs [15] and these are deposited in the collection of the Phytochemical Laboratory (CEBAS, CSIC, Murcia).

Sample preparation

The different standard aglycones were dissolved in methanol, acetonitrile or running buffer to be analysed by CE. The available flavonoid aglycones were grouped into six different groups (**1-6**) (table 1) according to their oxygenation pattern in order to study the influence of increasing methylation on CE behaviour.

Table 1. MECC analysis of different lipophilic methylated flavones.

Groups	Aglycone names	Structures	OMe/OH	Buffers		
				A	B	C
	sideritoflavone	5,3',4'-OH 6,7,8-OMe	3/3	8.01	11.26	6.70
	thymonin	5,6,4'-OH 7,8,3'-OMe	3/3	10.18	12.27	7.10
	menthoflavone	5,6-OH 7,8,3',4'-OMe	4/2	18.85 / 19.90	12.75	7.28
1	8-methoxycirsilineol	5,4'-OH 6,7,8,3'-OMe	4/2	18.94	20.62	15.69
	gardenin D	5,3'-OH 6,7,8,4'-OMe	4/2	19.85	22.31	17.28
	nobiletin	5,6,7,8,3',4'- OMe	6/0	19.13 / 19.53*	22.31/22.55*	17.60
	5-desmethilnobiletin	5-OH 6,7,8,3',4'-OMe	5/1	19.15 / 19.47*	25.06/25.83*	18.38
	scutellarein	5,6,7,4'-OH	0/4	7.03	12.57	7.17
	isoscutellarein	5,7,8,4'-OH	0/4	7.56	12.82	7.37
2	hispidulin	5,7,4'-OH 6-OMe	1/3	10.46	13.81	9.22
	cirsimaritin	5,4'-OH 6,7-OMe	2/2	14.36	21.44	16.30
	tetramethoxyflavone	5,6,7,4'-OMe	4/0	19.23/19.42*	28.71/29.74*	18.12
	salvigenin	5-OH 6,7,4'-OMe	3/1	19.32/19.70*	30.91/31.39*	18.80
	apigenin	5,7,4'-OH	0/3	12.54	14.68	10.51
3	acacetin	5,7-OH 4'-OMe	1/2	14.01	18.04	14.58
	genkwanin	5,4'-OH 7-OMe	1/2	14.69	18.22	14.80
	apigenin 7,4'-methyl ether	5-OH 7,4'-OMe	2/1	16.70 /17.44 *	25.63*	25.07
	cirsiliol	5,3',4'-OH 6,7-OMe	2/3	7.90	12.52	6.80
	nodifloretin	5,6,7,4'-OH-3'-OMe	1/4	8.63	13.01	8.70
	6-OH luteolin	5,6,7,3',4'-OH	0/5	10.12	14.27	9.28
	hypolaetin	5,7,8,3',4'-OH	0/5	10.42	14.43	9.66
4	nepetin	5,7,3',4'-OH 6-OMe	1/4	10.49	16.58	11.70
	cirsilineol	5,4'-OH 6,7,3'-OMe	3/2	17.16	21.79	15.73
	sinensetin	5,6,7,3',4'-OMe	5/0	17.25 *	22.02/22.29*	16.18
	eupatorin	5,3'-OH 6,7,4'-OMe	3/2	17.60*	24.13/24.42*	18.02
	5-demethylsinensetin	5-OH 6,7,3',4'-OMe	4/1	18.90*	27.30/27.78*	19.18
	thymusin	5,6,4'-OH 7,8-OMe	2/3	9.92	13.13	10.18
5	xanthomicrol	5,4'-OH 6,7,8-OMe	2/3	14.08	20.49	21.36
	tangeretin	5,6,7,8,4'-OMe	5/0	14.52/14.80*	24.64/25.52*	24.46
	gardenin B	5-OH 6,7,8,4'-OMe	4/1	14.90*	28.39/29.31*	26.16
	luteolin 7-methyl ether	5,3',4'-OH 7-OMe	1/3	7.76	9.92	8.17
6	luteolin	5,7,4',3'-OH	0/4	8.54	10.66	9.92
	chrysoeriol	5,7,4'-OH 3'-OMe	1/3	11.65	15.10	10.95
	diosmetin	5,7,3'-OH 4'-OMe	1/3	14.38	16.26	11.62

Values are migration times in minutes. Buffer conditions: buffer **A** 0.2M sodium borate (pH 8) 50 mM SDS and 10% MeOH; buffer **B** 0.1M sodium borate (pH 8) 30 mM SDS and 25 % MeOH; buffer **C** 0.1 M sodium borate (pH 8) 50 mM SDS and 10% ACN. The samples were dissolved in methanol in the separations with buffers **A** and **B** and in the running buffer in separation with buffer **C**. *Double or triple peaks.

Micellar electrokinetic capillary chromatography (MECC).

MECC separations were carried out using a Beckman P/ACE System 5000 apparatus equipped with a 87 cm X 75 μm I.D. (80 cm to detector) fused-silica capillary and a Diode Array Detector. The daily conditioning started by rinsing the column with methanol (5 minutes), then with 1M NaOH solution (5 minutes), 0.1M NaOH (5 minutes), distilled water (5 minutes) and ending with the running buffer (3 minutes). Between consecutive runs the capillary was flushed with 0.1M NaOH (3 minutes), water (2 minutes) and running buffer (3 minutes). All buffers were freshly prepared after 2 injections. The following running buffers were used for the analyses of the different flavonoid aglycones: (**A**) 0.2M sodium borate (pH 8) 50 mM SDS and 10% MeOH; (**B**) 0.1M sodium borate (pH 8) 30 mM SDS and 25 % MeOH; (**C**) 0.1M sodium borate (pH 8) 50 mM SDS and 10% ACN (SDS, Sigma). The voltage was 20 kV, with an average current of 55μA, 32 μA and 65 μA respectively for buffers **A**, **B** and **C**, and the samples were injected by hydrodynamic injection for 2 seconds. All electropherograms were recorded on a Merck-Hitachi (Darmstadt, Germany) integrator. The on-column detector was operated at 280 nm, and the UV spectra recorded with the diode array detector.

RESULTS AND DISCUSSION

Separation of lipophilic methylated flavone aglycones by CE.

Due to the lipophilic nature of these flavone aglycones, and to the reduced number of ionizable phenolic hydroxyls in many of their molecules, MECC was considered the technique of choice for the separation of these substances by CE. In fact, some preliminary assays were achieved with CZE, using 0.1 M sodium borate buffer (pH 9.5 and 10.5), and under these conditions no separations were obtained for the more methylated flavones, which eluted together very close to the electroosmotic flow (data not shown).

The first approach to the analysis of these flavone aglycones by MECC was attempted using the conditions recently described for the separation of honey flavonoid aglycones [11]. In this case 0.2 M sodium borate buffer (pH 8) with 50 mM SDS and 10% MeOH was used (buffer **A**). The migration times (MTs) for the different flavonoids analysed under these conditions are shown in table 1. In order to test the influence of increasing

methylation on the electrophoretic behaviour of these molecules, the available flavonoid aglycones were arranged, according to their oxygenation pattern, in six differents groups (table 1, groups **1-6**). With buffer **A**, the flavones with a methoxyl/hydroxyl ratio lower than 1, showed a good resolution, but the most lipophilic flavones (methoxy/hydroxyl ratio higher than 1) migrated together and showed broad peaks remaining unresolved. In addition, an unexpected effect was observed. The highly methoxylated flavones 5,6,7,4'-tetramethoxyflavone, gardenin B, tangeretin, salvigenin, nobiletin, 5-desmethylnobiletin, 5-hydroxy-6,7,3',4'-tetramethoxyflavone and sinensetin showed double and sometimes triple peaks (Figure 1). The double peaks observed for each lipophilic flavone showed the same UV spectra (recorded with the diode array detector) and demonstrated that all the peaks observed were produced by the same substance.

Increasing the percentage of methanol in the running buffer has been considered a convenient and useful procedure in order to improve the separation both in CZE and MECC [6,11,16-20]. By this reason the percentage of methanol added to the separation buffer was increased gradually to reach 25%. As the MTs became too large when increasing methanol concentration, the ionic strength of the buffer was reduced to 0.1 M sodium borate, in order to increase the electroosmotic flow, and therefore reduce the MTs of the different flavonoids. The SDS concentration was also reduced to 30 mM to contribute to this MTs reduction. Under these new conditions the aglycones were much better separated. The effect observed when increasing the methanol concentration is shown in figure 1. All structural groups were quite well resolved with buffer **B** (Table 1), but the most hydrophobic (methylated) flavones still showed double or triple peaks as it happened with 10% methanol .

In order to determinate which was the origin of these double peaks, the flavone salvigenin (5-hydroxy-6,7,4'-trimethoxyflavone) was analysed using different solvents to dissolve and inject the sample (methanol, isopropanol, acetonitrile, and buffers **B** and **C**), and three different buffers were assayed to achieve the separations (buffers **A**, **B** and **C**). The results obtained are shown in Table 2, and indicate that the use of methanol to dissolve the sample is one of the factors leading to double peak formation, since double peaks are observed in separations achieved with the three buffers when salvigenin is dissolved in methanol. Isopropanol gives the same results as methanol, as well as acetonitrile when buffers containing methanol are used (buffers **A** or

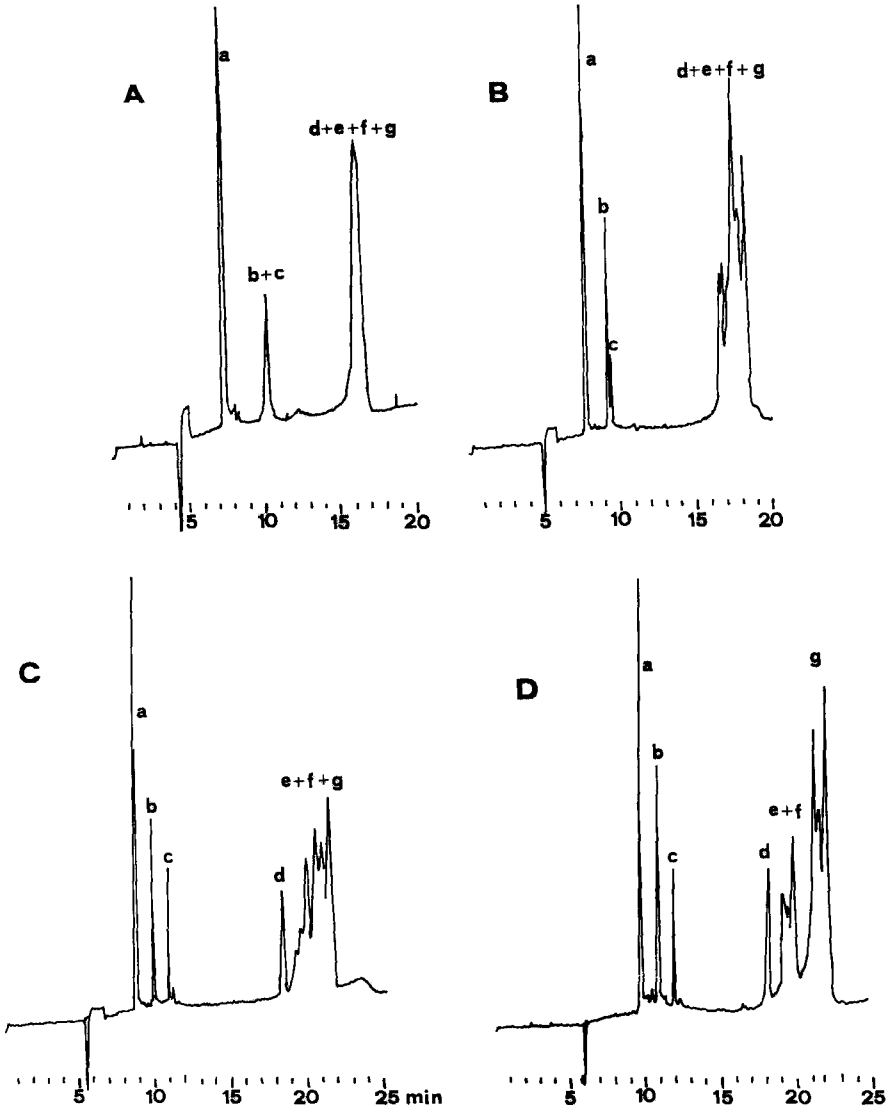

Fig. 1. Electropherograms of flavones included in group **1**. Influence of addtition of methanol to the buffer. (**A**) 0.2M sodium borate (pH 8), 50 mM SDS, 5% methanol; (**B**) 0.2M sodium borate (pH 8), 30 mM SDS, 10% methanol; (**C**) 0.2M sodium borate (pH 8), 30 mM SDS, 15% methanol; (**D**) 0.1M sodium borate, 30 mM SDS, 20% methanol; (**E**) 0.1M sodium borate, 30 mM SDS, 25% methanol. (**a**) sideritoflavone = 5,3',4'-OH-6,7,8-OMe; (**b**) thymonin = 5,6,4'-OH-7,8,3'-OMe; (**c**) 5,6-dihydroxy-6,7,3',4'-tetramethoxyflavone; (**d**) 8-methoxycirsilineol = 5,4'-OH-6,7,8,3'-OMe; (**e**) gardenin-D = 5,3'-OH-6,7,8,4'-OMe; (**f**) nobiletin = 5,6,7,8,3',4'-OMe; (**g**) 5-desmethylnobiletin = 5-OH-6,7,8,3',4'-OMe

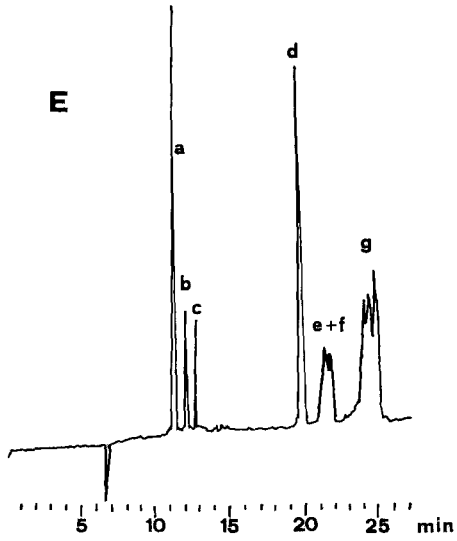

Fig. 1 (continued)

Table 2. Analysis of the flavone salvigenin (5-hydroxy-6,7,4'-trimethoxyflavone) under different conditions.

Sample solution	Separation buffer	Result
Methanol	Buffer A	Double peaks
Methanol	Buffer B	Double peaks
Methanol	Buffer C	Double peaks
Isopropanol	Buffer B	Double peaks
Acetonitrile	Buffer B	Double peaks
Buffer B	Buffer B	Double peaks
Acetonitrile	Buffer C	Single broad peaks
Buffer C	Buffer C	Single peaks with good resolution

B). When the sample was dissolved in buffer **B** and the analysis was achieved with the same buffer, double peaks were still observed.

These results show that when methanol, is present either as a sample solvent, or as an additive of the running buffer, the peak of salvigenin appears as a double peak. However, the use of buffers with acetonitrile (buffer **C**), dissolving the sample either with acetonitrile or with the running buffer **C**, produced single peaks, although very broad peaks were observed when the samples were dissolved in acetonitrile.

Multiple peaks formation could be explained by the partial precipitation of the lipophilic flavonoids in the running buffers, when the samples dissolved in methanol are injected in the buffer. In fact, if salvigenin is dissolved in methanol, and this sample is added to the different buffers used, a precipitation occurs. However, when acetonitrile is utilized as a sample solvent or as an organic modifier of the buffer, the solubility of the lipophilic flavones is higher.

As a summary, we can conclude that the best separations of these lipophilic flavonoids were observed when dissolving the samples in the acetonitrile containing buffer **C**, and using the same buffer to run the electrophoresis.

These conditions were then applied to the different flavone standards groups and the same effect was observed, leading to electropherograms with single sharp peaks as it can be seen in fig. 2.

Structure of flavonoid aglycones and electrophoretic migration in MECC

When analysing flavonoids by MECC, three different factors affect the electrophoretic migration; the hydrophobic interaction with micelles, which is the main factor, the ionization of free hydroxyls providing a negative charge to the molecule, and the complex formation with borate.

1- Hydrophobic interaction with micelles.

The flavone aglycones were analysed in the three buffers (**A**, **B** and **C**) to test the influence of different buffer compositions on their migration behaviour and as a general rule, as could be expected for a MECC separation, the higher the hydrophobic character of a flavone, the higher its MTs, since there is more interaction with the SDS micelles. This can be easily observed when comparing the methoxyl/hydroxyl ratio of the different

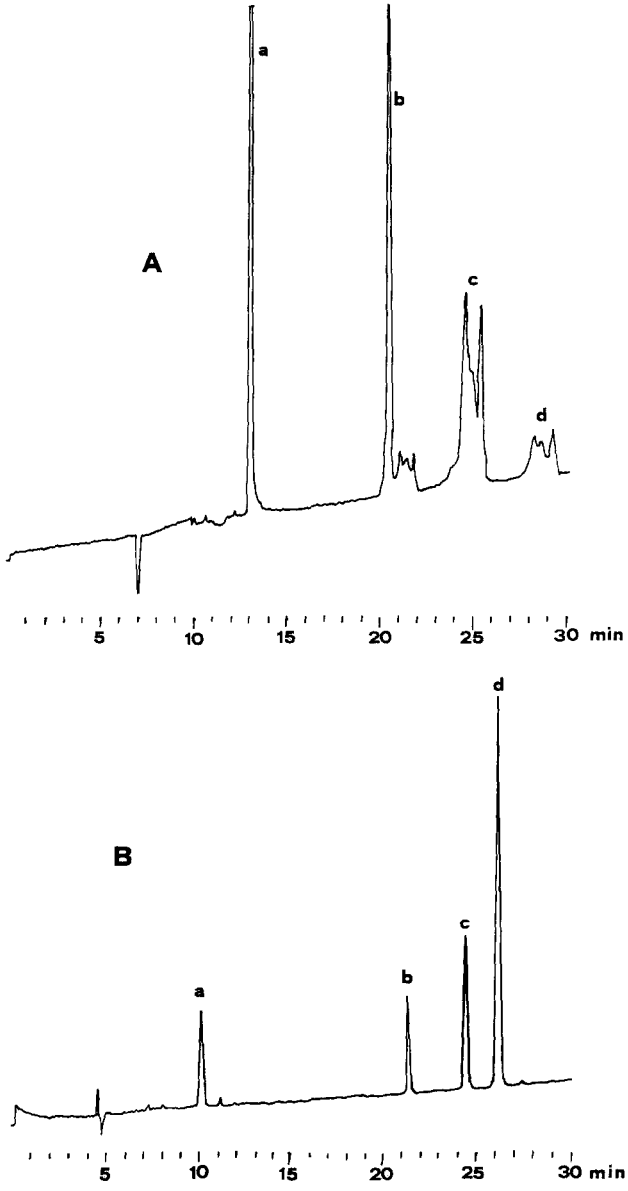

Fig. 2. Electropherograms of flavones included in group **5**. Influence of methanol on double peak formation. (**A**) running buffer: 0.1 M sodium borate (pH 8), 30 mM SDS, 25% methanol. Sample dissolved in methanol. (**B**) running buffer: 0.1 M sodium borate (pH 8), 50 mM SDS, 10% acetonitrile. Sample dissolved in running buffer. (**a**) thymusin = 5,6,4'-OH-7,8-OMe; (**b**) xanthomicrol = 5,4'-OH-6,7,8-OMe; (**c**) tangeretin = 5,6,7,8,4'-OMe; (**d**) gardenin-B = 5-OH-6,7,8,4'-OMe.

flavones with their MTs. In the different groups, when increasing this ratio the MTs increase. For instance, in group **2**, the substitution of one hydroxyl by one methoxyl in the molecule of scutellarein (5,6,7,4'-tetrahydroxyflavone) to give hispidulin (5,7,4'-trihydroxy-6-methoxyflavone) increases the MT in nearly 2 minutes (buffer **C**), and the further substitution of another hydroxyl to give cirsimaritin (5,4'-dihydroxy-6,7-dimethoxyflavone) increases the MT by 7 minutes. The introduction of another methyl ether at C-4' position to give salvigenin (5-hydroxy-6,7,4'-trimethoxyflavone) increases the MT by another 3 minutes. However, the substitution of the last hydroxyl to produce a completely methylated flavonoid (5,6,7,4'-tetramethoxyflavone) decreases its MT by nearly one minute in buffer **C**. The same effect is observed in groups **1**, **4** and **5**. The fully methylated nobiletin (group **1**), sinensetin (group **4**) and tangeretin (group **5**), migrate with shorter MTs than the corresponding flavones with a free hydroxyl in 5 position. A similar effect has previously been reported in the HPLC analysis of this type of compounds on reversed-phase columns [21]. In this case it was suggested that when a hydroxy group is present at position 5 (as in most of the flavones studied in this work), a strong internal hydrogen bond is formed between this group and the carbonyl group at position 4, and therefore the carbonyl, which is the strongest bond acceptor in a flavone, can no longer interact with the buffer [21-23]. Thus, the flavones with a methyl ether on the hydroxyl at 5, prevent this internal hydrogen bonding and allow the hydrophylic interaction of the carbonyl with the buffer, decreasing its MT. The same effect could explain why the 6-hydroxyflavone isomers migrate with shorter MTs than the 8-hydroxy isomers. For instance, the pair of isomers scutellarein (5,6,7,4'-tetrahydroxyflavone) and isoscutellarein (5,7,8;4'-tetrahydroxyflavone) and the isomers 6-hydroxyluteolin (5,6,7,3',4'-pentahydroxyflavone) and hypolaetin (5,7,8,3',4'-pentahydroxyflavone). This could be explained by an internal hydrogen bonding between the hydroxy groups at C-6 and C-5, which decrease the interaction described above between the latter hydroxy and the 4-keto group, and therefore decreasing MTs.

Isomers which are difficult to be separated by HPLC, are nicely separated by MECC due to the higher resolution of this technique. For instance the isomers showing the ring B substitution 3'-methoxy-4'-hydroxy and the isomeric forms 3'-hydroxy-4'-methoxy are very well separated. In all the compounds analysed, the isomers with a free hydroxyl in C-4' position (8-methoxycirsilineol, cirsilineol, and chrysoeriol) migrate with shorter MTs than

the corresponding isomers with free hydroxyl at C-3' position (gardenin-D, eupatorin and diosmetin respectively) (Table 1).

2- Electric effects

The migration order observed in the different groups is generally similar to that observed when analysing these substances in HPLC with reverse-phase columns [20]. However, some significant differences are observed, and could be explained by the electric effects, since this is an electrodriven separation. Therefore, flavones with free hydroxyls which are susceptible of ionization at the pH of the running buffer, will have a negative charge and therefore an electrophoretic migration to the anode (injection end of the capillary) and will show higher MTs. In this case the acidity (the pKa) of the different phenolic hydroxyls plays a crucial role in the separation. Phenolic hydroxyls with pKa values below 8 (the pH of the buffer) will be ionized and will suffer electrophoretic migration. For instance, the 7-methyl ether of luteolin (5,3',4'-trihydroxy-7-methoxyflavone) migrates with shorter MTs than luteolin (5,7,3',4'-tetrahydroxyflavone). This does not agree with the lipophilicity of the flavonoid, but in this case an electric effect is working in the separation. The most acidic phenolic hydroxyl in the flavonoid molecule is the hydroxyl at 7-position. The pKa for this hydroxyl in flavonoids with two free hydroxyls on ring B at C-3' and C-4', is generally below 8 (7.3 for quercetin), while in flavonoids with only one hydroxyl on ring B at C-4' the pKa values for the hydroxyl at 7 position is usually higher than 8 (8.2 for kaempferol) [4]. At pH 8 (the pH of the running buffer in these separations), when a free hydroxyl is present in C-7 of the flavone luteolin, this hydroxyl is ionized, and therefore has a negative charge which is not present in the compound with a methyl ether at this position, and this explains the higher MTs of luteolin with respect to its 7-methyl ether. The same effect could explain why cirsiliol (5,3',4'-trihydroxy-6,7-dimethoxyflavone) migrates with shorter MTs than 6-hydroxyluteolin (5,6,7,3',4'-pentahydroxyflavone).

However, when regarding the pair apigenin (5,7,4'-trihydroxyflavone) and genkwanin (5,4'-dihydroxy-7-methoxyflavone), the same effect is not observed, since in this type of compounds (flavonoids with a mono-oxigenated ring B), the pKa of the hydroxyl at C-7 is higher than 8 [4], and the hydroxyl at C-7 in apigenin is not ionized at pH 8, and therefore, the behaviour is similar to that observed in reversed-phase HPLC, and the MTs of apigenin 7-methyl ether (genkwanin) and the 4'-methyl ether (acacetin) are similar.

3- Borate complexation

Another possible factor which could affect the separation of flavonoids by MECC is the possibility of borate complexation. If a complex is formed with ortho-dihydroxyl groups, a negative charge is introduced in the molecule producing a retarding effect on the MT. Some examples illustrate this effect. For instance, nodifloretin (5,6,7,4'-tetrahydroxy-3'-methoxyflavone) migrates at 8.70 minutes in buffer C, while 6-hydroxyluteolin (5,6,7,3',4'-pentahydroxyflavone), which is more hydrophylic than nodifloretin and should interact less with the micelles eluting first, shows a migration time of 9.28 min. This could be explained by the formation of a borate complexation with the hydroxyls at C-3' and C-4', which provide additional negative charge, and therefore an increase in the MT for 6-hydroxyluteolin.

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SEPARATION OF BENZODIOXINIC ISOMERS IN LC. A MOLECULAR MODELING APPROACH FOR THE CHOICE OF THE STATIONARY PHASE

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ABSTRACT

Seven couples of position isomers on the aromatic ring of benzodioxinic compounds had to be separated. The tries made on classical systems in gas chromatography or liquid chromatography did not allow the separation. We have studied the structures of solutes by molecular modeling. Many molecular descriptors (lipophilic, electronic and steric) were calculated. Our objective was to compare the relative difference between the isomers in term of lipophilicity, electronic and steric properties and design the chromatographic system where these interactions are dominant. From these results, the porous graphitic carbon stationary phase was chosen and allowed a good separation of all the products.

INTRODUCTION

As part of our search for antiatherosclerotic agents [1,2] we needed 2-substituted-2,3-dihydro-1,4-benzodioxin (Fig. 1a) and 2-substituted-1,4-

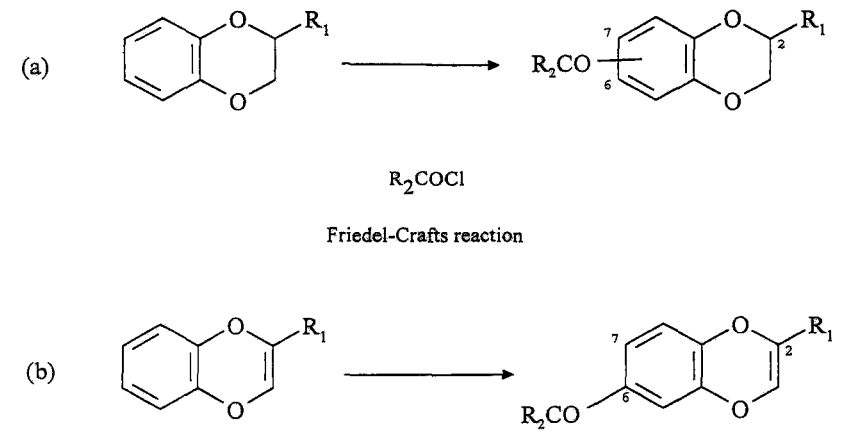


FIGURE 1 : The Friedel-Crafts acylation of Benzodixanic compounds.

benzodioxin (Fig. 1b) derivatives with acylated group on several positions of the aromatic ring [3]. The Friedel-Crafts acylation of saturated derivatives leads to an isomeric mixture of two monoacylated products at C_6 and C_7 positions with the 2,7 isomer as the main product (Fig. 1a). In contrast the same reaction using unsaturated precursors yields regioselectively the C_6 acetylated derivative (Fig. 1b).

In order to compare the reactivity depending on the substitution position, we converted the saturated isomeric mixture (Fig. 1a with $R_2 = CH_3$ and $R_1 = CN$) in a few steps to the 2,6 and 2,7 unsaturated analogues [3].

We used chromatographic analysis to study the regioselectivity of these reactions of acylation and to determine the isomeric ratio of each isomer identified by ^{1}H-NMR.

After various unsuccessful attempts on GC columns (no selectivity or no elution) and on LC columns (with bare silica in normal phase and octadecyl silica in reversed phase) we used molecular modeling to understand the retention of model compounds (R_1=CN). The results of this theoretical study allow us to

explain the difficulty in separating of these isomers on classical ODS stationary phase and the choice of porous graphitic carbon phase (PGC) to separate them.

The PGC column possesses a rigid, planar surface in addition to high electronic and charge transfer interactions and this is an advantage for the separation of our isomeric compounds [5].

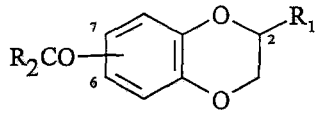
EXPERIMENTAL

1- Materials

The HPLC equipment comprised the following components: Gilson Model 302 pump (Villiers le Bel, France) and Rheodyne Model 7125 injection valve (Cotati, California, U.S.A); for UV detection at 254 nm, a Jasco Model 2550 spectrophotometer (Tokyo, Japan). Quasi-identical UV response of isomers at this wavelength was confirmed by an evaporative light scattering detector Model Sedex(ELS) 45 (Sedere, France) set on line. In fact, the narrow spread of the response factors of the ELS detector indicated that it is suitable for direct raw quantification [6]. Data were processed with a Shimadzu model CR 5A integrator/recorder (Kyoto, Japan).

HPLC analyses were carried out in normal phase on 250 x 4.6 mm i.d. column packed with 7 µm Zorbax Sil (DuPont, Wilmington, U.S.A); in reverse phase on 125 x 4 mm i.d. column packed with 5 µm Lichrospher 100 RP18 (Merck, Darmstadt, Germany); with various mobile phases on 150 x 4.6 mm i.d. column packed with Hypercarb (Shandon, Cergy-Pontoise, France). The first two systems do not give any selectivity between isomers.

Experiments by Gas Chromatography were carried out using a Varian GC Model 3700 (Les Ulis, France) on various columns: 3 m, 0.2% Silar 10C coated on 80/100 Graphpac-GC (Alltech, Templeuve, France) in packed glass column; 25 m 1 µm, 530 µm o.d., BP-1 fused silica column and 12 m 0.5 µm, 530 µm o.d., BPX70 fused silica column (SGE, Villeneuve St. Georges, France). On the first



Compounds	R_1	R_2
1	CN	CH3
2	CN	$(CH2)_3CH_3$
3	CO_2H	CH3
4	CH_2COCH_3	CH3
5	$CH_2OCO(CH_2)_2CH_3$	$(CH2)_3CH_3$
6	CH_2OCOCH_3	$(CH2)_3CH_3$

Compounds 7

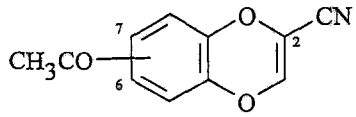


FIGURE 2 : The list of compounds.

column high adsorption of solutes precludes their elution. On both capillary columns no selectivity is obtained.

2- Mobile Phase and Reagents

The mobile phases, acetonitrile, methanol, water, methylene chloride (HPLC grade) and trifluoroacetic acid were obtained from Aldrich (St Quentin Fallavier, France).

Compounds 1-7 (Fig 2) were synthesised according to Thiéry *et al.* [3] by treatment of 2-substituted 2, 3 dihydro-1.4 benzodioxin with various acylchlorides

in presence of aluminium trichloride and carbon disulfide. In order to determine unambiguously the right position of the acetyl group, we prepared on the basis of literature data [4] and isolated in the pure form the 2,6 and 2,7 unsaturated derivatives.

3- Molecular modeling

We studied only the products substituted with CN group in position 2 and acetyl group in position 6 or 7 with or without the double bond (compounds 1 and 7). These model compounds were the easiest to study due to the small number of stable conformers.

Molecular structures were constructed from the input operating mode of the molecular modeling program Macromodel V3.5X [7] on Silicon Graphics Iris.

We carried out a systematic conformational search (MULTIC program) using the MM2 force field [8] with parametrisation for a isolated compound. All the conformations with good convergence were kept.

The molecular geometries of these conformations were optimised using the semi-empirical orbital program Mopac5 [9] (key-words: *am1, precise, polar*).

For each conformer i we calculated several descriptors D_i. The descriptor value D for a compound is then obtained from the values of this descriptor for all the stable conformations using equations 1 and 2.

For a descriptor value D and a conformation i (equation 1):

$$D = \sum_i Fi \times Di$$

where F_i is the molar fraction obtained from the Boltzman distribution at 25 °C (equation 2):

$$Fi = \frac{e^{-\Delta Ei/RT}}{\sum_i e^{-\Delta Ei/RT}}$$

ΔE_i is the energy difference between the conformer i and the most stable conformer.

We chose descriptors which have been used with success in other QSRR studies and also some other descriptors. These descriptors are:

surf and *vol*: respectively the Connolly surface and volume [10] (solvent accessible surface and volume) generated by program Molcad [11]. Specific properties such as molecular potential (*mep* or *mlp*) can be mapped on the Connolly surface.

mep : the molecular electrostatic potential [12] calculated on the Connolly surface.

mlp : the molecular lipophilic potential of the structure calculated on the Connolly surface. This potential is calculated using the Fauchère *et al* formula [13] and Ghose-Crippen's [14] atomic contribution.

The integrated value (*mepint* or *mlpint*) [15] is obtained by the algebraic sum on all the points computed by the Molcad program with the default density of the Connolly surface. This descriptor is therefore not only an electronic or lipophilic but also a geometric one.

oval : ovality (defined as the ratio of the actual surface area and its minimum surface area).

mtdip : the total dipole moment of the structure.

pol : the molecular polarisability calculated in an electric field.

homo : the energy level of the highest occupied molecular orbital.

lumo : the energy level of the lowest unoccupied molecular orbital.

RESULTS AND DISCUSSION

1- Quantitative Structure-Retention Relationships.

1-1 Molecular Modeling Study

In Table 1, we present the descriptor values of the two isomers (6-acetyl and 7-acetyl) of saturated compound 1. Each isomer has four stable conformers

Table 1

The calculated descriptors for isomers of compound 1.

	pol	*mf dip*	*plmint*	*pemint*	*surf*	*vol*	*oval*	*homo*	*lumo*
D 2-6	16.360	3.680	7.429	-485.491	214.829	204.867	1.279	-9.520	-0.628
D 2-7	16.390	3.615	7.445	-464.264	214.911	204.832	1.279	-9.518	-0.649
rd	0.030	0.066	0.016	21.227	0.083	0.035	0.001	0.002	0.021
% rd	0.16	1.78	0.21	4.37	0.04	0.02	0.05	0.02	3.38

D 2-6 : the descriptor value for isomer with acetyl in position 6.

D 2-7 : the descriptor value for isomer with acetyl in position 7.

rd : the absolute value of the difference on descriptors between the two isomers.

% rd : the relative difference value in percentage.

(two axial and two equatorial). The CN axial conformers are energetically the most favourable.

For each isomer, we calculated the value of each descriptor (according to equations 1 and 2). We also calculated the difference (**rd**) and the relative difference (**%rd**) between the two descriptors of 6-acetyl and 7-acetyl isomers.

The greater **%rd** values were noticed for *pemint* (4.37%), *lumo* (3.38%) and *mtdip* (1.78%). The others **%rd** are less than 0.21% (*pol, plmint, surf, vol* and *homo*).

In Table 2, we present the descriptor values of the two isomers (6-acetyl and 7-acetyl) of unsaturated compound 7. Due to the presence of the double bond, each isomer has only two stable conformers. We calculated **D, rd** and **%rd** as above. The main difference of this compound *vs* the compound 1 is its global shape. All the atoms of the rings and substituents (CN and acetyl) lie into a plane.

For compound 7, the greater **%rd** values were noticed for *mtdip* (14.3%), *pemint* (10.8%). The others **%rd** are less than 3% (*pol, plmint, surf, vol, oval, homo* and *lumo*).

For the calculation of lipophilic descriptors we do not use log P (calculated partition coefficient in octanol/water system using fragmental approach) as lipophilic descriptor because two position isomers have the same calculated log P. The integrated molecular lipophilic potential (*plmint*) calculated on Connolly surface is a lipophilic but also a geometric descriptor, consequently the *plmint* of two isomers may be different.

The difference between isomers in *plmint* (lipophilic descriptor), molecular volume and molecular surface ('bulk' descriptors) is very low. The difference is much greater for electronic descriptors like dipole moment and the integrated molecular electrostatic potential.

1-2 Correlation with Chromatographic Retention

When we tried to separate the isomers on apolar stationary phases (ODS and BP1) and polar stationary phases (silica and BPX70) in liquid and gas

Table 2

The calculated descriptors for isomers of compound 7.

	pol	*mt dip*	*pimint*	*pemint*	*surf*	*vol*	*oval*	*homo*	*lumo*
D 2-6	16.883	2.311	1.957	-364.852	209.724	196.047	1.285	-9.199	-1.014
D 2-7	16.955	2.643	2.007	-325.552	209.743	196.365	1.284	-9.203	-0.988
rd	0.072	0.331	0.050	39.300	0.019	0.318	0.001	0.003	0.026
% rd	0.4	14.3	2.6	10.8	0.0	0.2	0.1	0.0	2.6

D 2-6 : the descriptor value for isomer with acetyl in position 6.

D 2-7 : the descriptor value for isomer with acetyl in position 7.

rd : the absolute value of the difference on descriptors between the two isomers.

% rd : the relative difference value in percentage.

chromatography respectively, no selectivity was obtained. These results can be explained by the small differences between the lipophilic descriptors of the isomers.

Recently, Forgacs *et al* [16] studied the retention of 22 phenol derivatives on porous graphitized carbon (PGC) and octadecylsilica (ODS). They evaluated the relationships between retention and physicochemical parameters by multivariate analysis. Calculations proved that a marked difference can be detected between the retention characteristics of PGC and ODS columns, and that the electronic parameters of phenol derivatives have the highest impact on their retention.

Kaliszan *et al* [17] have used quantitative structure-retention relationships (QSRR) to analyse HPLC retention data for a set of non-congeneric aromatic solutes. They used graphitic carbon as stationary phase with hexane as eluent. The capacity factors were quantitatively related to structural information extracted from nineteen molecular descriptors of solutes by multivariate analysis. These QSRR studies using principal components provide evidence for the decisive role of specific, polar electronic interactions for the separation of solutes in PGC-hexane HPLC system.

The electronic descriptors are the ones with the greatest difference for the couple of isomers of our compounds (*mtdip* and *pemint* mainly).

According to these results, we tried to separate the isomers on Hypercarb column. The mobile phase used depends on the class of benzodioxinic compounds to be separated, saturated or unsaturated ones.

With a benzodioxinic saturated compound like solute 1, a better selectivity is obtained with acetonitrile-water-trifluoroacetic acid (57:42.7:0.3 %) as mobile phase. Using this mobile phase, unsaturated CN-substituted compounds as solute 7 are not eluted, they require a pure organic mobile phase. It is can be explained by the difference in the adsorption energy on the PGC planar surface and the surface of the adsorbed molecule. Compound 7 is much more planar than compound 1 thus it has a high interaction with PGC surface.

This qualitative study shows that we can design a better system to separate a set of compounds using molecular modeling and theoretical properties calculations.

2- Chromatographic Results

For the compounds having a moderately polar substituent such as $R_1 = CH_2OCOCH_3$ and $R_2 = CH_3$ (pair of solutes 4), a good selectivity is obtained on Hypercarb using an acetonitrile-water 85:15 (v/v) mixture as mobile phase, this packing being used as a "pure" reversed phase material (Chromatogram 4, Fig. 3).

When $R_1 = CH_2OCOCH_3$ and $R_2 = (CH_2)_2CH_3$ (pair of solutes 6), the retention increases considerably with such a mobile phase. For sufficiently selective elution of this pair of isomers, plain acetonitrile as mobile phase is required (Table 3; Chromatogram 6, Fig. 3). With the same eluent, isomers with $R_1 = CH_2OCO(CH_2)_2CH_3$ and $R_2 = (CH_2)_2CH_3$ (pair of solutes 5, Fig. 3) are more retained.

When R_1 substituent is more polar (CN, COOH) (pair of solutes 1 and 3 respectively), an electronic modifier (e.g. trifluoro acetic acid) is required to obtain a good shape of peak and to avoid peak tailing. In fact this is due to the presence of an electron pair of trifluoroacetic acid which acts as an electron donor and decreases the charge transfer between analyte and porous graphitic carbon. With 42.7 % of water content in the mobile phase, the pair of solutes 1 are separated (Table 3, Fig. 3), in retention time of 6.2 min. Using this mobile phase, unsaturated CN-substituted compounds are not eluted, showing the large difference between these two series.

To increase the hydrophobic character of CN-substituted isomers, $R_2 = (CH_2)_3CH_3$ (pair of solutes 2) replaced $R_2 = CH_3$ (solutes 1). Consequently a larger amount of organic modifier (acetonitrile) is required to elute the isomers with similar retention (Chromatogram 2, Fig. 3).

When the substituent is a polar group such as COOH (solutes 3), a better selectivity is obtained with a free-water mobile phase (Table 3; chromatogram 3,

Table 3

Selectivity of the separation of isomers.

Isomers mixture	α	Mobile phase
1	1.16	CH3CN-H2O-CF3COOH (57/42.7/0.3)
2	1.34	CH3CN-H2O-CF3COOH (200/50/1)
3	1.15	CH3CN-H2O (100/1)
4	1.25	CH3CN-H2O (85/15)
5	1.21	CH3CN
6	1.15	CH3CN
7	1.37	CH2Cl2

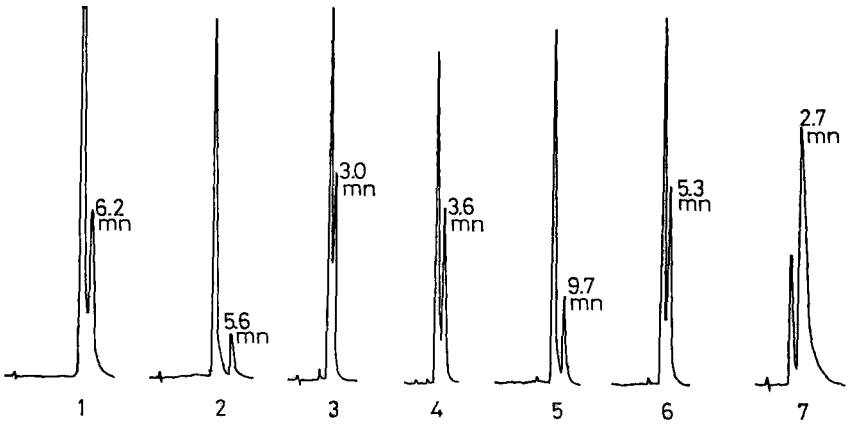


FIGURE 3 : The chromatograms of the separated isomers.

Fig. 3) and the comparison of the retention with that of the corresponding CN-substituted compound demonstrates the more hydrophobic character of the first derivative.

With the benzodioxinic derivatives where R_1 = CN and R_2 = CH_3 (solutes 7), the stronger interactions between solutes and porous graphitic carbon surface require a pure organic mobile phase (methylene chloride) for elution (Chromatogram 7, Fig. 3). Such a mobile phase is required by the strong adsorption potential of this material with these flat molecules. This illustrates the large choice of solvents used with porous graphitic carbon. Comparison of the behaviour of saturated and unsaturated CN-substituted compounds illustrates the great influence of the molecule's stereochemistry in the process of separation and we can note that for saturated compounds, the 2-7 isomer (the higher peak in chromatograms 1-6, Fig. 3) is less retained when for unsaturated ones the 2-7 isomer (the higher peak in chromatogram 7, Fig. 3) is more retained.

CONCLUSION

In this work, we have used molecular modeling to design a better chromatographic system to separate isomers. The Hypercarb was the best stationary phase used for the separation of these isomers. We have explained the separation on the PGC stationary phase by the large relative difference in the electronic properties of the isomers compared to their lipophilic ones.

This is the proof of the usefulness of molecular modeling in the rational choice of a chromatographic system.

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A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE QUANTITATION OF IMPURITIES IN AN NMDA ANTAGONIST USING EVAPORATIVE LIGHT SCATTERING DETECTION

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ABSTRACT

A reversed-phase high-performance liquid chromatography (HPLC) method utilizing an evaporative light scattering detector (ELSD) was developed for a new NMDA (N-methyl-D-aspartate) antagonist. This method permits quantitation of both the bulk drug substance purity and the related materials possible within the bulk drug substance. The method is compatible with LC/MS and the mass spectral data were obtained for each component in the bulk drug substance.

INTRODUCTION

Pharmaceutical compounds are routinely evaluated for bulk drug substance purity as well as content of possible impurities and degradation products. HPLC is currently the most common technique used to quantitate both bulk drug substance purity and nonvolatile impurities in a drug substance. Typically, HPLC with UV detection is chosen due to high sensitivity, ease of use and linear range.

Complications often arise in quantitation of unknown impurities since many of these entities may lack a UV chromophore or the chromophore may have vastly different properties when compared to the spectral properties of the parent drug substance. The recent introduction of the evaporative light scattering detector (ELSD) into the arena of HPLC detectors helps to resolve this nonequivalence response issue.

The ELSD has been introduced commercially and has gained acceptance as a sensitive universal detector.[1-2] The ELSD operates by nebulizing the volatile effluent from the HPLC column into a fine mist. The mist is then carried through a heated drift tube which evaporates the mobile phase and leaves behind the nonvolatile solute particles. The fine cloud of solute particles is carried at a high speed through a beam of light causing scattering which is detected. The amount of light scattered is dependent upon the size, shape and number of particles and therefore proportional to the concentration.

The ELSD is not influenced by the UV spectral properties of the solvents used for mobile phases, therefore the ELSD is not subject to baseline drift from gradient elution. The choice of acceptable solvents is expanded since the spectral background is not an issue with the ELSD. The ELSD is not affected with sample solvent interferences and the sample response is independent of the chemical structure of the solute. A limitation of the ELSD requires the complete volatilization of all mobile phase components. Addition of nonvolatile components to the mobile phase would cause an elevated background by the continuous generation of solid particles into the light source. The elevated background decreases the sensitivity of the detector for the sample components.

The current chromatographic literature demonstrates the applicability of the ELSD for use with phospholipids,[3-9] triglycerides, fats and fatty acid esters,[10-15] carbohydrates,[16-17] synthetic polymers,[18] steroids[19] and inorganic counter ions.[20] Recently, Lafrosse *et al* demonstrated the applicability of using the ELSD for pharmaceutical compounds.[21] While the HPLC/ELSD combination has been used for quantitation of drug entities, the lack of interest in the use of the technique for determination of impurities and degradation products is surprising. The universal capabilities of this detector lends itself to such uses.

The intent of this paper is to show the applicability of the ELSD to accurately detect and quantitate impurity levels in a pharmaceutical compound. Our laboratory recently developed a method to determine the purity of an NMDA antagonist with the structure shown in Figure 1. This chemical entity has insufficient UV chromophores and therefore a more sensitive means of detection was desired. Various UV and fluorescence derivatization schemes are possible but such manipulation introduces their own source of errors into analytical procedures. Furthermore, the lack of nonaqueous solubility of this NMDA antagonist limits the number of derivatization schemes that are possible. Gas chromatography (GC) could not be used because the compound decomposes at 283°C. This NMDA antagonist presented an ideal situation for employment of the ELSD. The detector technology should provide a means for quantitation of the main compound, as well as offering a means for determination of potential impurities in the drug substance. A high-low approach was used to quantitate impurity levels.[22] Quantitation was based directly on the peak area responses of the substances injected which is a principle of the ELSD, however, it should be noted that differing physical properties in samples (i.e., viscosity, refractive index, temperature sensitivity) can result in a different droplet size and subsequently different response for similar concentrations. The HPLC method for this NMDA antagonist was then run using LC/MS to identify the unknown impurities.

MATERIALS AND METHODS

Chemicals

The NMDA antagonist (Eli Lilly compound LY235959, [(-)-3S,4aR,6S,8aS-6-(phosphonomethyl)-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid]) was synthesized from m-tyrosine.[23] The bisphosphonic acid and 6-methyl impurities were also prepared within Eli Lilly and Company (Indianapolis, IN). Trifluoroacetic acid (TFA) A.R. was purchased from EM Science (Gibbstown, NJ). The acetonitrile (ACN) was HPLC grade and

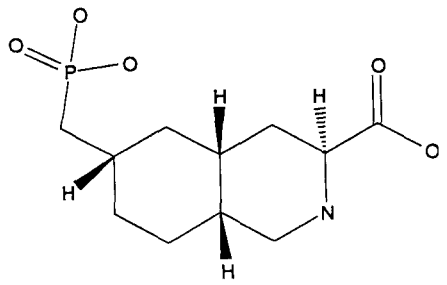

FIGURE 1. Structure of NMDA antagonist.

purchased from Mallinckrodt (Paris, KY). Water was deionized and filtered through a Milli-Q™ water purification system (Millipore, New Bedford, MA). NF grade nitrogen was used for the ELSD.

Apparatus

The HPLC system used for this study consisted of a Hewlett-Packard 1050 autoinjector, pump and UV detector (Wilmington, DE). A Varex IIA ELSD (Burtonsville, MD) was used in tandem with the UV detector set at 205 nm. A Zorbax® SB-Phenyl 250 x 4.6 mm I.D. column (Mac Mod Analytical, Chads Ford, PA) was used to separate the impurities from the NMDA antagonist. A Sciex API III LC/Ionspray Mass Spectrometer (Toronto, Ontario, Canada) was used to determine the mass of the impurities. A scan range of 150 to 600 daltons was used with a scan rate of 2.4 seconds/scan. The ionspray ionization mode was used with a post-column split ratio of 20:1 (20 μL/minute flow to the mass spectrometer source).

Assay and Chromatographic Conditions

A binary reversed-phase gradient system was used to ensure all impurities were eluted from of the column. The gradient profile and mobile phases are described in Table 1. Equilibration time before the next injection was 20 minutes.

TABLE I

Gradient System for the NMDA Antagonist Impurity Assay

Time (min.)	% A[a]	% B[b]
0.0	100	0
12.0	100	0
22.0	0	100
38.0	0	100
40.0	100	0

[a]A = 1% Acetonitrile/99% water pH adjusted to 2.4 with trifluoroacetic acid
[b]B = 60% Acetonitrile/40% water pH adjusted to 2.4 with trifluoroacetic acid

The mobile phase flow rate was set at 0.4 mL/min. The column temperature was ambient and injection volume was 50 µL. Impurity levels in the NMDA antagonist drug substance were assessed using a high-low approach.[22] Sample concentration of 3 mg/mL for the NMDA antagonist was prepared in mobile phase A as the high concentration sample. The sample was placed in an ultrasonic bath for approximately 5 minutes to enhance dissolution. A 1:10 dilution yielded a 0.3 mg/mL solution for the low concentration sample. The low concentration solution was then analyzed by HPLC followed by the high concentration solution.

RESULTS AND DISCUSSION

This method for the determination of impurities in the NMDA antagonist was validated for the parameters of linearity, precision, selectivity and limit of detection. The method uses a high-low chromatography approach to determine impurity levels. High-low chromatography is a sampling technique used to improve the detection limit of trace components in a bulk drug substance by extending the dynamic range of the detection system.[22] Prior to validation, the Varex IIA ELSD was optimized for nitrogen gas flow and temperature. The validation sample lot was then run on an LC/MS system where the impurities were identified.

ELSD Optimization

The ELSD is relatively independent of molecular functional groups within a chemical entity. To illustrate this, a UV detector and an ELSD were connected in series allowing for the superimposition of the two detector responses on a single chromatogram corresponding to the same sample injection. A mixture of a small aliquot of the mother liquor from the NMDA antagonist synthesis combined with 2 mg/mL of the NMDA antagonist bulk drug substance resulted in a situation where the main compound (NMDA antagonist) does not have a suitable chromophore and some of the impurities do (Figure 2). In this situation, the ELSD proves to be a more suitable detector than UV because the ELSD response is independent of the molecular function groups.

Optimization of the Varex IIA ELSD is critical for an impurity assay because sensitivity levels can be dramatically reduced under sub-optimal conditions. Operating conditions for the Varex IIA ELSD were optimized to obtain the greatest signal-to-noise ratio by controlling both the nitrogen gas flowing into the nebulizer and the temperature of the drift tube. The nitrogen gas flow rate into the nebulizer controls the droplet size which is critical for obtaining efficient vaporization of the mobile phase and maximum sensitivity of the detector. A nitrogen gas flow of 3.0 L/min was used for the validation. Figure 3 illustrates the effect of increasing nitrogen gas flow into the nebulizer on the NMDA antagonist peak signal-to-noise ratio. It is important to note that changing parameters in the HPLC assay would require reoptimization, however, maintaining the established validated HPLC conditions did not require recalibration of the detector over time.

It is necessary to completely evaporate the mobile phase prior to detection by the laser for maximum sensitivity. Figure 4 shows the effect of increasing the drift tube temperature on the NMDA antagonist peak signal-to-noise ratio. The optimum temperature of the drift tube used for this validation was 150°C. The effect on peak response caused by temperature adjustments to the drift tube is minor compared to changes in the nitrogen gas flow rate into the nebulizer.

Linearity

It is usual practice to perform linearity determinations over a wide range of sample concentrations to fully assess the linear dynamic range of the detection

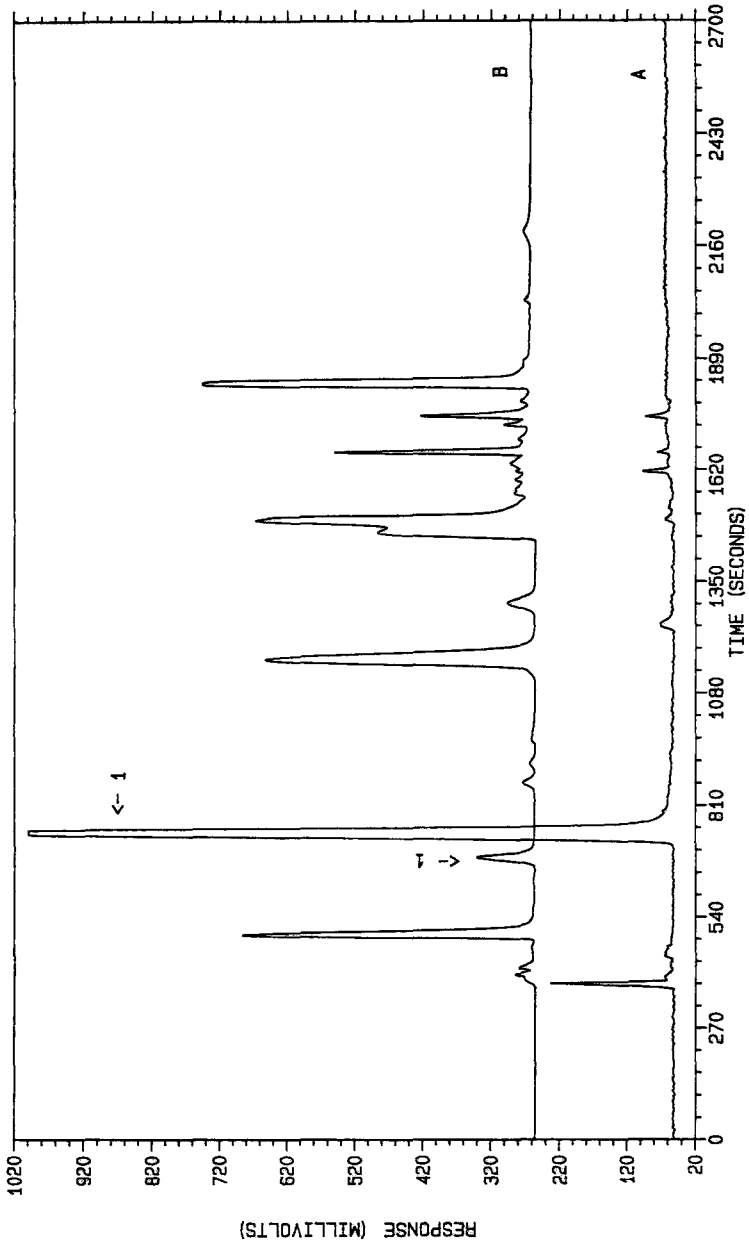

FIGURE 2. Sample injection of a mixture of a small aliquot of the mother liquor from the NMDA antagonist synthesis combined with 2 mg/mL of the NMDA antagonist as detected by ELSD (A) and UV (B). Peak 1 = NMDA antagonist drug substance.

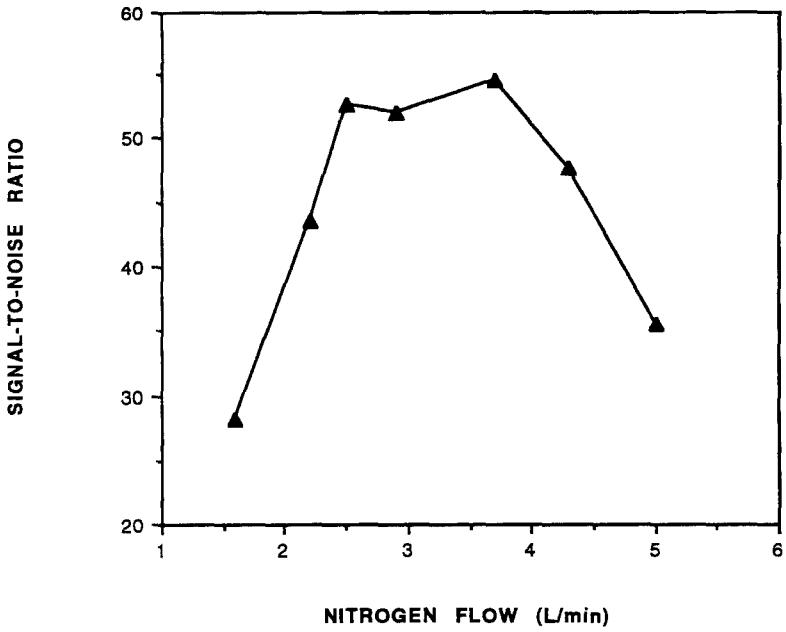

FIGURE 3. The effect of increasing nitrogen gas flow into the nebulizer on the NMDA antagonist peak signal-to-noise ratio.

system. Since a high-low approach is used, the linearity of the impurities in the high concentration sample and linearity of the NMDA antagonist in the low concentration sample were evaluated separately. The NMDA antagonist peak is off-scale for the high concentration samples and therefore cannot be evaluated. The three impurities detected in the sample lot were evaluated using six sample preparations over a concentration range of 1.0-4.3 mg/mL of the NMDA antagonist to establish the system linearity. These impurities peak areas were found to be linear with respect to increasing concentration. The corresponding correlation coefficients for peaks 1-3 (Figure 5) were 0.9997, 0.9938 and 0.9940, respectively.

The linearity of the NMDA antagonist peak was determined with the low sample concentration by injection of six samples representing a concentration range

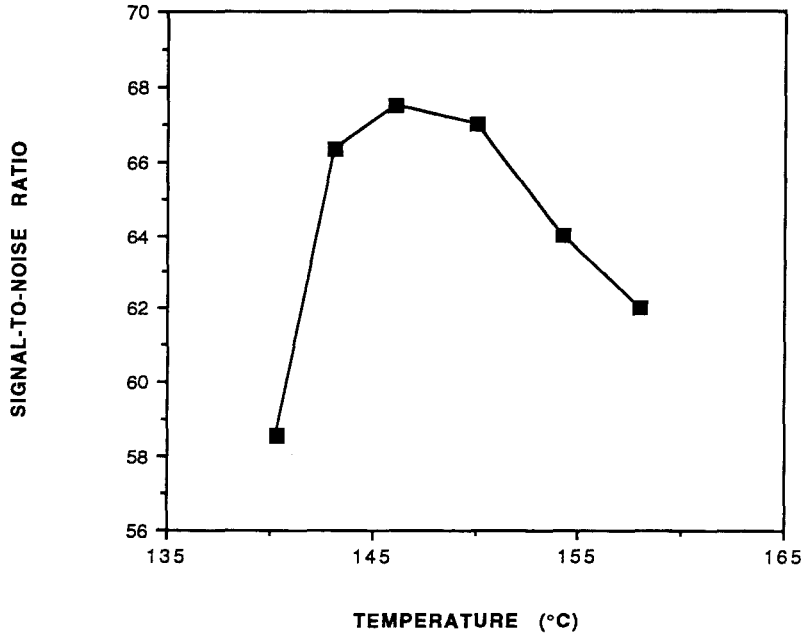

FIGURE 4. The effect of increasing the drift tube temperature on the NMDA antagonist peak signal-to-noise ratio.

of 0.10-0.43 mg/mL of the NMDA antagonist which corresponds to a 1:10 dilution of the high concentration samples. The NMDA antagonist peak areas were found to be linear with respect to increasing concentration. The resulting correlation coefficient was 0.9987.

Precision

The precision of the method was evaluated by injecting singly five individually prepared high concentration samples and the corresponding five low sample concentrations. The total impurity average for the five samples was determined to be 5.6% with an relative standard deviation of 1.3%. Figure 5 illustrates a typical sample chromatogram.

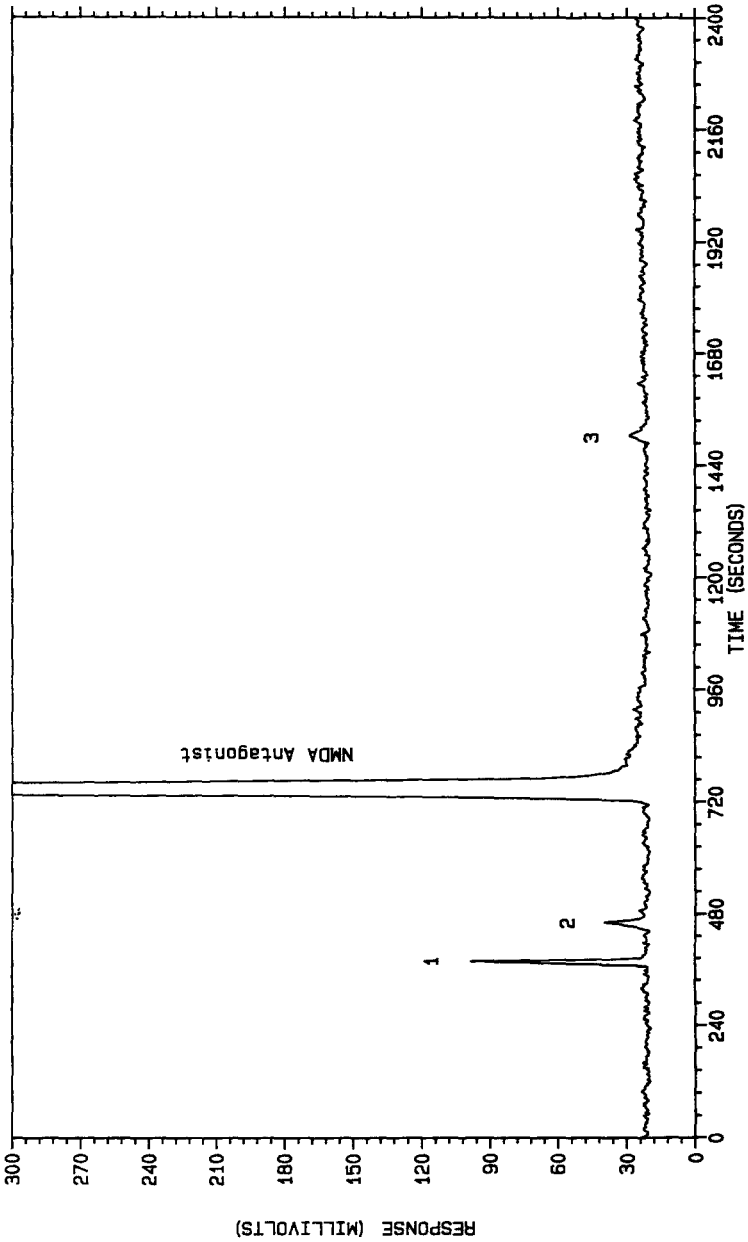

FIGURE 5. Sample chromatogram of NMDA antagonist drug substance high concentration prepared at 3 mg/mL. Peak 1 = bisphosphonic acid, peak 2 = unknown impurity, peak 3 = 6-methyl impurity. The NMDA antagonist peak is off-scale.

Selectivity

As part of the United States Pharmacopeia (USP) guidelines for validation, a method must be shown to have selectivity for the analyte of interest. For this method, selectivity was demonstrated by showing separation of all available impurities including starting materials, process intermediates and degradation products from the NMDA antagonist.

Limit of Detection (LOD)

The LOD is defined as the lowest concentration of a sample that can be clearly detected above baseline noise. The LOD was experimentally determined by making serial dilutions of each compound and then analyzing by HPLC. The LOD was determined to be 0.5 micrograms injected on column for the NMDA antagonist, 0.5 micrograms for bisphosphonic acid and 0.6 micrograms for the 6-methyl impurity. To maintain the LOD, the nebulizer must be cleaned periodically. Increased baseline noise, increased pressure reading and decreased peak response are indicators that the nebulizer is becoming contaminated.

Identification of Impurities

The LC/MS analyses were conducted using chromatographic conditions of the HPLC/ELSD experiments. Analyses were accomplished using a freshly prepared 20 μL injection volume of a 10 mg/mL solution of the NMDA antagonist. The three impurity peaks identified numerically and the NMDA antagonist peak in Figure 5 corresponded to the peaks observed with the LC/MS system.

Peak 1 corresponded very closely to the void volume and contained a variety of ions. The most prominent signal had an m/z of 176.9. Tetraethyl methylene bisphosphonate is used for a reaction in the synthesis. A by-product of this reaction would be bisphosphonic acid (see Figure 6) which has an m/z of 176.9 daltons. The retention time of the peak 1 impurity was then matched with an authentic sample of bisphosphonic acid using the HPLC/ELSD system. Bisphosphonic acid was also observed by ^{31}P-NMR in the NMDA antagonist sample validation lot. It is interesting to note that bisphosphonic acid was the major

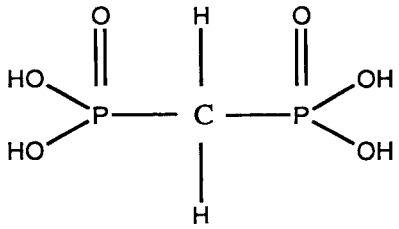

FIGURE 6. Structure of bisphosphonic acid.

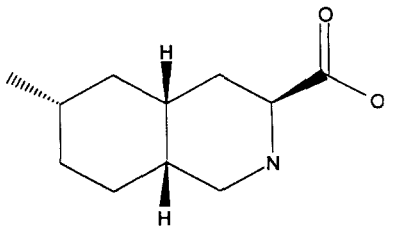

FIGURE 7. Structure of 6-methyl impurity.

impurity found in the validation lot yet it provided no UV response, further illustrating detection with UV could miss a source of impurity in a pharmaceutical compound.

Peak 2 occurred just after the void volume and contained primarily a component having m/z of 196.0. The structure of this impurity has not yet been confirmed.

Peak 3 is detected after completion of the gradient and yielded a very clean spectrum with a m/z of 198.2. This corresponded to the 6-methyl impurity (MW 197.3) which is a possible synthesis contaminant carried through from a previous step in the synthesis. The structure of the 6-methyl impurity is shown in Figure 7. An authentic sample of the 6-methyl impurity matched the retention time of that in the validation sample lot using the HPLC/ELSD system.

The NMDA antagonist (MW 277.3) peak provided the most intense signal and resulted in the expected m/z 278.3 ion (MH+). Another component with m/z of 554.9 was also observed. This signal was attributed to a gas phase dimer (M2H+) of the NMDA antagonist.

CONCLUSION

The applicability of a commercially available evaporative light scattering detector for the determination of impurities in a pharmaceutical compound has been demonstrated. The validation of this assay shows that the ELSD is an effective and practical alternative to conventional detectors. Linearity, precision, sensitivity and compatibility with HPLC have been shown to be more than adequate. Since the ELSD is capable of detecting many types of solutes regardless of functional groups, the versatility of an ELSD provides a practical tool for the analytical chemist.

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THE USE OF A NON-POROUS REVERSED PHASE COLUMN FOR RESOLUTION OF PORCINE INSULIN FROM LOW MOLECULAR WEIGHT AMIDES IN THE SAME MATRIX

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ABSTRACT

A rapid, reversed phase, high performance liquid chromatography (HPLC) method for the quantitation of porcine insulin has been developed and validated at Emisphere Technologies. Insulin is assayed with a C4 silica based column in a matrix containing a patented Proteinoid Oral Drug Delivery System (PODDS™) for the oral delivery of protein and non-protein drugs. This technology is based on low molecular weight, thermally condensed and acylated amino acids (proteinoids) forming pH dependent microspheres to encapsulate the insulin. Chemical modification of the proteinoids were shown to enhance drug delivery but interfered with the HPLC assay by co-eluting with the insulin. This resulted in distortions to the insulin peak shape reduceing the accuracy and precision of the quantitative data. This method was then re-developed and the interferences removed by employing a unique, non-porous, poly(styrenedivinylbenzene) (PSDVB) packing material. The absence of pores eliminates the column efficiency (**N**) and resolution (**R**) values important for the separation of small molecules which conform to the principals of partition chromatography. Consequently the small proteinoid molecules elute at the column void while large proteins

such as insulin, which conform to the principals of partition chromatography, elute at a later time. The result is complete resolution of large proteins from peptides and low molecular weight compounds present in the same matrix.

INTRODUCTION

A rapid, reversed phase, high performance liquid chromatographic (HPLC) method for the routine quantitative analysis of porcine insulin was currently developed and validated in our laboratory. Briefly, it employs a C4 silica based column and gradient elution with a ten minute run time (1). The matrix used for validating this method contains a very concentrated solution of various low molecular weight, thermally condensed (2) or acylated (3) amino acids synthesized at Emisphere Technologies. These protein-like compounds (proteinoids) are components of a unique Proteinoid Oral Drug Delivery System (PODDS™) technology used to encapsulate and orally deliver insulin, and other proteins.

Once the insulin is encapsulated the excess is removed by centrifugation, as reported by Milstein et al. (4). The HPLC method referred to above is then employed to quantitate the percent of encapsulated insulin. As part of an on-going research program, further chemical modifications to the proteinoids have improved their drug delivery attributes. Often these modifications also alter their physicochemical properties which frequently causes partial overlap of elution times with insulin in this assay. This overlap distorts the insulin peak shape and reduces the accuracy and precision of the quantitative data sometimes to the point of invalidating the results. Consequently, this quantitative insulin HPLC method required re-development study to regain the accuracy and precision needed to quantitate the percent of insulin encapsulated.

This report summarizes this new insulin HPLC method, the chromatographic problems encountered with the chemically modified

proteinoids and the steps taken to overcome them. They include altering the mobile phase composition, gradient profiles and use of two column packing with different selectivity. One packing consists of a diphenyl group chemically bonded to silica and the other consists of a non-porous, poly(styrenedivinylbenzene) (PSDVB).

MATERIALS

The HPLC pump (model 250), autosampler (model ISS 200), variable wavelength UV detector (model LC-95) and Turbochrom data acquisition software (version 3.3) used in this research were purchased from the Perkin-Elmer Corp. The C4 column was a Brownlee, 30 x 4.6 mm, 7 mcn, 300 Angstrom, Aquapore from Applied Biosystems. The diphenyl column was a Brownlee, 30 x 4.6 mm, 10 mcn, 300 Angstrom from Applied Biosystems. The non-porus PSDVB column was a PRP-Infinity, 30 x 4.1 mm, 5 mcn from Hamilton. Acetonitrile (ACN) and tetrahydrofuran (THF) were HPLC grade, purchased from EM Science. Potassium phosphate and porcine insulin were purchased from Sigma Chemical Co . The proteinoids used in the matrix were produced by Emisphere Technologies, Inc. Their concentration was 40 mg/ml in all cases. All water was deionized by a filtration system from Millipore.

METHODS

The origional method, developed and validated for the quantitative analysis of insulin used a C4 column and a linear gradient between two mobile phases (A and B) at a flow rate of 2.5 ml/min. Mobile phase A was composed of 10% THF and 90% aqueous 10 mM K_2HPO_4, pH 7.0. Mobile phase B was composed of ACN/THF(40:10) and 50% aqueous 10 mM K_2HPO_4, pH 7.0. The gradient conditions were from 40-70% B in 7 minutes

with a 3 minute hold. Detection was at 220 nm and the run time was 10 minutes. After it was discovered that the chemically modified proteinoids all had overlapping elution times with the insulin, variations in mobile phase composition were made and tested to eliminate this interference. These variations included changing the buffer strength to 5, 25, 50 and 100 mM K_2HPO_4, decreasing its pH to 3 and varying the percentage of ACN and THF in the gradient profiles. All of these attempts failed to completely resolve the insulin from the various proteinoids tested, resulting in this method no longer being valid.

The column selectivity was then changed to a diphenyl stationary phase using the first mobile phase composition and gradient profile. This attempt to adequately resolve insulin from all the proteinoids tested was unsuccessful. All of the successive mobile phase variations as described above also proved unsuccessful. The conditions found to provide the best, but still unacceptable, resolution were a two step linear gradient between the first mobile phase A and B compositions. The first step consisted of a 4 minute linear gradient from 10-35% B. The second step was a 3 minute linear gradient from 35-60% B with a 3 minute hold. The flow was 2.5 ml/min, detection was at 220 nm and the run time was 10 minutes.

Further attempts to adequately resolve the insulin from the proteinoids used a non-porous PSDVB column. The first mobile phase tried was the A and B compositions first described above. A two step linear gradient was successful in completely resolving the insulin from all proteinoids tested. The first step was a linear gradient from 10-70% B in 3 minutes followed by a 2 minute linear gradient from 70-85% B with a 5 minute hold. The flow was 2.5 ml/min, detection was at 220 nm and the run time was 10 minutes.

Precision and accuracy were determined for each column of different selectivity and the mobile phase gradient conditions adjusted to give the best

insulin resolution (**R**). A quantitative value for **R** was given in the USP, where $\mathbf{R} = 2(t_2-t_1)/W_2 + W_1$ (5). The criteria used for determining acceptable precision and accuracy limits for the data was taken from a recently published report on methods validation (6). These guidelines suggest no more than 15% coefficient of variation for precision and no more than 15% deviation from the nominal value for accuracy.

RESULTS

The partial over lap in elution times of the modified proteinoids with the insulin produced by the method employing the C4 column is shown in Figure 1. These elution effects cause the precision and accuracy values of a quality control standard to fall outside the accepted limits (Table 1). Modifying the mobile phase composition or its percentage of organic solvents is only partially successful in completely resolving the insulin from all the proteinoids tested. Changing the selectivity by the use of a phenyl bonded phase along with the use of various mobile phase compositions and gradient profiles is also unsuccessful in most cases (Figure 2). A non-porous PSDVB column, however, completely resolves insulin from all modified proteinoids tested using the original mobile phase composition and a rapid gradient profile (Figure 3). This non-porous support also provides the necessary resolution for acceptable precision and accuracy, even in high concentrations of proteinoid in the matrix.

DISCUSSION

The principles of partition chromatography demonstrate that increasing silica based column efficiency (**N**) results in an increased resolution (**R**) value. This is illustrated in Equation 1 which relates **R** to the column selectivity factor (a), capacity factor (k') and **N** (7,8).

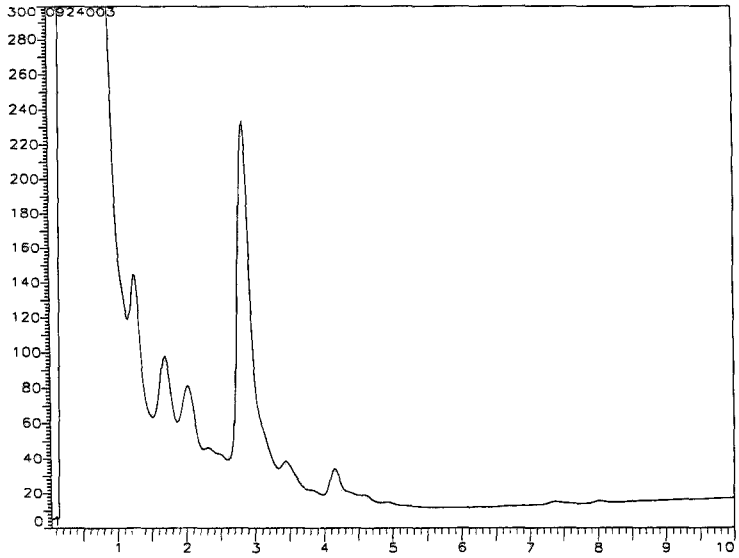

FIGURE 1. Chromatogram of modified proteinoid #E15 using the HPLC method requiring a 30 X 4.1 mm, 7 mcn, 300 Angstrom, C4 silica based column from Brownlee. Insulin retention time 4.1 minutes.

TABLE 1

Accuracy, Precision and Resolution values for Insulin were determined from repetitive injections of an aqueous standard consisting of 449 mcg/ml of insulin and 40 mg/ml of PODDS™ matrix E15.

HPLC Column	Accuracy Error (% deviation)	Precision (%CV)	Resolution (R)	n
C_4	84.7%	22.8%	0.66	2
Phenyl	3.9%	2.3%	1.20	4
Non-porous	1.0%	6.5%	2.85	4

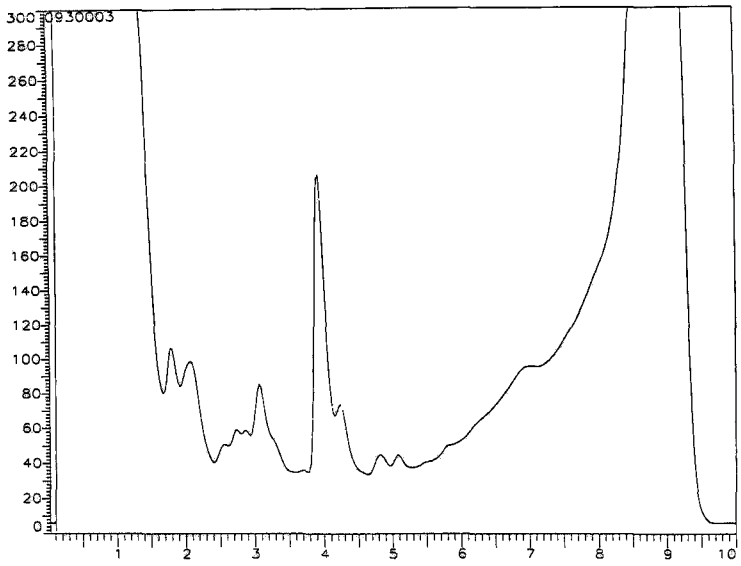

FIGURE 2. Chromatogram of modified proteinoid #E15 using the HPLC method requiring a 30 X 4.6 mm, 10 mcn, 300 Angstrom, diphenyl silica based column from Brownlee. Insulin retention time 5.0 minutes.

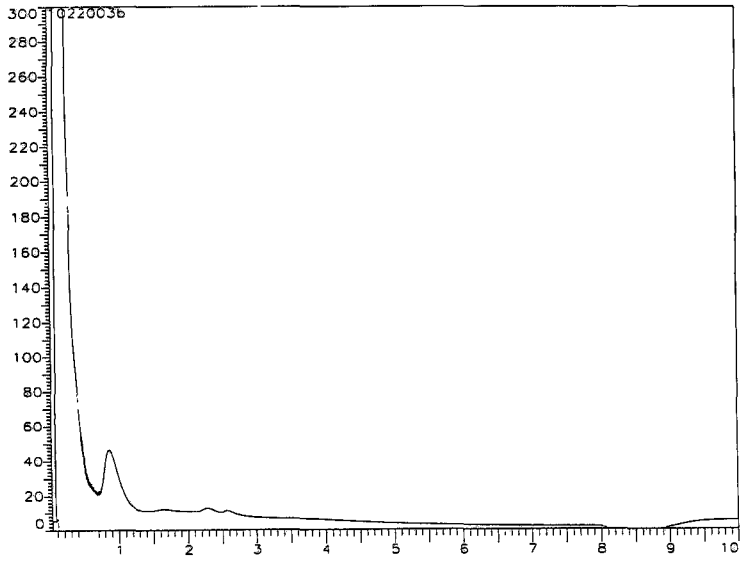

FIGURE 3. Chromatogram of modified proteinoid #E15 using the HPLC method requiring a 30 X 4.1 mm, 5 mcn, non-porous PSDVB based column from Hamilton. Insulin retention time 5.3 minutes.

$$R = (1/4)(a-1)N^{0.5}\,[k'/(1+k')] \quad (1)$$

The direct consequence of Equation 1 is that **R** is proportional to the square root of **N**. This suggests that high **N** values should be indicative of high **R** values for any given column (7,8). A numerical value for **N** may be determined from the retention time (t_R) and peak width at half-height ($W_{1/2}$) from Equation 2 (5).

$$N = 5.54(t_R/W_{1/2})^2 \quad (2)$$

A report by Pearson et al. (9) however, suggested that a common misconception is that a column of high **N** value will necessarily indicate superior **R** for proteins. Their comparison of different brands of silica having different calculated values for **N** showed that the column with the highest calculated **N** produced the poorest **R** of proteins with more than 30 residues. Conversely, the column with the lowest calculated **N** produced the greatest **R** value. It was concluded that increasing the **N** or plate number of silica-based columns, decreases the chromatographic **R** of proteins which suggests that they do not conform well to the principals of partition chromatography.

The separation and **R** of large proteins do however, conform well to the principals of adsorption chromatography (10). Separations based on these principals rely on the equilibria of the solute between the solvent and the hydrophobic coating of the support. According to this model, a protein is resolved by adsorbing onto the hydrophobic surface from the mobile phase and remaining adsorbed until it is displaced by a sufficiently high

concentration of organic solvent. The **R** of a mixture of large proteins, therefore is entirely dependent upon their ability to be adsorbed versus their solubility in an organic solvent and is independent of column **N** or plate number.

Another factor that must be considered for optimal protein **R** is the contribution of the silica pore size. Smaller pore silicas (<120 Angstroms) are reported to be inferior to large-pore-diameter supports for resolution and recovery of proteins. Pore sizes of 300 Angstroms offered the best resolution and recovery but was not indicative of success in every case (9). The principals of partition chromatography suggest that inefficient recovery and uneven pore size distribution may contribute to ghosting and band broadening sometimes produced by large proteins when chromaographed on small pore silica columns (7,8).

These principals and factors have led to the development and use of columns with lower calculated **N** values and large pore size for the chromatography of large proteins. These features allow uniform elution for proteins while decreasing the **R** of low molecular weight compounds causing them to elute closer to the column void. Many large pore silicas however, maintain enough **R** and uneven pore size distribution to adequately retain some small peptides and low molecular weight compounds. This may increase their elution time sometimes to the point of interfereing with the elution of large proteins. This was apparently the case in the methods using the 300 angstrom pore size C4 and diphenyl columns.

A PRP-Infinity, non-porous column was developed to eliminate all pore diffusion effects by reducing the value of **N** to zero which consequently reduces the value of **R** to zero in Equation 1. Since there is essentially zero **R**, those compounds which separate according to the principals of partition chromatography will elute at the void. Those compounds which separate by the principals of adsorption chromatography will be retained until they are

eluted by the appropriate amount of organic solvent. The method using the non-porous PRP-Infinity column described herein illustrates how these principles may be combined for the successful separation of a large protein from an abundance of low molecular weight compounds.

Since the PRP-Infinity column reduces the value of **N** to zero in Equation 1, the calculated **R** values between each of the columns has no value. This makes it difficult to represent the resolving ability of these columns in a numerical context. An appropriate solution is determining **R** values using the equation $R = 2(t_2-t_1)/W_2 + W_1$ (4). For each column, the retention time and peak width of the proteinoid peak eluting closest to the insulin peak may approximate t_1 and W_1. The elution time and peak width of the insulin peak then may be t_2 and W_2.

This non-porous technology proves very useful in this case for separating a large protein from a complex mixture of low molecular weight compounds with different physicochemical properties. This type of chromatography may be used to develop a method with no residual diffusion or ghosting effects in minimal development time. The result is a rugged, quantitative method capable of excellent separations in minimal run time.

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EFFECT OF DEXTRAN FILLING IN MACROPOROUS HEMA SORBENT ON ITS BEHAVIOR IN DYE-AFFINITY CHROMATOGRAPHY

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ABSTRACT

Macroporous and microparticulate poly(hydroxyethyl methacrylate) gel (HEMA) and HEMA containing pores filled with a dextran network were used as supports for immobilization of reactive dye Cibacron Blue 3G-A. The performance of both dextran-filled and non-filled HEMA sorbents, with various ligand densities, in dye-ligand affinity

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chromatography of several enzymes and of bovine albumin were studied. The obtained results show the positive effect of dextran filling on enzyme recovery and on binding capacity of the separation media.

INTRODUCTION

The poly(hydroxyethyl methacrylate)-based gel Separon HEMA (HEMA) belongs to the organic polymer supports suitable for HPLC of proteins [1-4]. It involves a large number of hydrophilic groups on its surface, what is condition for a good protein recoveries, and for introduction of various selective groups by a number of common methods. The application of HEMA for immobilization of different ligands, and subsequently for affinity chromatography, were presented by Taylor and Marenchic [5,6]. However, hydrocarbon polymer backbone chains of HEMA cause certain hydrophobicity of the material. It results in undesirable hydrophobic interactions of the HEMA sorbents with proteins which often prevent the direct application of HEMA in affinity-based separations. The hydrophility of HEMA surface can be increased by various low-molecular-weight, or better, polymeric modifiers.

In the present paper the use of macroporous and microparticulate HEMA particles with pores filled with crosslinked dextran (HEMA-D) as a support for high-performance affinity chromatograpfy (HPLAC) is described. The preparation, the permeation properties and the preliminary study of elution behaviour in dye-affinity chromatography of various dextran-filled HEMA sorbents were objects of study in our previous papers [7,13]. The dextran-HEMA composite sorbent showed suppressed nonspecific interaction with lactate dehydrogenase in dye-affinity chromatography, as compared with non-filled HEMA support. The aim of present work was to find out how the dextran filling effects the binding capacity of chromatographic media and the recovery of chromatographed enzymes, when different amount of reactive dye Cibacron Blue was bound to the supports. The positive effect of dextran filling on all chromatographic properties studied was found.

EXPERIMENTAL

Materials

Separon HEMA 1000, particle size 10 m was purchased from Tessek (Prague, Czech Republic). Dextran D-40, M_W=40 000 (Biotika, Slovenská Lupča, Slovak Republic) and 1,4-butanediol diglycidyl ether (Aldrich, Milwaukee, USA) were used for modification of sorbent. Cibacron Blue 3G-A (CB) was purchased from Ciba-Geigy (Basle, Switzerland). Lactate dehydrogenase (LDH; E.C.1.1.1.27) from beef flank muscle was prepared as crude lyophilizate (4 U/mg of lyophilizate) [8]. Other enzymes, such as LDH from rabbit muscle, malate dehydrogenase from pig heart (MDH; EC 1.1.1.37), glucose-6-phosphate dehydrogenase from yeast (G-6-PDH; EC 1.1.1.49) and glycerol kinase from yeast (GK; EC 2.7.1.30) were from Fluka (Buchs, Switzerland). Hexokinase from yeast (HK; EC 2.7.1.1.) and pyruvate kinase from rabbit muscle (PK; 2.7.1.40) were from Serva (Heidelberg, Germany). Bovine serum albumin was from SEVAC (Prague, Czech Republic), NADH from Reanal (Budapest, Hungary).

Apparatus

Equipment for gradient HPAC included LKB 2152 HPLC gradient programmer; LKB 2150 HPLC high pressure pump; LKB 2151 detector (all LKB, Bromma, Sweden), automatic injector and collector (Gilson typ 4, Viliers-Le-Bel, France). Date were collected and evaluated with Data Apex integrator software (Datapex, Prague, Czech Republic) programed for HPLC analysis . Columns were filled on Pneumatic HPLC pump (Knauer, Berlin, Germany). Spekol 11 and Specord M 400 (both from Carl Zeiss, Jena, Germany) were used for spectrophotometric determination of dyes, enzymes and proteins.

Methods

Dextran was incorporated into macropores of HEMA and subsequently cross-linked with 1,4-butanediol diglycidyl ether according

the procedure described previously [7,13]. The mass ratio of incorporated dextran vs. HEMA was 0.32 g/g. The amount of cross-linking agent added was 3.10-4 mol per gram of dextran.

Preparation of sorbents for scanning electron microscopy was performed using procedure of fixation with cacodylate buffer and glutaraldehyde as described by Casson and Emery [9]. The samples were examined in scanning BS 301 electron microscope (Tesla, Brno, Czech Republic).

The described technique of modifying of HEMA and HEMA-D with CB was used [7], where the mass ratio of sorbent : CB were 1 : 0.016-0.070. The degree of substitution was determined spectrophotometrically at 610 nm from the difference between the initial amount of CB in the reaction mixture and the residual amount of CB in the washing solution.

The activities of LDH, MDH, G-6-PDH, GK, HK and PK were determined spectrophotometrically at 340 nm (10) and the protein content was determined according to the method of Bradford at 595 nm [11].

Chromatographic experiments

Elution experiments

The described procedure [7] was applied in this case. The glass columns were filled with 0.3 g of dyed sorbents (1.5 x 1 cm I.D.) and equilibrated with 50 mmol/l phosphate buffer pH 7. The solutions of enzymes (ca 15 U of LDH/0.2 ml, 10 U of MDH/0.2 ml) or bovine serum albumin (ca 2 mg/0.5 ml) were loaded. The bound proteins were eluted with water solution of 1.3 mol/l KCl (LDH, MDH) or 0.5 mol/l KSCN (BSA). The flow-rate was 0.2 ml/min. The activity of enzymes and protein content were determined in 1 ml fractions.

Loading experiments

The same dyed columns as described above were used for loading experiments. The columns were saturated with solution of LDH from bovine muscle (18 U/ml). The saturation of LDH was controlled by

determination of activity of unbound LDH in 3 ml fractions. The bound LDH was eluted after the saturation with 1.3 mol/l solution of KCl. The recoveries of LDH activities were determined in all fractions.

HPAC experiments

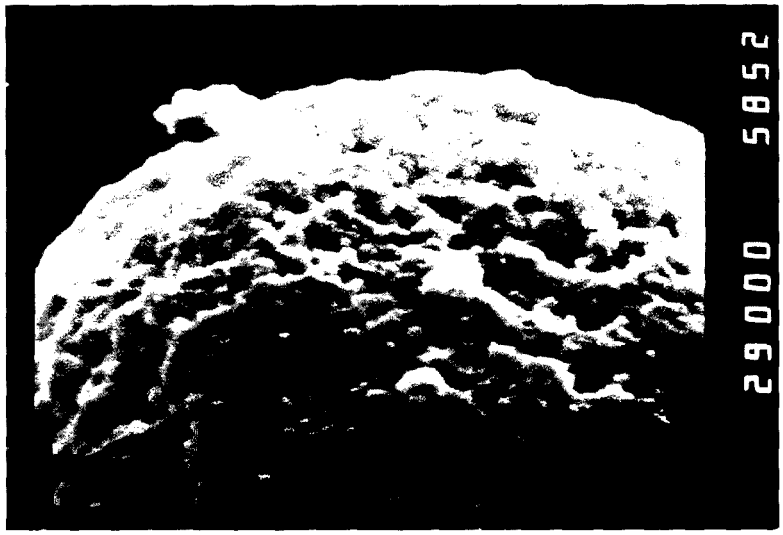
Mixtures of enzymes were separated on a CB-HEMA-D column (12.5 x 0.6 I.D.) with 16.03 μmol of immobilized CB per g of sorbent. 20 ml of enzymes from rabbit muscle (LDH and PK) or yeast enzymes solution (HK, GK and G–6–PDH) were loaded. The flow rate was 1 ml/min, pressure 2 - 6 MPa. The bound enzymes were eluted with a KCl gradient (0-1.5 mol/l). UV absorbance at 280 nm was monitored and the enzyme activities in 1.2 ml fractions were determined in the effluent.

RESULTS and DISCUSSION

Dextran gel was created within macropores of rigid microparticulate poly(hydroxyethyl methacrylate-co-ethylene dimethacrylate) resin (HEMA). The preparation and properties of dextran-filled HEMA supports (HEMA-D) were described more in detail previously [7,13]. The non-filled and dextran-filled HEMA supports were studied by scanning electron microscopy (SEM). The swollen dextran network usually can not withstand the conditions of SEM sample preparation. Therefore, the porous structure of incorporated dextran was fixed by the method of Casson and Emery [9] before electron microscopy study. Figure 1 shows the SEM electron micrographs of the particles of HEMA and HEMA-D. It is possible to see that filled HEMA has smoother surface with rare presence of macropores in comparison with non-filled one. No differences were seen if the fixing procedure was not applied to HEMA- D.

The incorporated dextran gel has its own porous structure with smaller pore size than those of original HEMA. The change in porous structure of support due to filling with dextran was seen on size-exclusion chromatographic calibration curves. It was found previously

A.



B.

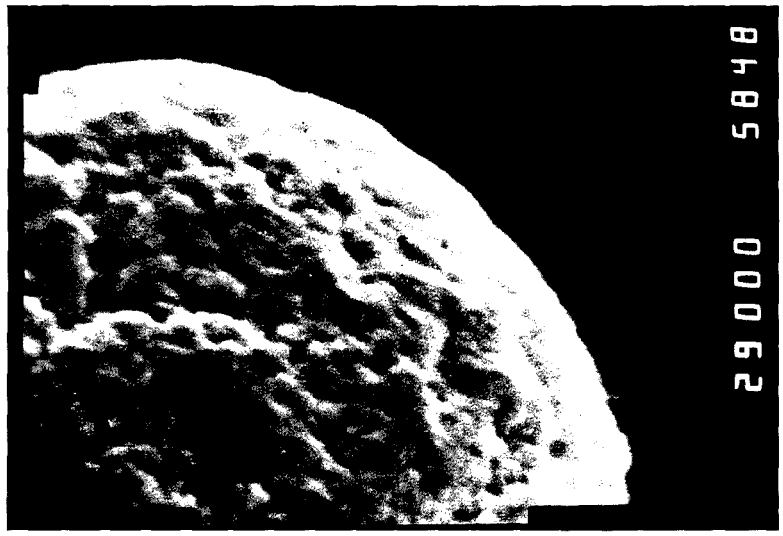


FIGURE 1. Scanning electron micrograph of HEMA (A) and HEMA-D (B). Original magnification: 29,000X, Present magniification 18,850X.

that permeation properties of dextran-filled HEMA depend on amounts of both incorporated dextran and crosslinking agent as well as on the porous structure of the starting HEMA [13]. The exclusion limits of HEMA and HEMA-D supports used in the study, as determined by SEC calibration for dextrans, were 1,000 and 400 kDa, respectively. SEC calibration showed that proteins with molecular weights under 700 kDa were still able to penetrate the incorporated dextran network.

Reactive dye Cibacron Blue (CB) was immobilized on both the original Separon HEMA 1000 (HEMA), and dextran-filled HEMA (HEMA-D), by the procedure described previously [7]. In the first series of experiments with dyed sorbents, we focused on the study of recoveries of selected proteins from both supports. Lactate dehydrogenase (LDH), malat dehydrogenase (MDH) and bovine serum albumin (BSA) were used for this purpose. The proteins were loaded at constant conditions and then eluted with equilibration and eluting buffers. The activities determined in effluents are summarized in Table 1. The differences in elution allows us to compare binding abilities of the sorbents studied. All proteins used in the study showed improved specific binding to the affinity ligands immobilized on dextran-filled HEMA, in comparison with non-filled support. This is probably due to suppression of non-specific interactions by dextran filling and improved accessibility of CB-ligands which are spread on the incorporated dextran network.

The loading characteristics of LDH expressed as breakthrough curves are shown in Figure 2. It shows that dextran- filled HEMA with immobilized CB can bind larger amount of enzyme than non-filled HEMA of about the same total ligand concentration.

Since amount of immobilized ligand seems to be a factor which significantly influences the effectivity of separation, the effect of this one on enzyme recovery was studied more in detail. Two different approaches were used in the recovery study. First, a constant amount of LDH from bovine muscle (15 U, ca 2.5 mg) was loaded (small loading - elution experiments on page 4)on all sorbents and eluted with both equilibration and eluting buffers. In another set of experiments, the sorbents were fully loaded (loading experiments on page 4) with LDH and recovered activities were determined. Recoveries, purification

TABLE 1

The recoveries of enzymes and BSA from CB-HEMA and CB-HEMA-D sorbents

Support	Dye content (μmol CB/g)	Recovery (%) Elution with							
		Equilibration buff.				1.3 M KCl			0.5 M KSCN
		LDH RM	LDH BM	MDH	BSA	LDH RM	LDH BM	MDH	BSA
CB-HEMA	9.6	0	3.4	2.4	44.0	68.0	68.2	62.4	20.1
CB-HEMA-D	7.6	1.7	1.2	0	60.6	68.6	94.3	87.0	36.1

factors and binding capacities of bovine muscle LDH for CB-HEMA and CB-HEMA-D sorbents of various ligand densities, as determined in these experiments, are summarized in Table 2. All columns were thoroughly washed with equilibration buffer after enzyme loading. Only a small part of enzyme activity (0.5-2.9%) was released in this step (at small loading).

The data in the Table 2 are obtained from the activities determined in effluent by using 1.3 M solution of KCl as eluting buffer. All of the important parameters of affinity sorbents such as recoveries, binding capacities, and purification factors are improved if dextran-filled support is used instead of bare HEMA. As expected, the binding capacities of both kinds of sorbents increased with increasing amount of ligand bound, however, the dextran-filled sorbents showed significantly higher binding capacities then the non-filled ones at the same dye content. The binding capacity related to dye content was about 26 U/ μmol CB in the case of CB-HEMA and about 42 U/ μmol CB for CB-HEMA-D

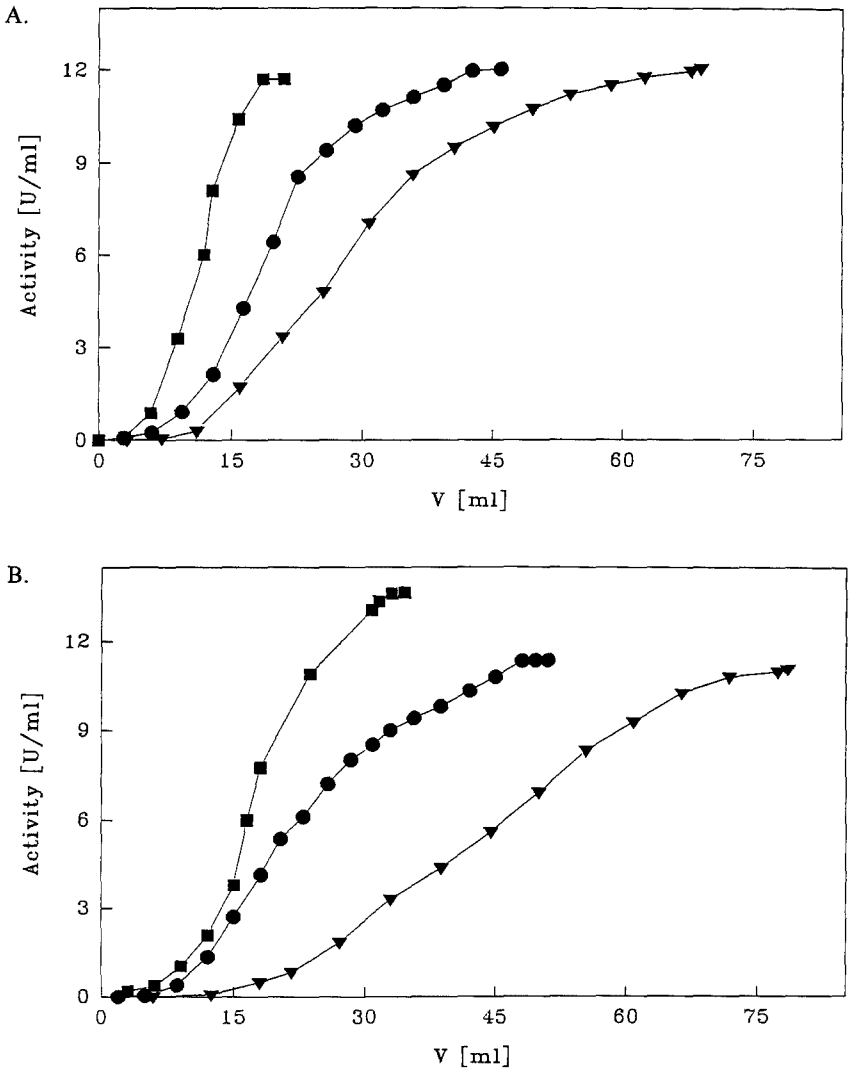


FIGURE 2. Breakthrough curves of LDH from rabbit muscle obtained on :

A - CB-HEMA
- ■ - 9.6 µmol CB/g
- ● - 14.4 µmol CB/g
- ▼ - 26.3 µmol CB/g

B - CB-HEMA-D
- ■ - 7.6 µmol CB/g
- ● - 15.9 µmol CB/g
- ▼ - 26.5 µmol CB/g

TABLE 2

Results from experiments with loading and elution of LDH-bovine muscle from CB-HEMA and CB-HEMA-D sorbents

		Small loading		Full loading	
Support	Dye content (μmol CB/g)	Recovery (%)	Purification factor	Recovery (%)	Binding cap. (U/mg)
CB1-HEMA	9.6	68.2	-	40.7	253.6
CB2-HEMA	14.4	86.0	9.3	60.4	390.0
CB3-HEMA	26.3	63.0	8.2	49.9	659.0
CB1-HEMA-D	7.6	94.3	21.4	82.3	342.6
CB2-HEMA-D	15.9	95.0	19.7	73.3	700.6
CB3-HEMA-D	26.5	78.7	14.1	44.7	942.3

sorbents. It means that the same number of affinity ligands can bind about 1.6 times higher amount of enzyme if the ligands are spread on the incorporated dextran network, in comparison with ligands bonded only on the surface of starting HEMA support. The higher capacity of CB-HEMA-D sorbents could be caused also by effect of stronger interaction of LDH with CB-dextran conjugate than CB.(14)

Figure 3 compares the recoveries and the binding capacities for the both small and full loadings of LDH. Even if the three-point-plots do not allow us to speak about dependency between the recovery and the binding capacity or dye content, respectively, some tendencies can be seen from such a graph. The interval between lower (full loading) and upper (small loading) curves corresponds to the recoveries which are possible to obtain for whole range of possible loadings for the both CB-HEMA and CB-HEMA-D sorbents. The recoveries are higher for dextran-filled sorbents, but the tendency of decreasing recovery at high concentration of immobilized dye can be seen for the both kinds of

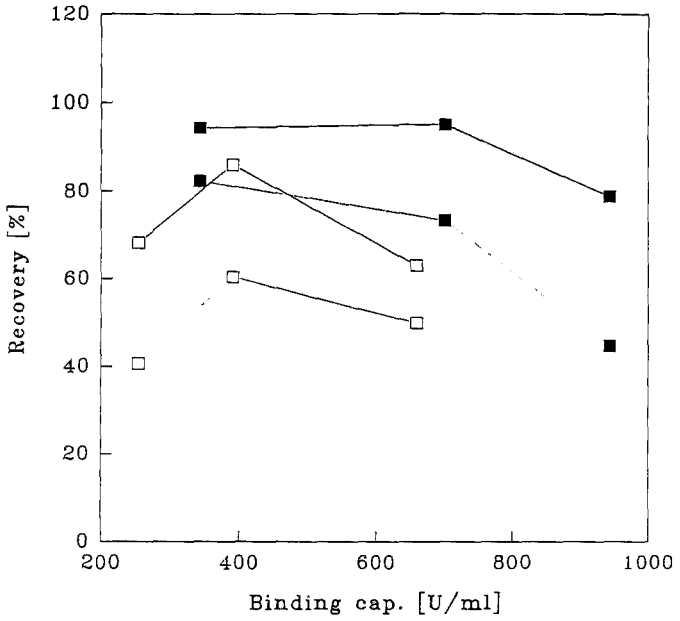


FIGURE 3. Graphical expression of the relation between the binding capacity of affinity sorbent and the enzymatic recovery of LDH. Upper curves corresponds to small loading of LDH, lower curves corresponds to full loading of LDH.

□ - CB-HEMA

■ - CB-HEMA-D

sorbents. It is probably due to multivalent interaction between enzyme molecules and affinity ligands that the binding capacity still increases with increasing amount of dye bound, however, the recovery begin to decrease at high ligand densities. Decrease in recovery of LDH from CB-HEMA sorbents with lower binding capacities may be caused by non-specific interaction of enzyme with support. It did not occur in the case of CB-HEMA-D sorbents which corresponds with our previous knowledge [7,13] that dextran filling suppress the hydrophobic interactions with HEMA surface.

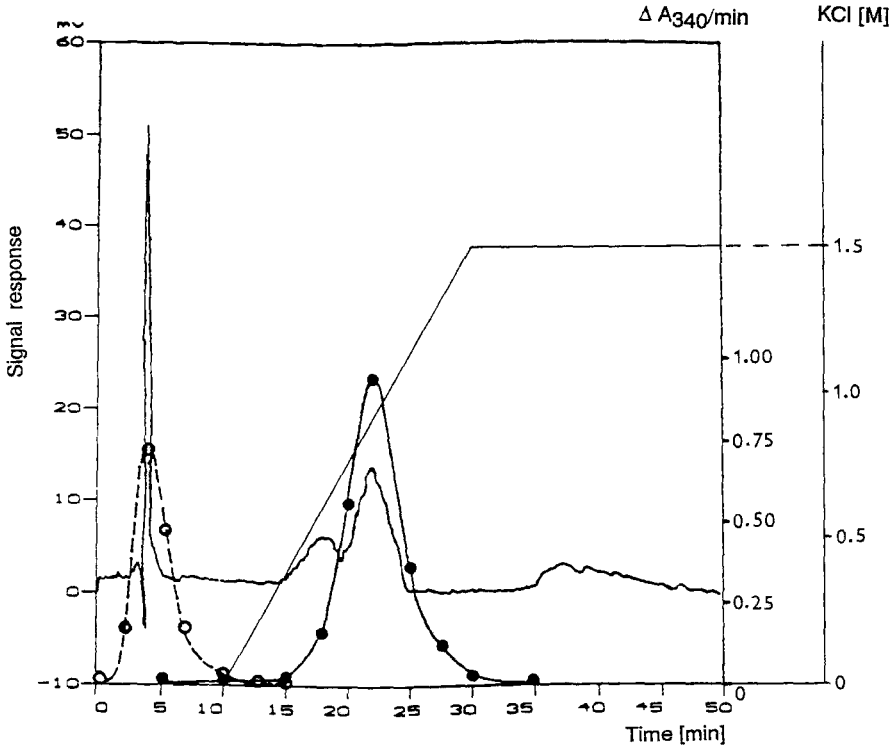


FIGURE 4. HPAC chromatogram of mixture of LDH and PK enzymes from rabbit muscle on CB-HEMA-D (16.03 mol CB/g) with a linear gradient of KCl concentration.

● - activity of LDH

○ - activity of PK

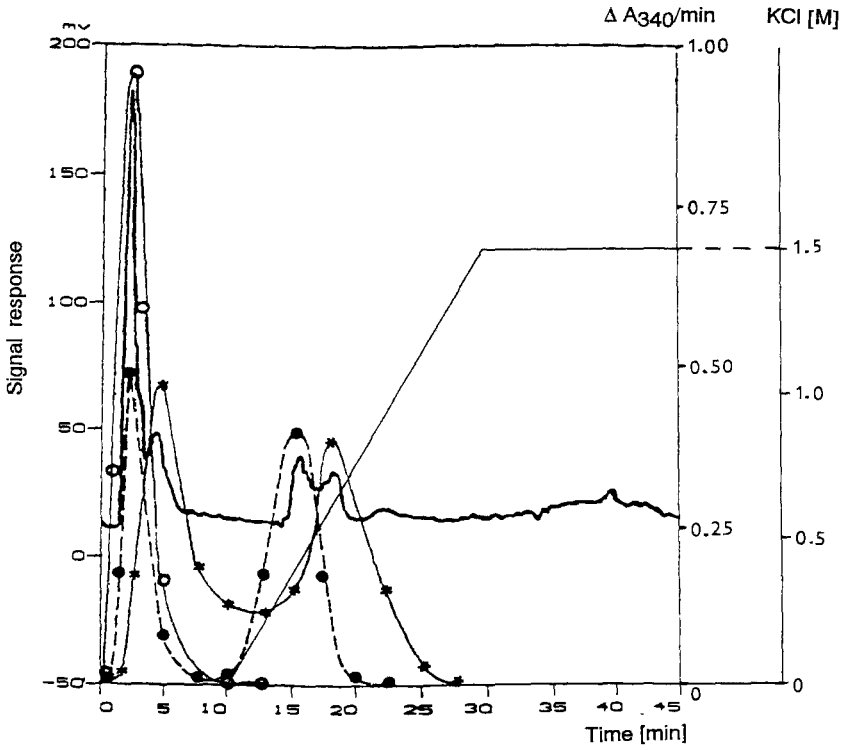


FIGURE 5. HPAC chromatogram of mixture of yeast enzymes GK, HK and G-6-PDH on CB-HEMA-D (16.03 mol CB/g) with a linear gradient of KCl concentration.

○ - activity of GK

● - activity of HK

✱ - activity of G-6-PDH

Columns packed with CB-HEMA-D sorbent containing 16.03 μmol CB/g were used for HPAC. Figure 4 shows separation of LDH and PK from rabbit muscle and Figure 5 shows HPAC of mixture yeast enzymes GK,HK and G–6–PDH. Both the UV- traces, and the activity assays, were used for detection of chromatographed enzymes. Several enzymes differing in the strength of affinity interaction with sorbent can be separated with high recoveries in a single injection.

CONCLUSION

The crosslinked dextran in macropores of HEMA-sorbent not only suppresses nonspecific sorption in dye-ligand chromatography, supports the LDH-CB interaction but also allows speading of the immobilized dye in the whole volume of the macropores. In such case, the ligands are better accessible for both molecules of chromatographed proteins and also a elution agent. As a result, an improved recovery of separated enzyme even at low ligand densities was obtained .

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REVERSED-PHASE HPLC RETENTION OF DEOXYRIBONUCLEOSIDES AS A FUNCTION OF MOBILE PHASE COMPOSITION

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ABSTRACT

Comparison of approaches describing retention of deoxynucleosides (dCyd, dUrd, dGuo, dThd, and dAdo) as a function of the mobile phase composition has been performed. The volume fraction (F) of organic modifier was changed from 0.05 to 0.30, and to 0.12 for methanol and acetonitrile, respectively. Linear regression has been carried out for the following equations: $\log k' = \log k'_w - S \times F$ (1), $\log k' = H + K \log(1/F)$ (2), $k' = A + B(1/F)$ (3), where k' is capacity factor, and $\log k'_w$, S, H, K, A and B are experimental coefficients. The best fits were obtained for the eqns. (1) and (3), and the poorest correlations were obtained for eqn. (2). The slope ratios $B_{methanol}/B_{acetonitrile}$ and $S_{acetonitrile}/S_{methanol}$ are changed in comparatively narrow ranges with average values of 2.86 and 2.30,

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respectively. Unlike the slope S of eqn. (1), the slope B of eqn. (3) can characterize the properties of solute and organic modifier simultaneously. For each solute, the intercepts A calculated for acetonitrile and methanol as organic modifiers are coincident closely . It is shown the validity of the coefficients of eqn. (3) to express the deoxyribonucleoside properties.

INTRODUCTION

Still now the theory of chromatographic retention of solutes under conditions of reversed-phase HPLC has not been worked out fully. An analysis of a number of approaches describing this phenomenon has been reported in the reviews [1,2]. It follows from the analysis, the the approaches developed have used some assumptions and can be applied only for qualitative predictions of solute chromatographic behaviour. Moreover, a majority of the relationships proposed has neither physical significance nor limited range of practical application.

For a given chromatographic column, the composition of the mobile phase determines the retention of solutes. The general approach assumes that reversed-phase retention obeys the linear relationship between log k′, where k′ is capacity factor, and volume fraction of organic modifier in the mobile phase [3]. It is really the true for a thermodynamically ideal binary solvent mixture . However, it was shown in [4] that methanol/water and acetonitrile/water mixtures are not ideal solutions. So the plots of log k′ vs. organic modifier fraction exhibit some curvature and are best fit by a quadratic equation [5], or they can be linearized by plotting vs. a spectroscopic function of the mobile phase: the so-called ET(30) index [1].

Nevertheless, even a formal description of solute chromatographic retention is very important and useful to systematize and to apply the

experimental data. The capacity factor data vs. content of organic modifier in the mobile phase could be regarded as a measure of the hydrophobic character of the solutes of biological, environmental and agricultural interests. Moreover, the optimum conditions for the gradient runs could be established on the basis of the precise description of solute movement in chromatographic column.

The chromatographic behaviour of 5 deoxyribonucleosides have been studied in [6], and it has been shown the non-coincidence of the experimental and extrapolated values of capacity factors with a mobile phase of pure water. The purpose of this work is to compare the different approaches describing retention of deoxyribonucleosides with respect to composition of mobile phase.

METHODOLOGY

Prediction of retention under reversed-phase HPLC is based usually on some expected dependences of capacity factor k′ on mobile phase composition. Also, there are a number of approaches to the effect of the mobile phase on chromatographic retention. More often it is discussed in literature a problem of the extrapolation of experimental data to estimate the value of capacity factor for water as mobile phase (k'_w) [7]. The value of k'_w serves as a good descriptor and predictor of the solute hydrophobicity in biological systems [2]. In our analysis the results of data interpolations were used advantageously to estimate the same properties of a solute.

The retention under reversed-phase HPLC conditions obeys the linear relationship [3]:

$$\log k' = \log k'_w - S \times F, \qquad (1)$$

where k′ refers to the solute capacity factor, k'_w is the value of k′ for water as mobile phase, F is the volume-fraction of organic modifier in the mobile phase, and S is a constant for a given solute and mobile phase composition.

Reversed phase slope and intercept values of eqn. (1) are regarded as a measure of the hydrophobic character of the solutes [8]. There are a number of papers reporting the use of eqn. (1) for the estimation of hydrophobic correlations. The peculiarities of eqn. (1) are discussed and reviewed elsewhere [1-8].

Due to the dependence of log k′ on the mobile phase composition, attempts have been made to find an alternative chromatographic parameter that is less dependent on the conditions and can be used as a continuous and universal scale. Kaibara and co-workers [9] defined the slope of log k′ vs. log(1/F) as a good indicator for hydrophobicity. We used this dependence in the next form:

$$\log k' = K \log(1/F) + H, \qquad (2)$$

where K and H are empirical coefficients.

The novel relationship between the capacity factor and organic modifier content in the eluent was proposed in [10]. This relationship has used an assumption that organic modifier adsorption is described by Langmuir isotherm. The final equation can be expressed as follows:

$$k' = A + B(1/F), \qquad (3)$$

where A and B are experimental coefficients, $A = k'_w h$, and $B = k'_w/b$, k'_w is the capacity factor in pure water, b is a constant of Langmuir equation characterizing the adsorption interaction between the organic modifier molecules and adsorbent surface, F is the organic modifier content in the mobile phase, and h is an empirical constant relating to the solute molecules and adsorbent surface interaction.

The eqn. (3) was obtained under a few of assumptions. But as it will be shown below, the deoxyribonucleoside chromatographic retention obeys the linear relationship between k' and 1/F.

All equations were linearized by LOTUS 123™. The correlation coefficients are noted as r^2.

RESULTS AND DISCUSSION

A chromatographic retention of five deoxyribonucleosides (dCyd, dUrd, dGuo, dThd, and dAdo) with respect to the mobile phase composition has been studied under isocratic conditions of reversed-phase HPLC. The Waters column (30x0.39 cm) packed by 10 μm μ-Bondapak C18 was used. The volume fraction (F) of organic modifier was changed from 0.05 to 0.30, and to 0.12 for methanol and acetonitrile, respectively. The elutions were performed by using an isocratic protocol at a flow rate of 1 ml/min. Methanol and acetonitrile were used as organic modifiers. The details of experimental part were described in [6].

In Figures 1-6, the experimental data and linear regression lines are shown for all approximations performed. With the exception of data of the eqn. (3), the slopes of plots obtained for different solutes remain fairly constant for each organic modifier, but the corresponding values of intercepts are changed strongly (Figures 1-4). Oppositely, the dependences of k' vs. 1/F plots are characterized by the different magnitudes of slopes for each solute (Figures 5-6).

Linear regression has been carried out according to the eqns. (1-3) for each solute and organic modifier. The slopes, intercepts, and correlation coefficients calculated are summarized in Table 1. Clearly

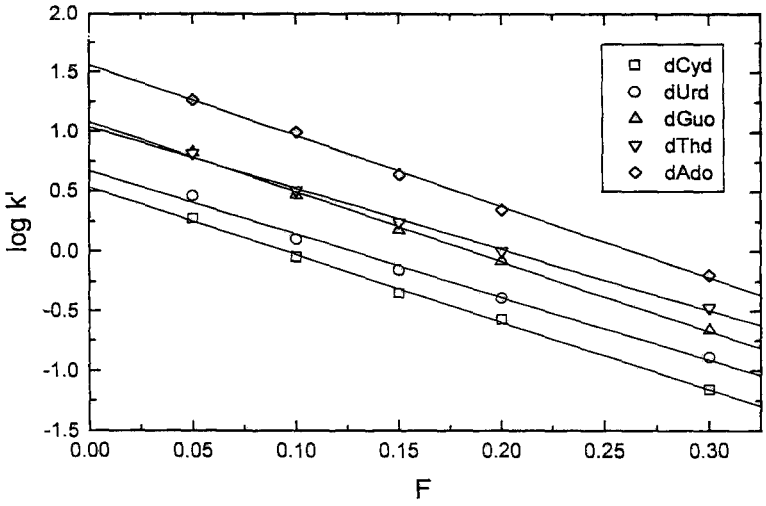

Figure 1. The plot of capacity factor vs. the methanol volume fraction approximated by using equation (1).

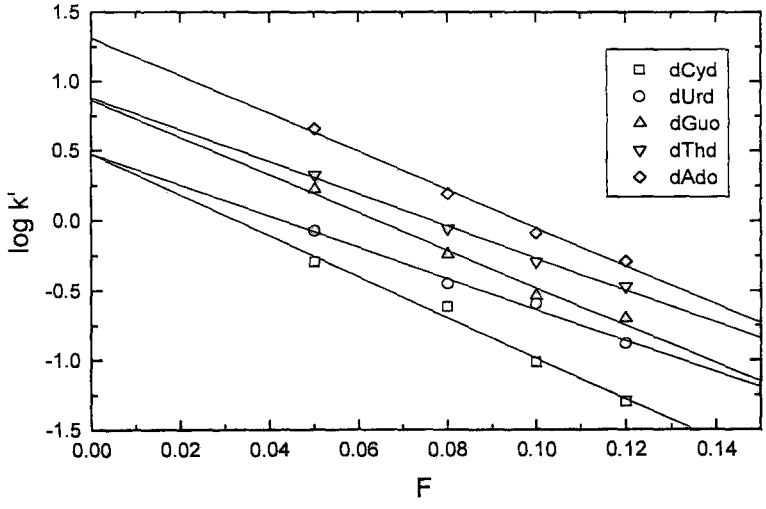

Figure 2. The plot of capacity factor vs. the acetonitrile volume fraction approximated by using equation (1).

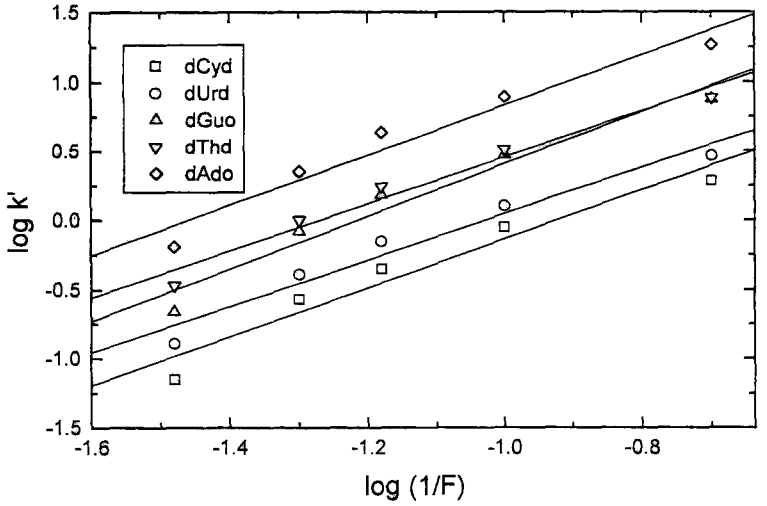

Figure 3. The plot of capacity factor vs. the methanol volume fraction approximated by using equation (2).

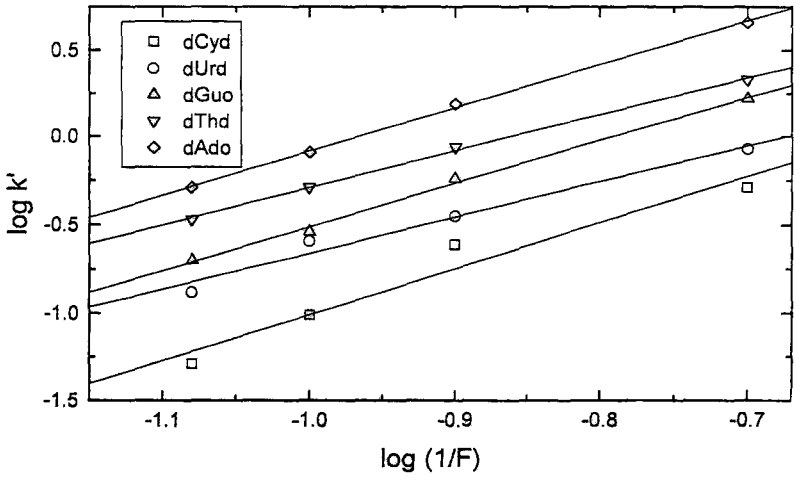

Figure 4. The plot of capacity factor vs. the acetonitrile volume fraction approximated by using equation (2).

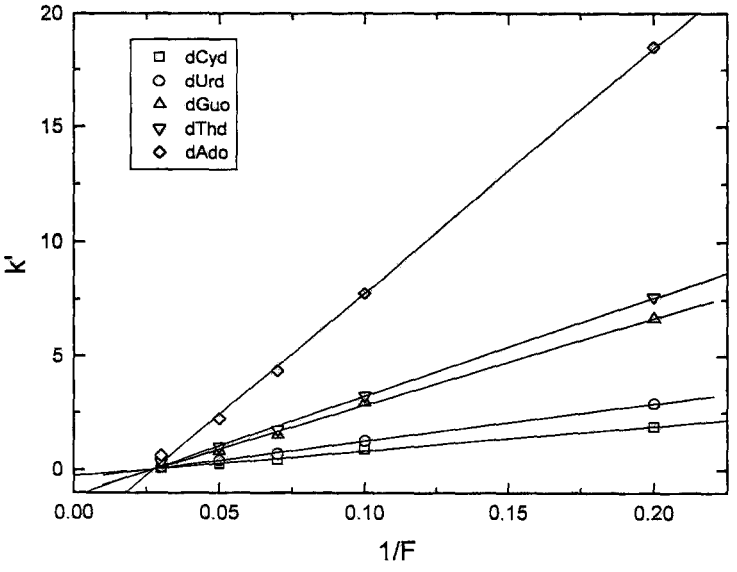

Figure 5. The plot of capacity factor vs. the methanol volume fraction approximated by using equation (3).

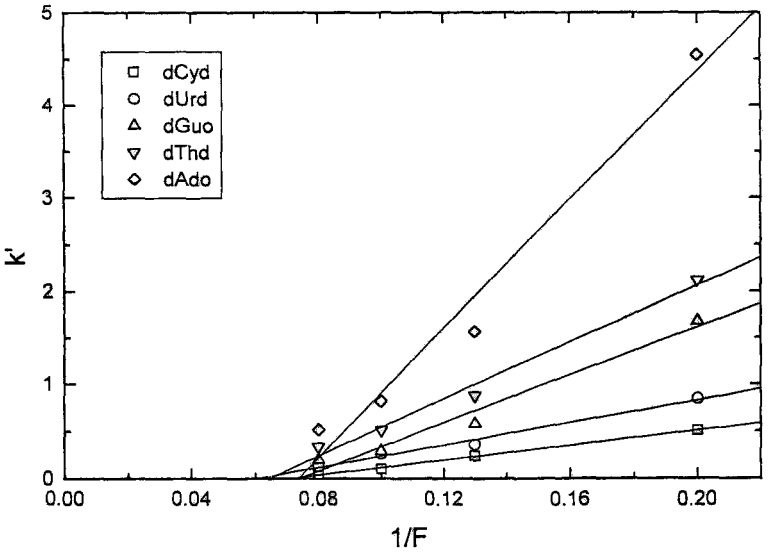

Figure 6. The plot of capacity factor vs. the acetonitrile volume fraction approximated by using equation (3).

TABLE 1
Slopes and Intercepts of Equations (1), (2), and (3)

Organic modifier	Solute	(1)			(2)			(3)		
		S	$\log k'_w$	r^2	K	H	r^2	B	A	r^2
Methanol	dCyd	-0.06	0.53	0.998	1.77	1.63	0.938	10.92	-0.27	0.997
	dUrd	-0.05	0.67	0.993	1.67	1.72	0.960	16.67	-0.42	1.000*
	dGuo	-0.06	1.11	0.995	1.89	2.30	0.956	38.87	-1.06	0.999
	dThd	-0.05	1.08	0.990	1.69	2.14	0.968	44.03	-1.17	1.000*
	dAdo	-0.06	1.51	0.996	1.81	2.64	0.950	107.46	-2.96	1.000*
Acetonitrile	dCyd	-0.15	0.48	0.984	2.61	1.80	0.945	3.99	-0.28	0.991
	dUrd	-0.11	0.48	0.990	2.01	1.35	0.974	6.04	-0.37	0.996
	dGuo	-0.13	0.86	0.985	2.46	1.95	0.996	13.09	-0.98	0.988
	dThd	-0.11	0.88	0.994	2.09	1.80	0.996	15.56	-1.02	0.996
	dAdo	-0.14	1.32	0.992	2.49	2.41	0.997	35.64	-2.67	0.999

*1.000 means $r^2 \geq 0.9995$

the best fits are obtained when the eqns. (1) and (3) were used to approximate the experimental data k' as a function of F. The correlation coefficients (r^2) are always higher than 0.990, with the exception of the three cases, where they are higher than 0.984 (see Table 1) .

The log k' vs. log(1/F) plots have poorest correlations. The eqn. (2) approximates in a good manner the data obtained for dGuo, dThd, and dAdo only with acetonitrile in the mobile phase (the correlation coefficients are more than 0.996). But these slopes and intercepts do not correlate with any properties of solutes or organic modifiers. Thus, for further analysis we will consider only correlations obtained by using the eqns. (1) and (3).

The slopes S of different solutes calculated according to (1) for each organic modifier are approximately coincided. Their ratio $S_{acetonitrile}/S_{methanol}$ is varied in comparatively narrow range from 2.09 to 2.61 with an average value of 2.30 (Table 2). In fact, these slopes are practically the same for different deoxyribonucleosides. This conclusion for deoxyuridine and its derivatives was reported in [11], where the slope values had not correlated to the hydrophobic properties of solutes. So the slopes of eqn. (1) characterize only the properties of organic modifier in the case of solute considered. The property of the log k'_w intercept to characterize the hydrophobicity of deoxynucleosides was described in [6] in details.

The correlation coefficients of linear regression obtained by using of the eqn. (3) in the most cases are exceeded the ones obtained for eqn. (1). It is obvious that eqn.(3) describes the experimental data for deoxynucleosides not worse than eqn. (1).

For a given organic modifier, the slopes B and intercepts A are changed strongly with respect to different solutes (see Table 1). It

TABLE 2

Ratios of Slopes Calculated for Methanol and Acetonitrile According to Equations (1) and (3)

Solute	dCyd	dUrd	dGuo	dThd	dAdo	AVG.*
$S_{acetonitrile}/S_{methanol}$	2.61	2.09	2.23	2.17	2.40	2.30
$B_{methanol}/B_{acetonitrile}$	2.74	2.76	2.96	2.83	3.01	2.86

* Average value of slope ratios

indicates that the experimental coefficients A and B characterize the properties of solutes and they probably relate with the value of k'_w. This conclusion is confirmed by an analysis of slopes of eqn. (3). The slopes B are different for deoxyribonucleosides studied. Their ratios $B_{methanol}/B_{acetonitrile}$ is varied in comparatively narrow range from 2.74 to 3.01 with an average value of 2.86 (Table 2). So the ratios of slopes S and B can characterize the properties of organic modifier only. Unlike the slopes of eqn. (1), the slopes of eqn. (3) express the properties of solutes and organic modifier simultaneously.

As seen from the data of Table 1, the intercepts A calculated for acetonitrile and for methanol as organic modifiers are closely coincident. These results are illustrated in Figure 7 by straight line arising from origin. The same results were obtained previously by using of eqn. (1) in the region of the small content of organic modifier [6].

Thus, it is shown the validity of both the slope and the intercept of eqn. (3) to express the properties of deoxyribonucleosides. But the range of application of eqn. (3) is not defined fully. As it follows

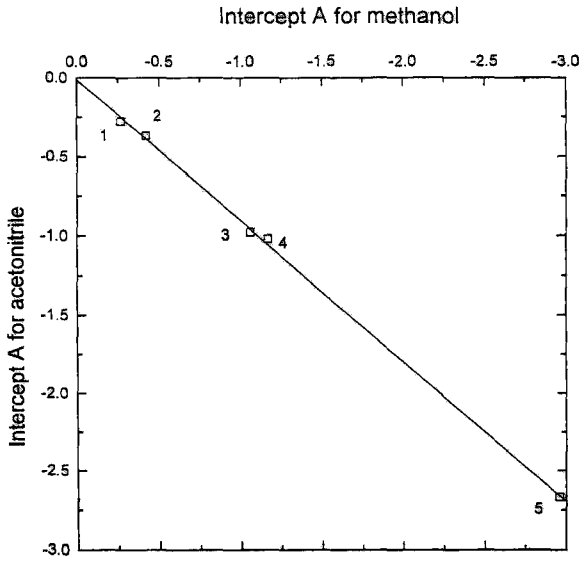

Figure 7. Comparison of intercepts A of equation (3) (deoxyribonucleosides: 1 = dCyd, 2 = dUrd, 3 = dGuo, 4 = dThd, and 5 = dAdo)

from an analysis of eqn. (3) [12], the features observed for deoxyribonucleosides are not the same for other substances. For example, the data of [7] have good correlations by using of equation (3), but the slopes of benzene solute calculated for different organic modifiers are equal.

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CHROMATOGRAPHIC BEHAVIOR OF DEOXYRIBONUCLEOSIDES WITH RESPECT TO ORGANIC MODIFIER CONTENT IN THE MOBILE PHASE

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ABSTRACT

A chromatographic behaviour of five deoxyribonucleosides (dCyd, dUrd, dGuo, dThd, and dAdo) has been studied under isocratic conditions of reversed-phase HPLC. The Waters column packed by 10 μm μ-Bondapak C18 was used. The volume fraction (F) of organic modifier was changed from 0.05 to 0.30, and to 0.12 for methanol and acetonitrile, respectively. Futhermore, the capacity factors with a mobile phase of 100 % water (k'_w) have been measured. Linear regression has been carried out for the log k′ vs. F data and the intercepts and the slopes of these plots were calculated.

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The slope magnitudes of different solutes were almost the same for each organic modifier. In all cases, the experimental values of k'_w were higher than extrapolated ones. For each solute, extrapolation of log k' vs. F gives practically the same intercept values for both organic modifiers. It was concluded that the intercepts calculated in the region of small content of organic modifier are independent on the nature of organic modifier and correlate with deoxyribonucleosides nature only.

INTRODUCTION

Deoxyribonucleosides are the important components of DNA, and they play an essential role in life-functions. Many of their derivatives have powerful physiological, antiviral and antitumor effects. In recent years the use of HPLC technique to analyse DNA-fragments has increased significantly. A number of publications describe the HPLC protocols to separate the mixtures of deoxynucleosides and other solutes of industrial, medical, and other interest [1-4].

Earlier the retention behaviour of deoxyuridine and its derivatives, and the dependence of the logarithm of capacity factor (log k') on the methanol concentration of the mobile phase were investigated under HPLC conditions elsewhere [5]. Correlations were studied between the retention data and physico-chemical parameters. The obtained multiple regression equations revealed that the hydrophobic (reversed-phase chromatographic retention) properties of the solutes have great influence on the DNA incorporation reaction. Previously the chromatographic selectivity of nucleosides and deoxynucleosides as a function of methanol concentration was reported by Gehrke et al. [6].

A review [1] underlines that the capacity factor with a mobile phase of 100 % water (k'_w) is the best measure of chromatographic retention and simultaneously a good predictor and descriptor of the solute hydrophobicity in biological systems. More often the

deoxynucleoside elutions were performed by using methanol and phosphate buffers as mobile phase components under gradient conditions [1-3]. This mode of chromatography can not give any sufficient information to estimate the values of k'_w. The rare data obtained under the isocratic mode of HPLC [2] do not allow us to extrapolate the value of log k'_w from the experimental results. So, the value of k'_w, which serves as a measure of the solute hydrophobic properties, is not defined accuracy for the deoxynucleosides.

The purpose of this work was to investigate the chromatographic behaviour of five deoxynucleosides on the mobile phase content of organic modifiers and to estimate more precisely the hydrophobic parameters k'_w of these substances.

THEORETICAL

The exact mechanism of solute retention in reversed-phase HPLC has been the subject of numerous studies [1, 5-8]. Ideally, knowledge of this mechanism would allow a priori prediction of solute retention and solute hydrophobic character in the case of reversed-phase HPLC. The difficulty in elucidating the mechanism of solute retention behaviour lies in the many interactions that a solute may undergo in both the stationary and mobile phases. For a given reversed-phase adsorbing material, the change in the mobile phase composition plays a primary role in the retention of compounds. The general approach assumes that retention under reversed-phase HPLC conditions obeys the linear relationship [7]:

$$\log k' = \log k'_w - S \times F \qquad (1)$$

where k′ refers to the solute capacity factor, k'_w is the value of k′ for water as mobile phase, F is the volume-fraction of organic modifier in the mobile phase, and S is a constant for a given solute and mobile-phase organic solvent.

The k'_w parameter is independent of any specific organic modifier effects, but it is dependent on the solute's structure and polar functionalities [8]. The k'_w value is difficult to measure directly. It is most often estimated as an intercept of the dependence (1) by the extrapolation of plot of log k′ vs. volume percent organic modifier.

Numerous papers have documented the validity of eqn.1, as well as modest deviations from this relationship [1, 5, 7-9]. While plots of log k′ vs. percent organic modifier often appear to be linear, they will always exhibit some curvature and are best fit by a quadratic equation [10]. Deviations from linearity were characterized with water-rich eluents, and good linear correlations were usually observed with mobile phases containing 30-50% of organic modifier. It was found in [5, 7-10] that the measured log k′ values of different solutes in the methanol/water and acetonitrile/water mixtures varied in a highly nonlinear manner vs. either percent or mole fraction of organic component. Johnson et al. showed in [9] that these curves can be linearized by plotting of log k′ values vs. a spectroscopic function of the mobile phase: the so-called ET(30) index. This approach and other ones were reviewed by Dorsey and Khaledi in [1].

Reversed phase slope and intercept values of eqn. (1) can be regarded as a measure of the hydrophobic character of the solutes. The intercept values are in the correlation to the partition coefficients of the compounds in the chromatographic stationary and mobile phase. The slope values can be regarded as a measure of the contact hydrophobic surface area of the solutes [5]. In all cases, the correct

measurements of the eqn. (1) parameters should be helpful to characterize the chromatographic and physico-chemical properties of the solutes. In this connection, it will be interesting to testify the validity of eqn. (1) in the region of the small content of organic modifier, and to compare the extrapolated values of k'_w of deoxyribonucleosides to the directly measured ones.

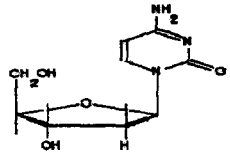
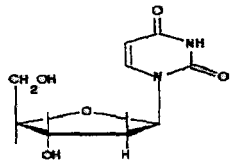
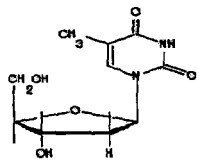
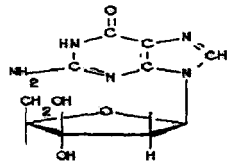
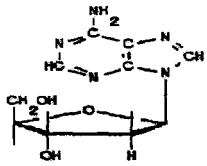
EXPERIMENTAL

Chemicals. The chromatographic behaviour of 5 deoxyribonucleosides with respect to composition of mobile phase was studied in this work. Table 1 gives the IUPAC names and the chemical structures of the solutes investigated. All deoxyribonucleosides were chromatographically pure and were purchased from Sigma (St.Louis, MO, U.S.A.). Standard solutions of solutes were prepared in the HPLC-grade water in 50 ppm concentration. HPLC-grade water, methanol and acetonitrile were obtained from Baker (Phillipsburg NJ, U.S.A.).

HPLC equipment. Throughout the study we used the following equipment: Waters Model 600 liquid chromatograph (Waters Associates, Milford, MA, U.S.A.) equipped with the Waters 600E Multisolvent Delivery System, a UV-visible tunable wavelength adsorbance detector (Waters 486), U6K injector (2 mℓ sample loop). The data aquisition system was CHROMATE (V.2.1, Interface Eng., Korea) installed in PC. A Waters column (30×0.39 cm) packed by μ-Bondapak C_{18} reversed phase material of 10 μm particle size was used.

Methods. The modifier concentration was ranged from 0 to 30% (v/v). Aliquots of 5 $\mu\ell$ were injected directly for HPLC analysis. The elutions were performed by using an isocratic protocol at a flow rate

TABLE 1
Structure of Deoxyribonucleosides Studied

Name	Structure	Abbreviation
2′-Deoxycytidine		dCyd
2′-Deoxyuridine		dUrd
2′-Deoxythymidine		dThd
2′-Deoxyguanosine		dGuo
2′-Deoxyadenosine		dAdo

of 1 ml/min. Absorbance was monitored at 254 nm with a sensitivity of 2 and 0,001 a.u.f.c. All separations were done at ambient laboratory temperature. The number of theoretical plates N was calculated for all both solutes and mobile phases. The average value of N was 5270, that corresponds to the N value reported in [2]. The dead volume was measured by introducing of 20 $\mu\ell$ of methanol, and the value of dead volume was equal to 2.95 ml.

RESULTS AND DISCUSSION

The experimental data of reversed-phase HPLC retention of five investigated deoxynucleosides in water-methanol and water-acetonitile mobile phases with respect to the content of organic modifier are presented in the Figures 1 and 2. In both cases, the retention of deoxynucleosides decreases with an increase in concentration of modifier in a semi-logarithmic relationship. Compared to methanol, acetonitrile offers approximately twice the elution power for nucleosides, but there is not any significant difference in their selectivity for separation of the nucleosides.

The slopes and intercepts of experimental dependences of log k′ of each solute vs. organic modifiers content were calculated according to eqn. (1). These coefficients and the values of k'_w experimentally measured by using a pure water as mobile phase are presented in the Tables 2 and 3. The empirical coefficients (log k'_w and S) of eqn.(1) were calculated with and without the experimental values of k'_w. The correlation coefficients (r^2) were always higher than 0.98, with the exception of dAdo data, calculated for both organic modifers by using the experimental value of k'_w.

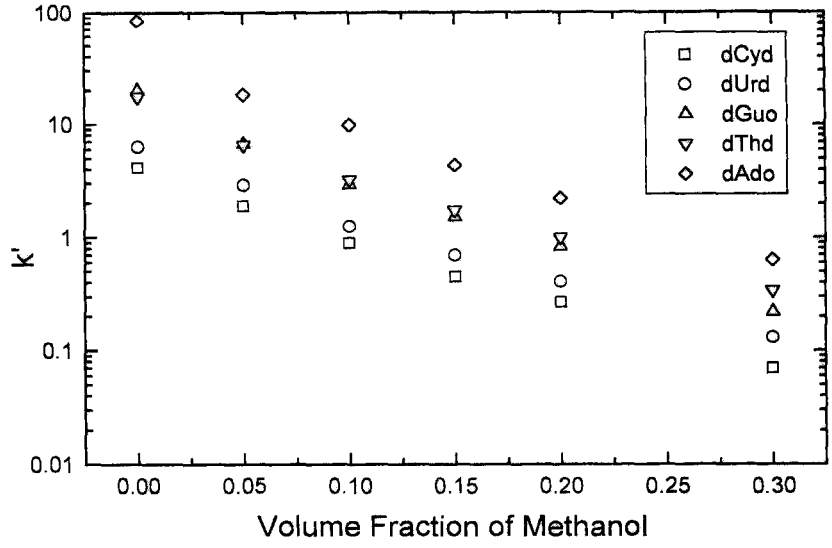


Figure 1. The plot of capacity factors against the methanol volume fraction for deoxyribonucleosides.

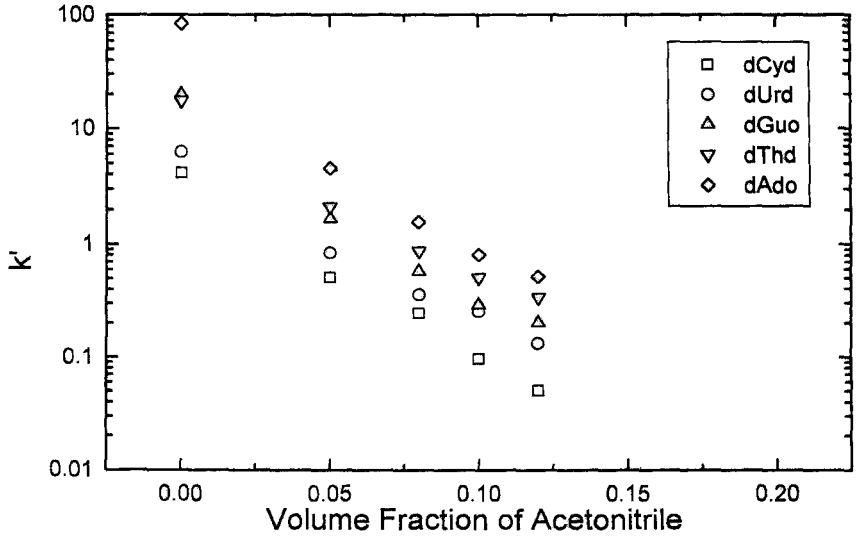


Figure 2. The plot of capacity factors against the acetonitrile volume fraction for deoxyribonucleosides.

TABLE 2
Intercepts (log k'_w) and Slopes (S) of Relationship (1) for Methanol as Organic Modifier

	Solute	log k'_w	S	r^2	k'_w(ext.)[1]	k'_w[2]
	dCyd	0.53	-0.06	0.998	3.39	4.13
	dUrd	0.67	-0.05	0.993	4.68	6.30
3)	dGuo	1.11	-0.06	0.995	12.88	19.31
	dThd	1.08	-0.05	0.990	12.02	17.59
	dAdo	1.51	-0.06	0.996	32.36	84.55
			$\overline{S_{ave}}$[5]=-0.06			
	dCyd	0.57	-0.06	0.999	3.71	
	dUrd	0.73	-0.06	0.991	5.37	
4)	dGuo	1.20	-0.06	0.990	15.85	
	dThd	1.16	-0.06	0.987	15.14	
	dAdo	1.71	-0.07	0.964	51.29	
			$\overline{S_{ave}}$[5]=-0.06			

1) extrapolated values
2) experimental values
3) calculations without the experimental value of k'_w
4) calculations with the experimental value of k'_w
5) averages

The slopes S of different solutes calculated for each organic modifier are approximately coincident. The average values of this slopes (see Tables 1 and 2) calculated with and without experimental values of k'_w are not significantly different, and changed from -0.13 to -0.16 for acetonitrile only. Thus, the slopes S characterize the properties of organic modifier in the case of deoxynucleoside elution under reversed-phase HPLC conditions.

TABLE 3
Intercepts (log k'_w) and Slopes (S) of Relationship (1) for Acetonitrile as Organic Modifier

	Solute	log k'_w	S	r^2	k'_w(ext.)[1]	k'_w[2]
	dCyd	0.48	-0.15	0.984	3.02	4.13
	dUrd	0.48	-0.11	0.990	3.02	6.30
3)	dGuo	0.86	-0.13	0.985	7.24	19.31
	dThd	0.88	-0.11	0.994	7.59	17.59
	dAdo	1.32	-0.14	0.992	20.89	84.55
			$\overline{S_{ave}}$[5]=-0.13			
	dCyd	0.58	-0.16	0.994	3.80	
	dUrd	0.72	-0.14	0.983	5.25	
4)	dGuo	1.19	-0.17	0.980	15.49	
	dThd	1.16	-0.14	0.982	14.45	
	dAdo	1.78	-0.19	0.969	60.26	
			$\overline{S_{ave}}$[5]=-0.16			

(For notations, see TABLE 2)

The difference in the slopes of each organic modifier can be explained by the competitive equilibrium between solute and solvent molecules for the sites on the stationary phase surface. Since the stationary phase possesses a non-polar surface, the less polar molecules will have a better affinity for this surface, and they will be adsorbed, therefore, more strongly. Acetonitrile is less polar solvent than methanol, it can be confirmed by the comparison of their polarity indexes and Hildebrand's solubility parameters. Moreover, unlike methanol, acetonitrile is a very weak hydrogen bond donor.

Due to these properties, it could be expected the preferential adsorption of acetonitrile molecules than methanol ones from aqueous mixtures on reversed-phase modified silica. This assumption was proofed experimentally. Even at 10% organic modifier concentration, acetonitrile was found to solvate the stationary phase to a much higher degree than methanol [11]. Thus, the two-fold higher slope of the curve plots that was found in the acetonitrile/water system could be explained by more preferable adsorption of acetonitrile molecules than methanol ones on the adsorbent surface.

The elution order of deoxynucleosides is the same in the different mobile phases (Figures 1,2). When the modifier concentration is less than 5%, the retention value increases as following: dCyd - dUrd - dThd -dGuo - dAdo. Here dCyd, dUrd, and dThd contain the pyrimidine bases, and dGuo and dAdo contain the purine bases. This result can be connected with an increasing of molecule size and, therefore, with an increasing of the surface area of solute molecule [9]. The retention order on Bondapak column in pure water corresponds probably to the hydrophobic properties of investigated deoxynucleosides, i.e. their affinity for this surface.

As seen in Figures 1 and 2, the elution order of dThd and dGuo is inversed when the methanol concentration in the eluent is increased from 5 to 10 % and more. The same phenomenon was reported in [2], where the elution order of dG and dT was changed by simply varying of the elution conditions. It must be noted that dG and dT represent a pair that is very difficult to separate. Perhaps, this is due to their specific interaction with the modifier molecules and free hydroxyl groups on silica surface.

The k'_w values extrapolated from the experimental dependences of log k' vs. organic modifier content do not coincide with the k'_w values measured directly. In fact, the experimental k'_w values are higher than

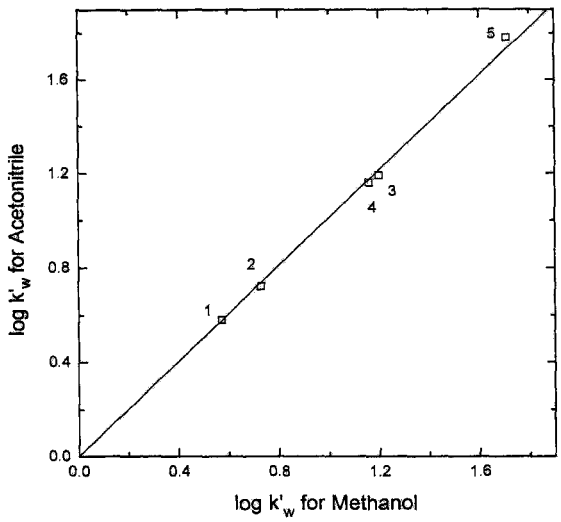


Figure 3. Comparison of intercepts calculated by using the experimental values of k'_w.
(deoxyribonucleosides: 1 = dCyd, 2 = dUrd, 3 = dGuo, 4 = dThd, and 5 = dAdo)

extrapolated ones. The disparities of experimental and extrapolated k'_w values are increased in acetonitrile as organic modifier. For example, the ratio of experimental and extrapolated k'_w values for dAdo is more than 4. The errors in the predicting the k'_w values are reduced when the experimental k'_w values are used for calculations. But in all cases, the difference between the experimental and extrapolated values of k'_w is sufficiently high.

Unexpected result has been obtained in this study. As seen in Figure 3, the dependence of the log k'_w intercepts calculated for acetonitrile vs. the log k'_w intercepts calculated for methanol as organic modifier is fitted by straight line arising from origin. That is, the intercepts of eqn.(1) calculated by using the experimental

values of k'_w for different organic modifiers are coincident. This fact is in contradiction with some data mentioned in review [1]. So, the intercepts calculated in the region of the small content of organic modifier are independent on the nature of organic modifier. Probably, the high level of a coincidence has an accidental character.

Thus, for each solute, the extrapolated and the experimental values of k'_w are not equal, but for different organic modifiers, the extrapolated k'_w values of the solute are the same. This important conclusion is confirmed indirectly by numerous regression correlations between the extrapolated values of k'_w and other hydrophobic and biological properties of solutes. The extrapolated values of k'_w measured in the regions of different content of organic modifier appear to be in the functional dependence on hydrophobic parameters of solutes. For example, the authors in [12] have obtained some good linear correlations for various groups of heteroaromatic compounds with eluents containing 50-70 % of methanol.

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FLUOROMETRIC HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR QUANTITATION OF NAPROXEN IN SERUM

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ABSTRACT

A simple, sensitive and reliable HPLC method for the determination of naproxen in serum is presented. Samples were prepared by a protein precipitation method using acetonitrile. A reversed-phase C-18 column was used with a mobile phase consisting of 47% acetonitrile and 53% pH 2.5 buffer solution. A fluorescence detection with excitation wavelength of 230 nm and emission wavelength of 370 nm provided high sensitivity and specificity with no interference from normal serum constituents. The limit of quantitation was 2.0 ng/ml with a CV of 10.6 %. The standard plots were highly linear ($r > 0.999$) over the range of 10.0-200.0 ng/ml. The average recovery was 97% and the CVs of inter- and intra-day variabilities were 2.0% and 1.2%, respectively, for the entire calibration range. The method was used for the measurement of the time course of naproxen in dog serum after administration of a topical gel.

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INTRODUCTION

Naproxen, (S)-6-methoxy-α-methyl-2-naphthaleneacetic acid, is a potent antiinflammatory agent often used for the symptomatic treatment of rheumatoid arthritis, ankylosing spondylitis, acute gout and related diseases [1,2]. The drug also possesses analgesic properties which can effectively relieve moderate pain following orthopedic, dental, or surgical procedures. It is well absorbed orally and has an elimination half-life of about 13 hrs in man. The bioavailability of naproxen after topical application was reported to be low [3], despite its high lipophilicity.

In contrast to an oral route, topical administration of nonsteroidal antiinflammatory drugs (NSAIDs) could offer therapeutic benefits while avoiding gastrointestinal side effects. Recently, it was shown that high drug levels in local tissues such as muscle, ligament and synovial fluid could be achieved after topical application of several NSAIDs [4,5] in animals. Since the amount of drug entering the systemic circulation through the skin is usually low due to the barrier function of the stratum corneum, pharmacokinetic studies of drug administered by a topical route require a highly sensitive analytical method.

Many HPLC methods for the quantitation of naproxen in biological samples have been reported, employing a different detection capability such as spectrophotometric [6,7,8], fluorescence [9,10] or electrochemical detection [11]. However, most of these methods either required a time consuming extraction procedure for the sample preparation or lacked the sensitivity and reproducibility needed for a complete pharmacokinetic study of this drug.

Generally, fluorometric methods of detection provide an excellent sensitivity, often exceeding the sensitivity of most spectrophotometric methods. It has been shown that fluorescence detection gave higher sensitivity than UV [6,10], diode-array [10], or GC-MS [10] method for the determination of naproxen. In addition, fluorometry offers an advantage of having less interferences in the chromatogram due to fewer fluorescent compounds present

in the biological fluids than UV absorbing molecules. Approximately 10 % of all absorbing compounds are known to emit radiation via fluorescence.

The purpose of this study was to develop a simple and sensitive HPLC method for the quantitation of naproxen in blood samples. The applicability of the assay was demonstrated by the complete elucidation of the time course of serum concentrations of naproxen in a dog after receiving a topical gel.

MATERIALS AND METHODS

Materials

Naproxen (Sigma Chemical Co., St.Louis, MO), phenylsalicylate (Eastman Organic Chemicals, Rochester, NY), potassium chloride (Merck & Co., Rahway, NJ), and hydrochloric acid (EM Science, Gibbstown, NJ) were analytical or reagent grade and used as received. HPLC-grade acetonitrile was purchased from J.T.Baker Inc. (Phillipsburg, NJ). Distilled water was filtered by means of a Millipore system (Continental Water Systems Corp., El Paso, TX).

Instrumentation

The chromatographic analysis was performed on a modular HPLC system consisting of a reciprocating pump (Beckman model 110A), equipped with a fluorescence detector (Kratos FS 970) and an integrator (Shimadzu C-R3A). The fluorometer was set at an excitation wavelength of 230 nm and an emission wavelength of 370 nm. The detector was operated at the sensitivity scale of 0.1 μA. Injections were made with a 50-μl sample loop using a syringe-loading injection system (Rheodyne model 7125). The analytes were eluted on the reverse phase C-18 column (250 x 4.6 mm), packed with Partisil 5 μm ODS-3 (Whatman^{R}) with a guard column (C-18, Perisorb 30 μm).

Mobile Phase

Acetonitrile and pH 2.5 hydrochloric acid buffer (0.04 M) were thoroughly mixed at a ratio of 47:53 (v/v) and degassed by sonication prior to

use. The mobile phase was pumped through an inlet filtering system at a flow rate of 1.2 ml/min at ambient temperature. The column was washed with the mobile phase by maintaining the flow rate of 0.2 ml/min overnight.

Stock Solutions

The stock solutions of naproxen and phenylsalicylate (internal standard) were prepared at the concentrations of 100 μg/ml and 1000 μg/ml, respectively, in the mobile phase. The working stock solutions of naproxen for the calibration and recovery studies were prepared by diluting the stock solution with the mobile phase to obtain the concentration range of 0.1 to 1.5 μg/ml.

Sample Preparation

Samples for the quantitation of naproxen in dog serum were prepared by precipitating proteins with acetonitrile. To 0.2 ml of dog serum (dosed or spiked) in a 5.0-ml glass test tube was added 20 μl of the stock solution of the internal standard and vortexed briefly. After adding 0.56 ml of acetonitrile, the mixture was vortexed for 30 sec and then centrifuged at 3500 rpm for 10 min. Fifty μl of the clear supernatant was loaded into the sample loop of the Rheodyne injector for quantitation.

Validation

Blank dog serum (0.2ml) in a 5-ml glass test tube was spiked with a varying amount of the working stock solution of naproxen and a constant volume of the phenylsalicylate stock solution to prepare seven concentrations within the calibration range of 10.0-200.0 ng/ml for naproxen and 100 μg/ml for the internal standard. The samples prepared according to the procedure described earlier were chromatographed, and the standard calibration graphs were constructed by plotting the peak height ratios of naproxen to internal standard against the respective concentrations of naproxen. For each of the intra-day (n=3) and inter-day (n=9) standard plots obtained, the correlation coefficient, slope and intercept

were determined. The reproducibility of the assay was evaluated by comparing the least-square linear regression data obtained from the replicate intra- and inter-day calibration plots. The accuracy of assay was periodically confirmed by analyzing several quality control samples during analyses of unknown samples.

Quantitation

The calibration plot for naproxen was constructed daily by analyzing spiked serum samples with known amounts of naproxen and internal standard. The concentrations of naproxen in unknown samples were calculated by interpolation of the calibration plot using the peak height ratios (naproxen/phenylsalicylate) obtained from the prepared samples.

Recovery Studies

The absolute recoveries of the assay were assessed by comparing the peak height ratios of naproxen over phenylsalicylate which were obtained from spiked dog serum samples of different naproxen concentrations (10.0-200.0 ng/ml) to the peak height ratios of the samples containing the equivalent amounts of the drug and internal standard (100 μg/ml) which were directly dissolved in the mobile phase. For each naproxen concentration used, three replicate samples were prepared and analyzed.

In Vivo Application

The proposed procedure was employed to determine the serum concentration versus time profile of naproxen after topical administration of naproxen in a gel (10 mg/kg) on the medial thigh of a beagle dog. Blood samples (4ml) were withdrawn from the jugular vein using vacutainer tubes containing clot activator at the intervals of 0, 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 hrs and were centrifuged at 2500 rpm for 8 min. The serum was stored in a screw-capped plastic vial at -20°C until assay within a week.

RESULTS AND DISCUSSION

The goodness of an HPLC method for the determination of drugs in biological fluids depends on many criteria such as the extraction efficiency, chromatographic quality, simplicity and reliability of the method, among others. It was found in this study that naproxen was highly extracted from dog serum by precipitating serum proteins with acetonitrile. However, a slow drop-wise addition of acetonitrile to the serum sample with continuous vortexing during protein precipitation was crucial for achieving high and consistent recovery of naproxen from the sample. This procedure may be necessary for the extraction of other drugs in serum, especially for those which are highly bound to serum proteins.

Fig 1 shows typical chromatograms for blank dog serum (1-A) and dog serum containing 6.25 ng/ml of naproxen (1-B). Under the conditions described, the peaks corresponding to naproxen and phenylsalicylate were well resolved, sharp and symmetrical with no interference from endogeneous materials at their respective retention times of 5 and 10 min, thus facilitating accurate measurement of the peak height ratios. It was found that the composition and pH of the mobile phase significantly affected the chromatogram of naproxen. Naproxen is a weak acid (pKa= 4.2), and therefore the peak retention time was longer at the mobile phase pH of 2.5 as compared to those obtained at higher pH values, which could be attributed to the greater adsorptivity of the acidic form of naproxen to the nonpolar stationary phase of the column. As the pH was increased, naproxen was eluted quickly along with several interfering peaks. On the other hand, increased peak broadening and tailing were observed as the volume ratio of acetonitrile in the mobile phase decreased below 47 %. Consequently, pH 2.5 and 47 % (v/v) acetonitrile were chosen as the optimal conditions of the mobile phase used in this assay.

The standard calibration graphs obtained by plotting the peak height ratios of naproxen to internal standard against the respective naproxen concentrations were highly linear over the range of 10.0-200.0 ng/ml. Using linear regression

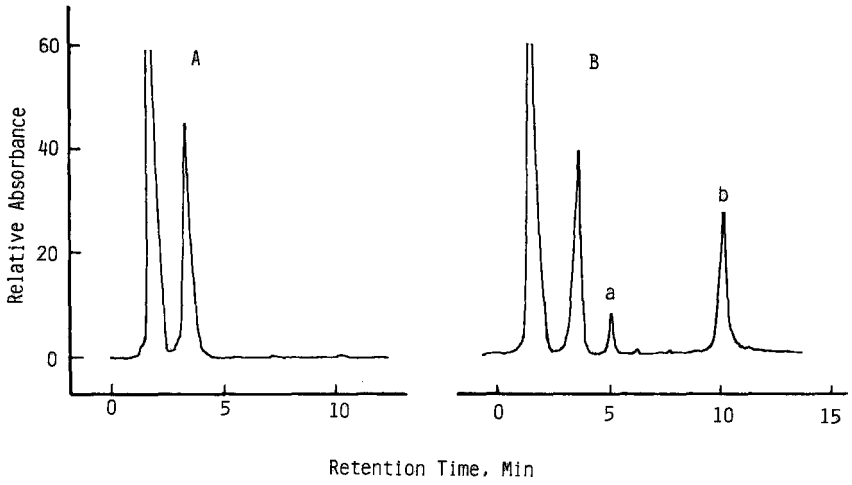

FIGURE 1. Chromatograms of naproxen in dog serum. A: blank dog serum; B: dog serum containing 6.25 ng/ml of naproxen (a) and internal standard (b).

analyses, the three standard plots obtained on the same day gave the average regression equation of $Y=-0.016+0.069X$ (CV of 1.59 % for the slope), where Y and X represent the peak height ratio and naproxen concentration, respectively. All of the intra- and inter-day standard graphs prepared for naproxen in dog serum had a correlation coefficient >0.999 (Tables 1 and 3).

The recovery and precision of the assay were assessed by analyzing replicate samples of inter-day and intra-day assays at each concentration used, and results were summarized in Tables 2 and 4. The overall mean recovery of naproxen from serum in the entire calibration range was 97.2 % with a CV of 2.0 %, when serum was deproteinized using 2.8 volume of acetonitrile per volume of serum. The recovery fell substantially when the volume ratio of acetonitrile to blood was less than 2.5. Acetonitrile has been widely used as a protein precipitant in extracting drugs for HPLC techniques because of its popularity as a mobile

TABLE 1

Intra-day Regression Data for the Standard Plots of Naproxen in Dog Serum

Standard plot	Slope[a]	Intercept	Correlation coefficient
1	0.0172	-0.0297	0.9994
2	0.0169	-0.0197	0.9997
3	0.0176	+0.0006	0.9998

[a] Mean slope (% CV) = 0.0172 ml/ng (1.59)

TABLE 2

Intra-day Variation of Naproxen Assay in Dog Serum

Concentration (ng/ml)	Mean peak height ratio	SD	CV (%)[a]
10.0	0.1773	0.0009	0.49
20.0	0.3333	0.0010	0.30
40.0	0.6613	0.0174	2.63
80.0	1.3198	0.0206	1.56
120.0	2.0766	0.0156	0.75
160.0	2.7926	0.0479	1.72
200.0	3.4152	0.0389	1.14

[a]Average CV = 1.3 %

phase component and high deproteinizing efficiency. The CVs for the inter-day and intra-day recoveries of naproxen in plasma were 2.0 % and 1.2 %, respectively, in the range of 10.0 ng/ml-200.0 ng/ml. The low coefficients of variation and high recovery indicate the good precision and accuracy of the present method.

The limit of quantitation and the limit of detection for naproxen in serum using the present method were 2.0 ng/ml (CV of 10.6 %) and 1.0 ng/ml (the signal/noise ratio of 3), respectively, when 0.2 ml of serum was used. The

TABLE 3

Inter-day Regression Data for the Standard Plots of Naproxen in Dog serum

Standard plot[a]	Slope[b]	Intercept	Correlation coefficient
1	0.0172	-0.0145	0.9998
2	0.0174	-0.0116	0.9999
3	0.0173	-0.0162	0.9997

[a] Prepared over a period of one week
[b] Mean slope (% CV) = 0.0173 (0.50)

TABLE 4

Inter-day Recovery and Precision Data for Naproxen Assay in Dog Serum

Naproxen added (ng/ml)	Mean peak height ratio	SD	CV (%)[a]	Recovered (ng/ml)	Recovery (%)[b]
10.0	0.1692	0.0071	4.18	9.0	90.0
20.0	0.3344	0.0063	1.88	18.9	94.4
40.0	0.6599	0.0108	1.64	38.8	97.1
80.0	1.3515	0.0307	2.27	80.3	100.4
120.0	2.0810	0.0231	1.11	119.3	99.4
160.0	2.7874	0.0348	1.25	158.9	99.3
200.0	3.4200	0.0463	1.35	199.8	99.9

[a]Average CV = 2.0 %
[b]Overall Mean Recovery = 97.2 %

sensitivity could be increased, for example, by increasing the volume of serum used, combined with the use of a higher volume of sample to be injected for chromatography. Concentrating the supernatant of deproteinized serum extract by evaporation could further enhance the sensitivity of the assay.

The method was successfully applied to determine the time course of naproxen concentrations in serum samples of a dog after receiving a topical dose of naproxen in a gel. The serum concentration versus time profile obtained with this assay is shown in Fig 2.

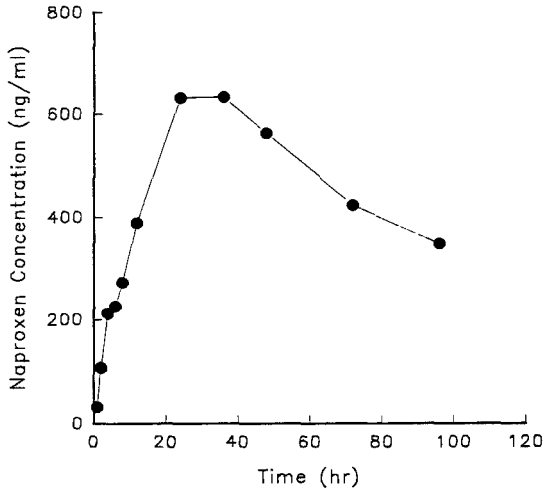

FIGURE 2. Serum concentration versus time profile of naproxen in a dog after administration of a topical gel (10mg/kg).

CONCLUSIONS

This report presented an improved HPLC method for the quantitation of naproxen in serum using a simple sample preparation procedure combined with the method of fluorometric detection. The method is accurate, sensitive and selective with no interference by endogeneous compounds. High and reproducible recoveries of the drug from serum were obtained due to the improved sample preparation procedure used. Since the method is simple and rapid, requiring no time consuming liquid or solid extraction procedures, it is recommended that this assay be used for routine therapeutic monitoring as well as for pharmacokinetic and bioavailability studies of naproxen in man and animals.

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DETERMINATION OF CEFTAZIDIME AND PYRIDINE BY HPLC: APPLICATION TO A VISCOUS EYE DROP FORMULATION

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ABSTRACT

A stability-indicating high performance liquid chromatographic (HPLC) method was developed to determine the cephalosporin antibiotic ceftazidime and its degradation product pyridine. The method was applied to analysis of an aseptically prepared viscous ceftazidime eye drop formulation (ceftazidime 5% w/v in *Sno Tears*). The chromatographic conditions were: Spherisorb 5μm hexyl column, 100 x 4.6mm; mobile phase acetonitrile:aqueous ammonium acetate 0.05M (7:93); flow rate 2ml/min; detector wavelength 254nm. The retention times of ceftazidime and pyridine were 1.3min and 3.8min respectively. The chromatographic precision of the method was typically 0.3-0.5% relative standard deviation (RSD) for ceftazidime. The recovery of ceftazidime from the eye drop was 101.3% and the within-day precision was 0.8% RSD. The limit of detection for pyridine was 2.4ng. Ceftazidime degraded by 7% over 7 days in the eye drops, with the production of pyridine accounting for 50% of the degraded antibiotic.

INTRODUCTION

The stability and compatibility of the third-generation cephalosporin eye drop ceftazidime has been studied in injection formulations by various reversed-phase HPLC methods. Hwang *et al.*[1] described a method for the analysis of ceftazidime in biological fluids which used a phenyl-bonded column. A number of authors have adopted this method for the analysis of ceftazidime in formulations[2-4].

The United States Pharmacopeia method for the analysis of ceftazidime injection[5] employs an ODS column. Various other methods employ this column type[6-9]. Other methods have used phenyl[10] and hexyl[11] columns.

A major decomposition pathway for cephalosporins is scission of the C-3 side chain[12]. In the case of ceftazidime, this would lead to the formation of pyridine. This product is potentially toxic and control of its levels in reconstituted injections and other formulations is desirable. None of the above methods allow its simultaneous determination with ceftazidime. Fabre and Kok[13] described an HPLC method for the detection of cephalosporins, including ceftazidime, and their degradation products. This depends upon post-column oxidation of the C-3 side-chain and would not be applicable to the determination of pyridine.

Cephalosporin or penicillin antibiotics have been used in eye drop formulations[14-16]. In the case of cefuroxime[15], a viscous artificial tears solution (*Sno Tears*) has been used as a vehicle in order to increase corneal residence time of the antibiotic.

This paper describes a reversed-phase HPLC method for the simultaneous determination of ceftazidime and pyridine and its application to a viscous ophthalmic formulation.

MATERIALS AND METHODS

Materials

Ceftazidime injection 1g (Fortum lots B1962NB and B3263BA) and ceftazidime pentahydrate standard lot AWS27C (84.8%w/v on a dried basis) were from Glaxo Laboratories, Uxbridge, UK. *Sno Tears* eye drops, containing polyvinyl alcohol 1.4%w/v (lots 209024 and 303038) were from Smith & Nephew Medical, Romford, UK.

Acetate buffers were prepared by mixing solutions of 0.05M ammonium acetate and acetic acid to the required pH.

All other chemicals and solvents were of analytical or HPLC grade.

Chromatographic Conditions

The HPLC system consisted of a CE1100 pump (Cecil Instruments, Cambridge, UK), SA6500 variable wavelength UV detector (Severn Analytical, Macclesfield, UK) and Peakmaster 3.2 data system (Harley Systems, Princes Risborough, UK). Samples were injected by an ASI-4 autosampler equipped with a Rheodyne 7010 injection valve (Talbot Instruments, Alderley Edge, UK).

A 100 x 4.6mm Spherisorb 5μm hexyl reversed-phase column was used, with a 10 x 4.6mm guard column of the same material (Hichrom, Reading, UK). The mobile phase was acetonitrile:0.05M ammonium acetate (7:93) at a flow rate of 2ml/min. The injection volume was 10μl and the detector wavelength was 254nm.

Sample Preparation

Samples were allowed to reach ambient laboratory temperature before processing. A 100μl sample of viscous ceftazidime eye drops was transferred with

an air-displacement pipette to a 25ml volumetric flask. The pipette tip was removed and rinsed into the flask with a jet of water. The flask was diluted to volume with water and the diluted solution sealed in an autosampler vial.

Standard Preparation

Ceftazidime pentahydrate standard, 100mg, was weighed into a 5ml volumetric flask. To this was added 1ml of a 1.2%w/v aqueous solution of sodium carbonate. When the ceftazidime pentahydrate had dissolved, the flask was diluted to volume with water. A 100μl aliquot of this solution was transferred, in duplicate, to 10ml volumetric flasks with the air-displacement pipette. The pipette tips were rinsed into each flask with water as for the sample. The flasks were diluted to volume with water and the solutions sealed in autosampler vials.

Ceftazidime was quantified by peak area measurement from duplicate injections of sample and standard solutions. Pyridine was estimated by measurement of its peak area from samples, calibrated with the corresponding ceftazidime peak and corrected using the relative response factor for pyridine with respect to ceftazidime. This factor was determined to be 1.4 in this chromatographic system.

System Suitability Tests

Each time the method was performed, the precision of chromatography was checked by chromatographing six replicates of a standard. The peak area should not have greater than 2% RSD. The mean peak area of duplicate injections of each of the two standards should not differ by more than 3%. A degraded solution was prepared by diluting 1ml of the initial standard solution with water; this was boiled

for approximately 5min and cooled. This solution was chromatographed at the start of each run to allow peaks to be identified and ensure adequate separation of degradation products from ceftazidime.

Preparation of Ceftazidime 5% w/v Eye Drops

The contents of a 1g vial of ceftazidime injection were dissolved in 20ml of either *Sno Tears* or water for injection, using aseptic technique in a laminar flow cabinet.

Eye drops for use in the recovery study were however prepared by mixing the contents of a 1g vial of ceftazidime injection and accurately weighing approximately half of the contents into a 10ml volumetric flask. This was dissolved in and diluted to volume with *Sno Tears*. The other half of the vial's contents was used as the standard: this was accurately weighed into a 10ml volumetric flask, then dissolved in and diluted to volume with water. Both solutions were further diluted as for preparation of the sample.

Forced Degradation

The stability-indicating potential of the method was assessed with solutions of 1.25mg/ml ceftazidime pentahydrate, from an injection vial, degraded in various aqueous solutions. The degradation conditions were: 1%w/v sodium carbonate, heated at 60°C for 5min; 0.1M sodium hydroxide maintained at ambient temperature for 30min; phosphoric acid, added dropwise to an aqueous ceftazidime solution, to give a pH of 1.0, heated at 60°C for 5min. The solutions were diluted 1:250 with 0.05M ammonium acetate solution before injection onto the chromatograph. Also, a simple aqueous solution of ceftazidime pentahydrate was boiled for 5min and was diluted 1:250 with water before chromatographing.

Confirmation of the stability-indicating capability of the method for use with viscous eye drops was obtained by chromatographing samples of formulation stored at 25°C for up to 17 days.

Stopped-Flow Scanning

The stopped-flow UV-spectra of the ceftazidime and pyridine peaks were obtained with a SA6503 detector (Severn Analytical, Macclesfield, UK).

RESULTS AND DISCUSSION

The degradation of the ceftazidime by any of the conditions employed lead to the production of a peak corresponding to pyridine and two other major degradation products which were not identified (Figure 1).

In the mobile-phase chosen for the method, the ceftazidime and pyridine peaks both had $_{max}$, by stop-flow scanning, of 254nm. This was chosen as the analytical wavelength.

A mobile-phase with an aqueous component pH of approximately 7.0, *i.e.* ammonium acetate solution with no pH modification, gave the best separation between ceftazidime and its degradation products (Figure 2). Increasing the concentration of buffer from 0.05M to 0.1M made no observable difference to peak shape or resolution.

Increasing flow rate caused decreased resolution between the ceftazidime and degradation product peaks (Table 1). However the symmetry of the ceftazidime peak increased with increasing flow-rate. A 2ml/min flow-rate was adopted since this gave adequate resolution of ceftazidime with a short analysis time, minimising sample degradation.

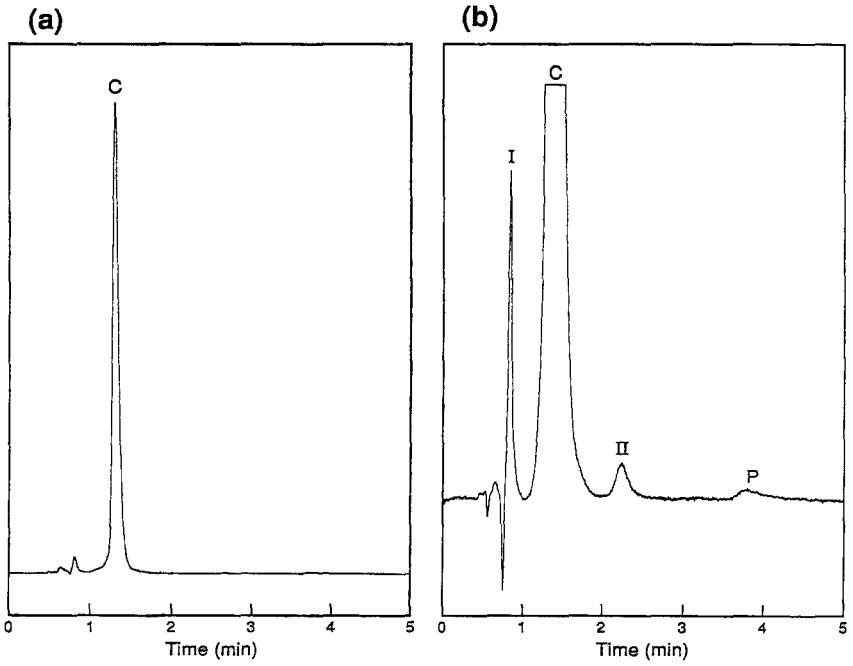

FIGURE 1: Chromatograms of (a) ceftazidime in eye drop formulation stored for 7 days at 7°C, (b) aqueous solution of ceftazidime, degraded by boiling. C = ceftazidime; P = pyridine; I, I I = unidentified degradation products.

Pyridine was confirmed as a degradation product of ceftazidime (Figure 3) from the retention time of the peak from a solution of ceftazidime degraded by boiling and from its stopped-flow UV spectrum, by comparison with the peak from an authentic pyridine standard.

Ceftazidime pentahydrate standard dissolved slowly in water. The addition of sodium carbonate to the standard solution caused rapid dissolution of the ceftazidime, giving a solution with a pH of approximately 7.0.

The chromatographic precision of the ceftazidime peak area, from system suitability testing, was typically in the range 0.3-0.5% RSD.

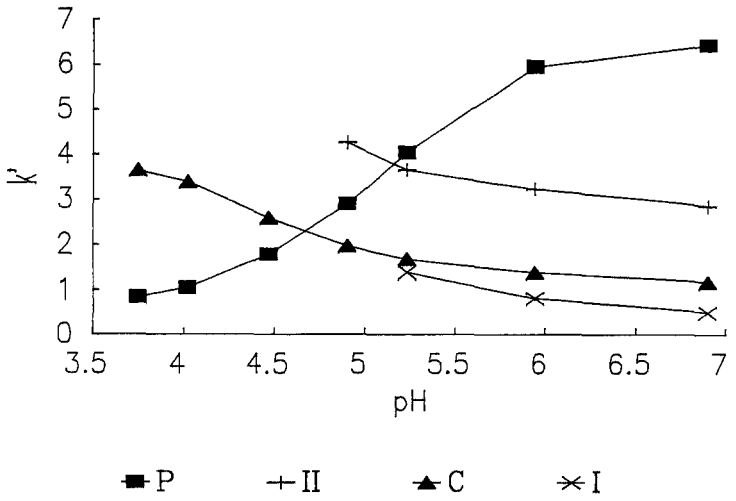

FIGURE 2: Effect of mobile phase pH on capacity factor (k').
C = ceftazidime; P = pyridine; I, I I = unidentified degradation products.

Table 1. Effect of Flow Rate on Peak Resolution and Symmetry.

Flow rate (ml/min)	Resolution factor[a] between ceftazidime and I[b]	Resolution factor[a] between ceftazidime and II[b]	Symmetry factor[a]
0.5	4.83	4.98	1.88
1.0	4.72	4.08	1.92
1.5	4.05	4.24	1.58
2.0	3.08	3.84	1.42

[a] As defined in British Pharmacopoeia 1993[17]

[b] See Figure 1

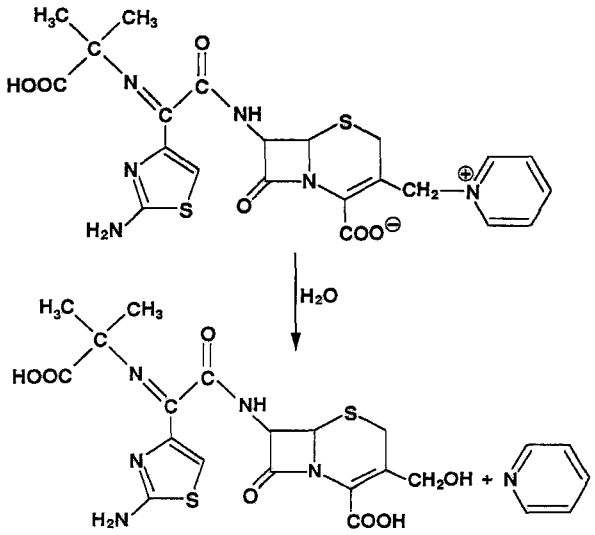

FIGURE 3: Potential scheme for the degradation of ceftazidime, forming pyridine.

The chromatography was linear for ceftazidime in the range 0.2-5.0μg injected; y = mx + c: m = 6.81×10^5 ($\pm 6.4 \times 10^3$, 95% confidence limit); c = -3.7×10^3 ($\pm 1.8 \times 10^4$, 95% confidence limit); n = 16; r = 0.9999. The chromatography was linear for pyridine in the range 0.0028-0.0282μg injected; y = mx + c: m = 4.53×10^5 ($\pm 1.1 \times 10^4$, 95% confidence limit); c = -2.35×10^2 ($\pm 1.9 \times 10^2$, 95% confidence limit); r = 0.9994. These data were obtained on separate days. In order to obtain an accurate estimate of the relative response factor of pyridine with respect to ceftazidime, a pyridine calibration curve was performed with concurrent calibration with a ceftazidime standard. This gave a value of 1.4.

The recovery of ceftazidime from the eye drops was 101.3%, and the within-day precision was 0.8% RSD.

The recovery of pyridine, spiked in the range equivalent to the degradation of 0.8-9.4% of the ceftazidime in the eye drops, was 93.1%, assessed by comparison of the slopes of the calibration curves for pyridine spiked into the formulation and for aqueous pyridine solutions. The detection limit for pyridine, judged on the basis of a peak of height four times greater than the baseline noise, was 2.4ng.

Diluted standard solutions degraded by 0.35%/h at room temperature and 0.20%/h in a refrigerator. Chromatography should therefore be performed immediately after solution preparation.

Storage of eye drops at 7°C for 7 days lead to a 7% reduction in the initial ceftazidime concentration. Pyridine levels increased to 500μg/ml, accounting for approximately 50% of the degraded ceftazidime. An unacceptable yellow colour developed after 7 days storage.

CONCLUSION

The method is rapid and stability-indicating and is accurate and precise enough for the determination of ceftazidime and pyridine in viscous eye drops. It is capable of adaptation to other formulations of ceftazidime.

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LIQUID CHROMATOGRAPHIC METHOD FOR THE CONCURRENT ANALYSIS OF SUCROSE POLYESTER, VITAMIN A PALMITATE, AND β-CAROTENE IN MARGARINE

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ABSTRACT

A liquid chromatographic method is described for the concurrent analysis of sucrose polyester (SPE), vitamin A and ß-carotene in margarine. The SPE and vitamins are separated from the acylglycerol by gel permeation chromatography (GPC). The GPC system is equipped with a refractive index and ultraviolet (UV) detector (313 nm) to monitor the peak elution. Following collection, the vitamins and SPE are analyzed by C-18 reverse phase chromatography. A ternary gradient of acetonitrile, methylene chloride and isopropanol with evaporative light scattering detection is used for SPE analysis. Vitamin A and ß-carotene are quantitated after isocratic elution with acetonitrile/methylene chloride/methanol (700/300/2, v/v/v) as mobile phase and detection at 313 nm and 436 nm, respectively. Well resolved, interference free chromatograms were obtained for each analyte.

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INTRODUCTION

Considerable interest has been shown recently in fat substitutes. One such substitute, olestra or sucrose polyester (SPE) has been the target of considerable analytical chromatography work by Tallmadge and Lin (1) and Chase et al. (2,3). Tallmadge and Lin (1) developed a liquid chromatography (LC) method based upon reverse phase nonaqueous chromatography and evaporative light scattering detection (ELSD) to quantitate olestra from olestra-lipid blends. Chase et al. (2) used gel permeation chromatography (GPC) to separate and collect SPE from the acylglycerol. The collected SPE fraction was then analyzed on a C-18 column with a ternary gradient, evaporative light scattering detection (ELSD), and quantitated in the presence of sucrose octaacetate as an internal standard.

The Chase et al. (2) procedure provided interference-free chromatograms with improved resolution between SPE and acylglycerols compared to the Tallmadge and Lin (1) procedure. Chase et al. applied their procedure to the analysis of SPE in salad dressings (2). Three different salad dressings containing SPE were studied. An extraction procedure developed by Landen (4,5) for fat soluble vitamins in infant formula was modified and applied to the salad dressings. The mean percent recovery (n=3) and standard deviation were 92.4 ± 2.6. GPC as a clean-up step is a valuable asset for studies with more complex matrices such as salad dressings. Pending approval of SPE as a fat substitute, a likely food to contain SPE would be margarine or a spread. This study applies the earlier work of Chase et al. (2,3) to a higher degree of matrix complexity since margarine tends to contain hydrogenated vegetable oils, liquid vegetable oil, lecithin, mono- and diacylglycerols, ß-carotene and vitamin A palmitate. The SPE, vitamin A palmitate and ß-carotene were isolated from the margarine by GPC and quantitated on a non-aqueous reverse phase LC system.

MATERIALS AND METHODS

Chemicals and Standards

The solvents, methylene chloride, acetonitrile and isopropanol were HPLC grade and obtained from J. T. Baker Inc. (Philipsburg, NJ, USA). Anhydrous magnesium sulfate was reagent grade and from J. T. Baker Inc.

Internal standard. A 500 mg portion of sucrose octaacetate (Aldrich Chemical Co., Milwaukee, WI, USA) was diluted to 250 mL with methylene chloride to give a concentration of 2000 µg/mL.

SPE standard. The SPE was synthesized with sucrose and the fatty acid methyl esters of soybean oil using the method of Boutte (6). A 125 mg portion of SPE was accurately weighed and diluted to 25 mL with the internal standard solution. A 3.0 mL aliquot was diluted to 10.0 mL with the internal standard to give a concentration of 1500 µg/mL.

Vitamin A standard. Approximately 38 mg of vitamin A palmitate (Fluka Bio Chemika, Switzerland) was dissolved in 50 mL of methylene chloride. The exact concentration was determined from the $E^{1\%}$ value of 975 (7) for vitamin A palmitate. Appropriate dilutions were made to give working standards with concentrations of 0.4, 0.8 and 1.5 µg/mL.

ß-Carotene standard. Approximately 20 mg of ß-carotene (Fluka Bio Chemika) was dissolved in 50 mL of methylene chloride. After determining the exact concentration from the $E^{1\%}$ value of 2620 (8) for ß-carotene, appropriate dilutions were made to give concentrations of 0.2, 0.4, and 0.7 µg/mL.

Instrumentation

Clean-up LC. The SPE was isolated from the acylglycerols in each sample by using four µStyragel 100 Å GPC columns (Waters Corp., Milford MA, USA) connected in series with a mobile phase of methylene chloride, injection volume of 250 µL, and a flow rate of 1.0 mL/min. The LC consisted of a Waters 510 pump, U6K injector, 440 detector (λ 313 nm), a 410 refractive index detector and a Kipp and Zonen (Delft, Holland) BD 41 dual pen strip chart recorder.

Analytical LC for SPE. The SPE was quantitated by injecting 50 µL on a Zorbax 4.6 mm X 25 cm, C-18, 5 µm column (Mac Mod Analytical, Chadds Ford, PA, USA). The LC consisted of a Constametric 4100 LC (Thermo Separations Products, Riviera Beach, FL, USA), a Waters 717 Plus autosampler, a Sedex 55 ELSD (Richard Scientific, Novato, CA., USA) and a Hewlett Packard (Avondale, PA, USA) model 3390 integrator. A flow rate of 1.0 to 1.5 mL/min was used with a gradient of methylene chloride, acetonitrile and isopropanol (2). The ELSD was set at 40°C, with a nebulizer pressure of 2.0 bar and a gain of 5.

Analytical LC for vitamins. Vitamin A palmitate and ß-carotene were quantitated by injecting 100 µL on a Zorbax 4.6 mm X 25 cm, C-18, 5 µm column (Mac Mod Analytical). The LC consisted of a Waters 510 pump, U6K injector, 440 dual channel detector (λ = 313 and 436 nm) and two Hewlett Packard model 3390 integrators. A flow rate of 1.0 mL/min was used with a mobile phase of acetonitrile/methylene chloride/methanol (700/300/2, v/v/v).

Analysis

A commercially available margarine was obtained at a local supermarket. Five portions of margarine were weighed out, four of which were blended with SPE to give a 5, 10, 20 and 30% level of SPE in the respective margarine. A fifth portion was used as a control sample.

Approximately, 10 g from each of the five portions of margarine was accurately weighed and dissolved in 50 mL of methylene chloride. After the margarine melted and dissipated in the methylene chloride at room temperature, 3 g of $MgSO_4$ was added and allowed to stand for two hours with frequent agitation. The mixture was filtered through a coarse porosity 60 mL fritted glass filter. The filtered solution was diluted to 100 mL with methylene chloride.

After establishing collection timeframes with standards of SPE, vitamin A palmitate and ß-carotene, the samples were injected onto the GPC system. At the appropriate times two fractions were collected for further analysis with one being for the SPE and the other for the vitamins. Each collected fraction was evaporated to dryness. The SPE fraction was reconstituted in the internal standard solution while the vitamin fraction was reconstituted in the mobile phase used for the analytical LC for vitamin A palmitate and ß-carotene. The amounts of each vitamin were reported as International Units/kg and were corrected for the amount of SPE added to each.

RESULTS AND DISCUSSION

This study combines the early work of Landen and Eitenmiller (9) with vitamin A palmitate and ß-carotene in margarines with the recent work of Chase et al. (2,3)

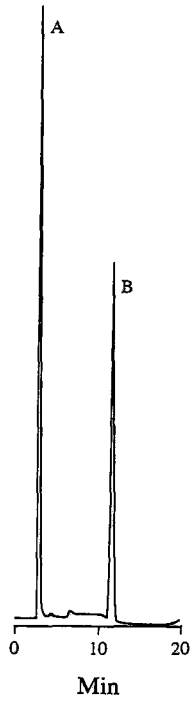

FIGURE 1: Reverse phase liquid chromatography chromatogram (C-18 column) of sucrose polyester (SPE) and the internal standard, sucrose octaacetate using ELSD with a pressure of 2.0 bar, temperature of 40°C and a gain of 5. A ternary gradient of methylene chloride, acetonitrile and isopropanol was used. The chromatogram was obtained after GPC clean-up step. A is the internal standard and B is the SPE peak.

with SPE. The procedure provides methodology for the concurrent analysis of SPE and vitamin A palmitate and ß-carotene from margarine type matrices.

Figure 1 illustrates the analytical LC chromatogram of the SPE fraction collected from the GPC column. Without GPC clean-up the SPE is poorly resolved as illustrated in Figure 2. In addition, without the use of GPC, we have observed the column pressure to gradually increase until the upper pressure limit is reached and the system shuts down. Excessive fat on the column will ultimately shorten the life of the column. Isolation of the SPE from the acylglycerol prior to determinative chromatography prevents column problems related to lipid injection.

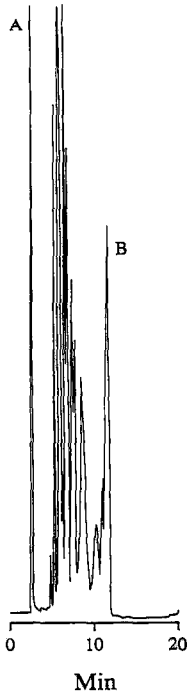

FIGURE 2: LC chromatogram of SPE and the internal standard. The conditions are the same as Fig. 1 except there was no GPC clean-up step. A is the internal standard and B is the SPE peak. Between A and B are the acylglycerols.

The chromatograms obtained for vitamin A palmitate and ß-carotene are illustrated in Figure 3. These chromatograms resemble classic examples of the method developed earlier by Landen (4, 5) and Landen and Eitenmiller (9). Concentrations of the vitamins were calculated by linear regression analysis. Since different wavelengths were used for vitamin A (313 nm) and ß-carotene (436 nm), a suitable internal standard that absorbs at both wavelengths and elutes at a time that does not interfere with either vitamin was not available. The peak purity of vitamin A palmitate and ß-carotene was confirmed by diode array comparison of the spectra of standard and sample peaks.

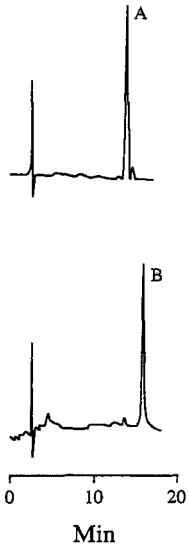

FIGURE 3: Reverse phase C-18 chromatogram of ß-carotene (A) and vitamin A palmitate (B). A mobile phase consisting of acetonitrile/methylene chloride/methanol (700/300/2, v/v/v) was used with λ 313 nm for vitamin A and λ 436 nm for ß-carotene.

Figure 4 shows the chromatogram used to identify and monitor the collection timeframes. One trace is from the UV detector (λ 313 nm) and the other is from the RI detector. The SPE peak eluted first, followed by the fat and finally vitamin A palmitate and ß-carotene. Therefore, the SPE and vitamin fractions are easily and efficiently isolated from the acylglycerols.

The amount of vitamin A palmitate and ß-carotene found is consistent from sample to sample as shown in Table 1. The average total vitamin A found was 36,934 IU/kg ± 1305 (cv 3.5), which was 92.3% of the manufacturers declared value. The amounts of vitamins found as listed in Table 1 were corrected for the SPE that was added. Table 1 also shows the percent recoveries for SPE of each sample, with an average of 104%. The linearity and detection limits have been previously calculated and discussed in the earlier work of Landen (4, 5), Landen and Eitenmiller (9) and Chase et al. (2,3).

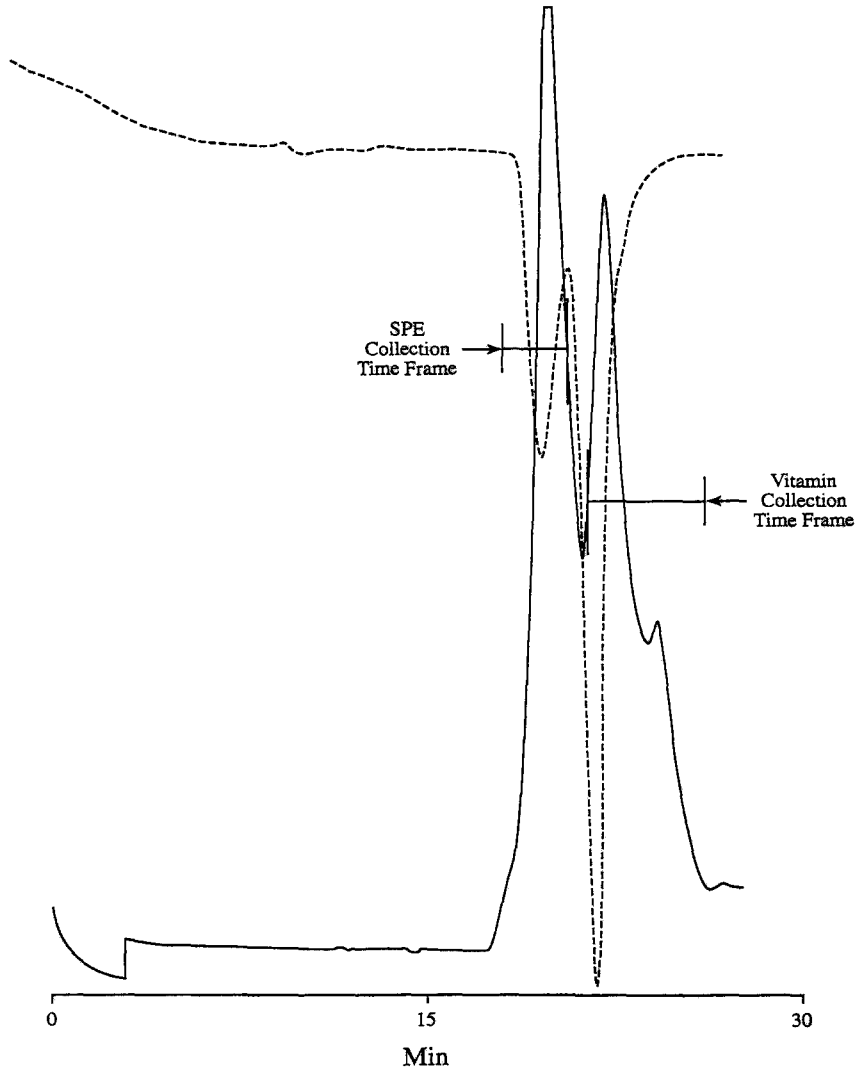

FIGURE 4: GPC chromatogram of the extract of SPE-margarine blend eluted with a mobile phase of methylene chloride. The solid trace is UV detection at λ 313 nm for vitamins and the broken trace is RI detection for SPE. Horizontal lines on the chromatograms indicate the collection points for the SPE and vitamins.

TABLE 1

Analysis of Vitamin A Palmitate, ß-Carotene and Sucrose Polyester (SPE) from SPE-Margarine Blends

	SAMPLE									
	Control		5% SPE		10% SPE		20% SPE		30% SPE	
Vit A (IU/kg):	25906	23763	25313	25509	25006	24943	25942	26715	26940	26521
ß-Carotene (IU/kg):	11446	10461	11873	11489	11491	10940	11572	11333	11777	11779
Total (IU/kg):	37352	34224	37186	36999	36496	35883	37514	38044	38716	38301
SPE Found (g/100g):	-	-	5.22	5.09	10.10	10.10	21.80	21.80	32.70	32.90
SPE Added (g/100g):	0	0	5.27	4.99	10.10	10.10	20.70	20.60	30.20	30.50
% SPE Recovered:	-	-	99.1	102	100	100	105	106	108	108

Note: The values found for the vitamins were corrected for the SPE that was added

This study provides an accurate method to assay not only the SPE content in a highly complex food matrix such as margarine but also the concurrent assay of vitamin A palmitate and ß-carotene.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(15), 3139-3146 (1995)

DETERMINATION OF COBALT(II) BY REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL AND SPECTROPHOTOMETRIC DETECTION

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Faculty of Engineering
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Bunkyo, Fukui 910, Japan

ABSTRACT

A reversed-phase liquid chromatographic method for the selective and sensitive determination of cobalt (II) with ethyl xanthate (EX) is described. The method is based on the pre-column derivatization of cobalt(III)-EX chelate, injection of the sample onto a C_{18} column, and elution with 4:1 acetonitrile/sodium acetate solution (0.02 mol dm^{-3}, pH 7.8) containing 0.02 mol dm^{-3} potassium nitrate as the mobile phase. The detection of the metal in eluate has been made by electrochemical (at +1.40 V *vs.* Ag/AgCl) and spectrophotometric (at 330 nm) methods. The detection limits (S/N=3) for cobalt(II) by the proposed method are 0.23 and 0.56 μg dm^{-3} for the electrochemical and spectrophotometric detection, respectively. Cobalt in pepperbush has been determined with satisfactory precision.

INTRODUCTION

In recent years, the separation and determination of metal ions by liquid chromatography (LC) has been increasing popular (1). These methods involve the formation of metal chelates with coordinating reagents such as diethyldithiocarbamate (2), β-diketones (3), quinolinol-8-ol (4), 4-(2-pyridylazo) resorcinols (5,6) and so on, followed by LC separation with spectrophotometric and electrochemical detections. It has been recognized that the spectrophotometric detection after LC separation of metal

chelates makes it possible to simultaneously determine multielments in a variety of samples. The electrochemical detection, on the other hand, is a very effective method for the selective determination of some components of interest, because only electroactive (oxidative or reductive) species can be detected amperometrically at a glassy carbon. Deficiencies in the use of the electrochemical detection arises from the necessary presence of excess electroactive ligand (*e.g.* dithiocarbamates) which has to be eliminated prior to electrochemical detection by an anion exchange column (7). Further, the slow decomposition of certain chelates under the conditions of the reversed-phase LC system is observed due to solubility problems. In order to overcome such problems, Bond et. al have used the exchange reaction with zinc bis-(2-hydroxyethyl)dithiocarbamate for automated monitoring of metal ions by LC with electrochemical detection (8).

In this work we describe the use of ethyl xanthate (EX) as an electroactive ligand for the electrochemical detection of the Co(III) chelate after the reversed-phase LC separation. The ligand has been utilized for normal-phase LC of such metal ions as Bi(III), As(III) and Sb(III) (9), but not for the reversed-phase LC system in which only the Co(III) chelate is the most stable than the other metals; the Co(III) chelate does not decompose in the C_{18} column even when the elution was made with the mobile phase containing no ligand. The results suggest that the use of EX as a precolumn derivatization achieves decrease of background current and detection limit, unchanged response and selective detection of the metal chelate at working electrodes. We have developed a selective and sensitive method for the determination of low levels of cobalt(III) by the reversed-phase LC with electrochemical detection as well as spectrophotometric one. It is demonstrated that the proposed method is virtually free from interferences such as Cu(II), Ni(II), Pb(II), Cd(II) and has been applied to the determination of cobalt in pepperbush (Standard Reference Material No.1 from the National Institute of Environmental Science, NIES 1, Japan).

EXPERIMENTAL

Apparatus

The liquid chromatograph used consisted of a Tosoh CCPD pump, a Rheodyne 7125 loop injector equipped with a 100 mm^3 sample loop, a Fuso Model HECS 318 High Sensitive Potentiostat equipped with a BAS LC-17A thin-layer electrochemical cell (a glassy carbon working electrode, a stainless steel auxiliary electrode and a Ag/AgCl reference electrode), a Shimadzu SPD-10A ultraviolet-visible spectrophotometric detector and Shimadzu Chromatopac CR-6A data processor. Tosoh TSK-Gel ODS 80TM chromatographic columns (250 x 4.6 mm i.d. and 150 x 4.6 mm i.d.) were used. A PAR 174A polarographic analyzer equipped with a Rika Denki RW-11 x-y recorder was used for differential-pulse voltammetric measurements. A Toa Denpa HM-30S pH meter was also used.

Reagents and Solutions

Acetonitrile was of LC grade from Kanto Chemicals. Potassium EX salt and metal ion standards for atomic absorption spectrometry were purchased from Wako Junyaku Chemicals. The water used was obtained from a Millipore Milli-Q water purification system. The mobile phase was 4:1 (v/v) acetonitrile/sodium acetate solution (0.02 mol dm^{-3}, pH 7.8) containing 0.02 mol dm^{-3} KNO_3. Potassium EX salt was dissolved in acetonitrile to give a concentration of 2 x 10^{-3} mol dm^{-3}. All other chemicals used were of analytical-reagent grade.

Procedure

A slightly alkaline solution (pH ca.9) containing Co(II) was taken in a 10-cm^3 mol dm^{-3} calibrated flask, and 5.0 cm^3 of 2 x 10^{-3} mol dm^{-3} potassium EX salt solution in acetonitrile was added. The total volume was made up to 10.0 cm^3 with water. A 100-mm^3 aliquot of the resultant solution was injected onto the analytical column, and eluted with the mobile phase at a flow rate of 1.0 cm^3 min^{-1}. The eluate after LC separation was detected by the electrochemical (+1.40 V *vs.* Ag/AgCl) and spectrophotometric (330 nm) detection methods. The amount of the metal was determined by measuring the peak height on the chromatogram.

Treatment of pepperbush sample

The sample was dried at 110 °C to obtain constant mass and was then weighed by 0.3790 g in this experiment. The weighed sample was transferred to a 500-cm^3 beaker, and treated with 10 cm^3 of nitric acid, 10 cm^3 of sulfuric acid and 30 cm^3 perchloric acid at 150-200°C. The solution was evaporated to almost dryness. The residue was dissolved in 10 cm^3 of 2 mol dm^{-3} hydrochloric acid, and the pH was then adjusted to *ca.* 9.0 by adding 5.0 cm^3 of 0.1 mol dm^{-3} sodium citrate and an appropriate volume of 6 mol dm^{-3} ammonia. The sample solution was made up to 100 cm^3 with water, and was mixed with 2 x 10^{-3} mol dm^{-3} EX solution in acetonitrile at a 1:1 volume ratio, as described in the section of procedure.

RESULTS AND DISCUSSION

Chromatographic studies of metal EX-chelates

Fig.1 shows a typical chromatogram for the Cu(II), Ni(II), Pb(II), Hg(II), Cd(II), Bi(III) and Co(III) chelates by the reversed-phase LC system using 4:1 acetonitrile/sodium acetate solution (0.02 mol dm^{-3}, pH 7.8) containing 10^{-3} mol dm^{-3} EX as the mobile phase. At the stage of the precolumn

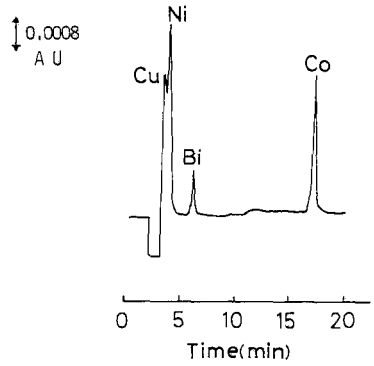

Fig.1 A chromatogram for metal-EX chelates by reversed-phase LC with spectrophotometric detection (330 nm): the mobile phase was 4:1 acetonitrile/sodium acetate solution (0.02 mol dm^{-3}, pH 7.8) containing 2 x 10^{-3} mol dm^{-3} EX and 0.02 mol dm^{-3} KNO_3; the metal concentration was 1.0 mg dm^{-3}; the analytical column was Tosoh ODS 80TM (4.6 x 250 mm).

derivatization, the labile Co(II) chelate was oxidized to the inert Co(III) derivative in the presence of air. It was found that the retention time of the metal chelates increased as the percentage of acetonitrile decreased. An acetonitrile content of 80% was chosen as the mobile phase, because the Bi(III) and Co(III) chelates were well separated from the others at retention times of 6 and 17 min, respectively. The two chromatographic peaks were unaffected even when the ligand was not present in the mobile phase; however, the other metal chelates gave no peak because they decomposed through the LC elution. The results indicate that the C_{18} column acts as a reactor to separate certain coexisting species. In addition, the detection limit could also be lowered as a result of decreased background noises on the chromatogram. In the present paper, we have studied the determination of Co(II) as the EX chelate by the reversed-phase LC separation using the mobile phase containing no ligand, followed by electrochemical and spectrophotometric detection.

Electrochemical detection after LC separation as the Co(III)-EX chelate

As described above, 4:1 acetonitrile/sodium acetate solution (0.02 mol dm^{-3}, pH 7.8) containing 0.02 mol dm^{-3} KNO_3 was selected as the best mobile phase for the LC separation of the Co(III)-EX chelate from the others. In the analysis of real samples, a C_{18} column (Tosoh ODS 80TM, 4.6 x 150 mm) was recommended in order to shorten the analytical time. Fig.2 shows a chromatogram obtained for a mixture of 0.1 μg cm^{-3} Cu(II), Ni(II), Bi(III), Pb(II), Cd(II), Hg(II) and Co(II) by the

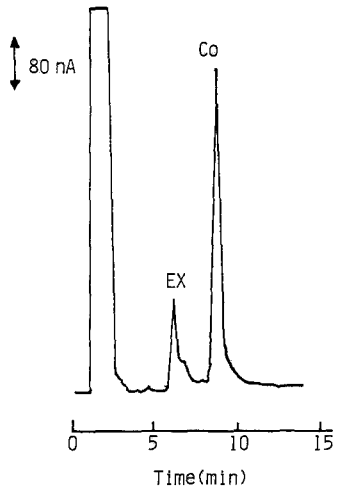

Fig.2 A chromatogram for the Co(III)-EX chelate by reversed-phase LC with electrochemical detection (+1.40 V *vs.* Ag/AgCl): the mobile phase was 4:1 acetonitrile/sodium acetate solution (0.02 mol dm^{-3}, pH 7.8) containing 0.02 mol dm^{-3} KNO_3; the metal concentration was 0.1 mg dm^{-3}; the analytical column was a Tosoh ODS 80 TM (4.6 x 150 mm).

reversed-phase LC with electrochemical detection +1.40 V *vs.* Ag/AgCl. Only Co(II) and excess ligand showed chromatographic responses at 9.3 and 6.5 min, respectively. Therefore, the proposed LC method is free from interferences due to the other metals. The differential-pulse voltammogram for the ligand EX in 9:1 acetonitrile/0.02 mol dm^{-3} sodium acetate solution was recorded in a conventional cell. The oxidation of EX to form thiuram disulfide was observed at +0.35 V *vs.* Ag/AgCl, above which potentials no further response of the ligand was seen. On the addition of Co(II) to the ligand solution, as shown in Fig.3, an oxidative response from Co(III) to Co(IV) appeared at +1.34 V *vs.* Ag/AgCl under the experimental conditions: scan rate 5 mV s^{-1}, modulation amplitude 50 mV, duration time between pulses 1s. The peak current for the Co(III) response increased with applied potentials commencing +1.30 V *vs.* Ag/AgCl. An applied potential of +1.40 V was chosen for the determination of Co(II) by the reversed-phase LC with electrochemical detection.

Calibration graphs and detection limits

According to the above procedure, the precolumn derivatization and sample injection of 100 mm^3 were made, followed by chromatographic elution. When the eluate was detected electrochemically at +1.40 V, the calibration graph of peak current *vs.* Co(II) concentration was linear in the range of

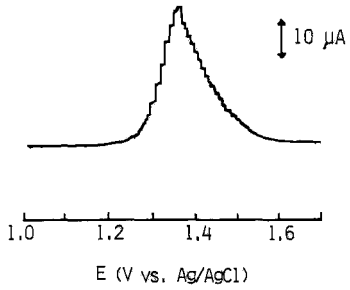

Fig.3 A differential-pulse voltammogram for the Co(III)-EX chelate in 9:1 acetonitrile/aqueous solution containing 0.05 mol dm^{-3} EX, 0.02 mol dm^{-3} sodium acetate and 0.02 mol dm^{-3} KNO_3; scan rate: 5 mV s^{-1}; modulation amplitude: 50 mV; pulse interval: 1 s; the metal concentration: 50 mg dm^{-3}.

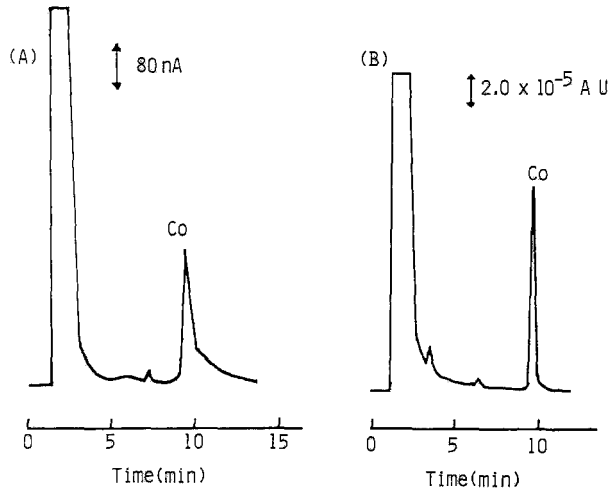

Fig.4 Determination of cobalt in pepperbush by the reversed-phase LC with electrochemical (A) and spectrophotometric detection (B); the experimental conditions are described in Fig.2 and the procedure of the experimental section.

0.5~200 μg dm^{-3}. The straight line can be expressed by the equation Y (peak current, nA) = 4.76 x 10^3 X(concentration, mg dm^{-3}) + 3.9, and the correlation coefficient (γ) was 0.9992. The detection limit was 0.23 μg dm^{-3} Co(II) at a signal-to-noise (S/N) ratio of 3.

When the spectrophotometric detection was made at 330 nm, the calibration graph was a straight line of Y (peak height, absorbance unit) = 3.06 x 10^{-3} X (concentration, mg dm^{-3}) + 4.16 x 10^{-6}, γ = 0.9994 in the concentration range of 1.0 ~ 200 μg dm^{-3}. The detection limit was 0.56 μg dm^{-3} at S/N = 3 (0.002 absorbance unit full scale).

Determination of cobalt in pepperbush

Pepperbush sample (NIES No.1) was analyzed by the proposed LC method. The chromatogram are shown in Fig.4 (A) and (B). Before injection, a 2.0-cm^3 aliquot of the sample solution (3.79 mg cm^{-3}) was mixed with the equal volume of 10^{-3} mol dm^{-3} EX in acetonitrile. The analytical results (n=5) obtained by the electrochemical and spectrophotometric detection were 22.8 ± 0.9 and 22.6 ± 0.5 μg g^{-1} respectively, which both coincided well with the certified value 23 ± 3 μg g^{-1}. The proposed method is very sensitive and free from interferences, and in addition it would be able to determine traces levels of cobalt in environmental and biological samples.

CONCLUSIONS

The results presented here indicate that the use of EX as a derivatization reagent is of analytical utility for the electrochemical and spectrophotometric detection after LC separation of metal species. The chromatographic system permits the oxidative detection of the Co(III) chelate to be discriminated against excess ligand in the mobile phase. As the result, the S/N ratio can be improved to obtain lowered detection limits. As seen from the successful application, we have also expected that the other real samples containing cobalt can be analyzed with good precision and accuracy.

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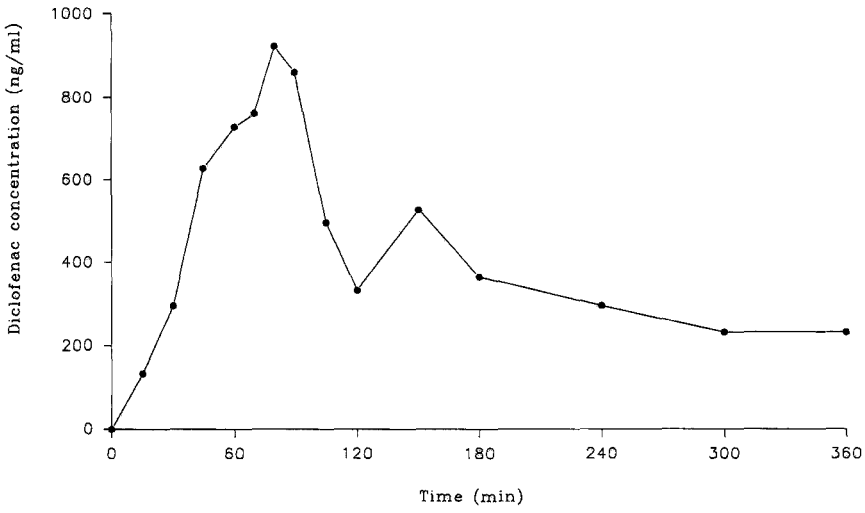
JOURNAL OF LIQUID CHROMATOGRAPHY, 18(15), 3147 (1995)

corrected 29 Jan. 96/A

ERRATUM

J. L. Mason, G. J. Hobbs, "A Rapid High Performance Liquid Chromatographic Assay for the Measurement of Diclofenac in Human Plasma," J. Liquid Chrom., 18(10), 2045-2058 (1995).

There is an error in Figure 3 (p. 2056); the y axis was incorrectly labelled. The y axis tick labels should be 0, 200, 400, 600, 800 and 1000. The corrected figure is shown below:



JOURNAL OF LIQUID CHROMATOGRAPHY, 18(15), 1349 (1995)

ERRATUM

corrected 29 Jan 96/A

Z. Grubišic-Gallot, Y. Gallot, and J. Sedláček, "Study of Polystyrene-block-poly(methyl Methacrylate) Micelles by Size Exclusion Chromatography/Low Angle Laser Light Scattering. Influence of Copolymer Composition and Molecular Weight," *J. Liquid Chrom.*, 18(12), 2291-2307 (1995).

Due to a printing error, the third author's name in the above-mentioned article was misspelled in the table of contents. It should have appeared as J. Sedláček, not J. SedláÒek.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(15), 3151 (1995)

ANNOUNCEMENT

1995 Frederick Conference on Capillary Electrophoresis Award Professor Shigeru Terabe to be Honored

Professor Shigeru Terabe, of the Himeji Institute of Technology in Japan, will be presented the Frederick Conference on Capillary Electrophoresis Award for outstanding contributions to the field of capillary electrophoresis. The award will be presented to him at the Sixth Annual Conference, which will convene on October 23 - 25, 1995 at Hood College in Frederick, Maryland, USA. More than 400 investigators and leaders in the field of capillary electrophoresis will be in attendance.

Dr. Terabe pioneered the development of micellar electrokinetic chromatography [*Anal. Chem., 56, 111-113 (1984)*] which made possible the separation of neutral molecules by capillary electrophoresis. Today, MEKC is an accepted technique for the separation of neutral as well as charged molecules.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(15), 3153 (1995)

ANNOUNCEMENT

BASIC PRINCIPLES OF HPLC
and
HPLC SYSTEM TROUBLESHOOTING

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

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

The following topics are covered in depth:

- Introduction to HPLC Theory
- Modes of HPLC Separation
- Developing and Controlling Resolution
- Mobile Phase Selection & Optimization
- Ion-Pairing Principles
- Gradient Elution Techniques
- Calibration & Quantitation
- Logical HPLC Troubleshooting

The instructor for the courses, Dr. Jack Cazes, is Editor-in-Chief of the Journal of Liquid Chromatography, of Instrumentation Science & Technology journal, and of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 30 years; he pioneered the development of modern HPLC technology. Dr. Cazes was also Professor-in-Charge of the ACS short course and the ACS audio course on Gel Permeation Chromatography for many years.

Details of these in-house courses may be obtained from Dr. Jack Cazes, Post Office Box 2180, Cherry Hill, NJ 08034-0162, USA; Tel: (609) 424-3505; FAX: (609) 751-8724.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(15), 3155-3161 (1995)

LIQUID CHROMATOGRAPHY CALENDAR

1995

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

SEPTEMBER 7 - 8: Engineering & Construction Contracting Conference, Phoenician Resort, Scottsdale, Arizona. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA

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

SEPTEMBER 18 - 21: Safety in Ammonia Plants & Related Facilities, Loews Ventana Canyon, Tucson, Arizona. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

SEPTEMBER 26 - 29: CCPS International Conference on Modelling & Mitigating the Consequences of Accidental Releases of Hazardous Materials, Fairmont Hotel, New Orleans, Louisiana. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

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

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

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

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

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

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

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

NOVEMBER 12 - 17: AIChE Annual Meeting, Fontainbleu Hotel, Miami Beach, Florida. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

NOVEMBER 14 - 16: Kemia'95: Finnish Chemical Congress, Helsinki Fair Center, Helsinki, Finland. Contact: The Associatioon of Finnish Chemical Societies, Hietaniemenkatu 2, FIN-00100 Helsinki, Finland.

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

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

1996

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

FEBRUARY 5 : Quality Auditing for the Industrila Laboratory - A One-Day Seminar, Saddle Brook, New Jersey. Contact: Beth Wrage, Marcel Dekker Professional Conferences, P. O. Box 2003, Murray Hill Station, New York, NY 10156, USA. Tel: 800-203-8782; FAX: 212-685-4540.

FEBRUARY 25 - 29: AIChE Spring National Meeting, Sheraton Hotel, New Orleans, Louisiana. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

MARCH 3 - 8: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

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

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

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

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

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San

Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JULY 14 - 18: 5th World Congress of Chemical Engineering, Marriott Hotel, San Diego, California. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 9 - 14: 31st Intersociety Energy Conversion Engineering Conference (co-sponsored with IEEE), Omni Shoreham Hotel, Washington, DC. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 17 - 20: 31st National Heat Transfer Conference, Westin Galleria, Houston, Texas. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

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

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

SEPTEMBER 9 - 12: Saftey in Ammonia Plants & Related Facilities, Westin at Copley Place, Boston, Massachusetts. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

OCTOBER 16 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

OCTOBER 24 - 26: 52nd Southwestern Regional Meeting, ACS, Houston, Texas. Contact: J. W. Hightower, Chem Eng Dept, Rice Univ, Houston, TX 77251, USA.

NOVEMBER 6 - 8: 31st Midwestern Regional Meeting, ACS, Sioux Falls, South Dakota. Contact: J. Rice, Chem Dept, S. Dakota State Univ, Shepard Hall Box 2202, Brookings, SD 57007-2202, USA.

NOVEMBER 9 - 12: 48th Southeast Regional ACS Meeting, Greenville,

South Carolina. Contact: H. C. Ramsey, BASF Corp., P. O. Drawer 3025, Anderson, SC 29624-3025, USA.

NOVEMBER 10 - 15: AIChE Annual Meeting, Palmer House, Chicago, Illinois. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

1997

APRIL 6 - 11: 213th ACS National Meeting, San Antonio, Texas. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

APRIL 14 - 19: Genes and Gene Families in Medical, Agricultural and Biological Research: 9th International Congress on Isozymes, sponsored by the Southwest Foundation for Biomedical Research, Hilton Palacio del Rio, San Antonio, Texas. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

SEPTEMBER 7 - 12: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1998

MARCH 29 - APRIL 3: 215th ACS National Meeting, St. Louis, Missouri. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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

1999

MARCH 21 - 26: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 27: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2000

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

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

2001

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2002

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