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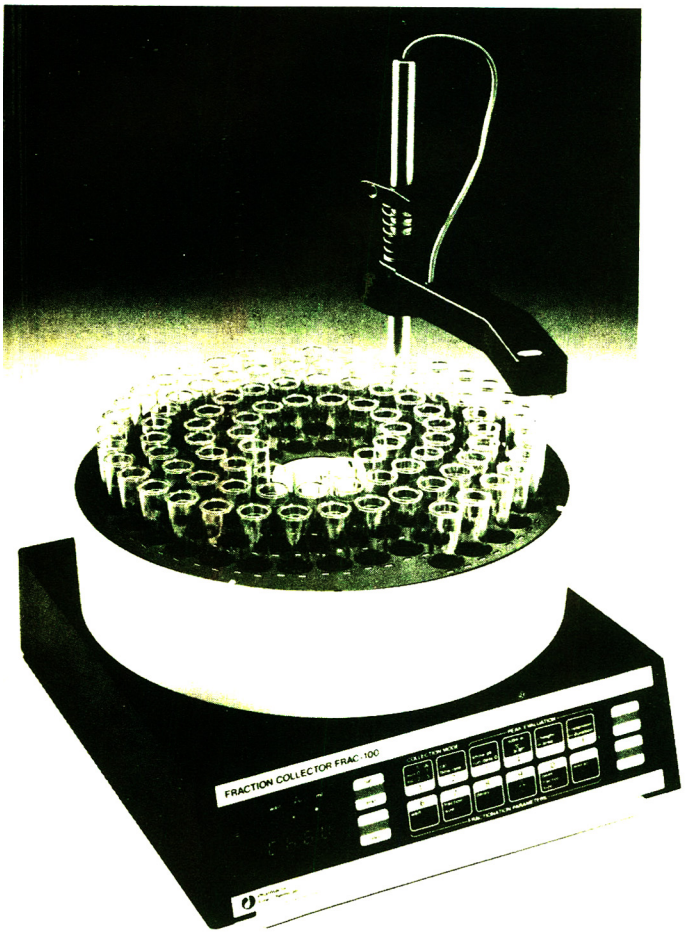

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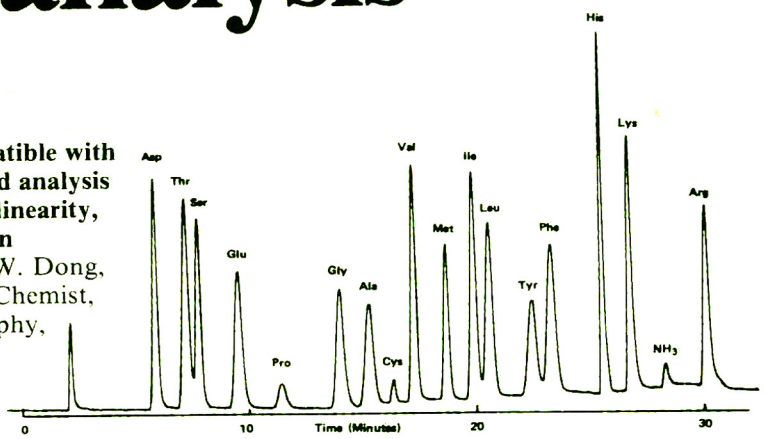

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(Due to limitations of space, only the plenary and keynote lectures from the first conference and the topics of the second conference are listed below.)

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SPECIAL ISSUE

INTERNATIONAL SYMPOSIUM ON HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE BIOLOGICAL SCIENCES

Melbourne (Australia), February 20-22, 1984

Guest Editor

MILTON T. W. HEARN

(Melbourne)

VOL. 336, NO. 1 JOURNAL OF CHROMATOGRAPHY DECEMBER 7, 1984

(Biomedical Applications, Vol. 37, No. 1)

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INTERNATIONAL SYMPOSIUM ON HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE BIOLOGICAL SCIENCES, MELBOURNE (AUSTRALIA), FEBRUARY 20—22, 1984

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FOREWORD

The International Symposium on High-Performance Liquid Chromatography in the Biological Sciences was held in Melbourne, Australia, from February 20th to 22nd, 1984. The Symposium was held under the auspices of The Australian Academy of Science, The Australian Biochemical Society, The Endocrine Society of Australia, The Royal Australian Chemical Institute, and The Australian Society for Medical Research. It is a pleasure to acknowledge the support of these professional organisations. I wish also to express my gratitude to the many participating scientists who ensured the success of this Symposium through the high quality of their scientific contributions. Many of the papers presented at the Symposium in lecture and poster sessions are included in this special issue of the *Journal of Chromatography*. The co-operation of Dr. Karel Macek, Editor of the *Journal of Chromatography, Biomedical Applications*, for his help in publishing these proceedings was much appreciated. The excellent support of the Symposium from various industrial and commercial organisations active in liquid chromatography on the international scene deserves special mention.

It is noteworthy that this Symposium represented the first occasion that an international meeting, dedicated to the theory and practice of modern liquid chromatography as it applies to the biological sciences, was held in the Southern Hemisphere and on the Australian continent. The strong interest in the Symposium shown by the many Australian investigators reflects their increasing awareness of the thetic view expressed many years ago by M.S. Tswett that "every scientific advance is an advance in methods". In this context, the Symposium proved a highly effective forum for all the participants to discuss state-of-the-art developments, to review current problems and to propose alternative approaches. No doubt this Symposium will stimulate further advances in liquid chromatography both in Australia and elsewhere.

MILTON T.W. HEARN
Symposium Chairman

Journal of Chromatography, 336 (1984) 3—14
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REVIEW

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF DNA

ROBERT D. WELLS

Department of Biochemistry, University of Alabama in Birmingham, Schools of Medicine and Dentistry, University Station, Birmingham, AL 35294 (U.S.A.)

CONTENTS

1. INTRODUCTION

The science community has witnessed a veritable explosion in our knowledge of many facets of DNA in the past eight years. Enormous advances have been made in our understanding of DNA structure and DNA—protein interactions, organization and expression as well as developmental regulation and gene transfer. These developments may be attributed to the establishment of innovative techniques in four areas: gene cloning, restriction enzymology, DNA sequencing, and high-performance liquid chromatography (HPLC) of restriction fragments. Each of the first three techniques will be reviewed briefly followed by a discussion of HPLC of DNA. The first three techniques are of such importance that Nobel Prizes have been awarded to the discoverers in recent years.

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1.1. Gene cloning

The development of gene cloning has been of paramount importance for our capacity to focus scientific attention of certain DNA segments for biochemical and genetic studies. The impact of gene cloning on present and future studies in the molecular biology of gene expression may be likened to the impact of chromatography on chemistry, biology, and physics at approximately the turn of the century. Simply stated, gene cloning is the process of inserting any given DNA segment (for example, from plant, viral, bacterial, or mammalian source) into a suitable vector which can be readily replicated and manipulated by genetic and biochemical techniques [1—5]. In general, investigators have used procaryotic cells as hosts although animal cells are used in some cases. Owing to the extreme power of cloning, it is possible to identify in pure form one given DNA fragment out of a population of many thousands of contaminating fragments. One requirement for successful gene cloning is a method of identification of the desired fragment. In general, this is accomplished by hybridization analyses, immunological identification of gene products, biochemical or genetic functions, etc.

It has been stated that virtually any DNA segment can be cloned today as long as the investigator has a procedure for identifying it. Virtually every leading biological journal today is comprised of a number of papers which embody studies on cloned molecules. Excellent reviews exist on the procedures involved [1—5] as well as on specific systems which have been cloned. A very brief listing of some of the thousands of gene systems which have been cloned are the following: DNA segments which undergo conformation transitions, bacteriophage promoters, animal tumor virus (SV40 and polyoma) promoters, ribosomal RNA genes, heat shock genes from monkey cells, promoters and hormone regulatory sequences in mouse mammary tumor viruses, immunoglobin genes, genes involved in recombination, nitrogen fixation genes from *Klebsiella*, cytochrome genes, growth hormone genes, human interferon genes, regulatory genes from *Dictyostelium*, genes for human lymphokines, histone genes, animal cell metallothionein genes, and human and murine antigen genes, just to name a few. Excellent reviews on these subjects exist [1, 2].

1.2. Restriction endonucleases

The second major development which has facilitated great strides in our recent understanding of DNA is the isolation and characterization of type II restriction endonuclease [6—9]. More than 210 restriction endonucleases have been purified and their recognition sites identified. These enzymes provide precise scalpels for dissecting DNA at specific base pair sequences. These tools are important for mapping sequences relative to each other and thereby aligning biochemical and genetic functions. Furthermore, these enzymes have been invaluable for DNA sequencing and gene cloning studies. Restriction enzymes have made possible experiments that were not even dreamed about just a few years ago.

Restriction endonucleases generally cleave base pair sequences that contain two-fold axes of symmetry. The types of ends which are produced are blunt ends (no nucleotide overhangs), two, three, or four nucleotide sticky ends. DNA fragments can be readily separated on the basis of length by gel electro-

phoresis. Even small differences (1—3%) in length between related DNA molecules can be readily detected by this procedure. The electrophoretic mobility of a DNA fragment is inversely proportional to the logarithm of the number of base pairs up to a certain limit. Whereas these gels have extremely high resolving power and sensitivity (a few micrograms), it is difficult to purify quantities of DNA fragments by this procedure (see HPLC description below).

1.3. Sequence determinations

DNA sequencing studies are routinely performed in a large number of laboratories by basically two procedures: the Maxam—Gilbert chemical cleavage method or the Sanger 2′,3′-dideoxy DNA polymerase method [6]. By either of these procedures, it is possible to routinely sequence approx. 200 base pairs per day per investigator. The largest intact genomes which have been sequenced to date include human mitochondrial DNA [10], bacteriophage lambda DNA [11], and adenovirus DNA [12]. The total number of base pairs known at the present time from all systems is over $2 \cdot 10^6$ and the number continues to rise at a rate of about $1 \cdot 10^6$ base pairs per year. The capacity of the DNA biochemists to readily establish the base pair sequence of

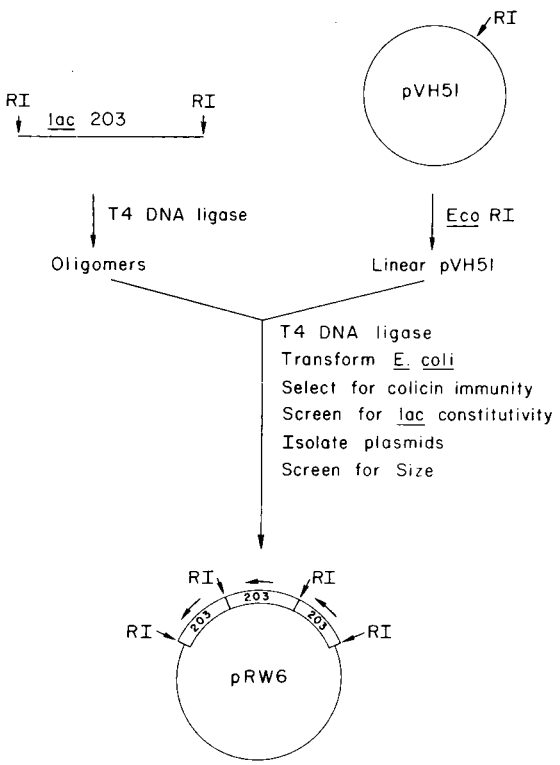

Fig. 1. Cloning of multiple inserts into vector. Multiple copies of the 203-bp fragment containing the *Escherichia coli* lactose operator and promoter were ligated to form oligomers. These oligomers were then ligated into the *Eco* RI site of pVH51. After suitable transformation and screening, it was possible to characterize a recombinant plasmid containing three copies of the 203-bp insert. All fragments are oriented in the same direction. For further details, see ref. 18.

a genome under study has transformed molecular genetics from the less precise field of genetics to the area of well characterized biochemical molecules. Today, a large number of DNAs are as precisely defined chemically as small peptides or steroids.

1.4. HPLC: interrelationship with other techniques

The fourth important technique is HPLC of DNA. This technique was originally developed in order to purify quantities of restriction fragments in order to perform spectroscopic and biochemical studies to evaluate the role of DNA structure in gene regulation [13]. Purifications were originally performed on RPC-5 [14—16], but this support has now been effectively replaced with NACS (Nucleic Acid Chromatography System, Bethesda Research Labs. trade name). These procedures have been invaluable for obtaining milligram quantities of restriction fragments for studying conformational features such as right-handed to left-handed structural transitions [17]. The most effective overall schemes for fragment purification and characterization involve the combined use of all four techniques (gene cloning, restriction enzymology, DNA sequencing, HPLC). Fig. 1 shows a general cloning scheme for a 203-bp (base pair) fragment containing a promoter and an operator for the *Escherichia coli* lactose gene. It is possible to clone multiple copies of this fragment, thus providing a higher yield of insert fragment for a given amount of plasmid. Fermentation technology is used [18, 19] to prepare pound quantities of cells containing the recombinant DNA. Procedures have been devised [18, 19] for isolating gram quantities of pure plasmid DNA from these cells. Thus, it is

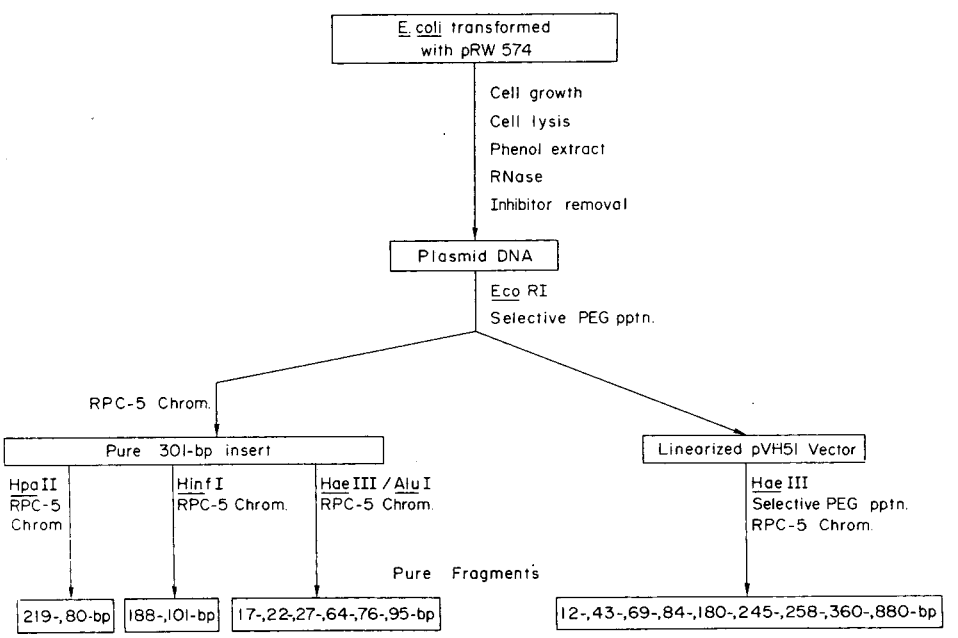

Fig. 2. Overview of purification schemes of restriction fragments. In addition to isolation of the pure 301-bp insert from pRW574 as shown, the 95-bp insert was purified from pRW554. Reprinted with permission from ref. 18.

possible to isolate milligram quantities of the fragment from the vector by HPLC [14—43].

An alternative general procedure is shown in Fig. 2; in this case, the insert, which is 301 bp in length was separated from the pVH51 vector by selective precipitation. Individual restriction fragments were resolved after suitable digestions of the 301-bp insert. When the linearized pVH51 vector was cleaved with a restriction enzyme, partial fractionation of the fragments into sub-classes could be accomplished by selective precipitation. Further fractionation then was accomplished on HPLC columns to obtain the pure individual fragments [18].

HPLC on RPC-5 or NACS is most useful in conjunction with gene cloning methods since recombinants can be devised which optimize the resolution of the column. However, cloning alone will not provide a single pure restriction fragment; another technique (HPLC) must be used. Thus, neither procedure alone is adequate.

2. HPLC OF DNA

HPLC of DNA has been reviewed previously [14, 16, 21, 44]. Thus, only an overview of the types of separations will be discussed. Previous reviews [14, 16, 21, 44] covered the literature on fractionations of oligonucleotides, tRNAs, and polynucleotides. This review will focus on duplex restriction fragments.

HPLC is the only efficient method available for isolating milligram quantities of DNA restriction fragments in homogeneous form. The technique is rapid, reliable, employs modest equipment, and can be used for a variety of applications (e.g. fractionation of fragments, separation of digested genomic DNA, isolation of plasmids from cell lysates, fractionation of short single-stranded oligonucleotides, fractionation of supercoiled DNA, transfer RNA, ribosomal RNA, separation of complementary strands of DNA restriction fragments, and other applications).

A number of biological studies have been performed on microgram amounts of fragments. For this purpose, it is acceptable to elute the fragments from a polyacrylamide gel. However, it is advantageous or necessary for other studies to have milligram quantities of a pure fragment. Some of these studies are: protein binding, ultraviolet (UV), Raman, and NMR spectroscopy, hydrodynamic studies, etc. The technique is optimum for fragments shorter than approx. 1000 bp but satisfactory resolution is achieved for longer fragments [14—44] if the size differential is sufficiently great.

The impressive feature of HPLC on RPC-5 or NACS is the scale of feasible purifications. Single-column fractionations have been routinely performed to give 10,000 times as much pure fragments as obtained by gel electrophoresis. Also, the order of elution of fragments is not always the same found by gel electrophoresis. Thus, RPC-5 column chromatography can completely resolve duplex restriction fragments of the same size in some cases [14, 16, 20, 21].

2.1. Fractionation of insert from vector

HPLC has been successfully used for the separation of up to 120 mg total of

UV-absorbing material when the goal is to separate a relatively small insert from a vector which is 10—20 times its size. Obviously, the resolving power of RPC-5 or NACS for large-scale preparations is significantly enhanced if it is possible to remove the major part of contaminating DNA by a fractional precipitation prior to the fine separation on HPLC.

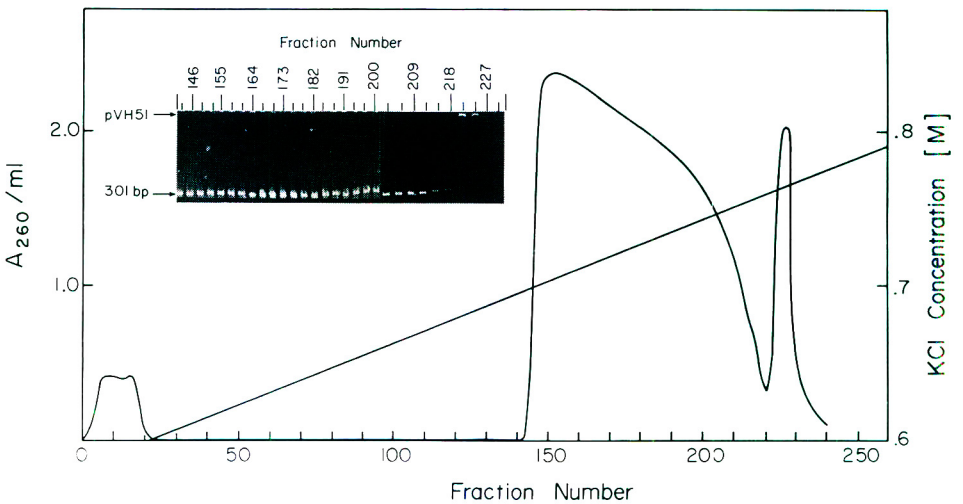

Fig. 3. Elution profile of the RPC-5 column fractionation of the 301-bp insert from the residual vector DNA. A 85 × 2.5 cm RPC-5 column thermostated at 45°C was loaded with 120 mg of UV-absorbing material and developed with a linear 3-l gradient from 0.5 to 0.8 *M* potassium chloride containing 10 m*M* Tris—HCl (pH 8.0) and 0.1 m*M* EDTA. Fractions (12 ml) were collected at a flow-rate of 1.7 ml/min. The insert shows the 5% polyacrylamide gel analysis of some of the UV-absorbing fractions. No DNA bands were found in the flow-through peak. The 301-bp fragment containing fractions were collected, evaporated until potassium chloride started to precipitate, then dialyzed against 10 m*M* Tris—HCl (pH 8.0) and 0.1 m*M* EDTA, further evaporated to a DNA concentration of 2 mg/ml, and redialyzed against the same buffer. The fragment was stored in this state at —20°C. The total amount of the 301-bp fragment purified in this step was 87.5 mg. Reprinted with permission from ref. 18.

Fig. 3 shows the separation of an *Eco* RI digest of pRW574 containing a tetrameric insertion of a 301-bp fragment originating from the *E. coli* lactose genetic control region [18]. The reaction products are thus a 301-bp fragment and the vector DNA which is approx. 3850 bp long [18]. Prior to chromatography on RPC-5 or NACS, the main amount of the vector DNA was removed by a fractionated precipitation using polyethylene glycol (PEG). The separation of the 301-bp fragment from the remaining vector DNA was complete and the yield of pure 301-bp DNA was 87.5 mg in this particular run. Similar separations with smaller amounts of DNA were also performed omitting the fractionated PEG precipitation.

2.2. Fragment purification

Fig. 4 shows a typical fractionation on an analytical system [20]. The sample was a *Hae* III digest of a miniCol E1 derivative, pRZ2, which gave 17 fragments ranging in size from 43 to 850 bp, including three 425-bp fragments.

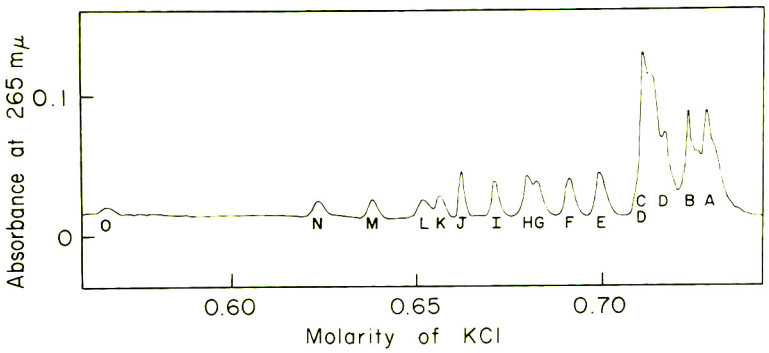

Fig. 4. Separation of DNA fragments generated by a *Hae* III digest of pRZ2 DNA on RPC-5. Fractionation of the digest was performed at 43°C and pH 6.8 using a 40 × 0.15 cm column. The column was eluted with a 40-ml gradient from 0.55 to 0.8 *M* potassium chloride containing 10 m*M* Tris—HCl, pH 6.8, and 0.1 m*M* EDTA. The flow-rate was around 0.22 ml/min. UV-absorbing fractions were analyzed on 5% polyacrylamide gels. The 789-bp fragment was loaded on every tube for calibration. The fragments are designated by letters, their sizes are the following: A 850; B 575; C 465; D 425 (three fragments); E 255; F 203; G 180; H 169; I 135; J 117; K 102; L 98; M 85; N 69; O 43; This figure shows the elution profile. Reprinted with permission from ref. 20.

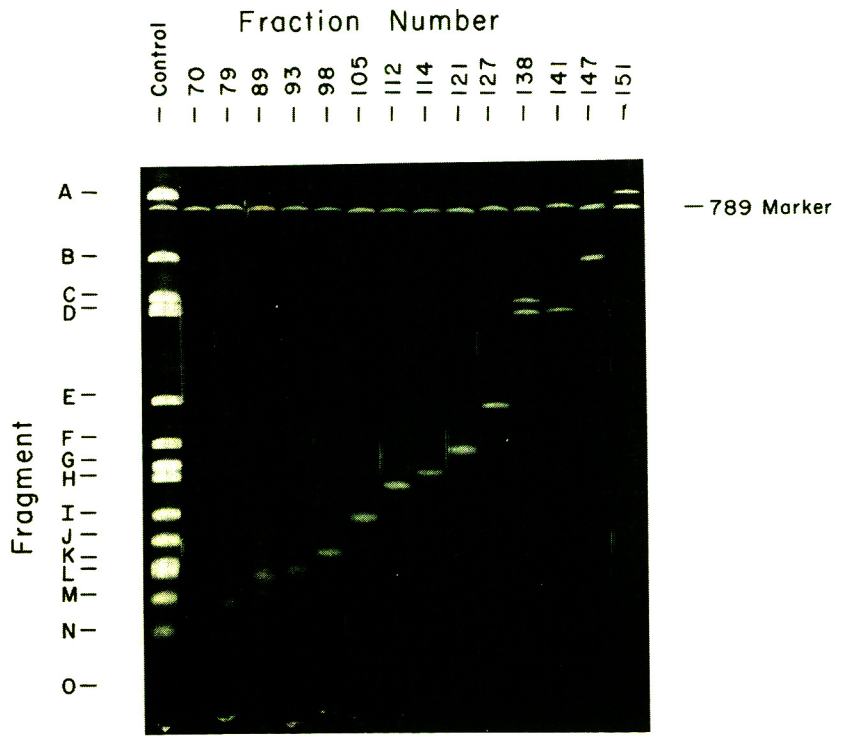

Fig. 5. Polyacrylamide gel analyses of elution pattern shown in Fig. 4. The contents of the fractions were determined by gel electrophoresis. Fragment sizes are listed in the legend to Fig. 4. Reprinted with permission from ref. 20.

Fig. 4 shows that virtually all 17 fragments can be separated from each other when using a potassium chloride gradient at pH 6.8 at 43°C. Even the 98- and 102-bp fragments, which differ in size by as little as 4%, were separated. Fig. 5 shows the polyacrylamide gel electrophoretic analyses of fractions from the HPLC column. Excellent resolution was observed to provide quite pure restriction fragments.

Fig. 6 demonstrates the excellent resolving power of HPLC for small double-stranded DNA fragments. The 301-bp fragment was digested with *Alu* I and *Hae* III to yield a stoichiometric mixture of 17-, 22-, 27-, 64-, 76-, and 95-bp fragments which were separated completely in one step on RPC-5 (or NACS). Although the load (approx. 0.6 mg DNA per ml RPC-5) for this separation is in the range of the upper capacity limit, the separation obtained under these conditions was nearly complete. All six fragments were separated with only very little overlap between the 64- and 76-bp fragments (peaks D and E).

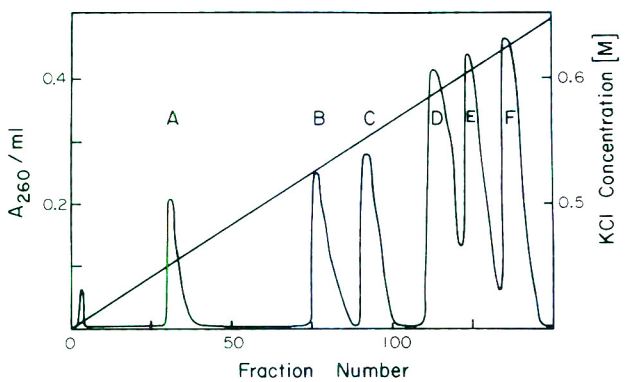

Fig. 6. RPC-5 elution profile of the *Hae* III/*Alu* I double digest of the 301-bp fragment. DNA (12 mg consisting of 17-, 22-, 64-, 76-, and 95-bp fragments was loaded onto a 25 cm × 1 cm RPC-5 column which was thermostated at 45°C. The column was developed with a 2-l gradient from 0.4 to 0.75 *M* potassium chloride containing 10 m*M* Tris—HCl (pH 8.0) and 0.1 m*M* EDTA. Fractions of 9 ml were collected at a flow-rate of 0.65 ml/min; 6% polyacrylamide gel analysis of the UV-absorbing fractions revealed that peaks A—F contained the pure fragments in the order of increasing size. The first small peak (fraction 3) does not contain DNA. Reprinted with permission from ref. 18.

There are many impressive features of HPLC of DNA as a general technique. The equipment is relatively inexpensive, the resins are stable and reusable, purifications are efficient and do not require extended time periods, reproducibility is excellent, recovery is extremely high (generally greater than 95%), and fragments purified by this method appear to contain no deleterious contaminants which inhibit further enzymatic, spectroscopic or biological studies. At least fifteen determinations have been applied to evaluate these properties [14, 16, 21, 44].

A number of factors have been evaluated which affect separations including the following: elution conditions, chain length, length and time of protruding ends, nucleotide composition, and other factors [14, 16, 21].

2.3. Separation of complementary strands

Under some conditions it is possible to separate the complementary strands

of modest size (less than approx. 500 bp) DNA fragments on alkaline HPLC columns. Fig. 7 shows the separation of the complementary strands of a 210-bp fragment by *Hind* II digestion of ϕX174 replicative form DNA. Purification of the duplex 210-bp fragment was described [14]. The strand-separation technique [14] is readily reproducible under the conditions tested (i.e. using 7.5 mg of duplex DNA fragment). In some cases, investigators have experienced difficulty in the fractionation of complementary strands of trace quantities of radioactively labeled DNA restriction fragments; however, others have made substantial progress in resolving these problems [44].

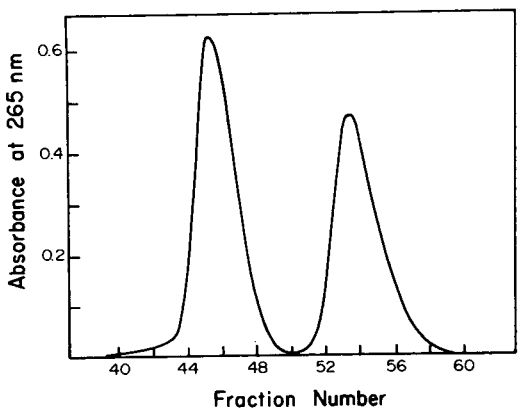

Fig. 7. Separation of the complementary strands of a 210-bp restriction fragment. The 210-bp *Hin* dII fragment from ϕX174 replicative form DNA was isolated by RPC-5 chromatography and dialyzed versus 0.25 *M* potassium chloride, 5 m*M* Tris (pH 7.4), 0.5 m*M* EDTA. The pH of the solution was raised to 12.5 by the addition of 1 *M* sodium hydroxide, and 7.5 μg of the fragment (in 0.2 ml) was loaded onto a 200 mm × 1.5 mm RPC-5 column equilibrated with 0.25 *M* potassium chloride, 12 m*M* sodium hydroxide (pH 12.5). All solutions were degassed immediately before use and kept under a nitrogen atmosphere to maintain the high pH. The column was washed with 2 ml of the equilibration buffer and was eluted with a 20 ml linear gradient of 0.90—0.95 *M* potassium chloride in 12 m*M* sodium hydroxide (pH 12.5) at 0.23 ml/min and 150 p.s.i. Fractions of 0.2 ml were collected. The elution profile was continuously monitored at 265 nm. The separated strands eluted between 0.92 and 0.93 *M* potassium chloride. Appropriate fractions were pooled and dialyzed, and aliquots were analyzed on 5% polyacrylamide gels. Reprinted with permission from ref. 14.

2.4. Recent developments

In the past several years, a number of important developments have been made. First, an alternate source of resin (NACS) was produced which is at least as good as the original RPC-5. Secondly, a fractionation of the resin material according to particle size into four classes (NACS-12, -20, -37, -52) was effected. This fractionation according to size has permitted development of resins which do not require high pressure. NACS-12 provides the highest resolution purification of nucleic acids but requires a high-pressure pump (requiring 1.3—33 bars). NACS-20 and -37 may be employed with a peristaltic pump and NACS-52 may be used in the gravity flow mode. Each of these fractionated resins has slightly different optimum uses. It is possible to screen clones, purify samples (such as after end labeling), concentrate samples, extract nucleic acids

from agarose or polyacrylamide gels, using minicolumns with gravity flow. Other separation may require a peristaltic pump or a high-pressure pump including separation of high-molecular-weight single-stranded DNAs, fractionation of complementary strands, fractionation of genomic DNAs, RNAs, or oligonucleotides.

Thirdly, prepacked columns have been developed which may be used in either a gravity flow mode or may be attached to a Pipetman for a wide variety of routine manipulations in the molecular biology laboratory, such as removal of small unreacted molecules from polynucleotide kinase or reverse transcriptase reactions, extraction of nucleic acids from gel pieces, or rapid screening of supercoiled DNA.

3. PROSPECTS FOR THE FUTURE

It is likely that this technique will be used much more widely in the future for a number of the following applications.

(1) In cases where it is necessary to isolate milligram quantities of defined segments of chromosomes, RPC-5 or NACS column chromatography is the only available high-resolution technique at the present time. Specific applications are the following. (a) The isolation of defined regions of complex chromosomes (such as eucaryotic chromosomal DNAs) in order to partially fractionate the genome. The fractionation may be followed by hybridization with specific probes such as RNA transcripts or viral nucleic acids. (b) The isolation of defined segments of genomes, such as operators, promoters and origins of DNA replication, etc., for biochemical and physical studies. (c) Isolation of regions of certain infectious agent genomes which cannot be cloned under the present NIH guidelines.

(2) The separation of some fragments from other fragments of similar chain length but with different base compositions. It is not possible to accomplish these separations, even on small scale, by polyacrylamide gel electrophoresis.

(3) The separation of some fragments which have approximately the same size (and therefore cannot be separated on gels) but one of which contains an *Eco* RI or *Hind* III end and the other has ends which are or behave like blunt ends.

(4) Separation of the complementary strands of DNA restriction fragments.

(5) The high-resolution fractionation of single-stranded oligonucleotides. To the best of our knowledge, this is the only high-resolution technique for separating quantities of oligonucleotides. Separations of up to approx. the 60 mer [44] have been observed in some cases (diethylaminoethyl cellulose chromatography loses resolution at approx. the 10—15 mer). These fractionations are very important for synthetic studies on oligonucleotides as well as for the isolation of single-stranded oligomers such as intermediates in DNA replication (i.e. RNA primers or short Okazaki fragments). For example, note the spectacular separations of Van Roode and Orgel [45] of oligomers which contain 2′—5′ internucleotide bonds from molecules with 3′—5′ bonds.

(6) As a probe for AT-rich fragments or for AT-rich regions within GC-rich fragments. In addition, present studies indicate that fragments which bind more tightly to the column contain known regulatory regions [14, 23, 25]. If further

work bears out this correlation, these properties could be used as an indication of biological function.

Potential future uses of RPC-5 or NACS are the following.

(1) The separation of DNAs which have small differences in their content of non-paired nucleotides, such as (a) DNA containing a short nick or gap. (b) Intermediates in DNA replication, such as growing fork regions which may contain several non-paired nucleotides. (c) Transcription complexes consisting of DNA template, which is predominantly in the duplex form, but with a nascent RNA still attached. (d) Intermediates in DNA recombination. (e) The separation of covalently closed circular DNAs containing different numbers of supercoiled turns. (f) Replicative form I from form II DNA. (g) DNAs with frayed ends (i.e. possibly produced purposely by Exo III or the lambda exonuclease) versus identical fragments which are fully double stranded.

(2) The separation of certain protein—nucleic acid complexes (such as nucleosomes or transcription complexes) from naked DNA.

A major commercial development of HPLC of DNA for the future will be in the area of automated analyses for developing routine clinical instruments. It is rather simple to automate the analyses of fragments, DNAs etc. from a column whereas it is rather difficult to automate the analyses from gels. Thus, it is likely that a variety of types of instruments will be developed for analyzing complex viral genomes, etc. using this methodology.

4. ACKNOWLEDGEMENTS

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PROPOSED MODEL FOR PEAK SPLITTING IN REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH COMPUTER PREDICTION OF ELUTED PEAK PROFILES

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SUMMARY

A simple retention model is advanced to explain the observation that a chromatographically pure solute can produce up to three discrete peaks under suitable mobile-phase conditions in reversed-phase, ion-pair high-performance liquid chromatography. This model is based on the proposal that peak splitting results from the interplay of two distinct retention mechanisms such as ion pairing or dynamic ion exchange. Transition between these two mechanisms is induced under certain mobile-phase conditions, for example the addition of suitable quantities of sodium sulphate to the eluent. The suggested model is used for the computer calculation of eluted peak profiles and these were found to closely resemble experimentally observed peak shapes.

INTRODUCTION

The retention mechanism which operates in ion-pair reversed-phase high-performance liquid chromatography (HPLC) is still a contentious issue, despite the fact that this technique has become well established and widely practised. This situation is undoubtedly due to the complexity of the chromatographic system involved, together with the difficulty of obtaining experimental data which unambiguously supports a single suggested mechanism.

In the simplest case, retention in ion-pair chromatography can be considered to result from the formation of neutral ion pairs in the mobile phase [1], with

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subsequent adsorption of these ion pairs onto the hydrophobic stationary phase. Alternatively the charged pairing ion can be envisaged to adsorb onto the stationary phase, resulting in the formation of a dynamic ion-exchange surface [2—4] which then can retain oppositely charged solutes by a conventional ion-exchange process.

The above two mechanisms can be considered to be extreme viewpoints and numerous alternative proposals have been made which embrace some elements of either or both of these mechanisms. These proposals include a combined desolvation and ion-exchange mechanism [5], an ion-interaction model [6] and a dynamic complex exchange model [7], all of which have been shown to be supported by experimental retention data. Knox and Hartwick [8] have pointed out that the mechanisms suggested for ion-pair chromatography involve kinetic processes and it is therefore inconclusive to use retention data (resulting from thermodynamic equilibria) to substantiate these proposed mechanisms. Kinetic effects in chromatography govern peak shape, therefore detailed study of peak shape in ion-pair chromatography may provide some insight into the mechanism operating.

In some earlier work [9, 10] in which we investigated certain selectivity effects arising from the addition of salts (such as sodium sulphate) to the mobile phase used for the separation of sympathomimetic amines by ion-pair chromatography, we observed severe peak distortion and peak splitting effects. These effects were observed for a large number of compounds and occurred for compounds which were proven to be chromatographically pure. We made the suggestion that peak splitting was the result of a composite interplay of two retention mechanisms, such as ion pairing and dynamic ion exchange, which were mutually competitive under certain conditions. This suggestion was based on considerable experimental data obtained by gas chromatography—mass spectrometry.

In this paper, we present a simple model to explain our experimental results, and this model is used for computer prediction of the distorted or split-peak profiles which we had observed.

THEORY

Development of a model

The experimental results obtained previously are summarized diagrammatically in Fig. 1, which shows that peak splitting could be induced by varying the concentration of either the added salt or the pairing ion in the mobile phase. The concentration range of salt or pairing ion over which peak splitting was observed was generally quite small and was strongly dependent on the nature of the solute used. In most cases, it is therefore quite unlikely that peak splitting would occur unless the chromatographer deliberately sought the appropriate conditions.

Analysis of eluted fractions corresponding to the three components of the split-peak profile in Fig. 1 revealed that the solute was present in each peak, but that ion pairs were present at appreciable concentrations only in the earliest eluting peak (A). Thus we have attributed this peak to an ion-pairing process and peak C was considered to result from an ion-exchange process. The

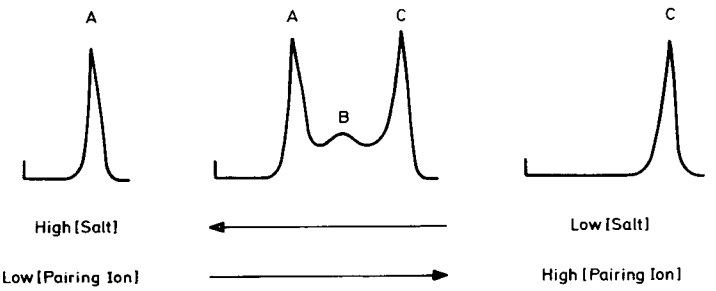


Fig. 1. Summary of experimental observations in ref. 9. Single peaks occur at the indicated conditions and peak splitting occurs at intermediate conditions. Peak A is considered to be the result of an ion-pairing mechanism, peak C is considered to result from dynamic ion exchange and peak B is attributed to an interplay of both mechanisms.

remaining peak (B) was generally quite small and was attributed to a composite interplay of the processes contributing to peaks A and C. Thus, under most conditions, a single retention mechanism operates, leading to formation of a single peak (A or C). Under exceptional conditions, particularly when a salt is added, peak splitting occurs owing to a transition in mechanism.

The simultaneous equilibria involved in the chromatographic retention process for a protonated base (B^+) in the presence of a pairing ion (A^-) can be represented as follows:

$$A_s^- + B^+ \rightleftharpoons (A_s^- B^+)_s \quad \text{(i)}$$

$$A_m^- + B^+ \rightleftharpoons (A_m^- B^+)_m \quad \text{(ii)}$$

$$(A_m^- B^+)_m \rightleftharpoons (A_m^- B^+)_s \quad \text{(iii)}$$

where subscripts m and s represent mobile and stationary phases, respectively.

In the case where a salt (for example sodium sulphate) is also added to the mobile phase, then the above equilibria would be influenced by competition between B^+ and sodium ions and to some extent, between A^- and sulphate ions. The data summarised in Fig. 1 supports the hypothesis that the dynamic ion-exchange process occurs mainly at low salt concentration while at high concentration the salt competes effectively with the protonated amines for the oppositely charged pairing ions which have adsorbed onto the C_{18} stationary phase. Under the latter conditions, ion-pair formation between the protonated amines and the pairing ions in the mobile phase is favoured. Thus, at low salt concentration the dominant species in solution is the protonated base (B^+), whereas at high salt concentration the neutral ion-paired species $(A_m^- B^+)_m$ is mainly involved. Between these two extremes, within the peak splitting range defined by the structure of the compound, interconversion between neutral $(A_m^- B^+)_m$ and charged species (B^+) can occur.

The model shown in Fig. 2 combines the essential requirements of the above hypothesis. The pairing ion A^- is present in both the mobile and stationary phases after the column has been equilibrated. In the absence of salt and at low salt concentrations, injected solute B^+ interacts with adsorbed pairing ion through an ion-exchange mechanism (process i). At high salt concentrations, competition from the salt cation diminishes the above process and the solute B^+ now interacts with pairing ions in the mobile phase. Neutral ion pairs are formed in the mobile phase (process ii) and these are then adsorbed onto the

stationary phase (process iii). The interconversion of the charged solute B^+ and the neutral ion pair (process ii) is assumed to be slower than the other processes.

Computer prediction of peak splitting patterns

In this section, the stochastic probability model given by Gidding's chromatographic theory [11, 12] was used. This theory, in its simplest form and within the context discussed here, gives the probability distribution for the relative times spent in the charged and ion-pair forms of the same amine compound when the reaction scheme is that denoted by process ii given in Fig. 2.

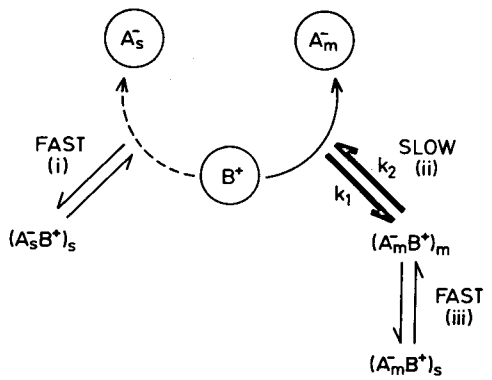


Fig. 2. Proposed model for peak splitting in reversed-phase ion-pair HPLC. B^+ is the protonated solute and A^- is the pairing ion. The subscripts m and s refer to the mobile and stationary phases, respectively. Three processes are identified in this scheme and they are denoted as i, ii and iii. The dashed curved arrow indicates the reaction pathway of B^+ in the dynamic ion-exchange mechanism, whereas the solid curved arrow is the pathway for the ion-pairing mechanism. The pathway followed is governed by the concentrations of salt and pairing ion in the mobile phase. k_1 and k_2 are the forward and reverse rate constants, respectively, for process ii.

The interconversion reaction considered here is

$$A_m^- + B^+ \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (A_m^-B^+)_m$$

where k_1 and k_2 are the rate constants for the forward and reverse reactions. For simplicity, the above reaction may be reduced to

$$B^+ \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (A_m^-B^+)_m$$

This equilibrium represents the conversion of the charged form of B (i.e. B^+) into a neutral form $(A_m^-B^+)_m$ of the same compound. The original fraction of molecules in the protonated form, B^+, can be denoted by α, and the fraction in the neutral form, $(A_m^-B^+)_m$, can be denoted by β.

If x is the fraction of time a molecule spends in one form, then the probability that this fraction is in the range $x + dx$ is given by $P_i(x)$, where $i = 1, 2$, representing either of the two forms.

Starting with one form, each species may undergo one of three possible reaction paths: the molecule starts in one form and after at least one reaction cycle ends up in exactly the same form; the molecule starts in one form and ends up in the second form; or finally, starting in one form, the molecule is unaffected. To designate these possibilities, subscript j is used, and the probability function becomes $P_i^j(x)$, where $i = 1, 2$ and $j = 1, 2, 3$.

The probability functions for the protonated form ($i = 1$) are:

$$P_1^1(x)dx = a \exp[-a(1-x) - bx]\ I\sqrt{4abx(1-x)}\ dx \tag{1}$$

$$P_1^2(x)dx = \left[\frac{ab(1-x)}{x}\right]^{1/2} \exp[-a(1-x) - bx]\ I\sqrt{4abx(1-x)}\ dx \tag{2}$$

$$P_1^3(x)dx = \exp(-a)\ dx \tag{3}$$

and the probability functions for the neutral form ($i = 2$) are

$$P_2^1(x)dx = b \exp[-a(1-x) - bx]\ I\sqrt{4abx(1-x)}\ dx \tag{4}$$

$$P_2^2(x)dx = \left[\frac{abx}{1-x}\right]^{1/2} \exp[-a(1-x) - bx]\ I\sqrt{4abx(1-x)}\ dx \tag{5}$$

$$P_2^3(x)dx = \exp(-b)\ dx \tag{6}$$

where $a = k_1 t$, $b = k_2 t$, and I is the Bessel function, expressed here as

$$I = \frac{\exp 2[abx(1-x)]^{1/2}}{4\pi^{1/2}[abx(1-x)]^{3/4}}$$

The overall concentration profile of an eluted solute is proportional to the final probability density, $P(x)$, which is obtained by properly weighting the above expressions.

$$P(x) = \alpha[P_1^1(x) + P_1^2(x)] + \beta[P_2^1(x) + P_2^2(x)] \tag{7}$$

$$P(x = 0) = \alpha \exp(-a) \tag{8}$$

$$P(x = 1) = \beta \exp(-b) \tag{9}$$

To make the last two discrete functions continuous, which is convenient for graphical purposes, Gaussian distributions were used with $x = 0$ and $x = 1$, respectively, and a finite standard deviation of $\sigma = 0.1$. Therefore, for $x \leqslant 0$

$$P(x = 0) = \alpha e^{-a} \cdot f(x) \tag{10}$$

where

$$f(x) = \int_{-\infty}^{0} \frac{1}{2\pi\sigma^2} \exp -1/2\left[\frac{x-\mu}{\sigma}\right]^2 dx$$

$\sigma = 0.1$ and $\mu = 0$

for $x \geqslant 1$

$$P(x = 1) = \beta e^{-b} \cdot f(x) \tag{11}$$

where $f(x)$ and σ are the same as in eqn. 10 (except the limit of integration is from 0 to $+\infty$) and $\mu = 1$.

A computer program was written for the calculation of the probability density function of the two interconverting species (with different retention times) under a variety of initial conditions.

EXPERIMENTAL

The liquid chromatograph, gas chromatograph and mass spectrometer used to obtain experimental data have been described previously [9], together with the procedures used. Computer simulation of peak splitting patterns was carried out using a Finnigan (Sunnyvale, CA) Model 6100 interactive data system, consisting of an Alpha LSI 100 Series computer (Irvine, CA, U.S.A.) and a Zeta Research (Lafayette, CA, U.S.A.) X—Y plotter. The program for calculation of the probability density function was written in Finnigan BASIC language [13] and copies are available from the authors on request.

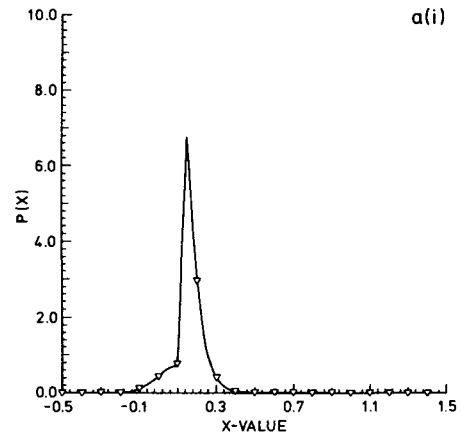
RESULTS AND DISCUSSION

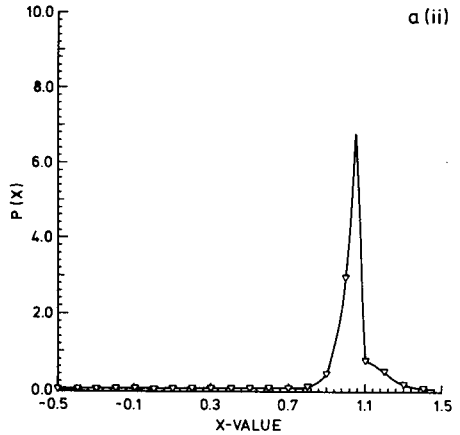
The parameters used to define the initial conditions in the solution of eqn. 7 above were a, b, α and β. Variation in a and b results from changes either in the rate constants (k_1 or k_2) or in the elapsed time, t. When t is constant, a and b are essentially the rate constants. With the rate constants fixed, an increase or decrease in t represents the calculation of a given chromatogram at respectively large or small time intervals. The rate constants (and hence a and b) are dependent on the nature of the solute. The parameters α and β define the fraction of solute molecules present in the charged (B^+) or neutral [$(A_m^-B^+)_m$] forms, respectively. Changes in α and β are considered to result directly from changes in the concentration of salt or pairing ion (A^-) added to the mobile

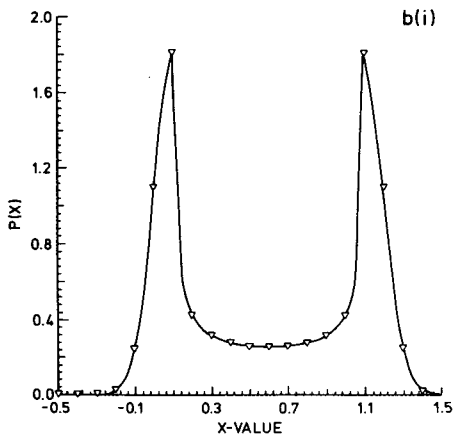
TABLE I

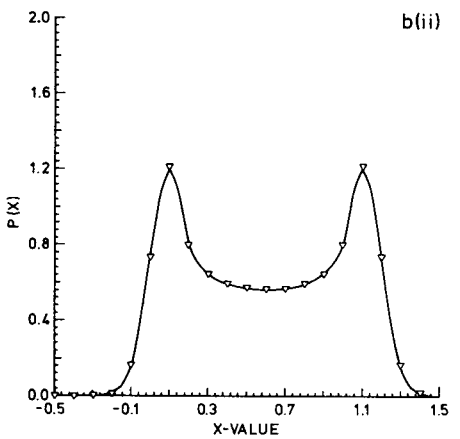
VALUES OF α, β, a AND b USED FOR CALCULATION OF THE PROBABILITY DENSITY FUNCTIONS (eqn. 7) DISPLAYED IN Fig. 3

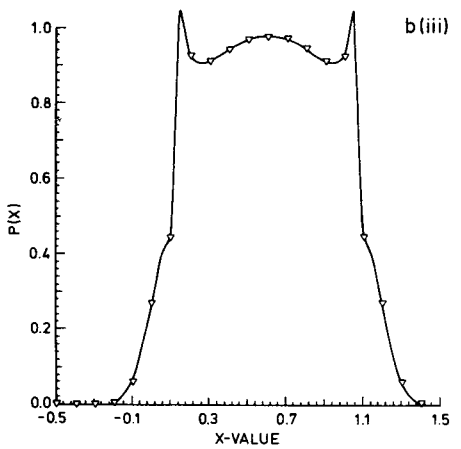
α	β	a	b	Fig. 3
0.5	0.5	1	30	a (i)
0.5	0.5	30	1	a (ii)
0.5	0.5	0.1	0.1	b (i)
0.5	0.5	0.5	0.5	b (ii)
0.5	0.5	1.5	1.5	b (iii)
0.5	0.5	4.0	4.0	b (iv)
0.35	0.65	0.5	0.05	c (i)
0.75	0.25	0.5	0.05	c (ii)
0.10	0.90	0.5	0.05	d (i)
0.10	0.90	0.005	0.005	d (ii)
0.35	0.65	1.5	1.5	e (i)
0.95	0.05	1.5	1.5	e (ii)
0.50	0.50	1	0.001	f (i)
0.50	0.50	5	0.005	f (ii)











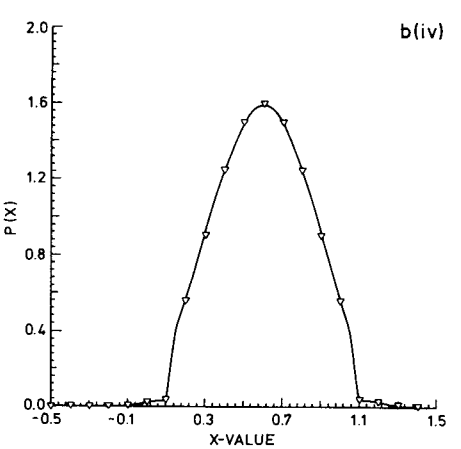


Fig. 3.

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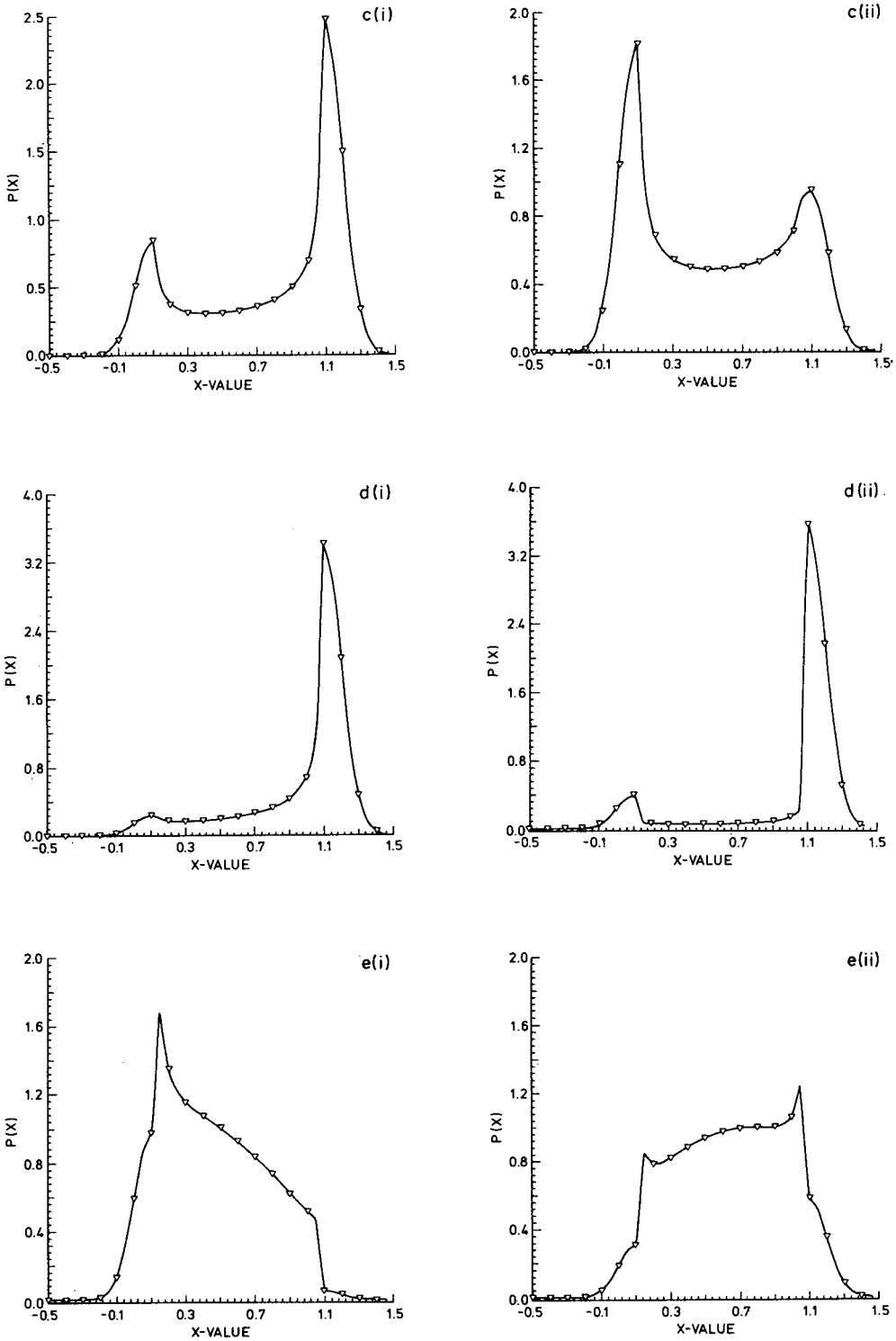

Fig. 3.

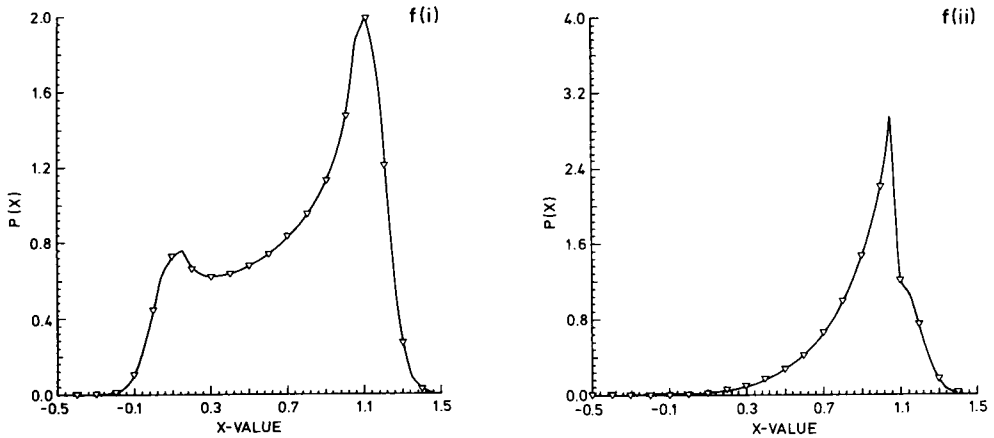

Fig. 3. Calculated probability density functions (eqn. 7) for the initial conditions indicated in Table I.

phase. Thus, selection of appropriate values of the parameters a, b, α and β can be used to simulate changes in mobile-phase composition or changes in forward and reverse rate constants for the interconversion reaction between the charged and neutral forms of the solute.

Table I lists the values used for a, b, α and β, whilst the corresponding probability density functions are shown in Fig. 3. These results indicate that the peak profile depends both on the initial proportions of the two species and on the magnitudes of a and b in the probability function.

When the initial proportions of the two species are equal, and a and b are equal or greater than 1, then only one peak results if a is grossly greater than b or vice versa. This is shown by Fig. 3a(i) and 3a(ii), where the two peaks are attributed to the charged and neutral species, respectively. These two peaks have been assigned slightly different retention times in accordance with experimentally observed behaviour [9]. In the symmetrical cases, where $\alpha = \beta$, and $a = b$, the principal consequence of increasing a and b is the reduction of peaks at either extreme and the filling in of the region between them [Fig. 3b(i)—3b(iv)]. When α and β are unequal and a and b are less than 1, the dominant peak of the resultant doublet depends on the initial proportions of the species B^+ and $A_m^-B^+$. This is shown by Fig. 3c(i) and 3c(ii) as well as 3d(i) and 3d(ii). In the latter cases the minor peak can easily be mistaken for an impurity peak. When a and b increase to more than 1, severe distortion of peaks is noted, as shown in Fig. 3e(i) and 3e(ii). Equal ratios of a:b do not guarantee the same peak splitting pattern; this is shown by Fig. 3f(i) and 3f(ii). Here, $\alpha = \beta$ and a:b ratios are equal, but the patterns obtained are dramatically different. Clearly, the absolute magnitude of a and b play a major part in determining the eventual splitting pattern.

The peak splitting patterns shown in Fig. 3 closely resemble those obtained experimentally [9], and identical matching can be achieved by careful adjustment of the input values of a, b, α and β. Whilst it is clear that further study is required for rigorous interpretation of the physical roles of the above parameters, the results obtained here strongly support the contention that the

observed peak splitting patterns are the result of an interconversion process. In the case studied, this interconversion is essentially regulated by mobile-phase concentrations of salt and pairing ion, and also by the nature of the solute used.

CONCLUSIONS

A simple model wherein changes in mobile-phase composition or solute type can produce peak splitting has been suggested in this paper. The model attributes peak splitting to an interconversion process between changed solute ions and neutral ion pairs in the mobile phase. When this model was used for the prediction of eluted peak profiles under a variety of mobile-phase conditions, the predicted profiles agreed closely with experimental results.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

LXIII*. REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CHARACTERISATION OF SEVERAL POLYPEPTIDE AND PROTEIN HORMONES

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SUMMARY

The chromatographic behaviour on alkylsilicas of a variety of hormonal proteins is described. Optimization of resolution and recovery of these protein hormones, which included porcine relaxins, human chorionic gonadotropin, human placental lactogen, pituitary derived growth hormone and adenohypophyseal glycoprotein hormones, was achieved by manipulation of both mobile and stationary phase parameters. With standard stainless-steel analytical columns (10—30 cm × 0.4 cm) packed with meso- or macro-porous *n*-alkylsilica supports these proteins can be readily fractionated at the semi-preparative level with separation times generally under 90 min using elution systems directly compatible with subsequent methods of primary structure determination or biological functional analysis. The effects of changes in several experimental parameters on peak symmetry, retention and recovery are described.

INTRODUCTION

At the present time reversed-phase high-performance liquid chromatography (RP-HPLC) is the most widely used liquid chromatographic technique for the separation at the micro- and semi-preparative level of peptides and small globular proteins [1]. Selectivity and retention of peptides and proteins on microparticulate chemically bonded alkylsilicas can be manipulated by a variety of mobile phase parameters including the chemical nature and con-

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centration of added pairing ion or buffer species, the pH, and the nature and concentration of the organic solvent modifier [1—9]. Under neat aqueous conditions peptides, polypeptides and proteins in general show considerable retention to alkylsilicas. Because of complex multi-site binding phenomena associated with the solute—stationary phase interaction, isocratic elution conditions with aquo—organic solvent combinations rarely permit adequate resolution and recovery of peptide or protein mixtures. However, gradients in organic solvent modifiers, pH and buffer ions allow rapid separation on bonded *n*-alkylsilicas of peptides and proteins encompassing a wide range of differences in hydrophobicities, molecular weight and subunit structure. In associated studies [4—9], we have reported rigorous procedures for the optimization of peptide resolution on porous microparticulate alkylsilicas. In this investigation these procedures have been applied to the RP-HPLC fractionation of a variety of hormonal peptides and proteins with the recovered fractions obtained in a form suitable for structural and biological characterization.

EXPERIMENTAL

Chemicals and reagents

Water was distilled and deionized using a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Acetonitrile was HPLC grade obtained from Waters Assoc. (Milford, MA, U.S.A.) or Burdick and Jackson Labs., (Muskegon, MI, U.S.A.). Orthophosphoric acid, acetic acid, trifluoroacetic acid, ammonium bicarbonate and ammonium acetate were AnalaR grade from BDH (Poole, U.K.) or May and Baker (Dagenham, U.K.). Triethylamine, trifluoroethanol and *tert.*-butanol were from Sigma (St. Louis, MO, U.S.A.). Partially purified porcine relaxins, which had been fractionated by the Sherwood and O'Byrne method [10], were a generous gift from Dr. R. Bradshaw, University of California at Davis, CA, U.S.A. Human chorionic gonadotropin (hCG), human placental lactogen (hPL), human growth hormone (hGH), human prolactin (hPrL), human follicle stimulating hormone (hFSH), human thyroid stimulating hormone (hTSH) and human luteinizing hormone (hLH) and their radio-iodinated analogues were prepared in this laboratory by established procedures. The human growth hormone 20K variant was isolated according to the method of Chapman et al. [11].

Apparatus

All chromatographic data were obtained on a Waters gradient elution system which consisted of two Model M6000A solvent delivery pumps, a M660 solvent programmer, a U6K universal sample injector, a Model M450 variable-wavelength UV monitor and a M720 data module. Sample injections were made with SGE syringes (Melbourne, Australia). The pH measurements were performed with a Radiometer PHM-64 meter equipped with a combination glass electrode. The μBondapak C_{18} and alkylphenyl columns were purchased from Waters Assoc. The in-house columns were made by bonding trimethylchlorosilane, *n*-butyldimethylchlorosilane and *n*-octyldimethylchlorosilane onto LiChrospher silica (Merck, Darmstadt, F.R.G.) of two nominal pore diameters,

10 nm and 50 nm and of nominal particle size 10 μm. The ligand density of the *n*-butyl- and *n*-octylsilica phases were 3.3, 3.8, 3.4 and 3.6 μmol/m^2, respectively. Radioactivity was determined on a Packard gamma spectrometer.

Methods

Bulk solvents and appropriate mobile phases were prepared and degassed as reported previously [5—8]. Columns were equilibrated for at least 30 min at 2 ml/min between gradient elution experiments and for at least 60 min for the isocratic elution experiments as well as for all systems containing *tert.*-butanol, trifluoroethanol and triethylamine. All chromatograms were prepared at 18°C. Amino acid analysis was performed with a Durrum D500 amino acid analyser, using standard hydrolysis conditions [12].

RESULTS AND DISCUSSION

RP-HPLC of porcine relaxins

Although relaxin was first described nearly sixty years ago by Hisaw and co-workers [13, 14], its role in human physiology remains to be completely elucidated. During gestation, relaxin levels increase in the ovaries and blood and produce relaxation of the symphysis, inhibition of uterine contractility, and softening of the cervix. Relaxin was first isolated from sow corpora lutea and later from other species [15—17] by a combination of conventional gel permeation and ion-exchange chromatographic procedures. Several reports have demonstrated [18—20] that the relaxin activity obtained by these conventional chromatographic procedures is shared by several low molecular weight basic proteins. The porcine relaxin preparations used in the present study were isolated by CM-cellulose chromatography based on the procedure of Sherwood and O'Byrne [10]. These preparations were believed to be micro-heterogeneous on the basis of several criteria including charge electrophoresis, isoelectric focusing and carboxypeptidase cleavage. The microheterogeneity was presumed to arise as a consequence of post-translational processing which leads to partial deletion of C-terminal argininyl peptides from the relaxin B-chain. The resolution on alkylsilicas under isocratic or gradient aquo—organic solvent conditions of such closely related polypeptides, differing mainly in arginine content, is known [1, 2] to be very responsive to pH and ionic additive effects. Selectivity advantages can be taken from these effects by employing ternary gradient elution systems derived, for example, from combinations of ammonium acetate, trifluoroacetic acid and an organic solvent modifier. A further advantage of such volatile systems is the relative ease by which the separated polypeptides can be recovered from the chromatographic fractions by lyophilisation. Based on preliminary experiments designed to examine band width, relative retention and recovery, a linear 0—50% acetonitrile gradient from 100 m*M* ammonium acetate, pH 7.0, to 15 m*M* trifluoroacetic acid, pH 2.0, was selected as a suitable elution system for the porcine relaxin preparations. The gradient slope and flow-rate were then chosen on the basis of relative resolution, i.e. from the peak capacity dependence on gradient *b*-values [7—9]. Fig. 1 shows the chromatographic profiles of three different relaxin preparations, obtained from the CM-cellulose ion-exchange stage,

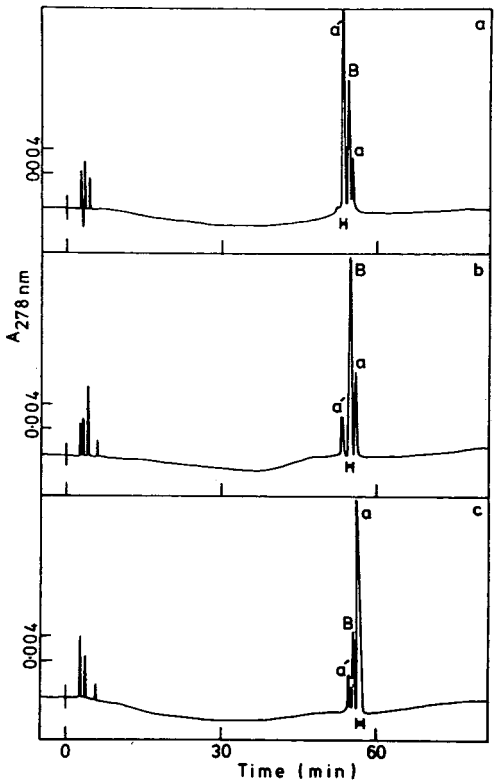

Fig. 1. Separation of porcine relaxin species by RP-HPLC. Chromatographic conditions: column, μBondapak alkylphenyl (30 × 0.4 cm, d_p 10 μm); flow-rate, 1.2 ml/min; linear 90-min gradient from 0 to 100% B, where solvent A was 100 m*M* ammonium acetate, pH 7.0, and solvent B was 50% acetonitrile—50% water—15 m*M* trifluoroacetic acid, pH 2.0; detection 278 nm at 0.04 a.u.f.s. Sample loadings: (a) CM-cellulose fractionated porcine relaxin a', 110 μg in 100 μl; (b) CM-cellulose fractionated porcine relaxin B, 125 μg in 100 μl; (c) CM-cellulose fractionated procine relaxin a, 115 μg in 100 μl. The bars under the peaks indicate fractions collected for amino acid analysis, and their compositions are given in Table I.

separated on a μBondapak alkylphenyl support under the above gradient elution conditions. Fractions corresponding to each profile were collected and subjected to amino acid analysis (Table I). From comparisons of the elution profiles and the composition analysis it was apparent that each of the CM-cellulose preparations contained three porcine relaxin species. When the results of the composition analyses of these three porcine relaxin species were compared with other published data it was evident that the material called relaxin "B" in this study corresponded to the porcine relaxin species sequenced by Schwabe et al. [21—23], the species labelled "B" by Frieden et al. [24], and the species designated "CM-B" by Sherwood and O'Byrne [10]. A feature of all of these porcine relaxin species, besides the other amino acids they share in common, is the presence of five arginine residues. The composition of the species designated "relaxin a" in Fig. 1 and Table I corresponded to the porcine relaxin preparation sequenced by James et

TABLE I

AMINO ACID COMPOSITIONS OF PORCINE RELAXIN SPECIES

Amino acid compositions of relaxin peptides from the chromatographic profiles shown in Fig. 1. Compositions are given for the main peaks only. The minor peaks were contaminants of either relaxins a, a′ or B. The data are presented as nmol of amino acid residue per nmol of lysine where the number of lysines per molecule was set equal to three. Total protein recovered in the collected fractions was: relaxin a′, 95 μg or 86%; relaxin B, 112 μg or 90%; relaxin a, 105 μg or 91%. nd = not determined.

Amino acid	Present study			Ref. 24			Ref. 10		Refs. 21—23	Ref. 25
	a′	B	a	A	B	C	CM-a	CM-b		
D	2.6	3.3	2.7	2.9	2.9	3.0	2.8	2.9	3	3
T	1.6	2.7	2.1	2.1	2.1	1.7	2.3	1.9	2	2
S	2.7	4.0	4.0	3.2	3.4	2.8	2.8	2.8	3	4
E	4.1	5.2	5.2	4.8	4.8	4.7	4.7	4.7	5	5
P	0	0	0	1.2	0	1.0	0	0	0	
G	2.7	4.6	4.9	3.8	3.6	3.4	3.6	3.2	3	4
A	1.8	3.0	2.7	2.5	2.6	3.1	2.7	2.3	2	2
C	nd	nd	nd	5.3	6.0	5.4	4.8	4.9	6	6
V	2.7	3.6	2.7	3.7	3.6	3.2	3.7	3.5	4	4
M	0.8	0.9	1.0	0.9	0.8	0.8	0.8	0.8	1	1
I	3.0	3.2	2.8	3.6	3.4	2.8	3.3	3.2	4	4
L	3.1	3.6	3.8	4.1	4.1	3.9	3.9	3.8	4	4
Y	0	0	0	0	0	0.2	0	0	0	0
F	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1	1
H	0	0	0	0	0	0.4	0	0	0	0
K	3.0	3.0	3.0	3.3	3.1	3.3	3.1	3.2	3	3
W	nd	nd	nd	1.6	1.4	1.0	nd	nd	2	2
R	3.9	5.3	6.0	5.6	5.0	3.9	5.3	4.5	5	6

al. [25], and that labelled "A" by Frieden et al. [24]. However, our relaxin "a" preparation lacked a proline residue as reported by Frieden et al. [24]. This relaxin "a" species contains six arginine residues. Finally, the composition of the species designated relaxin "a′" in Fig. 1 and Tabel I corresponded to the material labelled "C" by Frieden et al. [24], except again our preparation lacked the proline residue as reported by these workers. This relaxin "a′" species contains four arginine residues. It is obvious from our results and other published data (Table I) that there are a number of different but chemically related relaxin polypeptides which are only partially resolved by CM-cellulose fractionation, but which can be well separated under the above RP-HPLC conditions. Whether these three porcine relaxin species arise as breakdown products during the isolation procedure or are true biosynthetic products remains, however, to be clarified.

The conditions used to generate the chromatograms in Fig. 1 gave excellent recoveries as determined by quantitive amino acid analysis, for example, recoveries of 86% for relaxin "a′", 90% for relaxin "B", and 91% for relaxin "a", were obtained. As part of the preliminary experiments other chromatographic conditions were also examined, including gradients in acetonitrile with low pH mobile phases consisting of either 0.1% orthophosphoric acid or 0.1%

trifluoroacetic acid. Under these acidic conditions it was not possible to resolve the three porcine relaxin species on several different stationary supports (e.g. μBondapak C_{18}, μBondapak alkylphenyl, and *n*-octyl LiChrospher 100). Similar lack of discrimination of microheterogeneous relaxin forms has been observed by Reinig et al. [15], who used 10% acetic acid as the mobile phase buffer in the isolation of shark relaxin and its two subunit components on a μBondapak C_{18} support. In other experiments, we examined the efficacy of 200 m*M* ammonium bicarbonate at pH 7.8 as the primary mobile phase buffer, but resolution of the "a" and the "a'" relaxin species was incomplete (Fig. 2) even when shallow gradients in acetonitrile concentration (e.g. 0.12%/ml/min) were used, the resolution was inferior to the results shown in Fig. 1 with either μBondapak C_{18} or *n*-octyl LiChrospher 100 support material. Since the porcine relaxins are basic solutes it was anticipated that chromatographic selectivity could be enhanced further by the use of an anionic pairing ion such as heptanesulphonate at a concentration up to 15 mmol/dm^3 in the mobile phase. Previous studies [5, 12, 26, 27] have demonstrated that increased column selectivity generally occurs with unprotected peptides when low concentrations of anionic pairing ions are used with acidic (ca. pH 2—3) mobile phase conditions. However, the three porcine relaxin species coeluted (data not shown) from the three different stationary phases examined when heptanesulphonate concentrations of 10 mmol/dm^3 were employed with aquo—acetonitrile gradient conditions otherwise identical to those used for the separations shown in Fig. 1.

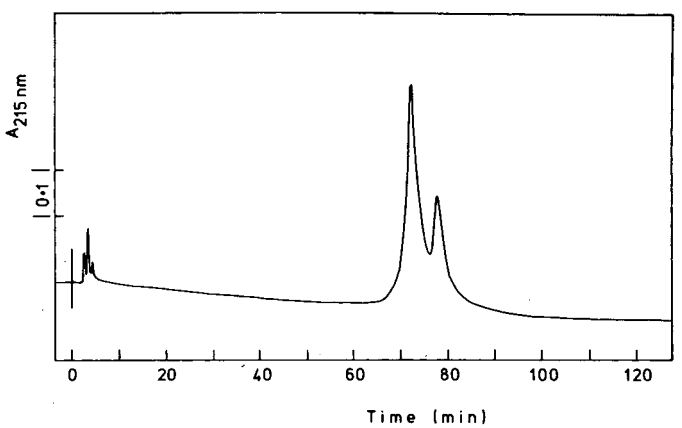

Fig. 2. Separation of CM-cellulose fractionated porcine relaxin a and porcine relaxin a' by RP-HPLC with an ammonium bicarbonate buffer in the mobile phase. Chromatographic conditions: column, C_8 alkyl chain bonded to 10 nm pore size LiChrospher silica, (15 × 0.4 cm, d_p 10 μm); flow-rate, 1.0 ml/min; linear 150-min gradient from 45 to 80% B, where solvent A was 200 m*M* ammonium bicarbonate—water, pH 7.8, and solvent B was 50% acetonitrile—50% water—200 m*M* ammonium bicarbonate; detection, 215 nm at 1.0 a.u.f.s.; sample loading, 125 μg of a mixture of porcine relaxins a and a' in a volume of 100 μl.

RP-HPLC of human placental polypeptide hormones

In pregnancy there are remarkable alterations in hormone production. The human placenta, for example, secretes large quantities of a variety of both steroid and polypeptide hormones, including chorionic gonadotropin (hCG)

and human placental lactogen (hPL). The glycoprotein hCG, in common with the pituitary glycoprotein hormones, possesses a quaternary structure characterized by two dissimilar polypeptide chains, designated α- and β-subunits. The α-subunit consists of 92 amino acids and is very similar in sequence to the α-subunits of the pituitary glycoprotein hormones [28, 29], while the β-chain consists of 145 amino acids [28, 30, 31] and shows some sequence homology with other glycoprotein hormone β-subunits [31]. In the human hCG has luteotropic activity and is believed to stimulate the foetal gonads to secrete steroids at a time prior to the secretion of LH by the foetal pituitary [32].

Fig. 3 shows the chromatographic profiles of preparations of hCG and its α- and β-subunits under mobile phase conditions identical to those used above for the separation of the porcine relaxin species. Although the α- and β-subunit preparations used in this investigation were believed by other criteria to be homogeneous [28, 29] each preparation gave two chromatographic peaks on

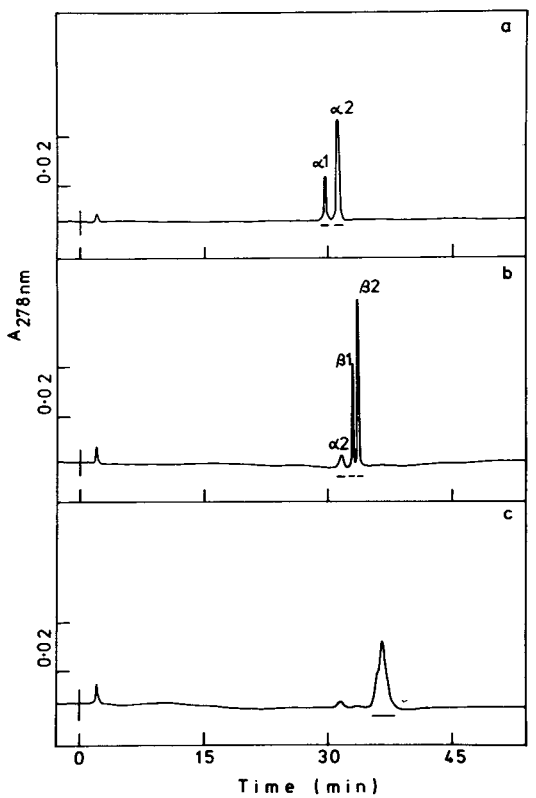

Fig. 3. RP-HPLC of preparations of human chorionic gonadotropin (hCG) and its α- and β-subunits. Chromatographic conditions: column, μBondapak alkylphenyl (30 × 0.4 cm, d_p 10 μm); flow-rate, 1.2 ml/min; linear 90-min gradient from 0 to 100% B, where solvent A was 100 m*M* ammonium acetate—water, pH 7.0, and solvent B was 60% acetonitrile—40% water—15 m*M* trifluoroacetic acid, pH 2.0; detection, 278 nm at 0.04 a.u.f.s. Samples: (a) 150 μg of hCG α-subunit in 100 μl; (b) 150 μg of hCG β-subunit in 100 μl; (c) 250 μg of hCG in 150 μl. Peaks underlined with bars were collected and analyzed for amino acid composition, and the data are summarized in Table II.

TABLE II

AMINO ACID COMPOSITIONS OF PLACENTAL HORMONES

Amino acid analysis of hCG, hCG subunits and hPL after chromatography on reversed-phase, microparticulate silica supports. Peaks were collected from the bar regions of the profiles in Figs. 3 and 4. For hCG the results are presented as nmol of amino acid residue per nmol of alanine where the integer value of alanine was set to 5 in the α-subunit, 8 in the β-subunit, and 13 for the intact hCG. Published composition values are from the amino acid sequence data given in ref. 29. Total recovery of protein was 126 μg or 83% for the hCG α-subunit preparation, 134 μg or 89% for the hCG β-subunit preparation, and 221 μg or 88% for the parent hCG molecule. For hPL the results are presented as nmol of amino acid residue per nmol of alanine where the integer value of alanine was set at 6. Published composition values for hPL are from refs. 36 and 37. Total recovery of protein was 159 μg or 91% of the starting material. nd = not determined.

Amino acid	hCG α-subunit			hCG β-subunit			hCG		hPL	
	1	2	predicted	1	2	predicted	observed	predicted	observed	predicted
D	6.4	6.3	6	12.4	11.1	11	17.3	17	20.2	23
T	8.1	8.3	8	9.7	9.5	10	17.9	18	11.9	13
S	7.9	8.0	8	11.5	10.9	13	19.9	21	16.9	18
E	9.8	10.1	9	9.7	9.1	9	19.2	18	20.1	24
P	9.4	9.1	7	23.1	25.1	22	31.1	29	6.9	5
G	5.0	5.0	4	9.3	8.6	8	14.1	12	6.9	7
A	5.0	5.0	5	8.0	8.0	8	13.0	13	nd	4
C	nd	nd	10	nd	nd	12	nd	22	nd	4
V	7.2	7.1	7	11.5	12.0	12	19.2	19	6.9	7
M	2.8	2.9	3	1.5	1.8	1	5.9	4	7.2	6
I	2.7	1.2	1	5.3	6.7	5	7.2	6	6.9	6
L	5.9	4.5	4	11.3	13.8	12	17.2	16	27.6	25
Y	5.7	3.6	4	5.0	5.0	3	8.6	7	9.1	8
F	6.0	4.0	4	1.9	2.1	2	8.1	6	13.3	11
H	2.7	3.1	3	2.0	1.1	1	4.3	4	6.6	7
K	6.0	6.2	6	4.6	4.0	4	9.9	10	9.1	9
W	nd	nd	0	nd	nd	0	nd	0	nd	1
R	3.2	3.3	3	11.5	11.5	12	15.3	15	11.2	10

RP-HPLC separation. Composition analysis of the collected fractions (Table II) showed that the two peaks corresponding to the α-subunit preparation had very similar amino acid content. Similarly, the two peaks generated from the β-subunit preparation also correlated closely in composition. These double peaks from each apparently homogeneous subunit may be due to either partially deamidation of the subunits during the isolation procedure, or alternatively to differences in the glycosylation state. As is evident from Fig. 3, these RP-HPLC procedures enable complete resolution of the two α-subunit isoforms from the two β-subunit isoforms and also from the parent hCG protein. Recoveries were 83%, 89% and 88% for the α-subunit, β-subunit and parent hCG, respectively. Even with small-pore alkylsilicas such as the μBondapak alkylphenyl support excellent peak shape can be achieved with the 100 m*M* ammonium acetate, pH 7.0, to 60% acetonitrile—15 m*M* trifluoroacetic acid, pH 2.0, elution condition where gradients in both composition and pH are achieved. These results can be contrasted with the observations of

Putterman et al. [33] who have reported a similar elution order pattern for the subunits (i.e. $t_g\alpha < t_g\beta$) but significantly lower resolution due to greater band broadening in the separation of hCG and its subunits by RP-HPLC procedures using a gradient in acetonitrile containing 0.1% trifluoroacetic acid alone as the mobile phase buffer, and a μBondapak C_{18} support.

Human placental lactogen (hPL) is a single-chain polypeptide hormone of 191 amino acids devoid of carbohydrate moieties [34]. There is considerable sequence homology between hPL and human growth hormone (HGH) with 162 residues of the primary sequence being identical in the two proteins [35—37]. Fig. 4 shows a further example of the utility of the ammonium acetate—trifluoroacetic acid system, namely, the chromatography of hPL, previously purified by established methods [36], on a μBondapak alkylphenyl support using the standard 0—50% acetonitrile gradient. Composition analysis of the collected peak fraction is given in Table II. The mass recovery of hPL was 91% of the protein loaded.

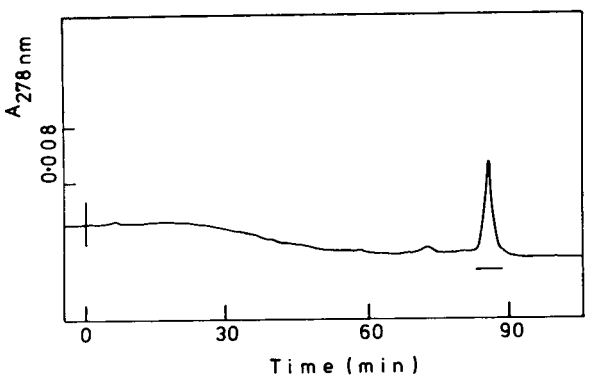

Fig. 4. RP-HPLC of human placental lactogen (hPL) preparation. Chromatographic conditions: column, μBondapak alkylphenyl (30 × 0.4 cm, d_p 10 μm); flow-rate, 1.2 ml/min; linear 90-min gradient from 0 to 100% B, where solvent A was 100 m*M* ammonium acetate—water, pH 7.0, and solvent B was 60% acetonitrile—40% water—15 m*M* trifluoroacetic acid, pH 2.0; detection, 278 nm at 0.04 a.u.f.s. Sample, 175 μg of hPL in 100 μl. The peak underlined was collected and analyzed for amino acid composition, and the data are given in Table II.

RP-HPLC of human pituitary polypeptide hormones

The human adenohypophysis contains several polypeptide hormones of established functional significance, including the two-subunit glycoproteins follicle stimulating hormone (hFSH), thyroid stimulating hormone (hTSH) and luteinising hormone (hLH), and the single-chain non-glycosylated proteins, growth hormone (hGH) and prolactin (hPrL). Our aim was to devise optimal chromatographic conditions for the recoveries of these protein hormones, compatible with subsequent structural or biological analysis. A stringent test of any chromatographic system is the requirement of high recovery of radiolabelled solutes such as radio-iodinated polypeptides which on a mass basis may be present in the sample to be loaded and the eluted fractions in only trace quantities.

Table III summarizes the recoveries of several different ^{125}I-labelled poly-

TABLE III

RECOVERIES OF RADIO-IODINATED POLYPEPTIDE HORMONES UNDER DIFFERENT ELUTION CONDITIONS

Key to the chromatographic conditions: Condition 1. μBondapak C_{18}, flow-rate 2.0 ml/min, linear 60-min gradient from 0 to 100% B, where solvent A was 15 m*M* orthophosphoric acid—water, pH 2.25, and solvent B was 50% acetonitrile—50% water—15 m*M* orthophosphoric acid. Condition 2. μBondapak C_{18}, flow-rate 2.0 ml/min, linear 60-min gradient from 0 to 100% B, where solvent A was 200 m*M* ammonium bicarbonate—water, pH 7.8, and solvent B was 50% acetonitrile—50% water—200 m*M* ammonium bicarbonate. Condition 3. μBondapak alkylphenyl, flow-rate 2.0 ml/min, linear 60-min gradient from 0 to 100% B, where solvent A was 200 m*M* ammonium acetate—water, pH 7.0, and solvent B was 60% acetonitrile—40% water—80 m*M* acetic acid, pH 3.5. Condition 4. C_1 alkyl chain bonded to 50 nm pore size LiChrospher silica, flow-rate 2.0 ml/min, linear 60-min gradient from 0 to 100% B, where solvent A was 200 m*M* ammonium bicarbonate—water, pH 7.8, and solvent B was 50% acetonitrile—50% water—200 m*M* ammonium bicarbonate. All of the μBondapak columns were of the same dimensions, 30 × 0.4 cm with d_p 10 μm, while the C_1 bonded phase was 7.5 × 0.4 cm, d_p 10 μm. In all experiments 1-min fractions were collected and counted in a γ-counter.

Chromatographic condition	^{125}I-labelled hormone	Recovery (%)
1	hGH	10
	hPrL	50
2	hGH	75
	hPrL	61
	hTSH	82
	hFSH	85
	hLH	65
3	hGH	60
	hPrL	97
	hTSH	94
	hFSH	99
	hLH	95
4	hTSH	85
	hLH	98

peptide hormones under a variety of chromatographic conditions. One of the constraints which must be applied to the reversed-phase chromatography of glycoprotein hormones such as FSH, TSH and LH, concerns the choice of the mobile phase pH. At inappropriate mobile phase pH values dissociation of the native glycoprotein hormones to their respective subunits may occur. In comparison the single-chain hormones such as hGH and hPrL are relatively stable for short periods in mildly acidic conditions, such as 0.1% phosphoric or 0.1% trifluoroacetic acid, but over some acidic pH ranges these proteins exhibit low solubility or may partially deamidate [12]. Load-dependent recovery effects may be a further complication in ultramicropreparative RP-HPLC separations of trace quantities of these radiolabelled polypeptide hormones. The results obtained with radio-iodinated hGH and hPrL with a μBondapak C_{18} column and a gradient of acetonitrile containing 0.1% orthophosphoric acid as

the mobile phase ionic additive (elution condition 1 in Table III) are typical examples. Thus, with sample loadings of [^{125}I]hGH or [^{125}I]hPrL equivalent to ca. 5 pg of protein the recovery of [^{125}I]hGH was 10% whilst the recovery of hPrL was 50%. The same chromatographic conditions have previously given good recoveries (as assessed by quantitative amino acid analysis) of hGH (ca. 90%) when the sample loaded was in the range 5 μg to 3 mg [38]. Other groups [39, 40] have also reported similar observations regarding load-related recoveries of certain proteins chromatographed on reversed-phase alkylsilicas.

Volatile mobile phases are commonly used for preparative separations because of their ease of removal. In previous studies [12, 38, 41] we have extensively used ammonium bicarbonate as a mobile phase buffer in the RP-HPLC of peptides and proteins. Using the same μBondapak C_{18} column as employed with the 0.1% orthophosphoric acid elution system, the recoveries of radio-iodinated HGH and hPrL improved significantly up to 75% and 61% respectively with 200 m*M* ammonium bicarbonate as the mobile phase buffer with a 0—50% acetonitrile gradient (chromatographic condition 2 in Table III). The recoveries of the radio-iodinated glycoprotein hormones hTSH, hFSH and hLH ranged from 65% to 85% under these chromatographic conditions. Fig. 5

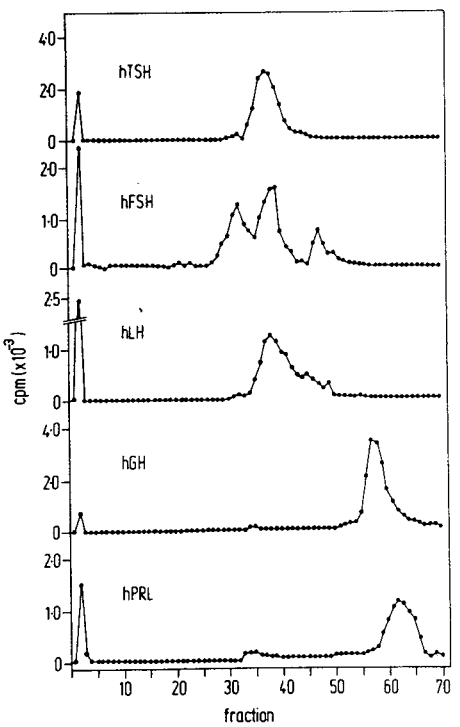

Fig. 5. RP-HPLC of iodinated pituitary polypeptide hormones under constant pH elution conditions. Chromatographic conditions: column, μBondapak C_{18} (30 × 0.4 cm, d_p 10 μm); flow-rate, 2.0 ml/min; linear 60-min gradient from 0 to 100% B, where solvent A was 200 m*M* ammonium bicarbonate—water, pH 7.8, and solvent B was 50% acetonitrile—50% water—200 m*M* ammonium bicarbonate. Fractions were collected at 1-min intervals and counted directly in a γ-spectrometer. Recoveries of labelled hormones are given in Table III.

shows the elution profiles obtained under these chromatographic conditions for these five polypeptide hormones. As discussed elsewhere [42] the peak broadening observed for these radio-iodinated hormones appears to be a consequence of several factors. First, iodination of proteins which contain several accessible tyrosine residues leads to the generation of multiple iodinated species which may be only partially resolvable under a particular chromatographic condition. Second, pituitary glycoprotein hormones in particular exhibit considerable microheterogeneity in terms of their charge and glycosylation patterns [43]. Third, peak shapes for globular proteins above 20 Kdaltons are very responsive to mobile phase mediated phenomena which affect the diffusion coefficients of these solute as well as the kinetic resistance to migration of the solutes onto and off the alkylsilica support. We have discussed the contribution of resistance to mass transfer between the mobile and stationary phases for protein solutes in a previous report [44]. Under otherwise fixed eluent conditions for solutes with small diffusion coefficients such as proteins, peak shape may be improved by reducing both particle diameter of the support and flow-rate, as well as by improving the mass transfer properties of the stationary phase. The latter requirement may be only partly achieved by increasing the pore size of the support [44]. Much more significant control over band broadening due to stationary phase effects can be achieved when less heterogeneously bonded or dynamically coated non-polar surfaces are employed thereby reducing the extent of multiple-site interaction with complex protein solutes [44, 45]. The participation of all of these factors can give rise to apparent peak broadening in the gradient elution RP-HPLC of these radio-iodinated protein hormones. It was thus of interest to examine the chromatographic behaviour of individual fractions on rechromatography. In all cases examined rechromatography under identical conditions of single fractions from the experiments shown in Fig. 5, resulted in the radio-iodinated component(s) eluting with unchanged retention, i.e. at the same fraction number if the flow-rate and fraction size was constant, but with significantly improved peak shape in many cases equivalent to that observed for the unlabelled parent protein. There are two precautions regarding the use of high molarity ammonium bicarbonate as a mobile phase buffer for protein separations by RP-HPLC which should be mentioned. First, its solution pH of 7.8 is close to or above the upper pH limit of some commercial silica-based supports. Second, it is our experience that highly basic proteins may exhibit very low recoveries with this buffer composition and pH due to titration of cationic side chains on the proteins resulting in a less polar species [5, 26, 27] and titration of weak acidic groups on the alkylsilica support resulting in non-ideal reversed-phase behaviour. Chromatographic conditions consisting of gradients in acetonitrile concentration and pH similar to those described for the porcine relaxins and hCG were also found effective for the separations of several pituitary polypeptide hormones. For example, with linear gradients of 100 m*M* ammonium acetate, pH 7.0, to 60% acetonitrile in 80 m*M* acetic acid, pH 3.5, recoveries of several radio-iodinated proteins were over 90% except for hGH (Table III) although significant band broadening was still evident (Fig. 6) in all cases.

The above results are in accord with our previous findings [4—9, 12] which

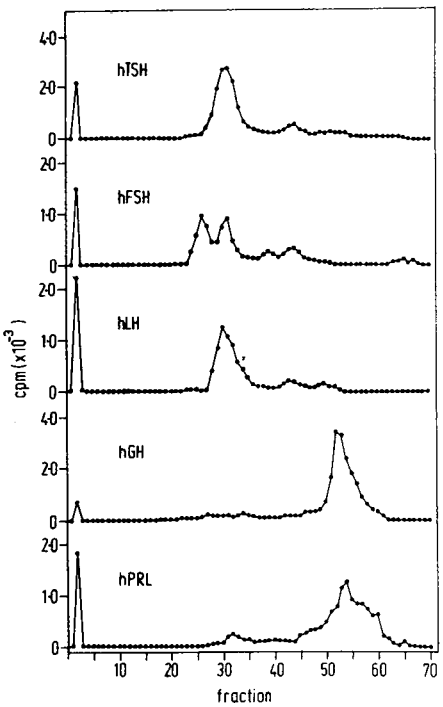

Fig. 6. RP-HPLC of iodinated pituitary polypeptide hormones, under pH and solvent gradient conditions. Chromatographic conditions: column, μBondapak alkylphenyl (30 × 0.4 cm, d_p 10 μm); flow-rate, 2.0 ml/min; linear 60 min gradient from 0 to 100% B, where solvent A was 200 m*M* ammonium acetate—water, pH 7.0, and solvent B was 60% acetonitrile—40% water—80 m*M* acetic acid, pH 3.5. 80 m*M* acetic acid is equivalent to ca. 0.5% solution. Fractions were collected at 1-min intervals and counted in a γ-spectrometer. Recoveries of labelled hormones are given in Table III.

demonstrated that retention of polypeptides and proteins on alkylsilicas is to a very large extent dominated by mobile phase effects and in particular by the choice and concentration of the organic solvent modifier. In common with many other globular proteins, the retention of the pituitary protein hormones to alkylsilicas is very responsive to small changes in the water content of binary or ternary aquo—organic solvent mobile phase combinations. For example both the 22-Kdalton hGH and the 20-Kdalton hGH variant exhibited characteristic bimodal retention dependencies on the concentration of the organic solvent modifier when eluted with water—acetonitrile elution systems from butyl- and octyl-bonded silicas of nominal pore sizes of 10 nm and 50 nm (Fig. 7). As can be seen from Fig. 7, with these two proteins there is clearly a significant pore size effect. Even when total column porosities and column packing densities are taken into account, at a given k' value both these proteins elute from the small-pore *n*-butyl- or *n*-octylsilica at a lower acetonitrile concentration than from the corresponding large-pore *n*-alkylsilica. In this retention behaviour, these hGH polypeptides differ from the glycoprotein hormones or other globular proteins such as lysozyme, trypsin or phosphorylase a [46]. Over the range of regular reversed-phase elution behaviour, the pituitary protein

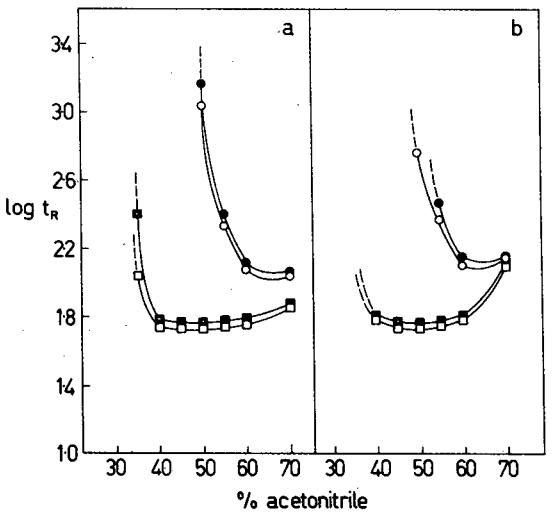

Fig. 7. Dependence of retention of 22K human growth hormone (a) and the 20K human growth hormone variant (b) on the mobile phase acetonitrile concentration. Chromatographic conditions: mobile phase, 50 m*M* NaH_2PO_4—15 m*M* H_3PO_4, pH 2.3, with the mobile phase acetonitrile concentration adjusted between 0 and 70%; flow-rate, 1.0 ml/min; columns, four supports each of a nominal 10-μm particle size but differing in alkyl chain length and particle porosity were used according to the key: □ = C_4 Si-100, ■ = C_8 Si-100, ○ = C_4 Si-500, ● = C_8 Si-500. The same column dimensions (15 × 0.4 cm) were used for each support material.

hormones exhibited curvilinear plots between the logarithmic capacity factor, log k', and the mole fraction of the organic solvent modifier, ϕ, with tangent slopes typical of globular proteins in this molecular weight range, i.e. with S-values between 60 and 80. One consequence of the pronounced retention dependencies of these proteins on the organic solvent content in RP-HPLC systems is that apparent retention times are nearly independent of column length under otherwise fixed gradient elution conditions. For example, under the 200 m*M* ammonium bicarbonate—acetonitrile elution conditions (condition 4 in Table III) the relative retention of the radio-iodinated protein hormones chromatographed on C-1, C-4 and C-18 bonded alkylsilica of 50 nm nominal pore diameter packed into columns of 10 cm length were almost identical to those observed with 30-cm columns of the same internal diameter packed with the same stationary phase.

Finally, the influence of polar modifiers on the resolution and recovery of these proteins was examined. Previously we have shown [47] that polar modifiers such as *tert.*-butanol in low concentrations reduce the retention of small peptides on alkylsilicas presumably by dynamically modifying the stationary phase surface. Addition of *tert.*-butanol or trifluoroethanol at a concentration of 1% (v/v) in the mobile phase (for example condition 2 in Table III) caused a reduction in the recovery of [^{125}I]hGH and [^{125}I]hPrL from 60—70% to about 10—15% of the amount loaded, compared to the corresponding elution condition lacking these polar modifiers as well as reduced resolution. Similarly, addition of 25 m*M* triethylamine at acidic (pH 3.0) and neutral (pH 7.8) conditions did not enhance recoveries as anticipated

on the basis of a decrease in silanol effects although peak shape was improved.

In summary, chromatographic conditions compatible with the RP-HPLC of several polypeptide and protein hormones have been developed. These separation conditions permit rapid high-resolution fractionation of pituitary derived protein hormones and components of placental or ovarian origin. Extension of these studies to large-scale purification of these biologically important proteins will be reported elsewhere [48].

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MEASUREMENT AND CHROMATOGRAPHIC CHARACTERIZATION OF VASOACTIVE INTESTINAL PEPTIDE FROM GUINEA-PIG ENTERIC NERVES

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SUMMARY

The material exhibiting immunoreactivity for vasoactive intestinal peptide in guinea-pig enteric nerves has been characterized by high-performance liquid chromatography in three modes: reversed-phase, cation-exchange and gel permeation. In each case a major portion of the material contained in acetic acid extracts of guinea-pig gut showed the same chromatographic properties as the synthetic porcine peptide of defined amino acid sequence. It is therefore concluded that this immunoreactive material is authentic vasoactive intestinal peptide. The study illustrates a number of the problems encountered in attempting to characterize, and measure reliably, peptides in tissue extracts.

INTRODUCTION

Vasoactive intestinal peptide (VIP) is one of a number of peptides that have been found in, and isolated from, the enteric nervous system [1]. It was first isolated from porcine gut [2] and the amino acid sequence of the peptide from this source has been determined [3]. The amino acid sequence of the VIP-like material in bovine [4], avian [5], human [6] and rat [7] gut has also been determined and has been found to be identical to porcine VIP, although small amounts of apparently variant molecular forms have been reported in a number of species [8, 9].

VIP-like immunoreactivity (VIP-LI) is also present in the guinea-pig small intestine, but it appears to differ from porcine VIP. It has been shown by immunohistochemical techniques, using antisera raised against porcine VIP, to be contained only in nerves [10, 11]. Radioimmunoassay estimation of concentrations in tissue extracts, however, have varied as much as 100-fold [1, 12].

The chromatographic behaviour of guinea-pig VIP-LI has also been reported

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to differ from porcine VIP [12]. Gel filtration chromatography on Sephadex G-50 indicated that the molecular size of guinea-pig VIP-LI was the same as porcine standard, but ion-exchange chromatography on CM-Sephadex indicated that the guinea-pig material was more acidic than the porcine standard.

In the present work, the nature of the VIP-LI material in guinea-pig enteric nerves has been investigated using high-performance liquid chromatography (HPLC). Acid extracts of guinea-pig gut have been analysed in the reversed-phase mode using two different solvent systems, and in the cation-exchange and gel permeation modes.

EXPERIMENTAL

All chromatographic analyses were conducted on a Varian 5020 binary gradient liquid chromatograph equipped with a UV-5 absorbance detector operating at 215 nm. Fractions were collected from the chromatograph using an LKB 2112 Redirac fraction collector. Separations were accomplished on a MicroPak MCH-10 (300 × 4 mm I.D.) column and a MicroPak TSK G2000SW (300 × 7.5 mm I.D.) column, both purchased from Varian Assoc., and on a SynChropak CM300 (250 × 4.1 mm I.D.) column, purchased from SynChrom (U.S.A.). Chromatographic-grade acetonitrile and methanol were purchased from Waters Assoc. Synthetic porcine VIP was purchased from Peninsula Labs. (U.S.A.). All other chemicals were purchased from Ajax Chemicals (Australia).

Tissue processing and extraction

Guinea-pigs (200—300 g) were killed by a blow to the head and bled out. The small intestine, between 20 and 80 cm proximal to the ileo—caecal junction, was taken immediately, opened along the mesenteric border and rinsed in phosphate-buffered saline (PBS) (0.15 *M* sodium chloride, 0.01 *M* sodium phosphate buffer, pH 7.2) to remove the contents. The tissue was then blotted dry on paper towel, wrapped in a piece of pre-weighed aluminium foil and snap-frozen in liquid nitrogen. The sample was weighed frozen and stored at −70°C until it could be extracted.

Frozen tissue was dropped into 5 vols. of boiling 2.0 *M* aqueous acetic acid (with or without 0.01 *M* 2-mercaptoethanol) and was boiled for a further 15 min. The mixture was then cooled in ice and homogenized with 10-sec bursts of a Polytron homogenizer on setting 5 three times. The homogenate was allowed to stand overnight at 4°C and was then centrifuged at 10,000 *g* for 10 min at 4°C. The clear supernatant was decanted, weighed and stored at −70°C until required.

Preparation of tissue extracts for HPLC analysis

Tissue extracts were prepared for chromatographic analysis by the following procedure. Duplicate aliquots (50 μl) were removed and dried down by vacuum centrifugation for estimation of VIP-LI concentration by radioimmunoassay. A portion of the extract (2—3 ml) was washed three times with an equal volume of diethyl ether to remove lipids, and was then loaded onto a pre-wetted Sep-Pak C_{18} cartridge (Waters Assoc.). The cartridge was washed with 20 ml of water to desalt the sample, and the peptides were then eluted with

1—2 ml of methanol. The methanolic eluate was evaporated to dryness and the residue reconstituted in 200 μl of 2.0 *M* aqueous acetic acid for injection into the chromatograph.

Chromatographic analysis of tissue extracts

Reversed-phase analysis was carried out on the MicroPak MCH-10 column, using either of the following solvent gradients: (A) acetonitrile in acid saline (0.15 *M* sodium chloride, pH to 2.1 with hydrochloric acid), 0—2.5 min at 0% acetonitrile, 2.5—5 min 0—10% acetonitrile, 5—45 min 10—40% acetonitrile, 45—50 min 40—60% acetonitrile [13]; or (B) acetonitrile in aqueous trifluoroacetic acid (TFA, 0.5%, v/v, pH 1.9), 0—5 min at 25% acetonitrile, 5—40 min 25—80% acetonitrile, 40—50 min at 80% acetonitrile. All gradient steps were linear and total flow-rate was maintained at 1.0 ml/min for both gradient systems.

Cation-exchange chromatography was carried out on the SynChropak CM300 column, eluting with a gradient of 1.0 *M* potassium chloride in potassium phosphate buffer (0.1 *M*, pH 4.8) containing 10% acetonitrile. The gradient consisted of two linear steps: 0—5 min at phosphate buffer only, 5—30 min 0—50% potassium chloride in phosphate buffer. Flow-rate was maintained at 1.0 ml/min.

Gel permeation chromatography was carried out on the MicroPak TSK G2000SW column, eluting with sodium phosphate buffer (0.2 *M*, pH 2.1) at a flow-rate of 1.0 ml/min.

In all cases 0.5-min fractions were collected from the chromatograph. Glass collection tubes contained 100 μl of bovine serum albumin solution (1%, w/v, in distilled water of fraction V powder from Sigma) to reduce adsorption of peptides to the glass surface.

Fractions collected from gradient analyses (viz. reversed-phase and cation-exchange) were dried down in a vacuum concentrator (Savant Instruments, U.S.A.) and then reconstituted in either distilled water (from acid saline and cation-exchange gradients) or radioimmunoassay buffer (from TFA gradients) before aliquots were taken for measurement of VIP-LI by radioimmunoassay. Fractions collected from the gel permeation column were aliquoted directly into the assay, with an equivalent aliquot of elution buffer added to the standard curve tubes.

The elution position of porcine VIP standard was determined by injection of either 5 μg of standard on-column and monitoring the eluate for absorbance at 215 nm, or by injection of 5 ng on-column and monitoring collected fractions for VIP-LI by radioimmunoassay.

When required, oxidized porcine standard was produced by treating 5 ng of standard in 100 μl of 2.0 *M* acetic acid with 20 μl of hydrogen peroxide (30%, v/v) for 60 min at room temperature prior to injection into the chromatograph.

The recovery of VIP-LI through the pre-column purification and chromatography steps was measured as the proportion of immunoreactive material present in the extract that was recovered in the collected fractions. The recovery from the chromatograph alone was estimated by measuring collected immunoreactive material after injection of standards. After running standards, columns were cleaned by extensive washing with the appropriate solvents (i.e.

blank gradients or elution buffer) until no immunoreactivity could be detected.

Radioimmunoassay

Tissue concentration of VIP-LI was determined from the extracts. Duplicate aliquots were dried down at room temperature in the vacuum concentrator and were reconstituted in assay buffer (0.04 *M* sodium phosphate buffer, pH 7.2, containing 0.15 *M* sodium chloride, 0.01 *M* EDTA and 0.25% bovine serum albumin). VIP-LI was assayed using antiserum 7913 (from Dr. J.H. Walsh) at a final dilution of 1 in 200,000 and using ^{125}I-labelled porcine VIP, iodinated by the iodogen method [14], as tracer. Under these conditions, the assay gave 35% binding of tracer in the absence of VIP standard and measured over the range 10—1000 pg of VIP per tube (100 pg of VIP giving 50% displacement of bound tracer).

The concentration of VIP-LI in fractions collected from the chromatograph was determined as described above.

The cross-reactivity of oxidized porcine VIP in the assay was determined by oxidation of 5 μg of standard with hydrogen peroxide as described above, and then construction of a standard curve using this material (appropriately diluted in assay buffer) together with a standard curve using the normal standard.

RESULTS

Reversed-phase chromatography

Analysis of porcine VIP (pVIP) standard on the reversed-phase column, using a gradient of acetonitrile in acid saline as described above, gave a single sharp peak with retention time of 38.5 min (Fig. 1A). Recovery of peptide from the chromatograph was 95% as measured by radioimmunoassay. Analysis of an extract of guinea-pig small intestine (extracted with 2.0 *M* acetic acid only) under the same conditions gave one major peak of VIP-LI, corresponding in retention time to pVIP and accounting for only 55% of recovered material, together with five other peaks, all eluting earlier (Fig. 2A). Total recovery of immunoreactive material from the chromatograph was 82% of that originally present in the extract. Oxidation of pVIP with hydrogen peroxide resulted in the formation of multiple immunoreactive peaks when chromatographed under these conditions (Fig. 2B), including peaks corresponding to those seen in the extract. Total recovery of immunoreactive material was 110%. The inclusion of 0.01 *M* 2-mercaptoethanol in the extraction medium resulted in only one peak of immunoreactivity being detected, corresponding to 57% of immunoreactive material present in the extract, and this peak corresponded to the elution position of authentic porcine standard (Fig. 3A).

Reversed-phase analysis of pVIP on the same column but with a different gradient of acetonitrile in aqueous TFA also gave a single peak for this material, but with a retention time of 18 min (Fig. 1B). Recovery of immunoreactive peptide was quantitative. Analysis of the extract containing 2-mercaptoethanol with this system gave one peak of VIP-LI, corresponding in retention time to the pVIP standard (Fig. 3B), and with 65% of immunoreactive material originally present in the extract being recovered.

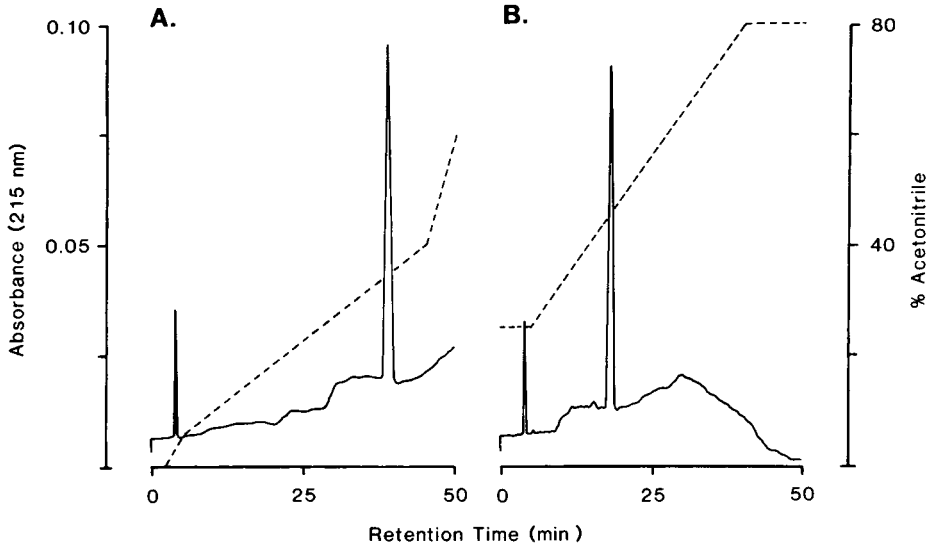

Fig. 1. Reversed-phase HPLC analysis of synthetic porcine VIP. Starting buffers were (A) 0.15 *M* sodium chloride (pH 2.1 with hydrochloric acid), and (B) 0.5%, v/v, TFA (pH 1.9), and the elution gradient (- - -) is expressed in terms of percentage acetonitrile (right hand ordinate). The peptide (5 μg) was injected onto the column and its elution position detected by its absorbance at 215 nm (left hand ordinate) (—).

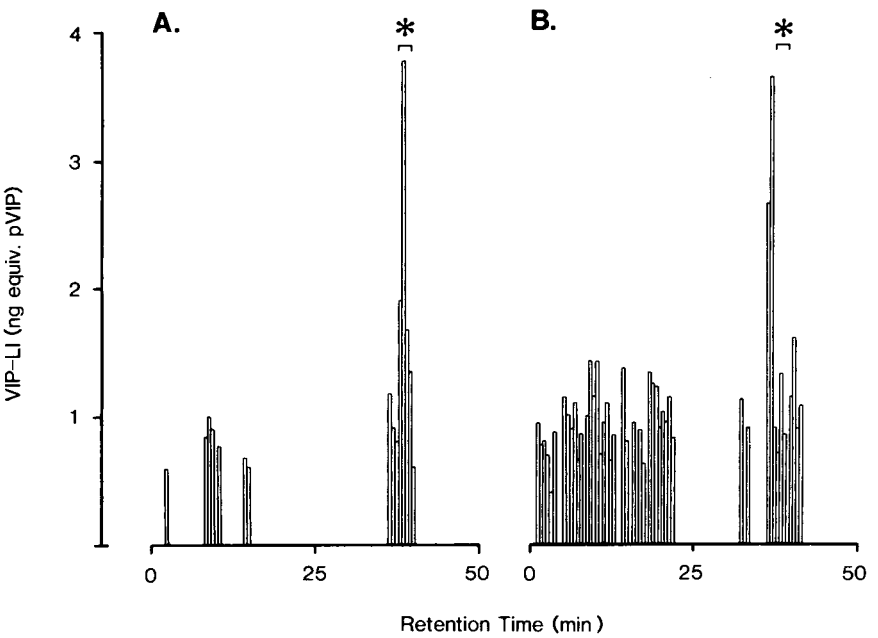

Fig. 2. Reversed-phase analysis of guinea-pig gut extract and oxidized porcine VIP. The histograms show VIP-like immunoreactive material recovered after reversed-phase HPLC analysis (using a gradient of acetonitrile in acid saline) of (A) a 2.0 *M* acetic acid extract of guinea-pig ileum, and (B) oxidized porcine VIP standard. The elution position of authentic pVIP is shown (*). The ordinate gives the concentration of immunoreactive material in ng equiv. of pVIP per 0.5-ml fraction.

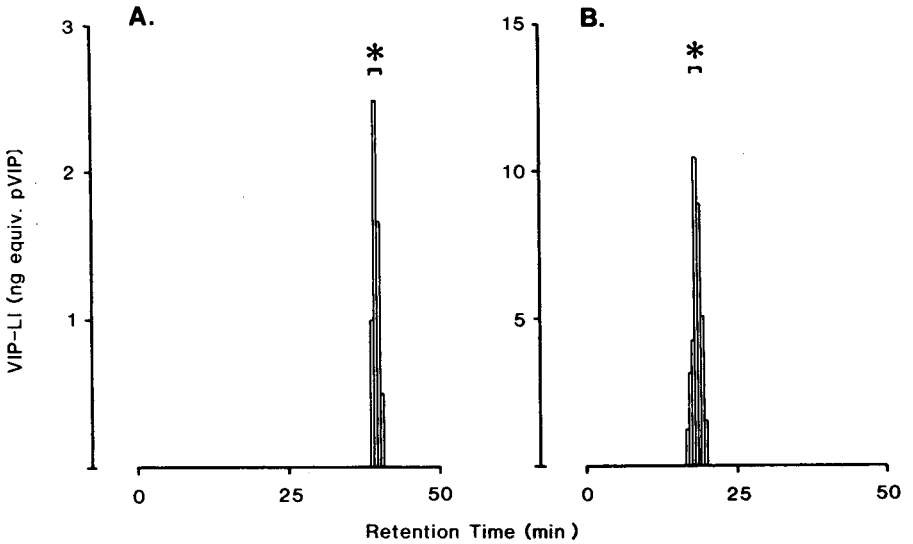

Fig. 3. Reversed-phase analysis of oxidation-protected guinea-pig gut extract. The histograms show VIP-like immunoreactive material recovered after reversed-phase HPLC analysis of a 2.0 *M* acetic acid extract (containing 0.01 *M* 2-mercaptoethanol) of guinea-pig ileum using a gradient (as shown in Fig. 1) of acetonitrile in (A) 0.15 *M* sodium chloride (pH 2.1 with hydrochloric acid), and (B) 0.5%, v/v, TFA (pH 1.9). The elution position of authentic pVIP is shown (*). The ordinates give the concentration of immunoreactive material in ng equiv. of pVIP per 0.5-ml fraction.

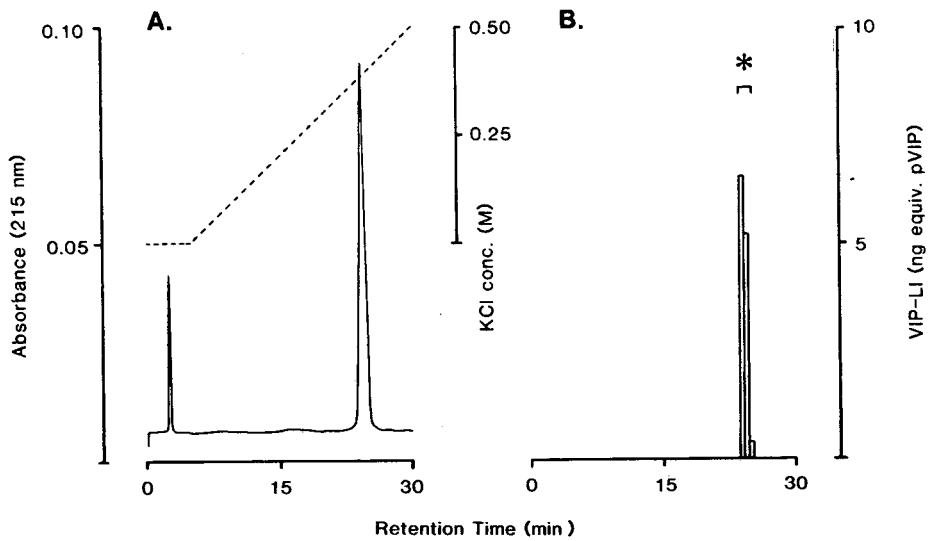

Fig. 4. Cation-exchange analysis of porcine VIP and guinea-pig gut extract. (A) Analysis of pVIP standard using a gradient of increasing potassium chloride concentration in 0.1 *M* phosphate buffer (pH 4.8), containing 10% acetonitrile. (- - -) Gradient profile (inset ordinate); (—) elution profile monitored by absorbance of the eluate at 215 nm (left hand ordinate), baseline-corrected. The amount of peptide injected on-column was 5 μg. (B) Histogram of VIP-like immunoreactivity recovered after cation-exchange analysis, using the above-mentioned gradient, of a 2.0 *M* acetic acid extract (containing 0.01 *M* 2-mercaptoethanol) of guinea-pig ileum. The concentration of material recovered is expressed in terms of ng equiv. of pVIP per 0.5-ml fraction (right hand ordinate). The elution position of authentic pVIP is shown (*).

Cation-exchange chromatography

Analysis of pVIP on the cation-exchange column, using an increasing gradient of potassium chloride in phosphate buffer, gave a single peak for the peptide (which was recovered quantitatively) with a retention time of 24.5 min (Fig. 4A). Analysis of an extract of guinea-pig small intestine, protected from oxidation with 2-mercaptoethanol, showed only one peak of immunoreactive material, eluting with the same retention time as authentic standard (Fig. 4B). Recovery of immunoreactive material was 70%.

Gel permeation chromatography

Analysis of pVIP on the gel permeation column showed only one peak for the peptide, eluting with K_0 = 0.65 (Fig. 5A), which was recovered quantitatively. Analysis of an extract of guinea-pig small intestine (extracted with aqueous acetic acid containing 2-mercaptoethanol) showed only one peak of immunoreactive material, again eluting in the same retention volume as the authentic standard (Fig. 5B). Recovery of immunoreactive material was 64%.

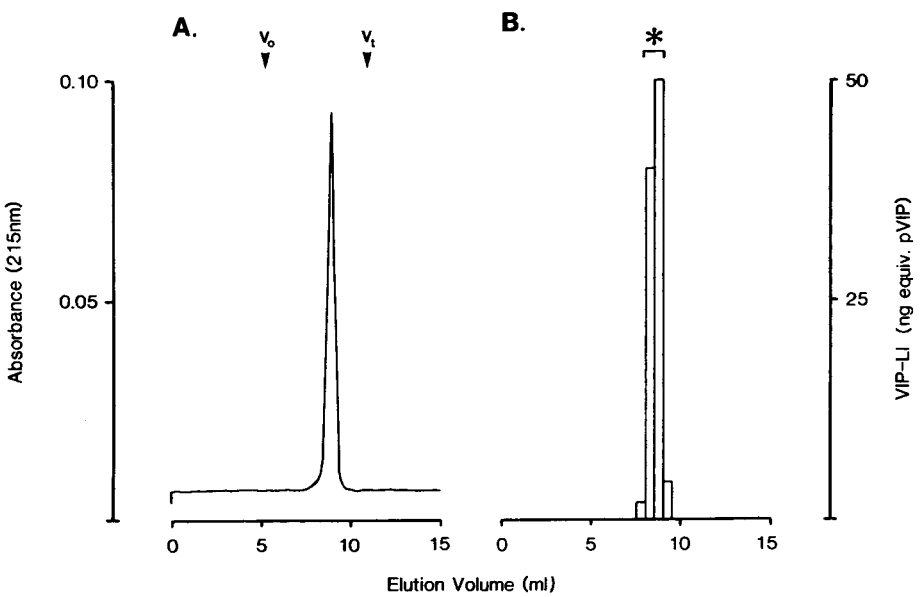

Fig. 5. Gel permeation analysis of porcine VIP and guinea-pig gut extract. (A) Analysis of pVIP standard eluted in 0.2 *M* sodium phosphate buffer (pH 2.1). The peptide (5 μg) was injected onto the column and its elution position detected by its absorbance at 215 nm (left hand ordinate). The void volume (V_0) and total volume (V_t) of the column are shown. (B) Histogram of VIP-like immunoreactivity recovered after gel permeation analysis of a 2.0 *M* acetic acid extract (containing 0.01 *M* 2-mercaptoethanol) of guinea-pig ileum. The elution position of authentic pVIP is shown (*). The right hand ordinate gives the concentration of immunoreactive material in ng equiv. per 0.5-ml fraction.

Radioimmunoassay of extracts

The mean concentration (± standard error) of immunoreactive material in extracts of whole wall of the guinea-pig small intestine was 66 ± 4 pmol/g wet weight of tissue for six animals.

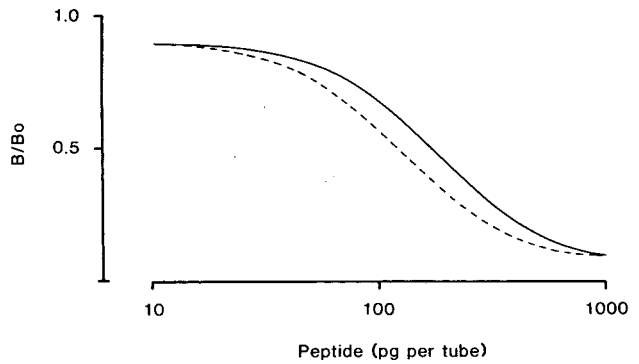

Fig. 6. Standard curves for radioimmunoassay of pVIP (—) and oxidized pVIP (- - -), plotted over the range 10—1000 pg of peptide per assay tube (abscissa). Binding of tracer (B) is plotted on the ordinate as a fraction of the amount bound in the absence of exogenous peptide (B_0). Displacement of 50% of bound tracer occurred with 170 pg of pVIP and 120 pg of oxidized pVIP.

The cross-reactivity of oxidized pVIP standard in the assay was found to be 140%, as shown in Fig. 6.

DISCUSSION

HPLC analysis indicates that the VIP-like immunoreactive material in extracts of the guinea-pig small intestine is identical to porcine VIP. Thus in the guinea-pig, which has been used extensively in studies of the physiological effects of neuropeptides, this clarification of the nature of the VIP-like immunoreactivity in nerves in the gut should lead to a better understanding of its postulated biological role as an enteric neurotransmitter [15].

The presence of an anti-oxidant such as 2-mercaptoethanol in the extraction medium has already been shown to be essential to maintain the chemical integrity of easily oxidized peptides [16], and this also appears to be the case for VIP. In the absence of an anti-oxidant, the apparent recovery of immunoreactive material is higher than when such a compound is included in the extraction medium (82% compared with 57%). However, only slightly more than half of this material chromatographs in the same place as the porcine standard. This apparent increase in recovery implies that the chemically altered forms of VIP (presumably oxidation products) are recognized and measured by this antiserum with greater efficacy than the native compound. That this explanation is correct is also indicated by the relative apparent recoveries of standard (95%) and oxidized standard (110%) from the chromatograph, and it was confirmed by comparison of standard curves constructed using both oxidized and native porcine standard (see Fig. 6). This difference in interaction has been noted for other, easily oxidized peptides, particularly methionine enkephalin [17], and may arise because of unintentional oxidation of a proportion of the peptide molecules during coupling to carrier molecules. Thus the antiserum raised in this case would be a mixture of antibodies directed at oxidation products in addition to the native peptide.

The reliability with which a particular antiserum can be used to measure, in

tissue extracts, the peptide against which it was raised will, then, depend upon two major factors: first, how well the chemical integrity of the peptide is maintained during conjugation to carrier, and secondly, how well the peptide's chemical integrity is maintained during extraction. VIP in particular is not only susceptible to chemical change during extraction, but has also been shown to be altered by some chromatography solvents [18].

A third factor which must also be taken into account is the cross-reactivity of the antiserum with peptides sharing some sequence homology with the antigen. Polyclonal antisera raised against large peptides (such as VIP) will contain components showing an immunochemical response to multiple sites in the amino acid sequence of the peptide, and consequently will increase the opportunities for cross-reactivity with other peptides. Thus precursors and/or metabolites of the peptide in question may contribute at least a portion of the measured tissue levels of immunoreactive material, and other unrelated peptides with limited sequence homology may similarly result in over-estimates of tissue concentrations.

Such differences in immunogenecity and chemical form may explain the large variation in reported tissue concentrations of VIP-LI in guinea-pig gut [1, 12], and suggest caution in the comparison of values obtained using different antisera and differing methods of extraction. Thus careful characterization of antisera, and the use of physicochemical techniques such as HPLC to separate peptides on the basis of their chemistry, are essential to the preliminary characterization of peptides. Isolation, purification and sequencing provide the ultimate chemical characterization of a peptide, and allow a better understanding of its biological role.

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SEPARATION OF TRYPTIC PHOSPHOPEPTIDES OF RIBOSOMAL ORIGIN BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Phosphorylation sites for cyclic AMP-dependent kinase in ribosomal proteins and their synthetic analogues were converted to tryptic phosphopeptides and analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) using gradients of acetonitrile in water and 0.1% trifluoroacetic acid. Tryptic variants differing by only NH_2-terminal basic amino acid residues or phosphoryl groups were not always well resolved under these conditions. The different phospho forms could be resolved by RP-HPLC in phosphate buffers at pH 7.0. A combination of gel permeation chromatography, RP-HPLC and thin-layer cellulose mapping was found to be the most effective strategy for the absolute purification of tryptic phosphopeptides from crude tryptic digests.

INTRODUCTION

A prominent early event following the stimulation of mammalian cells with various polypeptide growth factors is the phosphorylation of ribosomal protein S6 at multiple sites [1—8]. Identification of the growth-associated S6 kinases requires knowledge of their specific phosphorylation sites. The sites can be selectively cleaved from whole ribosomes with trypsin [8—11]; this strategy has led to the isolation and characterisation of the sites for cyclic AMP-dependent protein S6 kinases [9, 11] (for other S6 kinases see ref. 12). In rat liver ribosomes, these and other sites appeared to be clustered within the sequence Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala-Ser-Thr-Ser-Lys-Ser-Glu-Glu-Ser-Gln-Lys [11]. The first serine is the preferred site for cyclic AMP-dependent protein kinase, with the second and third serines being phosphorylated at high concentrations of the kinase in vitro [9, 11].

Tryptic digestion of S6 phosphorylated in this region generates a complex mixture of structurally related phosphopeptides [9, 11]. The variety of peptides originating from the same region is attributable to the clustering of sites within the region, the presence of several potential tryptic sites and the inhibi-

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tory influence of neighbouring phosphoryl groups on certain tryptic cleavages [11].

The isolation and characterisation of each of the tryptic phospho-derivatives are necessary steps in the interpretation of phosphorylation patterns for the different S6 kinases. An important purification step has been reversed-phase high-performance liquid chromatography (RP-HPLC) using C_{18}-bonded micro-silica columns [13—16] eluted with gradients of acetonitrile in water containing the hydrophobic ion-pairing reagent trifluoroacetic acid (TFA) [15, 16]. Under these conditions, the order of elution of small peptides (<20 residues) is closely correlated with their amino acid compositions [16]. Predicted retention coefficients for individual residues [16—18] suggest that it should be possible to resolve peptides of the types encountered in tryptic digests of phosphorylated S6 that differ in structure by only a single basic or phosphorylated amino acid residue. However, the statistically determined retention coefficients do not allow for interactions within sequences and the overall conformational features of peptides that might also influence retention.

Here we investigate the potential of RP-HPLC for resolving structurally related tryptic variants of S6. The elution characteristics of synthetic peptide analogues of the region of S6 containing the phosphorylation sites for cyclic AMP-dependent kinase were also studied. The effects of phosphoryl groups on column retention were investigated using material phosphorylated with cyclic AMP-dependent protein kinase in vitro.

EXPERIMENTAL

Phosphopeptides of ribosomal origin

Liver ribosomes were prepared as in ref. 9 from 10—20 weeks old male Wistar rats which had been starved overnight. The ribosomes were phosphorylated with [γ-^{32}P]ATP and the purified catalytic subunit of cyclic AMP-dependent protein kinase from beef heart (a gift from Dr. B.E. Kemp) under conditions resulting in the transfer of approximately 2 mol phosphate per mol S6 [11]. Partial tryptic peptides containing the phosphorylation sites were selectively cleaved from whole ribosomes by mild tryptic digestion [9, 11] and the peptides fractionated according to their size by gel permeation chromatography on Sephadex G-25 in 1 *M* acetic acid [9].

Synthetic peptides

The peptides were synthesised by B.E. Kemp using the Merrifield solid phase synthesis procedure [20] in a Beckman 990 synthesiser. Peptides S6-2 and KS6-2 were prepared as the COOH-terminal amide form whereas S6-1 and KS6-1 and S6-Y were in the free COOH-terminal form. The general properties of the peptides and their abilities to serve as substrates for cyclic AMP-dependent kinase will be detailed separately [19]. The stoichiometry of peptide phosphorylation was determined by binding to phosphocellulose papers and counting the radioactivity by liquid scintillation spectrometry [21]. The assignment of phosphorylation sites was made on the basis of isoelectric focusing properties of tryptic peptides [9, 11] and data obtained from automated sequence analysis [22]. Tryptic digestions of phosphorylated peptides were performed in 0.1 *M* NH_4HCO_3 for 16 h at 30°C (trypsin, 50 μg ml^{-1}).

HPLC

Peptides were analysed by RP-HPLC using a Varian delivery system and UV monitor set at 214 nm with a μBondapak C_{18} column (Waters Assoc.). In standard analyses (Table I), the column was eluted with a linear gradient of aqueous acetonitrile containing 0.1% trifluoroacetic acid (TFA), with the acetonitrile concentration increasing at a rate of 1% min^{-1}. The flow-rate was constant at 1 ml min^{-1}. Samples were injected 1 min prior to the commencement of the gradient. ^{32}P-radioactivity associated with phosphorylated peptides was monitored by Cerenkov counting of HPLC fractions (1 ml) in a liquid scintillation spectrometer. The elution position of each peptide was expressed as retention time (t_R). Determinations of t_R were performed in triplicate; the S.E. in each case was less than 0.1 min. The recoveries of phosphopeptides in fractions eluted from HPLC columns were generally > 90%. However, the overall yields of the more hydrophobic phosphopeptides (e.g. the Peak II compared with the Peak I peptides described in Fig. 1) were appreciably lower due to selective losses (up to 80%) during lyophilisation steps.

TABLE I

RP-HPLC OF RIBOSOMAL TRYPTIC PEPTIDES AND THEIR SYNTHETIC ANALOGUES

Peptide	Sequence***	t_R (min)
Ribosomal*		
$T2_A$	RRLS(P)S(P)LR	20.0
$T2_B$	RLS(P)S(P)LR	22.5
$T2_C$	RLS(P)SLR	22.0**
$T1_A$	RLS(P)S(P)LRAS(P)TSK	22.5
$T1_C$	RLS(P)S(P)LRASTSKSEESQK	19.0
Synthetic*		
KS6-2	KRRLSSLRASTSKS(NH_2)	20.8
	KRRLS(P)SLRASTSKS(NH_2)	20.8
S6-2	RRLSSLRASTSKS(NH_2)	20.7
KS6-1	KRRLSSLRA	22.6
S6-1	RRLSSLRA	23.2
	RRLS(P)SLRA	22.9
	RRLS(P)SLR	22.0
	RLS(P)SLR	22.3**
	LSS(P)LR	19.4
	LSSLR	
S6-Y	KRRASSLKA	19.5

*5 nmol peptide analysed with a linear gradient of acetonitrile in aqueous 0.1% TFA increasing at an acetonitrile concentration of 1% min^{-1}.

**Different μBondapak columns were used for the analyses of ribosomal and synthetic peptides, hence the reason for the slight difference in t_R for the RLS(P)SLR species.

***Amino acid residues are designated by the single-letter code of Dayhoff [26]; the positions of phosphoryl serine residues are denoted by S(P).

Different phosphorylated forms of peptides were separated by RP-HPLC using a μBondapak C_{18} column eluted with gradients of acetonitrile in aqueous potassium phosphate buffer at pH 7.0. Solution A was 50 m*M* potassium phosphate. The percentage of solution B was increased as described in the legends to Figs. 6 and 7.

Other analytical procedures

Two-dimensional mapping of phosphopeptides was carried out on thin-layer cellulose plates (Macherey-Nagel) using electrophoresis at pH 4.4 [23] as the first dimension and chromatography in butanol—acetic acid—water—pyridine (15:3:12:10) as the second dimension [24]. Amino acid analyses were performed on an LKB Biochrom 4400 amino acid analyser and automated Edman sequence analyses on an Applied Biosystems Model 470A gas—liquid phase sequencer.

RESULTS

Isolation of tryptic peptides of ribosomal origin

RP-HPLC using a Waters C_{18} μBondapak column with gradients of acetonitrile in water and 0.1% (v/v) TFA was only partially effective for resolving tryptic peptides of ribosomal origin [9, 11]. The major problems encountered related to the difficulty of separating individual species within families of structurally related tryptic variants containing multiple phosphorylation sites. This is illustrated with the case of the larger phosphopeptides released during mild

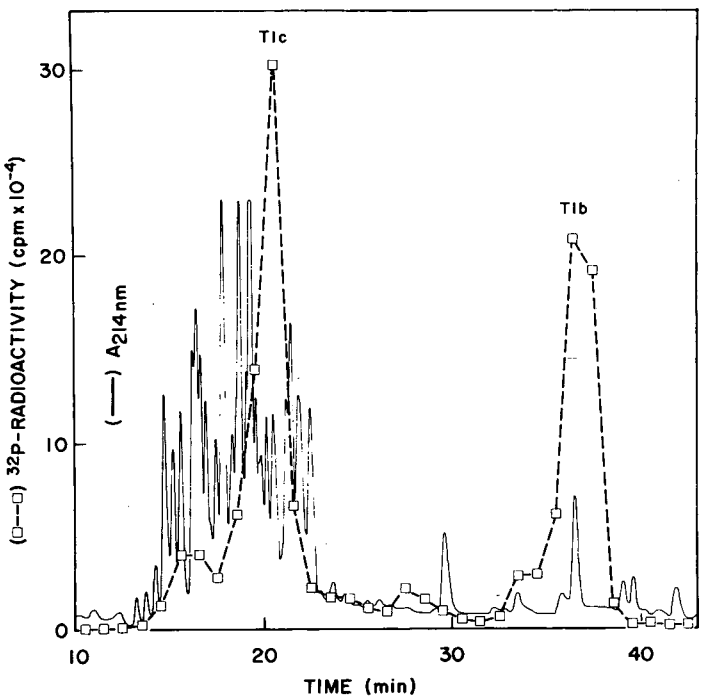

Fig. 1. Fractionation of phosphopeptides selectively cleaved from ribosomes by mild tryptic digestion. Phosphorylated ribosomes (50 nmol) were digested with trypsin for 20 sec, and the large phosphopeptide fraction (V_e/V_0 = 1.2—1.35) obtained by gel filtration on Sephadex G25 as described in ref. 11. The phosphopeptides were analysed by RP-HPLC using a discontinuous gradient of acetonitrile in 0.1% TFA; the concentration of acetonitrile increased at 1.5% min^{-1} from 0 to 10 min after injection and at 0.5% min^{-1} from 10 to 50 min. The phosphopeptide fractions denoted as T1c and T1b in the figure are referred to as the Peak I and Peak II fractions, respectively, in the text.

tryptic digestion of ribosomes phosphorylated with cyclic AMP-dependent protein kinase.

The tryptic peptides were sized by gel chromatography on Sephadex G-25. The larger peptides (V_e/V_0 = 1.20—1.35) [11] were analysed by RP-HPLC using a gradient of acetonitrile increasing at 0.5% min^{-1} in water—0.1% TFA (Fig. 1). Two major peaks of ^{32}P-labelled peptides were eluted with retention times of 24 min (Peak I) and 33 min (Peak II) (cf. the retention times quoted in Table I which were determined with acetonitrile gradients increasing at 1% min^{-1}).

The peak I fraction has been shown previously to contain three species of phosphopeptides [11]. These were only partially resolved by further HPLC using a discontinuous gradient of acetonitrile in aqueous 0.1% TFA (Fig. 2). Complete resolution of the three phosphopeptides was eventually achieved by two-dimensional mapping on thin-layer cellulose plates (Fig. 3a). The thin-layer cellulose step also resolved the radioactive species from several non-phosphorylated species which coeluted with the phosphopeptides during RP-HPLC (Fig. 3a). Structural analyses showed that the three phosphorylated species represented the mono-, di- and triphospho derivatives of the sequence Arg-Leu-Ser-Ser-Leu-Arg-Ala-Ser-Thr-Ser-Lys-Ser-Glu-Glu-Ser-Gln-(Lys) originating from ribosomal S6 [11].

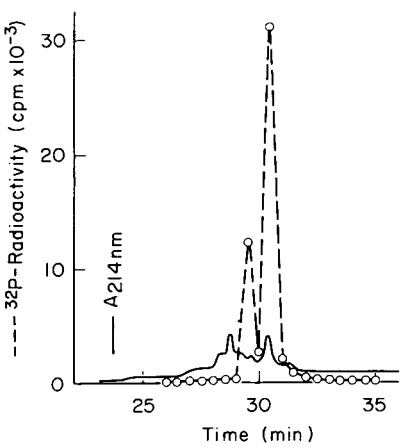

Fig. 2. The Peak I (T1c) phosphopeptides (sample size ca. 2 nmol) described in Fig. 1 were analysed by RP-HPLC using a discontinuous gradient of acetonitrile in aqueous 0.1% TFA; the acetonitrile concentration increased from 0 to 12% during the period from 0 to 10 min after injection, remained constant at 12% from 10 to 20 min, increased from 12 to 15% from 20 to 23 min and, thereafter, was held constant at 15%.

The Peak II phosphopeptides eluted as a single broad peak during further RP-HPLC analyses using various forms of discontinuous gradients of acetonitrile, water and 0.1% TFA (not illustrated). However, thin-layer cellulose mapping showed that several species of phosphopeptides were present in the Peak II fraction (Fig. 3b).

The major species (spot 4, Fig. 3b) was only recovered in sufficient quantities for microsequence analysis by automated Edman degradation. The first

13 residues were identified as Ala-Ile-Thr-Gly-Ala-Ser-Leu-Ala-Asp-Ile-Met-Ala-Lys. Several observations showed that the other species were structurally related tryptic variants of the spot 4 sequence. First, the number of species was reduced to two by further tryptic digestion (50 μg trypsin per millilitre for 8 h at

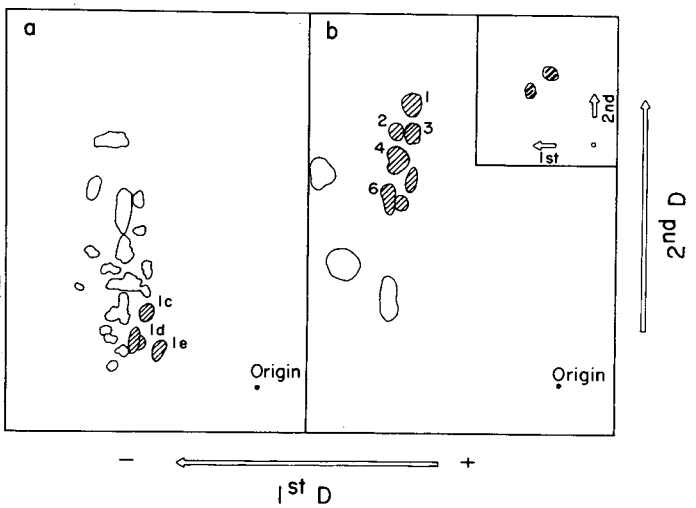

Fig. 3. Thin-layer cellulose mapping of ribosomal tryptic peptides. a and b, Peaks I and II phosphopeptide fractions, respectively, as defined in Fig. 1; insert in b, Peak II peptide fraction subdigested with trypsin as described in the text. The open areas describe peptide spots stained with fluorescamine whereas the hatched-in areas represent ^{32}P-labelled phosphopeptides detected by autoradiography.

TABLE II

COMPOSITIONS OF THE TRYPTIC DERIVATIVES OF PEAK II PHOSPHOPEPTIDES

Hydrolyses were done in 5.7 *M* HCl—5 m*M* phenol for 24 h at 110°C. Serine and threonine were corrected for 10% and 5% destruction, respectively. Results were expressed as mol amino acid per mol peptide after assuming that T1b and T1b′ represented 13- and 14-residue peptides, respectively. Impurities <0.2 mol are omitted. The values in parentheses represent the composition of T1b reported previously [9].

Amino acid	Peptide*	
	T1b	T1b′
Asp/Asn	0.7 (1.4)	1.3
Thr	1.0 (1.1)	0.7
Ser	1.5 (1.2)	1.4
Glu/Gln	0.5 (0.9)	0.6
Gly	1.7 (1.2)	1.9
Ala	2.8 (3.6)	2.7
Val	0.7 (0.7)	0.6
Met	1.2 (0.2)	1.0
Ile	1.0 (1.6)	0.9
Leu	0.7 (1.1)	1.3
Lys	1.2 (1.0)	1.7

*Peak II peptides isolated by RP-HPLC (Fig. 1) were subdigested with trypsin and the resulting phosphopeptides isolate by thin-layer cellulose mapping (Fig. 3b, insert).

30°C) of the original Peak II peptides. The two species could not be resolved by RP-HPLC but were clearly resolved by thin-layer cellulose mapping (Fig. 3b, insert). While the amino acid compositions of the peptides indicated that they were still not pure, it was apparent that the two species were closely related probably differing by only a single lysine residue (Table II). The compositions also showed that the two species were related to the spot 4 sequence identified in the undigested Peak II fraction (Fig. 3b) and to the previously isolated peptide T1b [9].

Elution characteristics of synthetic analogues of ribosomal S6

The investigation described above has provided examples of two problems often encountered during the purification of tryptic derivatives of ribosomal phosphoproteins by RP-HPLC. The problems relate to the unsatisfactory resolution of structurally related peptides differing only by number of phosphoryl groups or by terminal basic amino acid residues. To gain a further understanding of these problems, the chromatographic properties of a range of structurally related synthetic peptide analogues of the region of ribosomal S6 giving rise to the Peak I tryptic peptides (see Fig. 1 and Table I) were investigated. In some cases, the peptides were phosphorylated with the cyclic AMP-dependent protein kinase under conditions in which the first serine residue in the peptides was fully phosphorylated [19].

Terminal basic residues

The influence of additional NH_2-terminal lysine residues was investigated by comparing the elution characteristics of the pairs of related synthetic peptides S6-1/KS6-1 and S6-2/KS6-2 using linear gradients of acetonitrile in water and

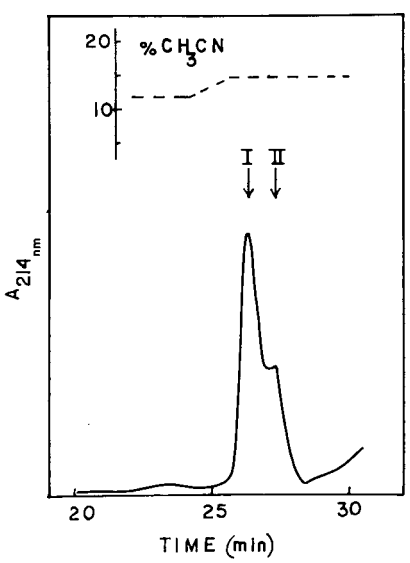

Fig. 4. Resolution of synthetic peptides KS6-2 and S6-2 by RP-HPLC using the discontinuous gradient of acetonitrile (in 0.1% TFA) described in the figure. The order of elution was S6-2 (10 nmol injected) followed by KS6-2 (5 nmol).

0.1% TFA (Table I). The addition of a lysine residue to the S6-1 sequence (to make KS6-1) decreased the t_R by 0.4 min. However, the addition of lysine to the larger S6-2 sequence slightly increased the t_R (by 0.1 min). The influence of the additional lysine in the latter case was more apparent when a mixture of the peptides was partially resolved using a discontinuous gradient of acetonitrile in water and 0.1% TFA (Fig. 4).

The inconsistent effects of NH_2-terminal basic residues was even more apparent with smaller phosphopeptides generated by tryptic digestion of phosphorylated S6-1 (Fig. 5, Table I). In particular, the Leu-Ser-Ser(PO_4)-Leu-Arg and Arg-Leu-Ser(PO_4)-Ser-Leu-Arg species were well resolved (t_R values differed by 2.9 min). However, the presence of a second NH_2-terminal arginine to make the Arg-Arg-Leu-Ser(PO_4)-Ser-Leu-Arg species actually decreased the t_R by 0.3 min.

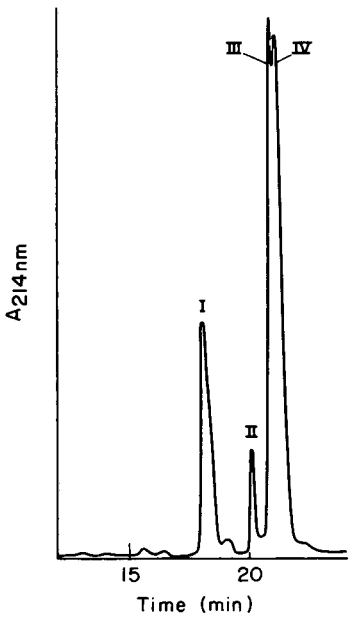

Fig. 5. Tryptic digest of phosphorylated synthetic peptide S6-1 (10 nmol) analysed by RP-HPLC in a discontinuous gradient of acetonitrile and aqueous 0.1% TFA; the acetonitrile concentration increased at 2% min^{-1} during the initial 5 min and at 0.5% min^{-1} thereafter. The ^{32}P-radioactivity was associated with Peaks I, III and IV which were found by automated Edman degradation analyses to have the amino acid sequences Leu-Ser-Ser-Leu-Arg, Arg-Arg-Leu-Ser-Ser-Leu-Arg and Arg-Leu-Ser-Ser-Leu-Arg, respectively. Isoelectric focusing analysis in polyacrylamide gels [9] showed that the phosphopeptides were predominantly in the monophosphorylated forms; the position of the phosphate was assigned to the first serine residue on the basis of the appearance of ^{32}P-radioactivity and serine adducts in the cleavage products at each cycle of the Edman degradation [22].

Effects of phosphoryl groups in the TFA system

The phosphorylation of synthetic peptides either slightly decreased or had no effect on the t_R values during RP-HPLC using acetonitrile gradients in 0.1% TFA (Table I). For example, the t_R for the phospho form of S6-1 was just

0.3 min less than that for unphosphorylated S6-1 whereas with the larger S6-2 and KS6-2 species the phosphorylated and unphosphorylated forms appeared to coelute. In what appears to be an unusual case, the t_R for the ribosomal peptide Arg-Leu-Ser(PO_4)-Ser(PO_4)-Leu-Arg was actually greater by 0.5 min than that for the monophospho form of the same peptide [9] (Table I).

Resolution of phosphopeptides using phosphate buffers of pH 7

The different phosphorylated and unphosphorylated forms of peptides were resolved by RP-HPLC on a C_{18} column using acetonitrile gradients in aqueous buffers at pH 7.0 to maximise the negative influence of charged phosphoryl groups (Figs. 6 and 7). Both potassium phosphate and triethylammonium phosphate (TEAP) buffers [13] (at 50 m*M*) have been employed successfully although the peptide peaks under these conditions were generally broader than those eluted with 0.1% TFA conditions. TEAP buffers gave sharper peptide peaks particularly with late-eluting peptides; however, potassium buffers were preferred because of the relative absence of impurities which interfered with UV monitoring at high sensitivity settings. Peptides isolated in potassium phosphate gradients were desalted as required by re-chromatography using aqueous acetonitrile gradients in 0.1% TFA.

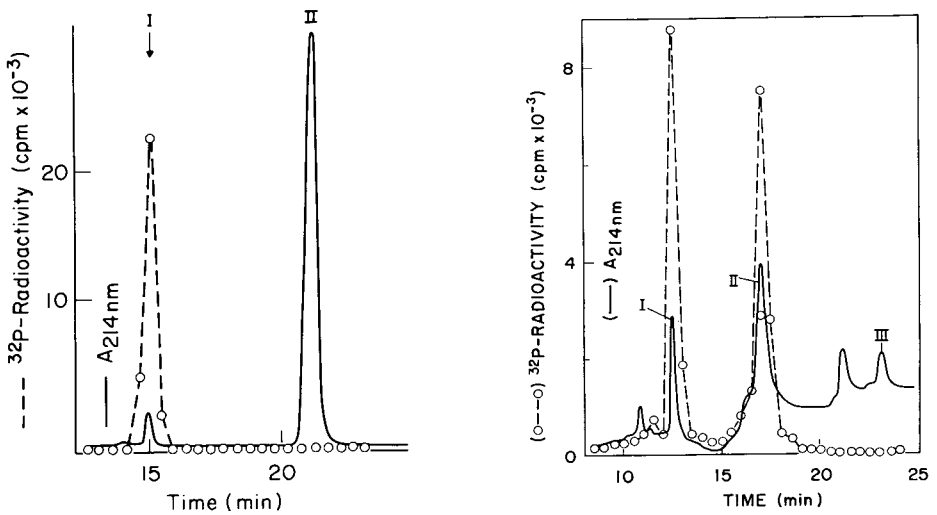

Fig. 6. Separation of phosphorylated and unphosphorylated forms of a tryptic derivative of S6-1 by RP-HPLC. The peaks containing the Leu-Ser-Ser-Leu-Arg peptides (Peak I; Fig. 4) were resolved using a gradient of acetonitrile in aqueous 50 m*M* potassium phosphate, pH 7.0. The acetonitrile concentration increased at 1% min^{-1} for the initial 7.5 min and at 0.25% min^{-1} therafter. The flow-rate was held constant at 1 ml min^{-1}. Both the peptides resolved (Peaks I and II) were found to have the amino acid sequence Leu-Ser-Ser-Leu-Arg by automated Edman degradation analyses.

Fig. 7. Analysis of phosphorylated synthetic peptide S6-Y (Table I) by RP-HPLC using phosphate-buffered gradients of acetonitrile gradients. The stoichiometry of phosphorylation of the peptide was 1.4 mol phosphate per mol peptide. The acetonitrile concentration increased at 1.5% min^{-1}. The flow-rate was constant at 2 ml min^{-1}.

The effectiveness of the procedure, using 50 m*M* potassium phosphate buffer with a gradient of acetonitrile increasing at 0.25% min^{-1}, is illustrated for the monophosphorylated and unphosphorylated forms of the pentapeptide Leu-Ser-Ser-Leu-Arg generated during trypsin digestion of phosphorylated S6-1 (Fig. 6). Mono- and diphospho forms of peptides were also well resolved. For example, preparations of the peptide S6-Y (Table I) phosphorylated with the cyclic AMP-dependent protein kinase (stoichiometry, 1.4 mol phosphate per mol peptide), yielded two peaks of phosphopeptides (Fig. 7), which were identified as the monophospho (early eluting peak) and diphospho (late eluting) forms according to their specific radioactivities. In this experiment, the rate of increase of the acetonitrile gradient was increased to 1.5% min^{-1}, to counter the problem of decreasing peak sharpness encountered with larger and more basic peptides.

DISCUSSION AND CONCLUSIONS

RP-HPLC has been used effectively in the purification of tryptic phosphopeptides originating from ribosomal S6. However, structurally similar derivatives differing only by single basic residues or phosphoryl groups were not always well resolved under the TFA buffer conditions employed.

Phosphoryl groups have been found previously to appreciably decrease retention of small peptides using TFA [16] as well as heptafluorobutyric acid [16] and *n*-hexanesulphonic acid [25] as ion-pairing reagents. Similarly, the association of sulphate groups with tyrosine residues has been observed to appreciably decrease the retention of peptides in RP-HPLC under acidic conditions [17]. The relatively small influence of phosphoryl groups on retention of ribosomal peptides may be at least partly due to intramolecular ion pairing between phosphoryl and closely situated guanidinium groups. Such an interaction could explain the apparently anomalous case where the diphospho-hexapeptide Arg-Leu-Ser(PO_4)-Ser(PO_4)-Leu-Arg was retained more than the monophospho derivative of the same sequence (Table I) [9].

The minimal effects of basic residues on retention were puzzling in view of the potential ion pairing between these residues and TFA ions leading to increased retention. Meek and Rossetti [17] have suggested that a concentration of 0.1% TFA is insufficient for efficient ion pairing between basic residues and TFA ions. However, the addition of arginine at the NH_2-terminus of the monophosphorylated Leu-Ser-Ser-Leu-Arg peptide increased retention appreciably (Table I) as might have been expected if ion pairing had occurred. In contradiction, the extension of the same sequence with a second NH_2-terminal arginine residue slightly decreased retention suggesting that ion pairing with TFA ions may be less effective in the region of adjacent arginine residues. A similar phenomenon could explain the minimal effects of lysine residues added to the NH_2-terminal of the S6-1 and S6-2 synthetic peptide analogues of S6 (Table I).

The results of this investigation show that RP-HPLC with acetonitrile gradients in TFA and phosphate buffers can be used to resolve many of the tryptic variants likely to be generated from phosphorylated S6. However, the difficulties in resolving certain peptides differing only in single basic amino acid and

phosphoryl residues has led to the use of thin-layer cellulose mapping (following the RP-HPLC step) to check sample purity and, where required, as the final step in the purification of ribosomal tryptic phosphopeptides.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND STUDIES OF NEUROPHYSIN—NEUROHYPOPHYSIAL HORMONE PATHWAYS

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SUMMARY

High-performance liquid chromatography (HPLC) is being used extensively to characterize active polypeptides, precursor processing mechanisms, and cooperative peptide—protein noncovalent complexes in neuroendocrine pathways for neurohypophysial peptide hormones, oxytocin and vasopressin, and the hormone-associated proteins, neurophysins. Reversed-phase and ion-exchange HPLC polypeptide mapping have been used to detect the hormones, associated proteins, and other molecular forms containing these. This mapping has provided a means not only to isolate these molecules when present in micro amounts but also ultimately to identify anatomical sites which contain the neurophysin/hormone molecular pathways and to define the relatedness of polypeptide forms contained in different pathways. Reversed-phase HPLC also has provided a means to study proteolytic precursor processing, both to isolate synthetic and semisynthetic polypeptides prepared for use as substrates in processing reactions and eventually to study the polypeptides and intermediates produced by these reactions. Finally, bioaffinity HPLC is being evaluated as a separatory and analytical tool. The latter includes its use to characterize the noncovalent peptide—protein and protein—protein interactions which occur among the molecular forms of the neurophysin/hormone pathways. These experiments typify the impact of HPLC for both analytical and preparative separations in studies of biologically active peptides and proteins.

INTRODUCTION

The neurohypophysial peptide hormones, oxytocin and vasopressin, and the associated small proteins, neurophysins, comprise molecular components of pathways which produce neuroendocrine-active polypeptides for delivery to target sites [1—10]. In the classical, central nervous system neuronal pathway,

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the hormones are made biosynthetically in cell bodies within the hypothalamus, each with one of the two major neurophysins as parts of a single-chain hormone/neurophysin biosynthetic precursor. Folded precursors are packaged in neurosecretory granules and translocated axonally to the neurohypophysis, with concomitant proteolytic processing to generate hormone—neurophysin noncovalent complexes. The complexes remain in the granules at the nerve terminals, as storage forms, until exocytotic release into the circulation, and the hormones subsequently are delivered to receptors at peripheral targets. The scheme of Fig. 1 gives an overall view of the molecular events, leading from biosynthetic precursors to processed polypeptides, which occur in such a pathway. The occurrence of neurophysin/hormone pathways in anatomical sites other than neurons leading from hypothalamus to neurohypophysis, and the physiological meaning of these other pathways, are receiving increased attention. In addition, the molecular mechanisms defining neurophysin/hormone pathways, including structural transitions leading from biosynthetic precursors to biologically active peptides, noncovalent interactions between polypeptide components, and the relatedness of these mechanisms among different pathways, continue to be much-studied subjects.

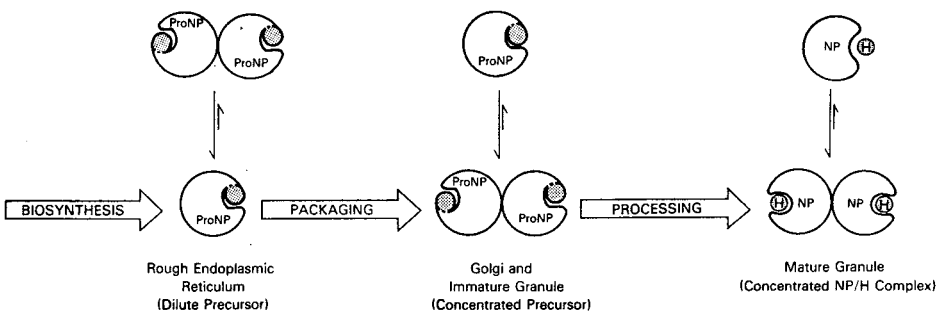

Fig. 1. Schematic diagram depicting the molecular events occurring in neurophysin/neurohypophysial hormone (NP/H) pathways. Overall processes of biosynthesis, packaging into neurosecretory granules, and proteolytic processing of neurophysin/hormone precursors are shown. Precursors are viewed as folded to form intramolecular interactions between hormone and neurophysin domains, with these molecules able to self-associate. The latter interaction may modulate the concentrations of hormones within granules as well as precursor processing events. Proteolytic processing within granules leads to mature hormone—neurophysin noncovalent complexes which also self-associate to an extent which is enhanced by hormone binding. The molecular events viewed to occur in such pathways are based largely on characterization of the most abundant case, hypothalamo-neurohypophysial neurons. The degree of similarity of this scheme to molecular mechanisms in other hormone/neurophysin pathways is not yet fully established. (Reproduced from ref. 11, with permission.)

MOLECULAR COMPONENTS OF MULTIPLE NEUROENDOCRINE PATHWAYS

Recent use of reversed-phase (RP-HPLC) and ion-exchange HPLC for protein and peptide mapping has allowed micro-detection and -isolation of neurophysins, hormones, and molecularly related species. For example, RP-HPLC has provided a valuable tool for detecting neurophysins and differentiating their isoforms, as shown in Fig. 2 for fractionation of bovine

posterior pituitary extracts on a C_8 column. Here, at least two major neurophysin II (vasopressin-associated) and four major neurophysin I (oxytocin-associated) species have been detected. The pattern of isoforms seen in these maps is relatively similar from preparation to preparation (see Fig. 2). Similar isoform separation has been accomplished with C_3 and C_{18} reversed-phase matrices as well as a Pharmacia Mono Q ion-exchange column. All of the above major RP-HPLC isoforms, and several of the minor ones eluting in between, are active, as judged by their ability to bind to peptide ligand affinity columns. The assignment of species as bovine I versus II has been made by comparing amino acid compositions with those expected from the known bovine neurophysin sequences [13, 14]. However, the precise structural differences between species within each of the neurophysin I and II families remain to be established fully. In this regard, limited proteolysis followed by RP-HPLC peptide mapping and analysis of isolated peptide fragments is being used. The data obtained so far suggest that at least some of the microheterogeneity within the neurophysin I family is due to proteolytic truncation at the carboxyl terminus; such truncation perhaps ensues upon prolonged storage of the neurophysins in secretory granules.

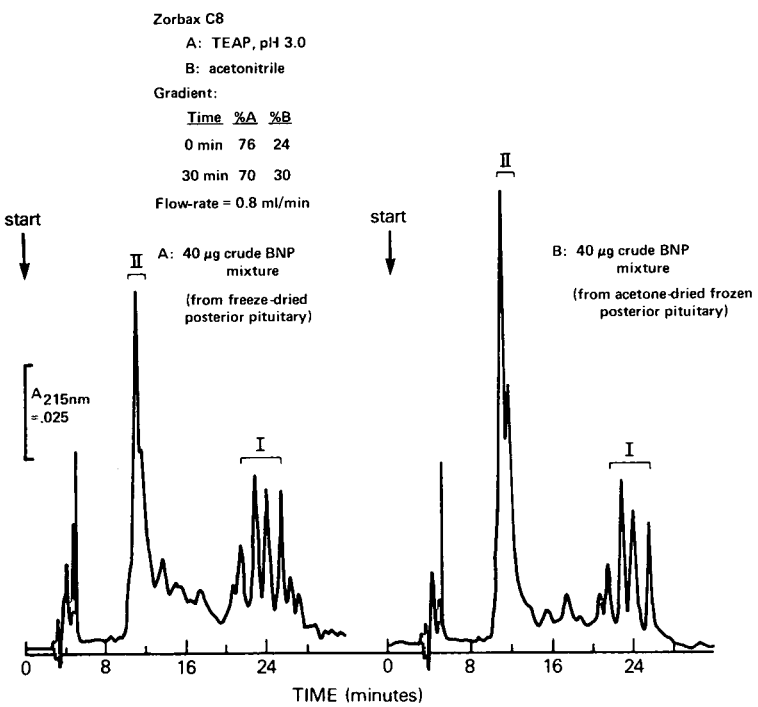

Fig. 2. Reversed-phase HPLC separation of bovine neurophysin (BNP) isoforms. Crude neurophysins were obtained by conventional methods of acid extraction of either (A) freeze-dried posterior pituitaries or (B) acetone-dried frozen posterior pituitaries, salting out of extracts with sodium chloride, and fractionation of dissolved precipitates on Sephadex G-75 in 1 *M* formic acid [12]. Neurophysin fractions from the gel exclusion separations were dissolved in triethylammonium phosphate (TEAP), pH 3.0, and separated on an analytical Zorbax C8 column (Dupont, No. 6119, 25 × 0.46 cm, 6 µm particle size) as denoted. Designations of peaks as neurophysin I- and II-related are based on comparisons of amino acid analyses of individual peaks.

Overall, our recent experience with C_8 and other matrices, along with previous reports with C_{18} [15, 16], emphasize the value of HPLC for detection of neurophysins and neurohypophysial hormones from posterior pituitary extracts. Given the particularly high resolving power of reversed-phase separations such as for the C_8 matrix in Fig. 2, it is encouraging that the proteins obtained from the C_8 fractionation remain functionally active after exposure to the solvents used.

RP-HPLC polypeptide mapping has been used to examine extracts obtained from ovary and peripheral nerve tissue as a means to demonstrate the presence in these anatomical sites of hormone- and neurophysin-related molecular species [17, 18]. For example, RP-HPLC fractionation of bovine ovary extract and analysis of eluted fractions by radioimmunoassay with oxytocin and bovine neurophysin I antibodies has yielded elution profiles such as shown in Fig. 3. The correspondence of elution positions of major immunoreactive species with autentic oxytocin and bovine neurophysin I strongly suggests that substantial amounts of intact or close-to-intact forms of the hormone/neurophysin pair are present in the ovary. This observation agrees with previous reports of oxytocin in the ovary [21, 22]. A developing view from data such as these is that oxytocin and its associated neurophysin are derived by a pathway independent of sources in peripheral circulation. This view is supported by the finding, using an analysis similar to that in Fig. 2, that far more neurophysin I

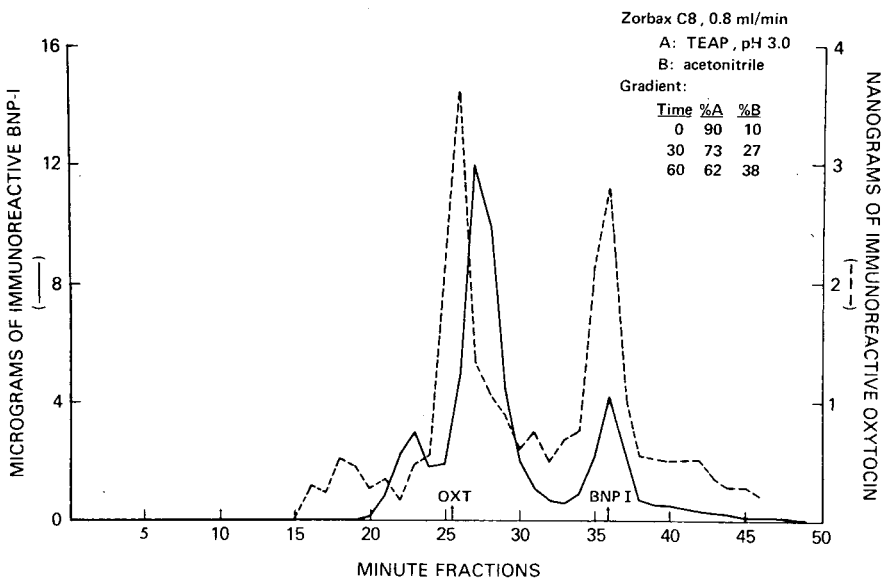

Fig. 3. Reversed-phase HPLC identification of oxytocin and oxytocin-associated neurophysin (neurophysin I) in extracts from bovine ovary tissue. Acid extracts of fresh bovine ovaries were fractionated using a C-18 Sep Pak (Waters), with adsorption in 0.1 *M* acetic acid and elution with ethanol—acetic acid—water (90:4:6, v/v) [19]. The latter fraction (from 0.4 g ovary tissue), after solvent removal, was fractionated on an analytical Zorbax C8 column (No. 6119) as denoted in the figure. Dried fractions were assayed for competition either with [^{125}I]bovine neurophysin I for binding to anti-bovine neurophysin I or with [^{125}I]oxytocin for binding to anti-oxytocin. Radioimmunoassays were carried out by methods similar to those described before [20].

than II is present in ovary extracts and that these relatively large amounts are unlikely to be derived by uptake from the circulation. Also, chromatograms such as that in Fig. 3 show the presence of hormonal immunoreactivity in a peak eluting close to neurophysin, a peak which may represent a larger, precursor-like form of oxytocin (based on elution characteristics of semi-synthetic oxytocin—neurophysin precursor [23]). Such data as the above indicate that a neurophysin/hormone pathway separate from the classical pathway of hypothalamus-to-neurohypophysis generates ovarian oxytocin. One view currently being examined is that the biosynthesis-processing pathway may occur within the ovary itself.

PATHWAYS OF PRECURSOR PROCESSING

While the structure of hormone/neurophysin precursor (see Fig. 4) now has been defined through studies by in vitro translation [24], pulse-labelling [7], and, ultimately, molecular cloning [25], the proteolytic mechanisms by which such precursors are converted to active peptide forms are yet to be defined rigorously. A major constraint has been the lack of readily available precursor in amounts suitable for use in assays for isolating processing enzymes and studying enzymic reactions with the natural substrate or intermediate.

An approach currently being used to alleviate this constraint is the chemical synthesis of sequences which represent likely substrate species. One sequence which has been synthesized chemically is the dodecapeptide, oxytocin-Gly-Lys-Arg (Fig. 4). This peptide represents a possible processing intermediate, derived by proteolytic cleavage at the dibasic sequence, which itself would be converted further to mature oxytocin by a combination of carboxypeptidase and α-amidating activities. As with solid phase synthesized peptides in general, reversed-phase HPLC has been effective in purifying oxytocin-Gly-Lys-Arg from closely related peptides which contaminate the crude synthetic product.

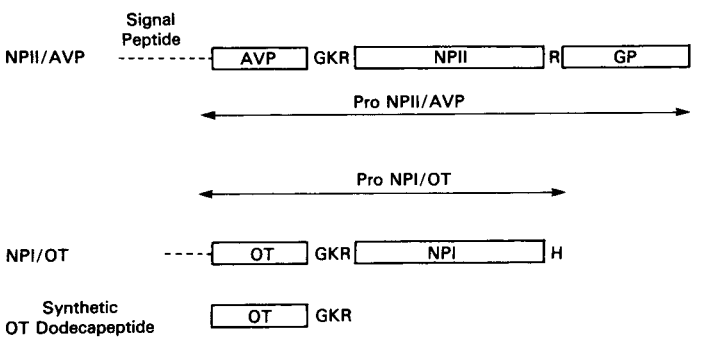

Fig. 4. Schematic diagram of the structure of bovine neurophysin/neurohypophysial hormone biosynthetic precursors [7, 24, 25]. The precursor structures were deduced from nucleotide sequences of cloned c-DNA's. The in vivo-occurring "pro" forms [7] are shown, in addition to the presence of N-terminal signal peptide regions which are contained in in vitro translation products [24, 25]. The synthetic oxytocin dodecapeptide, made as a substrate to detect and characterize processing reactions in the hormone—neurophysin spacer region [26], also is shown. Abbreviations: NPI = neurophysin I; NPII = neurophysin II; AVP = arginine vasopressin; OT = oxytocin; GKR = Gly-Lys-Arg; GP = glycopeptide. (Reproduced from ref. 26, with permission.)

Beyond purification, reversed-phase HPLC mapping is being used as an analytical tool to follow the proteolytic degradation of this peptide by neurosecretory granule extracts [26].

A second tactic in examining molecular properties and processing of precursors has been to prepare semisynthetic precursors by recombining synthetic hormone and mature neurophysin pieces. Recent data [23] have shown that this can be accomplished by chemical coupling of the above-defined oxytocin-Gly-Lys-Arg (as an active ester) with bovine neurophysin I (blocked at ϵ-amino groups). The semisynthetic product, which is distinguishable from native precursor only in its lack of the C-terminal His extension (see Fig. 4), has been isolated from coupling reactions by reversed-phase HPLC using a C_{18} column. It has affinity chromatographic properties, including lack of binding to immobilized peptide ligand but significant retardation via self-association on immobilized neurophysin, consistent with its identity as a precursor-like molecule. These findings suggest the promise to produce and isolate molecules suitable for detection and ultimate isolation of processing enzymes and determination of structural features of precursors which control the processing reactions.

MECHANISMS AND FUNCTION OF COOPERATIVE HORMONE—NEUROPHYSIN INTERACTIONS

Noncovalent neurophysin—hormone complexes produced by precursor proteolysis exhibit cooperative interactions, including neurophysin dimerization, higher hormone binding affinity of dimers versus monomers, and higher hormonal affinity of singly liganded than unliganded dimers. These interrelationships are shown in Fig. 5. Recent data have shown that the biosynthetic

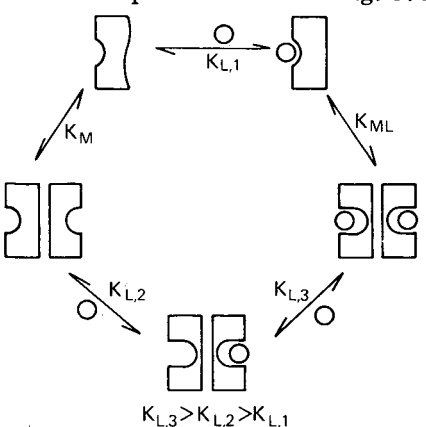

Fig. 5. Scheme of cooperative relationship between peptide ligand (○) binding and protein self-association in the neurophysin/hormone system. $K_{L,1}$, $K_{L,2}$, and $K_{L,3}$ are affinity constants of ligand for neurophysin monomer, unliganded dimer, and singly liganded dimer, respectively. K_M and K_{ML} are affinity constants for self-association of, respectively, unliganded and liganded neurophysin monomers. The scheme denotes the relationship between intermolecular hormone binding and self-association occurring in mature hormone—neurophysin noncovalent complexes. A similar relationship pertains for precursor between intramolecular hormone domain—neurophysin domain interaction and precursor self-association. (Reproduced from ref. 10, with permission.)

precursors self-associate and that the degree of self-association is enhanced over that expected by unliganded monomers [11, 23]. These and other data suggest that the precursor molecules effect hormone—neurophysin binding as an intramolecular domain—domain interaction and, in addition, self-association as an intermolecular interaction enhanced by the intramolecular hormonal interaction. Thus, the noncovalent interactions of the mature neurophysin—hormone complexes can be thought of as vestigial expressions of binding processes that initially are features of biosynthetic precursor structure. In any case, any cogent description of the structural transitions leading from precursors to active peptides in neurophysin/hormone pathways will be aided by an understanding of the inter- and intramolecular interactions which occur at different stages of the pathway.

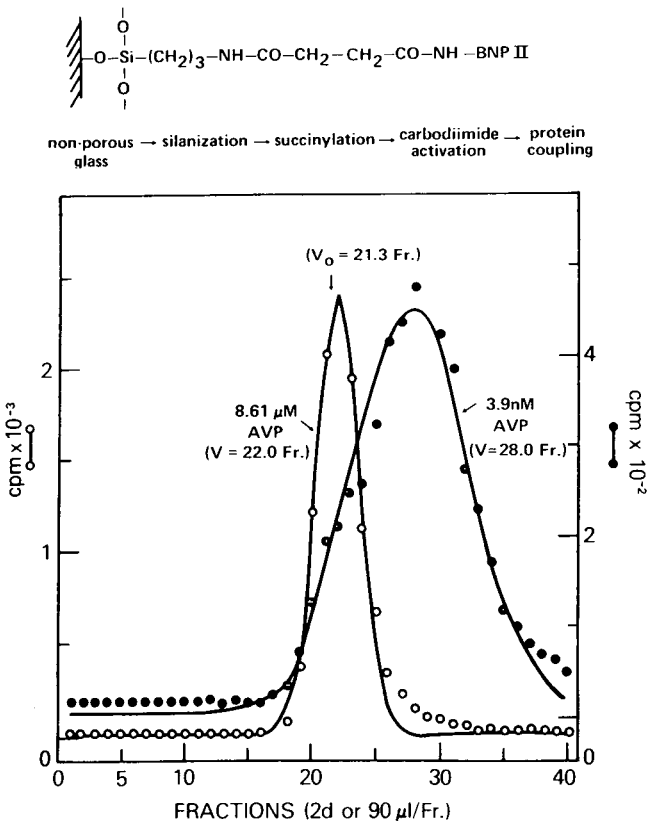

Fig. 6. Analytical bioaffinity HPLC of vasopressin on glass-immobilized bovine neurophysin II. Top: schematic structure of bovine neurophysin immobilized onto non-porous glass. Protein was immobilized via amino groups using a reaction scheme, outlined in the figure, that was developed previously [29]. Bottom: bioaffinity HPLC zonal elutions of tritiated arginine vasopressin on neurophysin—glass matrix in 0.4 *M* ammonium acetate, pH 5.7. The matrix was packed into an empty analytical 25 × 0.46 cm steel column and incorporated into a Varian 5000 liquid chromatograph. [^{3}H]Vasopressin elution, at 0.2 ml/min, was monitored by scintillation counting of collected fractions (2 drops or 90 μl each). Elution volumes, *V*, were calculated by computer-assisted fit of elution data to Gaussian distributions. Elution profiles are shown for two concentrations of vasopressin, 8.61 μ*M* and 3.9 n*M*, which represent the initial concentrations of tritiated peptide in the zone injected onto the affinity column.

We have carried out studies to analyze surface recognition processes in the neurophysin/hormone system by analytical affinity chromatography, and therein have become interested in the use of bioaffinity HPLC. Previous experiments have shown that agarose-immobilized peptides and neurophysins can be used to define quantitative relationships between self-association and hormone binding equilibrium constants [27, 28]. Such analytical chromatography in principle should benefit from the improved instrumentational features afforded by bioaffinity HPLC, including reproducibility in repetitive elution experiments, detection accuracy, possible use of narrow-bore or microbore miniaturizing configurations, and use of non-porous matrices. Recently, glass-immobilized neurophysin has been prepared and tested for use as a tool for quantitative bioaffinity HPLC. As shown in Fig. 6, neurophysin immobilized on non-porous glass beads is functionally active, such that vasopressin is retarded specifically. Interestingly, in this initial work the degree of retardation of vasopressin has been found to be dependent on the amount of hormone in the initial zone, a behavior expected if the amount of mobile interactant is significant compared to the amount of immobilized interactant [30]. Chromatographic data such as in Fig. 6 are being evaluated to calculate both rate and equilibrium constants for the vaspopressive—neurophysin interaction. In general, tests with glass-immobilized neurophysins are being used to help evaluate the use of bioaffinity HPLC as both an analytical and, ultimately, preparative tool.

CONCLUDING COMMENTS

The continuing development of HPLC has led to progressively greater impact of this methodology in separations of peptides and proteins. In many cases, a high-performance method has replaced an existing conventional chromatographic method mainly because the HPLC alternative could accomplish the separation better (more conveniently, with improved resolution, etc.). But, new capability not feasible with conventional separations also has marked the evolution of HPLC. In this regard, application of HPLC to studies of neurophysin/neurohypophysial hormone pathways perhaps is typical. The sensitivity and separatory power of reversed-phase HPLC has had important benefits both in detecting neurophysin isoforms not normally separated in conventional liquid chromatographic preparations from classical anatomical sites (e.g. the neurohypophysis) and in isolating peptide and protein species present in micro amounts (often difficult to separate preparatively by conventional chromatography) in such non-classical sites as the ovary and peripheral nerves. The increased resolving power and ability to fractionate and recover micro quantities by reversed-phase HPLC also have been beneficial in purification and analysis of synthetic precursor fragments and close-to-intact semisynthetic precursors. In the case of bioaffinity HPLC, the advantages over conventional affinity chromatography are as yet not fully defined. But, results so far suggest that analytical and high-sensitivity preparative affinity chromatographic separations both will benefit from application of HPLC technology.

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MACROSCALE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND INSTRUMENTAL IDENTIFICATION OF COMPONENTS OF DIETHYLAMINOETHYL MURINE EPIDERMAL GROWTH FACTOR

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SUMMARY

Murine epidermal growth factor (m-EGF), a polypeptide produced as a chromatographically homogeneous peak on diethylaminoethyl (DEAE) cellulose by the method of Savage and co-workers, and characterised as a single compound, has been shown by ourselves and several other groups to be a mixture. The present contribution extends our previously reported work and discusses the separation of this material, termed DEAE—m-EGF, into its components by preparative ion-pair reversed-phase high-performance liquid chromatography (RP-HPLC) on C_{18} μBondapak in quantities up to 50 mg per run. Isocratic elution was used and the mobile phase was acetonitrile—water (26:74, v/v, 0.04 *M* in triethylamine acetate); pH was 5.6, temperature 40°C, and detection was by ultraviolet absorption at 254 nm, and (for some runs) by differential refractometry. Seven significant peaks, four major, three minor, were detected. Of the major peaks, two designated α- and β-EGF, constituted 70% of the total mass and were the most important to our work. Each of the eluted peaks was recovered by lyophilisation, and this product checked for homogeneity by ion-pair RP-HPLC on a C_{18} μBondapak analytical column, with ultraviolet detection as before. All recovered peaks were found to be homogeneous by this criterion.

These chromatographically homogeneous compounds were investigated by modern physicochemical instrumentation to determine their structure. The molecular weight of each of the species was determined by fast atom bombardment mass spectrometry. High-field proton magnetic resonance at 270 MHz provided structural and conformational information. Polarimetry and ultraviolet absorption were also used to characterise the compound. α-EGF, for example, had a molecular weight of 6040 corresponding to the 53 amino acid residue peptide previously designated EGF; β-EGF had a molecular weight of 5930. This molecular weight differential of 110 suggested the hypothesis that β-EGF was a 52 residue peptide corresponding to α-EGF minus the terminal asparagine at position 1. Proton mag-

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netic resonance difference spectroscopy (β spectrum subtracted from α) provided powerful confirmatory evidence for this hypothesis.

All materials recovered from RP-HPLC were tested in the sheep and found to retain their biological activity.

INTRODUCTION

This paper presents the results of an investigation into a problem in biological chemistry, a field in which the compounds tend to be of large molecular weight and of uncertain stability, and thus difficult to separate and characterise. We are here stressing the characterisation of the separated compounds by sophisticated physicochemical (structure-determining) instrumentation such as high-field proton magnetic resonance (HF-PMR) and fast atom bombardment mass spectrometry (FAB-MS).

In addition to the intrinsic scientific interest in separating and characterising these compounds, we had a practical problem in mind in that the compound had physiological properties which we wished to utilise, and to do this we needed accurately measured doses, and this in turn required that each compound be pure and that its properties be known. Additionally, for this biological testing [1] the compound was required to be available in amounts up to 500 mg per experimental run.

The compound of interest to us was murine epidermal growth factor (m-EGF), a polypeptide obtained (largely) by the method of Savage and co-workers [2, 3] from the submaxillary glands of mice (Fig. 1). For the work reported here, the starting material, recovered from the diethylaminoethyl (DEAE) cellulose column as a single peak and therefore regarded as homogeneous, was DEAE—m-EGF. This product, characterised as a single compound [4, 5], has been reported to have 53 amino acid residues and a calculated molecular weight of 6040, and the molecule has three disulphide bonds (Fig.

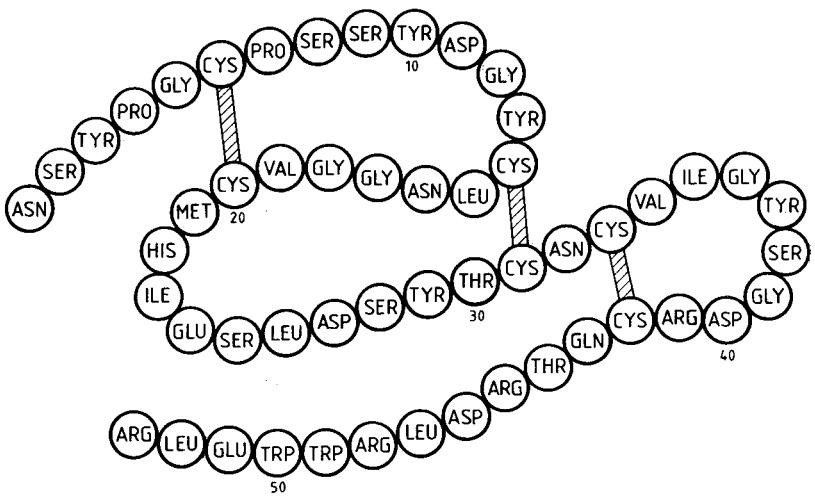

Fig. 1. The amino acid sequence of m-α-EGF showing disulphide bonds [3, 5]. m-α-EGF is the 1—53 residue peptide (MW 6040); m-β-EGF is the 2—53 residue peptide (MW 5926).

1). For such a composition there are 10^{60} linear combinations alone, and this presents a potentially formidable problem in characterisation especially for the enzymatically synthesised or genetically engineered materials we subsequently expected to investigate. In the preliminary biological testing, prior to a tritium labelling experiment [6], the DEAE—m-EGF was checked for purity by reversed-phase high-performance liquid chromatography (RP-HPLC) on a C_{18} μBondapak column with acetonitrile—water (26:74) containing 10 g/l PIC D-4, a commercial ion-pair reagent (Waters Assoc.), at pH 3. It became apparent that the apparently homogeneous DEAE—m-EGF was a mixture [6]. Further work by ourselves [7, 8] and others [9—11] using a variety of RP-HPLC systems confirms this unequivocally.

At this stage in the investigation there were two broad areas of the problematic: (i) How can the DEAE peak be separated by HPLC into its components in at least 50-mg quantities per run with retention of biological activity? and, (ii) what are the characteristics of the compounds separated?

For the HPLC system ion-pair RP-HPLC was chosen because stable chemically bonded reversed-phase columns were available at relatively low cost, and concentrations of counter ion and organic modifier and ionic strength could all be easily changed to enhance separation selectivity. Additionally, aqueous samples, which were expected from a further phase of the biological investigation, are easily handled by this technique.

The decision to use RP-HPLC for the preparative separations, together with the further decision to recover the compounds by lyophilisation, ncessitated additional investigation of separation methods since, for example, the PIC D-4 reagent (above) was involatile. A further matter of concern was whether analytical methods (microgram amounts) could be scaled up to preparative (milligram) amounts, with adequate column resolution and loading. Since the compounds were required for subsequent biological investigations, tests on retention of biological activity were, ipso facto, part of the programme.

Since biological activity is dependent upon molecular structure, instrumental techniques, sensitive to changes in molecular structure, were used to characterise the compounds. FAB-MS, which has recently been extended to polar compounds larger than 6000 daltons [12], was used to determine the molecular weight of the recovered compounds. Changes in molecular structure (e.g. loss of an amino acid residue) are immediately detectable by such a technique. Some approach to the empirical formula, and thus the composition of the molecule, is also available by mass spectrometric techniques [13]. HF-PMR probes the environment of every hydrogen in the molecule, and yields spectra (usually overlapping for such complex compounds) which are especially sensitive to composition and conformation. Each of the recovered compounds was examined by HF-PMR as an aid to determining its structure (by peak—structure correlations) and to determine the difference between closely related compounds, e.g. by difference spectroscopy.

Other physical properties, such as ultraviolet absorption and optical activity, which are more characteristic of compound classes than individual compounds, were also determined.

EXPERIMENTAL

Chemicals and reagents

High-performance liquid chromatography. All water used in the project was deionised and then glass-distilled. Acetonitrile, orthophosphoric acid (85%) and acetic acid were AR grade from Ajax Chemicals (Sydney, Australia). Triethylamine (TEA) was from E. Merck (Darmstadt, F.R.G.).

PIC D-4 mobile phase modifier was from Waters Assoc. (Milford, MA, U.S.A.).

Mobile phase. This mobile phase, which consisted of an acetonitrile–water mixture (26:74), 0.04 *M* in triethylamine acetate and with a pH of 5.6, was used for all the preparative separations and also for the analytical runs unless otherwise noted. It was prepared by dissolving the triethylamine in water, adjusting the pH to 5.6 with acetic acid, filtering through a Millipore 0.5-μm PTFE filter and then degassing under vacuum. Acetonitrile was filtered (Millipore, 0.5 μm), degassed under vacuum, and the organic and aqueous phases were mixed.

For the alternative mobile phase, the PIC D-4 was dissolved in water, the solution degassed and mixed with degassed acetonitrile to yield a mobile phase of acetonitrile–water (26:74, v/v) and containing 10 g/l PIC D-4. The solution was adjusted to pH 3 using orthophosphoric acid. This mobile phase was used in some of the initial analytical work and in an alternative RP-HPLC procedure for checking the homogeneity of recovered peaks.

Proton magnetic resonance. The deuterium oxide was 99.75% ^2H_2O and was obtained from the Australian Institute of Nuclear Science and Engineering (Lucas Heights, Australia). The sodium deuteroxide (NaO^2H) was 40% (w/v) in ^2H_2O and was supplied by Merck Sharpe & Dohme (Quebec, Canada).

Apparatus

High performance liquid chromatography. All chromatography data were collected isocratically using a Waters Assoc. HPLC system equipped with a Model 6000A solvent delivery system, a Model U6K universal injector (2 ml max.), and a Model 440 fixed-wavelength, dual-channel ultraviolet absorption detector.

The differential refractive index detector was a Perkin-Elmer Model LC-25 (Norwalk, CT, U.S.A.) equipped with a water jacket for temperature control. The prepacked columns (Waters Assoc.) were stainless steel, 30 cm in length and either 3.9 mm (analytical) or 7.8 mm (preparative) I.D. The reversed-phase column packing was C_{18} μBondapak (10 μm particle size), and a guard column was also packed with this material. The columns were water-jacketed and they and the differential refractive index detector were temperature-controlled to better than 0.5°C by a Braun Thermomix Model 1420 (Braun, Melsungen, F.R.G.). Control specification was ±0.02°C at 37°C.

The recorder was a Servoscribe 2 (Smiths, U.K.) with dual channels and switched range voltage input. It was operated on the 10 mV range. A Radiometer Model 26 pH meter (Copenhagen, Denmark) with glass–calomel electrodes was used and was calibrated by the two-buffer method.

Analytical samples were injected using a 25-μl SGE syringe (Melbourne,

Australia). Preparative samples were injected with a tuberculin (1 ml) syringe with a modified needle.

Nuclear (proton) magnetic resonance. The instrument used was a Bruker HFX-270 and was operated in the Fourier transform mode at 270 MHz to acquire proton spectra; 5-mm sample tubes were used (Bruker, Rheinstetten, F.R.G.).

Fast atom bombardment mass spectrometry. The instrument used was a VG analytical ZAB-HF mass spectrometer equipped with a fast atom bombardment source using xenon at 8 keV, a high-field magnet, and a mass marker calibrated against caesium iodide cluster ions [14] (VG Analytical, Manchester, U.K.).

Polarimeter. Optical activity was determined on a Perkin Elmer-Model 241 polarimeter (Bodenseewerk Perkin-Elmer, Überlingen, F.R.G.) equipped with a sodium and a mercury lamp.

Mercury lines were selectable by filter wheel. Accuracy of the polarimeter was 0.002°, repeatability 0.002°, and readout accuracy 0.001°.

Ultraviolet spectrophotometer. The instrument used was a Gilford Model 2600 (Gilford Instrument Labs., OH, U.S.A.) with a wavelength accuracy of 0.1 nm, a wavelength repeatability of better than 0.1 nm, and a photometric precision of better than 0.002 A. The instrument was equipped with automatic wavelength calibration using the 360.9-nm peak of holmium oxide. Matched quartz sample cells of 1 cm path length were used.

Lyophilisation apparatus. The lyophilisation apparatus was an Edwards Modulyo refrigerated unit equipped with a Pirani 10 vacuum gauge (Edwards, Crawley, U.K.).

Methods

High-performance liquid chromatography. Using the mobile phase containing TEA, all RP-HPLC separations, analytical and preparative, were carried out with the C_{18} μBondapak column (and the RI detector) at 40°C. Before initial use, and regularly during use, the plate count of each column was determined using uracil-acenaphthene and the 5-sigma peak measuring method of Waters Assoc. [15]. Initially, the analytical column had 2500, and the preparative column, 5900 theoretical plates. Before sample injection each column was equilibrated to the mobile phase for 60 min at the operating flow-rate.

For separations standardised conditions were used throughout. All samples were dissolved in the mobile phase. Analytical samples were made up to a concentration of 1 μg/μl and 25 μl were injected; column flow-rate was 1.5 ml/min. For preparative separations, 50 mg of DEAE—m-EGF was dissolved in 2 ml of the mobile phase, filtered through an 0.5-μm PTFE Millex-SR filter, transferred to the U6K injection loop in 2 × 1-ml injections and thence to the column. Isocratic elution was carried out at a constant flow-rate of 0.8 ml/min. These conditions had been established by previous experimentation (not reported here) to be optimal for the separation of α- and β-EGF with good separation efficiency and column loading. This method is largely a single-column version of the analytical method of Martrisian et al. [11] scaled up 1000-fold, and optimised to our requirements.

Detection of the eluted peaks was by ultraviolet (UV) at 254 nm and by dif-

ferential refractometry. Samples were collected from the detector flow cell outlet using 0.23-mm I.D. steel tubing to minimise dead volume, using the delay time (2.5 sec) previously established with methylene blue dye. To ensure minimal cross-contamination, peaks were collected after the rise from the baseline and before the return to it.

To recover the compounds the eluates were transferred to lyophilisation flasks, frozen in a dry ice—ethanol bath, and lyophilised on an Edwards refrigerated unit overnight. To check separation efficiency, a sample of each of the lyophilised products was dissolved in the mobile phase (concentration 1 μg/μl) and 4 × 25 μl were injected and chromatographed under identical conditions to the separation and on the same column. All products were found to yield a single peak. The products were also examined by HF-PMR and found to contain a coupled triplet—quartet (1.1 and 2.75 ppm) ascribed to TEA, hypothesised to be complexed to the EGF peptides since it was not removed by lyophilisation for four days. The TEA was removed by careful addition of 0.01 *M* sodium hydroxide to an aqueous solution of the product to a pH of 8.5, and the product recovered by further lyophilisation for 24 h. The products were again checked for homogeneity by RP-HPLC, and again run on HF-PMR. They were found to be homogeneous and free from complexed TEA. At the conclusion of each set of separation runs the material from each identical peak was composited, dissolved in water and lyophilised to provide uniform bulk batches. These bulk batches were checked by chromatography as before, and also examined by HF-PMR. It was on these bulk batches, stored under nitrogen in sealed containers, under refrigeration, that the biological testing and characterisations were carried out.

As an analytical back-up, RP-HPLC using the PIC D-4 mobile phase was carried out, under identical conditions to those described previously, except that ambient temperature was used. This alternative RP-HPLC confirmed that the peaks were homogeneous.

Proton magnetic resonance. For ^{1}H spectra, the compounds were dissolved in 0.4 ml of ^2H_2O, transferred to an 0.5-ml nuclear magnetic resonance (NMR) tube and the pH was adjusted in situ to 8—8.5 with NaO^2H.

For ^{1}H data acquisition, the spectrometer was operated in the Fourier mode at 270 MHz using a sweep window of 3600 Hz and collecting into 4 K of memory at an acquisition time of 0.54 sec. An 0.2-sec gated pulse was used to suppress the water resonance prior to data acquisition. Data processing involved the use of a resolution enhancement function (usually trapezoidal multiplication) followed by zero filling to 8 K of memory. The reference used was the internal H^2HO peak at 4.74 ppm, and all chemical shifts are in ppm. Output was to a chart recorder (X—Y plotter).

Difference spectrum. For the difference spectrum of α- minus β-EGF separate spectra of α-EGF and β-EGF were acquired sequentially, as above, with the spectrometer in the absorption mode. The samples were prepared and the spectra run with careful attention to detail so that all parameters and variables (pH, concentration, data acquisition) were identical. The separate spectra were then subtracted electronically using the elimination (approach to zero) of selected reference lines to monitor the process. The difference spectrum was then recorded as above.

Fast atom bombardment mass spectrometry. Samples were dissolved in 1 *M* hydrochloric acid, and a 1-μl aliquot was transferred onto a trace of glycerol on the stainless-steel stage of the mass spectrometer. Ionisation was by xenon atoms at 8 keV, and the scans were run at the reduced accelerating voltage of 4 kV to increase mass range [12, 16]. These scans were acquired under mass control from 6500 to 5000 a.m.u. and data output was obtained on an on-line oscillographic recorder. The mass marker was calibrated using caesium iodide cluster ions [14]. Mass assignments for the compounds were made on peak centroids.

Polarimetry. Optical activity (specific rotation) was determined in aqueous solution at a concentration of 1 mg/ml in a 1-dm tube at 589 nm (Na^2H line) and at 578 nm (Hg line). D-Glucose (stabilised with benzoic acid) was used to check the polarimeter (α_D + 50°).

The polarimetry was carried out on duplicate samples read in triplicate. The instrument was "re-zeroed" and checked with the standard glucose between each set of sample readings. Room temperature was 22°C.

Specific rotation = $[\alpha]_\lambda^{22^\circ} = \frac{100\alpha}{lc}$, where $[\alpha]$ is specific rotation, α is measured angle of rotation, l is path length of cell in dm, c is concentration in g per 100 ml, and λ is wavelength used.

Ultraviolet absorption. UV measurements were made on aqueous samples (pH 8—8.5) at a concentration of 0.2 mg/ml in matched quartz cells of 1 cm path length with water in the reference beam. Scans were between a wavelength of 220 and 320 nm, with step size of 0.100 nm and slit width of 0.22 mm.

$E_{1\,cm}^{1\%} = \frac{A}{bc}$ where A is the measured absorbance, b the path length of cell in cm, and c is the concentration in g per 100 ml.

RESULTS AND DISCUSSION

Separations

The separation achieved by RP-HPLC on C_{18} μBondapak using acetonitrile—water (26:74), 0.04 *M* in triethylamine acetate as the mobile phase, is shown in Fig. 2. Virtual baseline resolution has been achieved for the major components, α- and β-EGF, which were those of most interest to the biological programme. Of the two minor peaks of interest the one that eluted between 40 and 44 min was designated pre-α and the one that eluted between 58 and 63 min post-β.

Identical resolution was achieved during preparative runs with 50-mg column loading. This was monitored on the peak troughs of the UV trace though the UV detectors were saturated on the peak tops. Re-injection of a sample from each of the lyophilised peaks confirmed their homogeneity, and thus also confirmed that resolution was being maintained. Differential refractometry (as an approach to mass detection) indicated that no UV inactive peaks were being eluted. Of the DEAE—m-EGF injected 7% was recovered as pre-α, 49% as α, 20% as β, and 6% as post-β: this together with that in minor peaks gave a recovery of 95% by weight. This method of RP-HPLC has thus proved efficacious for the preparative separation of those components of DEAE—m-EGF of interest to our biological programme.

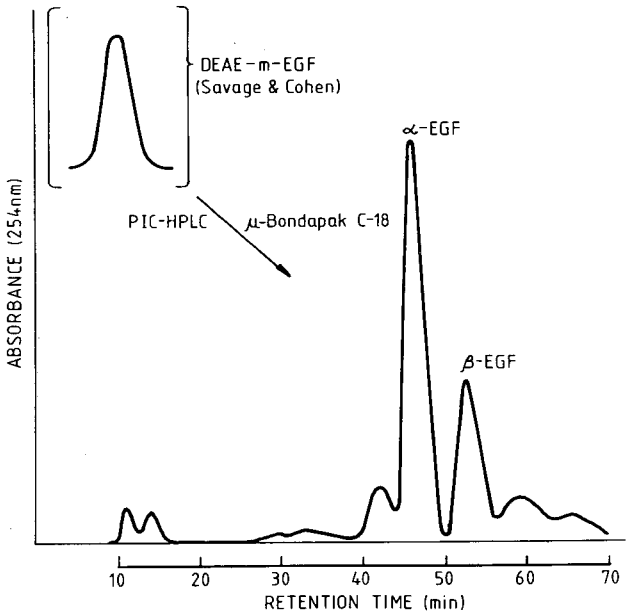

Fig. 2. Isocratic elution of DEAE—m-EGF on a C_{18} μBondapak column (300 × 7.8 mm). Conditions: flow-rate 0.8 ml/min; temperature 40°C; solvent acetonitrile—water (26:74), 0.04 *M* in triethylamine acetate, pH 5.6. Sample size was 100 μg injected in a total volume of 100 μl. Absorbance at 254 nm, 0.05 a.u.f.s.

The next problem to be addressed was whether the biological activity of interest to us had been retained, qualitatively and quantitatively, during RP-HPLC treatment. Possibilities for reactions resulting in such loss included: irreversible complexing of the TEA counter ion to the peptide; conformational changes and cleavage of labile amino acid residues, particularly those at terminal positions. Welinder et al. [17] reported that separation of iodinated insulin species from insulin (a polypeptide with similarities to EGF) on C_{18} columns by RP-HPLC resulted in loss of in vitro biological activity in that there was a loss of binding ability to isolated adipocytes. These authors suggested the possibility that the loss in activity was due to the extremely non-physiological conditions under which HPLC is performed: high pressure, shear forces, organic solvents, and the hydrophobic chromatographic matrix. They further suggested the possibility that the F_3CCOO^- ion from the triethylammonium trifluoracetate—acetonitrile buffer could irreversibly bind to the arginine residues, but no experimental investigation of this important hypothesis appears to have been undertaken. To the contrary, however, Smith et al. [18] reported, without citing supporting experimental evidence, that RP-HPLC on C_3 and C_{18} columns did not inactivate EGF which retained biological and immunological activity for more than one year at −20°C in the eluent solvent.

These findings may not be as conflicting as they appear, since biological activity is complex and for compounds such as insulin and EGF the mechanism of action is unknown, and though it is of course structure-related, it may involve only a part of the molecule. It seems likely that changes in biological activity depend on the nature of the test and on whether it is in vivo or in vitro.

In EGF we were interested in in vivo activity (defleecing action in the sheep) and extensive quantitative testing was carried out in a collaborative experiment using over 50 sheep on the compounds from RP-HPLC. DEAE—m-EGF from the same bulk batch, but not processed by RP-HPLC was used as a control. This investigation showed that, within the limits of experimental error, all materials were of the same potency, i.e. RP-HPLC did not deactivate the compound for this test. These results will be presented in detail elsewhere [1].

Characterisation

The difficulties associated with characterisation of polypeptides of the size of EGF is illustrated by the report of Johnson [19] on the characterisation of human insulin prepared by recombinant DNA technology. In all they used twelve discrete techniques including separation methods, instrumental techniques and biological (in vivo and in vitro) testing. For our purposes we used, principally, FAB-MS and HF-PMR. Both of these techniques are sensitive to changes in molecular structure. HF-PMR, in particular, was used to monitor the product at all stages of separation and recovery.

Proton magnetic resonance

PMR examination showed the product from RP-HPLC contained TEA after the first lyophilisation (Fig. 3). This assignment was made on the basis of the coupled triplet—quartet at 1.1 and 2.75 ppm (referenced to H^2HO at 4.74) which is typical of the ethyl group in amines. This TEA was found to be so stably bound to the peptide molecule that it could not be removed even by extended lyophilisation (four days). The complexed TEA was removed by treatment of an aqueous solution of the lyophilised material with 0.01 *M* sodium hydroxide to pH 8.5 and the compound recovered by a further lyophilisation (Figs. 4 and 5).

Because of its complex overlapping nature the proton spectrum constitutes

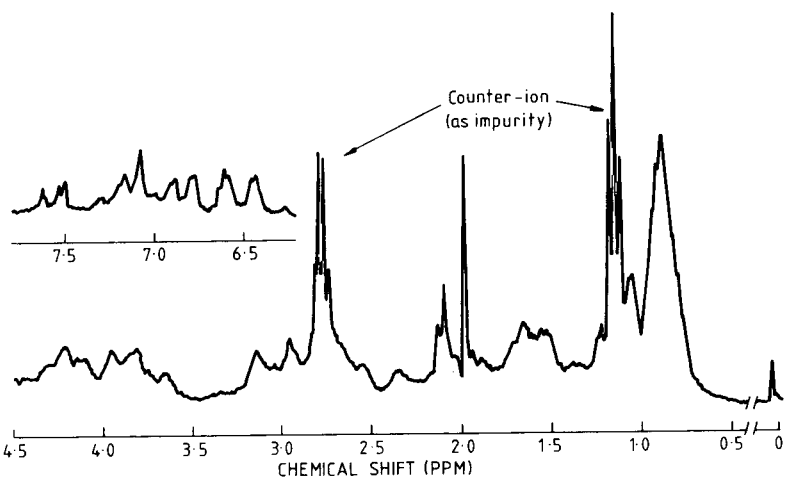

Fig. 3. PMR spectrum at 270 MHz of impure α-EGF ex stage 1 of lyophilisation (PIC-HPLC); 1 mg EGF in 0.5 ml ^2H_2O at pH 8.5. Coupled triplet—quartet at 1.1 ppm due to complexed TEA counter ion. Chemical shifts in ppm referenced to H^2HO at 4.74 ppm.

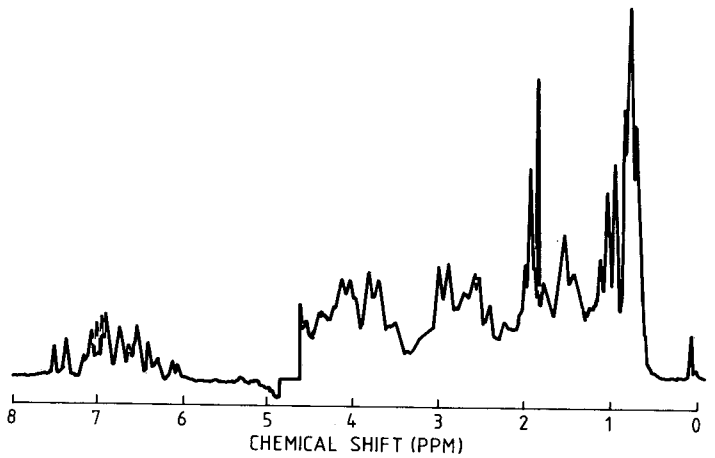

Fig. 4. PMR spectrum at 270 MHz of pure α-EGF ex PIC-HPLC from final stage of lyophilisation (desalted); 1 mg in 0.5 ml ^2H_2O at pH 8.5. Chemical shifts in ppm referenced to H^2HO at 4.74 ppm.

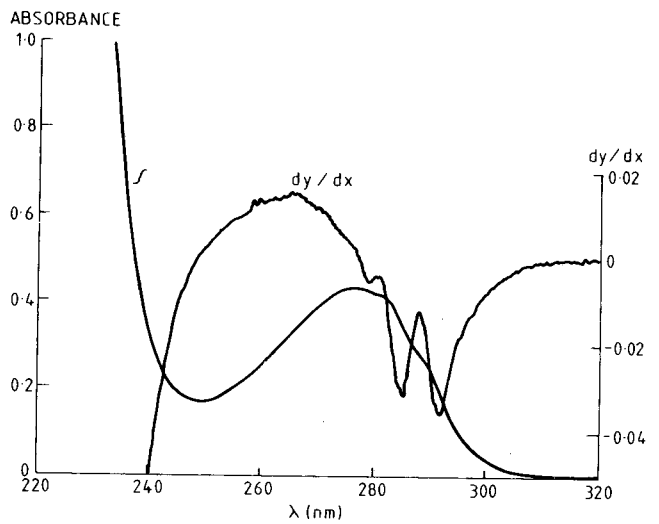

Fig. 5. The UV spectrum of m-α-EGF in water [ex (BBV) PIC-HPLC, C_{18}]. Concentration 0.2 mg/ml. Scanned range 200—320 nm. Cell path length 1 cm. Absorbance maximum value at 277.1 nm, 0.432 a.u. The first derivative (dy/dx) is also plotted for this sample.

a fingerprint of the molecule since it has a contribution from all hydrogens in the molecule except for those in readily exchangeable positions such as amide groups. Compound comparisons were facilitated by the peak—structure correlations which were undertaken concurrently [8, 20] and supplemented latterly by the report of De Marco et al. [21] who have assigned the peak—structure relationships in the aromatic regions (6—8 ppm). In our PMR studies we found no evidence that either the composition of the recovered material nor its conformation was altered by the separation and recovery processes.

Difference spectra ($\alpha - \beta$)

The only PMR absorption signal remaining in the difference spectrum of α- minus β-EGF was that in the region 2.55 ppm assigned [8, 20] to the terminal asparagine at position No. 1 of α-EGF (Fig. 1). The absorption signal was positive, thus indicating that asparagine was present in α-EGF but absent in β-EGF. Confirmatory evidence for the loss of this asparagine in β-EGF was given by the spectral dispersion lines for proline (No. 4) at 3.5 and 3.7 ppm and for tyrosine (No. 3) at 6.5 and 7.0 ppm indicating non-coincidence of these peaks in the α- and β-spectrum and thus indicating a greater degree of mobility in the moiety in the 2–4 region consequent on the loss of the asparagine terminal. The reduction to zero of all other peaks in the spectrum shows that in these regions, α and β are identical in composition and conformation. The hypothesis that α- and β-EGF differ by one amino acid (asparagine) was tested and confirmed by the molecular weight determined by FAB-MS.

Fast atom bombardment mass spectrometry

For the characterisation of a compound, the molecular weight is probably the single most important physical property. However, for polar, non-volatile, thermally unstable compounds of molecular weight in excess of 6000, such as the polypeptides of this study, it can only be obtained by the recently developed technique of FAB-MS [22]. For these compounds the instrument was equipped with a high-field magnet, and was run at reduced accelerating voltage to increase the mass range despite the resultant decreased sensitivity.

TABLE I

SUMMARY OF RESULTS

All compounds biologically active in the sheep.

Compound	Optical activity* 22°C	Absorptivity** (λ_{max} in nm)	Molar absorptivity	FAB-MS molecular weight (m/z)	Remarks
DEAE—m-EGF	—104.5° (mixture)	24.19 (276.6)	14611 (mixture)	6040 [Peaks at 6040 (α) and 5926 (β)]	
α-EGF	—88.2°	20.30 (277.1)	12261	6040	1—53 Residues (Fig. 1)
β-EGF	—88.4°	21.42 (277.0)	12693	5930	2—53 Residues (Fig. 1). Loss of asparagine terminal; confirmed by NMR
Pre-α-EGF	—95.5°	22.31 (277)	13475	6040	NMR spectrum similar to α-EGF
Post-β-EGF	—77.3°	21.56 (277)	12582	5836	3—53 Residues (Fig. 1). Possible loss of serine terminal from β-EGF

*$[\alpha]_{578\ nm}^{22°C}$.

**$E^{1\%}_{1\ cm}$.

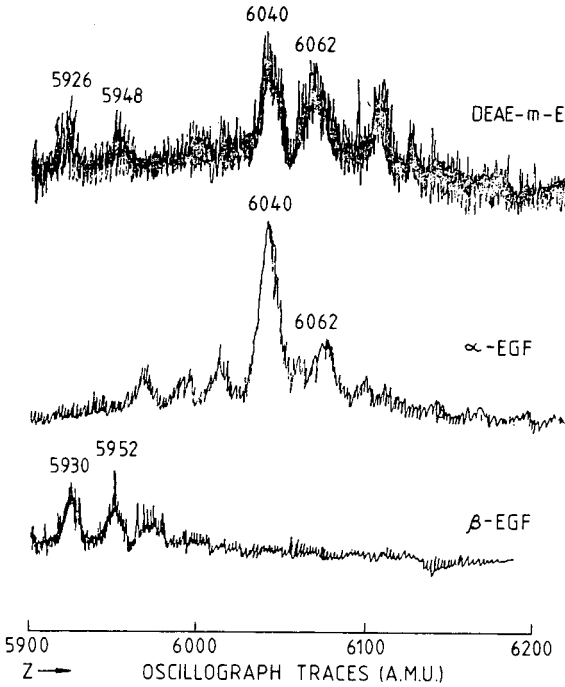

Fig. 6. Molecular ion for α-, β-, and DEAE—m-EGF obtained by FAB-MS on a VG ZAB-HF mass spectrometer (M-Scan, U.K.); ionisation by xenon at 8 keV, accelerating voltage 4 kV. Results: *m/z* for α-EGF, 6040 a.m.u.; for β-EGF, 5930 a.m.u.; DEAE—m-EGF was a mixture mainly of α- and β-EGF.

FAB-MS spectra were run on all four of the major compounds recovered and on the original DEAE—m-EGF (Table I). Fig. 6 shows the spectrum of DEAE—m-EGF together with that of the major components α- and β-EGF; the peaks 22 a.m.u. above the molecular ion is the sodiated adduct. The molecular weight of 6040 as determined by FAB-MS for the compound designated α shows this compound to be the 53 amino acid residue peptide of calculated molecular weight 6040 previously called m-EGF.

The molecular weight of 5930 for the β compound, a differential of 110 a.m.u. between it and α, confirms the hypothesis derived from difference HF-PMR that β-EGF differs from α-EGF only in the absence of the terminal asparagine (No. 1), i.e. β-EGF is the 2—53 residue compound (Fig. 1).

Other physical properties

Optical activity and UV absorption results on the compounds, shown to be homogeneous by RP-HPLC and characterised by FAB-MS and HF-PMR, are presented in Table I and Fig. 5. As would be expected from compounds of nearly identical structure, the values are also closely similar.

CONCLUSION

DEAE—m-EGF has been separated on a preparative scale, with retention

of biological activity, into four major and three minor components. The four major peaks have been characterized using FAB-MS and HF-PMR as well as by optical activity and UV absorption. No investigation, however, was made into the origin of the separated components which may, for example, be artifacts arising from proteolysis during recovery from the mouse as has recently been reported [18].

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Mr. P. Van Dooren of CSIRO Prospect provided the sample of DEAE—m-EGF. M-Scan, U.K., ran the FAB mass spectra as a commercial venture.

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PARTIAL PURIFICATION OF A SPECIFIC INHIBITOR OF THE INSULIN-LIKE GROWTH FACTORS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We report here preliminary data using reversed-phase high-performance liquid chromatography for the purification of a specific inhibitor (a molecular weight 16,000—18,000 protein) of the insulin-like growth factor (IGF) or somatomedin family. Crude inhibitor prepared from Cohn fraction IV-1 of human serum was first partially purified using an IGF/CH-Sepharose 4B affinity column. Following elution of the bound inhibitor and resuspension in 0.1% aqueous trifluoroacetic acid (mobile phase A), it was injected (100 μl; 2.0 mg protein) onto a Brownlee Aquapore RP-300 column. Application of a linear gradient from 0% to 100% mobile phase B (45% isopropanol—0.1% trifluoroacetic acid) resulted in elution of two peaks of inhibitor activity between 31% and 34% isopropanol associated with a major homogeneous protein peak and a minor heterogeneous protein peak. No inhibitor was recovered when an acetonitrile gradient was used instead of isopropanol, indicating that the inhibitor is very hydrophobic. These data suggest that high-performance liquid chromatography offers a simple procedure for the potential purification of IGF inhibitor(s) from normal human serum.

INTRODUCTION

The insulin-like growth factors (IGF) or somatomedins (Sm) [IGF-I/SmC, IGF-II, SmA] are a family of circulating polypeptide growth factors with a marked, but not absolute, dependence on serum levels of growth hormone (GH) [1]. IGF-I/SmC has been shown to mediate the actions of GH on skeletal growth of hypophysectomized rats [2]. A variety of specific and non-specific inhibitors of the IGFs have also been reported in several severe catabolic states in humans and experimental animals [1]. Recently, we have identified, for the first time in normal human serum, a quite specific inhibitor of many of the actions of the IGFs [3] (Table I). This suggests that under normal physiological circumstances normal growth may well be dependent on an adequate balance

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TABLE I

PHYSICOCHEMICAL AND BIOLOGICAL CHARACTERISTICS OF IGF INHIBITOR

(1)	Proteinaceous, M_r approx. 18,000, pI 4.0—4.4 Acid-stable, heat-labile
(2)	Causes competitive inhibition of (a) IGF cartilage sulphation activity (b) IGF adipocyte non-suppressible insulin-like activity (c) IGF binding to placental and adipocyte receptors
(3)	Mechanism of action via direct interaction (binding) with IGF
(4)	Effects are dose-dependent and specific for the IGF/Sm family of growth factors

between these positive (IGF/Sm) and negative (inhibitor) factors. In order to substantiate this hypothesis one requires better characterization and identification of the inhibitor(s) together with the establishment of a specific and quantitative assay. Thus, we have attempted to further purify this substance from fractions of normal human serum and report here the results of our initial studies using reversed-phase high-performance liquid chromatographic (HPLC) techniques.

EXPERIMENTAL

Materials

Sephadex G-75 and CH-Sepharose 4B were from Pharmacia (Upsala, Sweden). Bio-Gel P-30 was from Bio-Rad (Richmond, CA, U.S.A.). Trifluoroacetic acid (TFA) was from BDH (Poole, England) and HPLC-grade isopropanol was from Ajax Chemicals (Sydney, Australia). Ultrafiltration membranes were from Amicon (Lexington, MA, U.S.A.).

Preparation of a crude inhibitor fraction

A crude IGF inhibitor fraction was prepared from Cohn fraction IV-1 of human serum as reported previously [3]. This involved initial ion-exchange chromatography on SP-Sephadex C-25, with application at pH 5.5 followed by sequential batch-wise elutions at pH 5.5, 6.5 and 9.7. This final elution fraction, which contained both the active IGF species as well as the inhibitor, was ultrafiltered (Amicon UM-2 membranes), dialysed and lyophilized. This fraction was redissolved in 1% formic acid (pH 2.3) prior to sequential gel chromatography under acid conditions on Sephadex G-75 (120 × 4.2 cm) and Bio-Gel P-30 (48 × 2.3 cm) columns. This latter chromatography step separates the IGFs (M_r approx. 7500; K_{av} 0.4—0.9) from the inhibitor (M_r 16,000—18,000; K_{av} 0.1—0.4) which was dialysed and lyophilized.

Affinity chromatography of inhibitor

Previous studies (unpublished) had indicated that the mechanism of action of the inhibitor was via a direct interaction with the IGFs. Thus, this property was utilized to further purify the inhibitor on an IGF/CH-Sepharose 4B affinity

column. A mixed preparation of IGFs was prepared by the method of Svoboda et al. [4] and separated from inhibitor activity by Bio-Gel P-30 chromatography in 1% formic acid as described above. The IGF was covalently linked to CH-Sepharose 4B according to the manufacturer's recommendations. The Bio-Gel P-30 inhibitor fraction was resuspended in Krebs—Henseleit bicarbonate buffer pH 7.4 (25 m*M*) and mixed with the IGF/CH-Sepharose. The mixture was placed in a glass column, stoppered and rotated end-over-end at 4°C for 16 h to allow IGF—inhibitor interaction to reach equilibrium. The column was then attached to a fraction collector and unbound proteins eluted with Krebs—Henseleit buffer until $A_{280\,nm}$ reached baseline (30 × 1-ml fractions). Bound proteins were then eluted with 1% formic acid (20 × 1-ml fractions). Acid fractions 3—8 contained inhibitor activity and were pooled, dialysed and lyophilized.

High-performance liquid chromatography

The lyophilized inhibitor was redissolved in 0.1% (v/v) aqueous TFA and an aliquot (100 μl; 2—3 mg protein) was injected onto a Brownlee Aquapore RP-300 (10 μm) column (25 cm × 4.5 mm) (Brownlee Labs, Santa Clara, CA, U.S.A.) attached to a Waters liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.). The system was equipped with dual Model 6000A solvent delivery systems, a Model 660 solvent programmer, a Model 441 absorbance detector, a Gilson (Villiers le Bel, France) Microcol TDC80 fraction collector and an Omniscribe (Houston Instruments, Austin, TX, U.S.A.) dual-pen recorder. Mobile phase A was a 0.1% (v/v) aqueous TFA solution. A linear gradient from 0% to 100% mobile phase B (45% isopropanol—0.1% TFA) was applied over 60 min at a flow-rate of 0.7 ml/min. Fractions (1 min) were collected, the protein elution profile was monitored at $A_{215\,nm}$ and the samples then dried in vacuo at 37°C and resuspended in Krebs—Henseleit buffer prior to assay.

Assay for inhibitor activity

Inhibitor activity was assessed by the ability of samples to inhibit IGF activity in a standard IGF/Sm bioassay system. This bioassay measures the stimulation by IGF of [^{14}C]glucose incorporation into [^{14}C]lipid by isolated rat adipocytes [5]. The effect of inhibitor on IGF activity in this system is dose-dependent and specific [3] but is essentially a qualitative rather than quantitative measurement of inhibitor activity. To facilitate the assay of many HPLC fractions, only one dose level (in duplicate) of each fraction was assayed against a single dose of a standard crude preparation of IGF activity (i.e. equivalent to 20%, v/v, of an acid—ethanol extract of a pool of normal human serum [5]). Inhibitor activity has been expressed as the percentage inhibition of the stimulation by that standard dose of IGF.

RESULTS AND DISCUSSION

The HPLC protein elution profile, plotted against the fraction number and percentage isopropanol is shown in Fig. 1A. Fig. 1B illustrates the IGF inhibitor activity profile. The inhibitory activity was eluted from the HPLC column as two peaks of activity between 31% and 34% isopropanol and

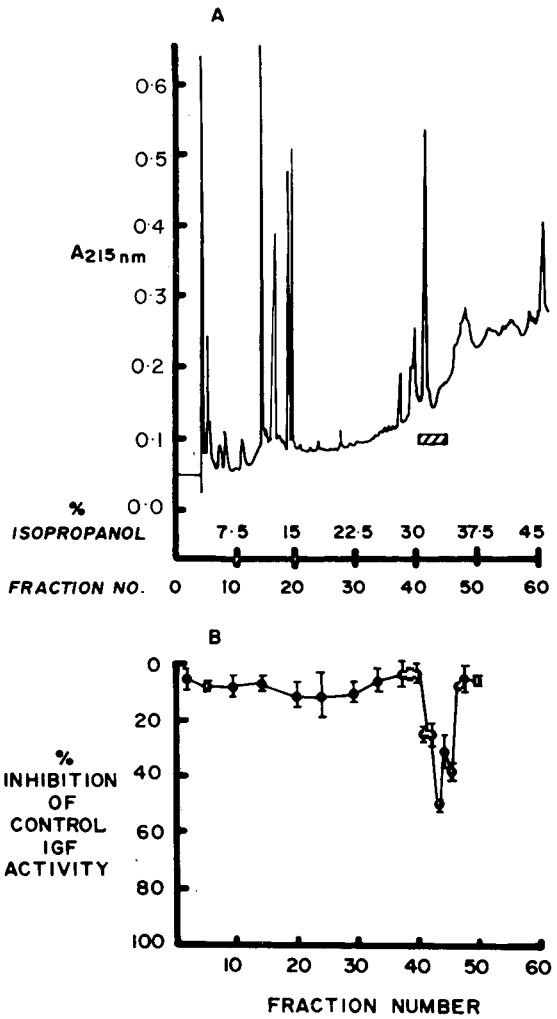

Fig. 1. HPLC of IGF affinity purified inhibitor. (A) Protein elution profile ($A_{215\,nm}$) with ▨ representing inhibitor activity profile as shown in B. Inhibitor activity was measured in pools of five consecutive fractions across the profile except for fractions 41—45 which were assayed individually. Each vertical bar represents the range of the duplicate determinations on each fraction (or pool). HPLC was carried out as described in the Experimental section.

corresponded to a major, apparently homogeneous, protein peak and a minor heterogeneous peak in Fig. 1A. Since the method of assay currently used is not entirely quantitative it is difficult to adequately assess recoveries. However, in other similar experiments (data not shown, but for example see Fig. 2B) the sum of the inhibitor activity present in HPLC fractions was equivalent to that of the activity of the aliquot of the original inhibitor sample applied to the HPLC column, suggesting a substantial recovery.

An IGF inhibitor can also be isolated from Cohn fraction IV-1 of human serum by methods other than that described above in the Experimental section, providing one includes the Bio-Gel P-30 acid chromatography. This step separates the inhibitor from the stimulatory IGF species and has been used to

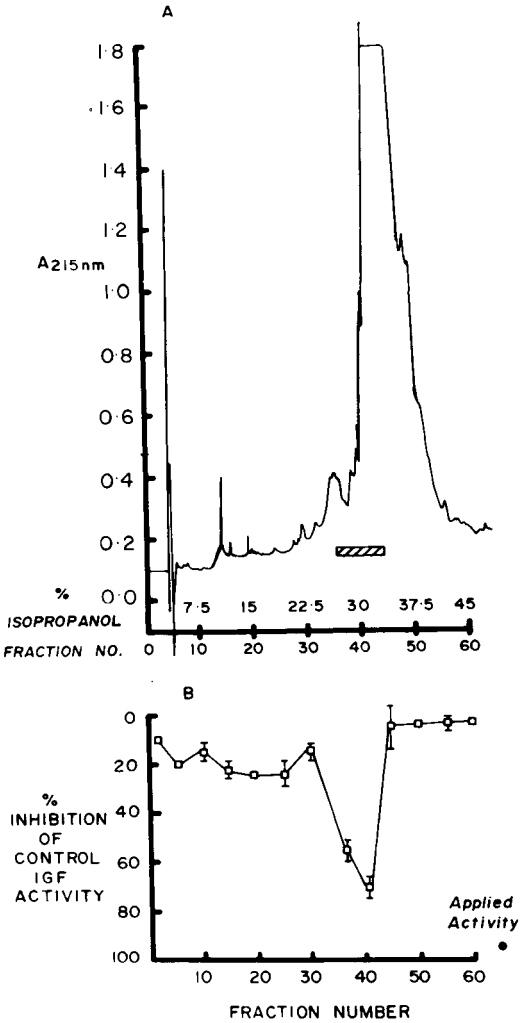

Fig. 2. HPLC of inhibitor without prior IGF affinity purification. (A) Protein elution profile ($A_{215\ nm}$) with ▨ representing inhibitor activity profile as shown in B. Inhibitor activity was measured in pools of five consecutive fractions across the profile. HPLC was carried out as described in the Experimental section.

isolate inhibitor from the recently reported IGF purification method of Svoboda et al. [4]. This inhibitor preparation was applied to the same HPLC system described above, although without prior IGF affinity purification. Because this was a much cruder preparation, the chromatogram (Fig. 2A) revealed that most of the protein ran as a large, broad peak between 30% and 40% isopropanol. However, the inhibitor activity was eluted in a very similar position as that previously described (Fig. 1) although it was recovered as a broader peak with activity spanning 27—34% (Fig. 2B). The activity was associated with the leading edge of the large heterogeneous protein peak. This data suggests that the IGF inhibitors isolated by the two methods [3, 4] are similar if not identical.

The reversed-phase HPLC conditions outlined here were achieved after studies using a variety of ion-pairing reagents (TFA, triethanolamine phosphate, ammonium hydrogen carbonate) and column packings (C_8, C_{18}, CN) together with acetonitrile gradients up to concentrations of 80%. In none of these experiments was inhibitor activity recoverable, despite the fact that inhibitor is stable to such acetonitrile concentrations (unpublished data). This would suggest that the inhibitor is quite hydrophobic. The IGF affinity column has also been used to isolate inhibitor(s) of IGF activity from other sources (e.g. directly from serum; liver extracts). However, the HPLC conditions used above did not result in recovery of inhibitory activity and other conditions are being examined for the purification of these particular species.

The significance of the presence in serum of such a specific inhibitor(s) of the IGF/Sm family is currently uncertain. The data presented here, however, do indicate that, together with IGF affinity purification, HPLC offers a simple procedure for the potential purification of this protein and should provide sufficient material to allow both the development of a direct and quantitative assay (e.g. a radioimmunoassay) for the inhibitor, and a study of its potential physiological role in modulating IGF/Sm stimulation of growth.

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RAPID ANALYSIS OF AMINO ACIDS USING PRE-COLUMN DERIVATIZATION

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SUMMARY

A new approach to the pre-column derivatization and analysis of amino acids is described. The method is based upon formation of a phenylthiocarbamyl derivative of the amino acids. The derivatization method is rapid, efficient, sensitive, and specific for the analysis of primary and secondary amino acids in protein hydrolyzates. The liquid chromatographic system allows for the rapid, bonded-phase separation with ultraviolet detection of the common amino acids with 12-min analysis time and a 1-pmol sensitivity.

INTRODUCTION

Amino acid analysis was pioneered by Moore et al. [1]. The approach for this analysis involved a separation on a sulfonated cation-exchange resin using a series of buffers as the eluent. Detection of the separated amino acids was done by colorimetry via a post-column reaction with ninhydrin [2]. This approach has been the mainstay in the protein laboratory for over the last twenty years.

Recently, conventional liquid chromatographs have been configured specifically for amino acid analysis [3]. These approaches have been variations of the classical analysis of amino acids using separation by high-performance ion-exchange resins [3] followed by derivatization with ninhydrin [4], fluorescamine [5, 6], or *o*-phthalaldehyde/2-mercaptoethanol [7—9]. In all of these liquid chromatographic (LC) approaches the post-column derivatization has required special reaction chambers as part of the chromatographic system. While this is an adequate approach it necessarily dedicates the apparatus to one type of analysis and the ion-exchange separation takes approx. 1 h.

Another LC approach to amino acid analysis has been to derivatize the amino acids before the separation. This has been referred to as pre-column derivatization. After the derivative is formed, the separation occurs on a conventional high-performance reversed-phase column. Dansyl (Dns) [10, 11] or phenylthiohydantoin (PTH) [12] derivatives of amino acids are two examples

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of pre-column derivatization methods. In general, the pre-column derivatization technique, especially with reversed-phase columns, offers greater efficiency, ease of use, and higher speed of analysis than the conventional ion-exchange techniques. The LC analysis of the PTH or Dns derivatives of amino acids was relatively rapid and showed good efficiency and sensitivity; however, these techniques suffer from a lack of derivative stability, less than quantitative yields (PTH) or interference from reagent peaks (Dns).

One reagent, *o*-phthalaldehyde (OPA), has been shown to meet many, but not all, of the requirements for a pre-column derivatization agent for primary amino acids [13, 14]. Most primary amino acids form a single, unique, substituted isoindole product that can be detected by fluorescence. However, OPA does not react with secondary amino acids and this can be a significant drawback.

This paper will report on a new approach to the pre-column derivatization and analysis of amino acids. The method is based upon formation of a phenylthiocarbamyl (PTC) derivative of the amino acids which was first demonstrated by Koop et al. [15] for the analysis of free amino acids liberated by carboxypeptidase Y digestion of peptides from cytochrome P-450. We have modified the technique for the application to free amino acids from acid-hydrolyzed proteins. The new method will be shown to be a rapid, efficient, sensitive, and specific technique for the analysis of primary and secondary amino acids in protein hydrolyzates. The method allows for the rapid, bonded-phase separation with ultraviolet detection of the common amino acids with 12-min analysis time and a 1-pmol sensitivity.

EXPERIMENTAL

Materials

Acetonitrile, triethylamine (TEA), phenylisothiocyanate (PITC) and amino acid standards (Pierce H) were obtained from Waters Assoc. (Milford, MA, U.S.A.). Constant boiling hydrochloric acid (Sequanal™ grade) was obtained from Pierce (Rockville, IL, U.S.A.). Sodium acetate was purchased from Mallinkrodt (St. Louis, MO, U.S.A.). Glacial acetic acid was reagent grade from J.T. Baker (Phillipsburg, NJ, U.S.A.). High-purity water was supplied by a Milli-Q™ purification system (Millipore, Bedford, MA, U.S.A.) that was fed with a supply of reversed-osmosis purified tap water. Hydrolysis and derivatization tubes were Pyrex™ brand (Corning, Corning, NY, U.S.A.), while the vacuum vials and resealable enclosures were from Waters Assoc. Proteins were from a variety of sources.

Sample preparation hardware

A new sample preparation module, the Pico-Tag™ Workstation (Waters Assoc.), was developed [16]. The unit incorporates two separate functions in a compact design for the batchwise preparation of protein and peptide hydrolyzates and for derivatization of the free amino acids. A high-efficiency vacuum pump, equipped with cold and acid traps, is connected to a manifold that allows virtually leak-free attachment of a specially designed vacuum vial

(Waters Assoc.) containing twelve 50 × 6 mm sample tubes. The vacuum vials are equipped with a resealable PTFE closure that is drilled through to allow a vacuum to be applied. The use of hydrochloric acid/water vapor for hydrolysis, rather than placing 6 *M* hydrochloric acid in the sample tubes, allows for a cleaner hydrolysis and faster sample drying after hydrolysis. Condensed hydrochloric acid is kept out of the sample tubes by the bulged sides of the vacuum vial. Removal of trace levels of oxygen from hydrolysis vials is easily accomplished by alternate evacuating and nitrogen flushing steps using the manifold controls on the Workstation. In this manner, twelve samples can be sealed under vacuum in less than 5 min.

The Workstation is also equipped with a four-chamber oven with variable-temperature control. Up to four of the vacuum vials, or 48 samples, can be processed simultaneously.

Hydrolysis of proteins and peptides

Standard proteins and peptides were dissolved in water or 0.1 *M* hydrochloric acid. Stock solutions contained 1.0 mg/ml, and for high-sensitivity work the stock solutions were diluted to 20 μg/ml. A volume corresponding to 0.1 to 5.0 μg was pipetted into a 50 × 6 mm tube, and up to twelve tubes were placed in the vacuum vial. The vial was then attached to the Workstation manifold, and the solvent removed under vacuum. After drying, the vacuum was released and 200 μl of constant boiling hydrochloric acid containing 1% (v/v) phenol was pipetted into the bottom of the vacuum vial (not the hydrolysis tubes). The vacuum vial was then reattached to the manifold, evacuated and sealed under vacuum (130—260 Pa). Samples were hydrolyzed in the Workstation either at 150°C for 1 h or at 108°C for 24 h. After hydrolysis, the outside of the 50 × 6 mm tubes were wiped clean and the residual hydrochloric acid inside the vacuum vial removed under vacuum.

Derivatization of amino acids with PITC

Standards of free amino acids, in a mix (Pierce H) or individual samples, containing up to 25 nmol of each amino acid were placed in the 50 × 6 mm tubes and dried under vacuum. Free amino acids and hydrolyzed samples were dried down again after adding 10—20 μl of ethanol—water—TEA (2:2:1) to each tube. When the vacuum reached 8—10 Pa (about 2—3 min), the samples were ready for derivatization.

The derivatization reagent was made fresh daily and consisted of ethanol—TEA—water—PITC (7:1:1:1). The PITC was stored at −20°C under nitrogen to prevent breakdown products from forming. To make up 300 μl of reagent, enough for twelve samples, 210 μl of ethanol were mixed thoroughly with 30 μl each of PITC, TEA, and water. PTC amino acids were formed by adding 20 μl of reagent to the dried samples and sealing them in the vacuum vials for 20 min at room temperature. The reagents were then removed under vacuum using the Workstation.

Using the conditions and Workstation previously described, the reaction of free amino acids with PITC is essentially complete after 20 min at ambient temperature. Longer reaction times and higher temperature do not result in increased yield of any amino acids; however, decreased yields of the acidic amino acids, glutamic and aspartic acids, were observed at elevated tempera-

ture. Furthermore, neither higher reagent concentration or addition of more reagent after 20 min gave increased response.

Reaction times as short as 10 min could be used on a standard amino acid mixture with little change in yield, but an occasional protein sample exhibited anomalous low yields for certain polar residues (e.g. aspartic acid and histidine) with a shorter period of reaction. These slower kinetics might be caused by relatively poor solubility in the largely organic reaction medium.

Substitution of TEA with other tertiary amines was studied. Both trimethylamine and pyridine were used either as the sole amine or in concert with TEA. However, the replacement of part or all of the TEA yielded significant amounts of interference peaks in the chromatographic analysis, especially in high-sensitivity studies. Even the use of redistilled pyridine posed problems in low-level analyses.

Chromatography

The system was a Model ALC 204 liquid chromatograph (Waters Assoc.) which consisted of two Waters M6000A solvent delivery systems and an M440 fixed-wavelength detector (254 nm) controlled with an M720 controller. The temperature was controlled within ± 1°C with a column heater (Waters Assoc.). Samples were injected in volumes ranging from 1 to 40 μl using an M710B WISP™ auto injector (Waters Assoc.). The columns were application-specified Pico-Tag columns, packed in 15 cm × 3.9 mm hardware, and quality-controlled for rapid, high-efficiency, bonded-phase separations. Eluents were kept under a blanket of helium with an Eluent Stabilization System (Waters Assoc.). The solvent system consisted of two eluents: (A) an aqueous buffer and (B) 60% acetonitrile in water. The typical buffer was 0.14 *M* sodium acetate containing 0.5 ml/l TEA and titrated to pH 6.35 with glacial acetic acid. A gradient which was run for the separation consisted of 10% B traversing to 51% B in 10 min using a convex curve (number 5). After this, a washing step was programmed to 100% B so that any residual sample components would be cleaned from the column.

RESULTS AND DISCUSSION

Resolution and analysis time

The amino acid standards were derivatized using the procedure previously described. The separation of the amino acids at the 250-pmol level is shown in Fig. 1. All of the amino acids are well resolved. The gradient shape was chosen to optimize the spacing of the separated peaks in the minimum analysis time. A more complete discussion of the development of the separation is given elsewhere [17].

Other common amino acids can be analyzed using the same conditions. PTC-carboxymethyl cysteine elutes at 2.7 min and PTC-hydroxyproline at 3.1 min, both well resolved from other derivatives. PTC-tryptophan elutes after PTC-phenylalanine and is also well resolved. Yet to be studied are the derivatives of asparagine, glutamine and the amino sugars that are often found in protein hydrolyzates. Reagent purity is important and if dirty or old TEA reagents are used there can be spurious peaks occurring between peak number

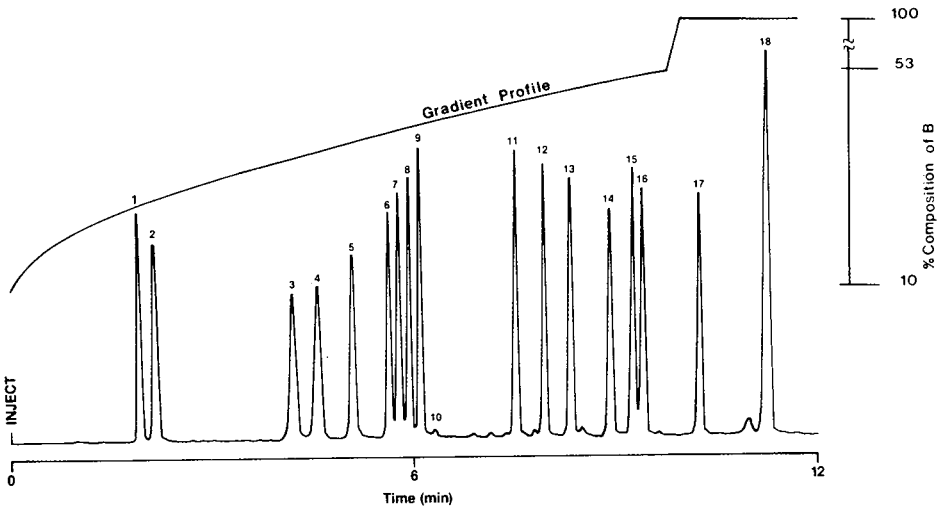

Fig. 1. Separation of amino acid standards (Pierce H). Eluent A: 0.14 *M* sodium acetate, 0.5 ml TEA, pH 6.4; eluent B: 60% acetonitrile in water; gradient: 10% B to 53% B in 10 min on curve 5; flow-rate: 1.0 ml/min; column: Pico-Tag analysis column; detector: ultraviolet (254 nm) at 0.1 a.u.f.s. For peak identification see Table I; 10 = ammonia.

13 and 14. The peak between peaks 17 and 18 is an unknown peak and is present in the analysis of a blank derivative.

Derivative stability

After the reagent is removed under vacuum the derivatized amino acids can be stored dry and frozen for several weeks with no significant degradation. Dissolved in solution prior to injection into the LC system, degradation will occur if the samples are not kept cold. At ambient temperature a 5% drop in response will occur for cystine, valine, and isoleucine after 6 h. After 10 h under the same conditions, most of the other derivatives have lost 5% in response. If kept cold, no noticeable loss in chromatographic response occurred after three days.

Response linearity and reproducibility

For any chromatographic analysis, it is important to demonstrate reaction linearity in the range of analytical interest. Amounts varying from 200 to 5000 pmol were derivatized, and 10% of the total sample was analyzed. Samples were run in triplicate, and the average areas were calculated. The data are plotted in Fig. 2, and show that linear response is indeed obtained in the range 20—500 pmol. Correlation coefficients for these data exceeded 0.999. The poorest linearity was for cystine. The reason for this is unknown at present.

In order to determine the reproducibility of the entire chromatographic procedure several analyses were performed. Individual samples of the amino acid standard mixture were derivatized and analyzed on the liquid chromatograph. The results are shown in Table I. The data have been normalized to the internal standard norleucine. As can be seen the retention time had an average

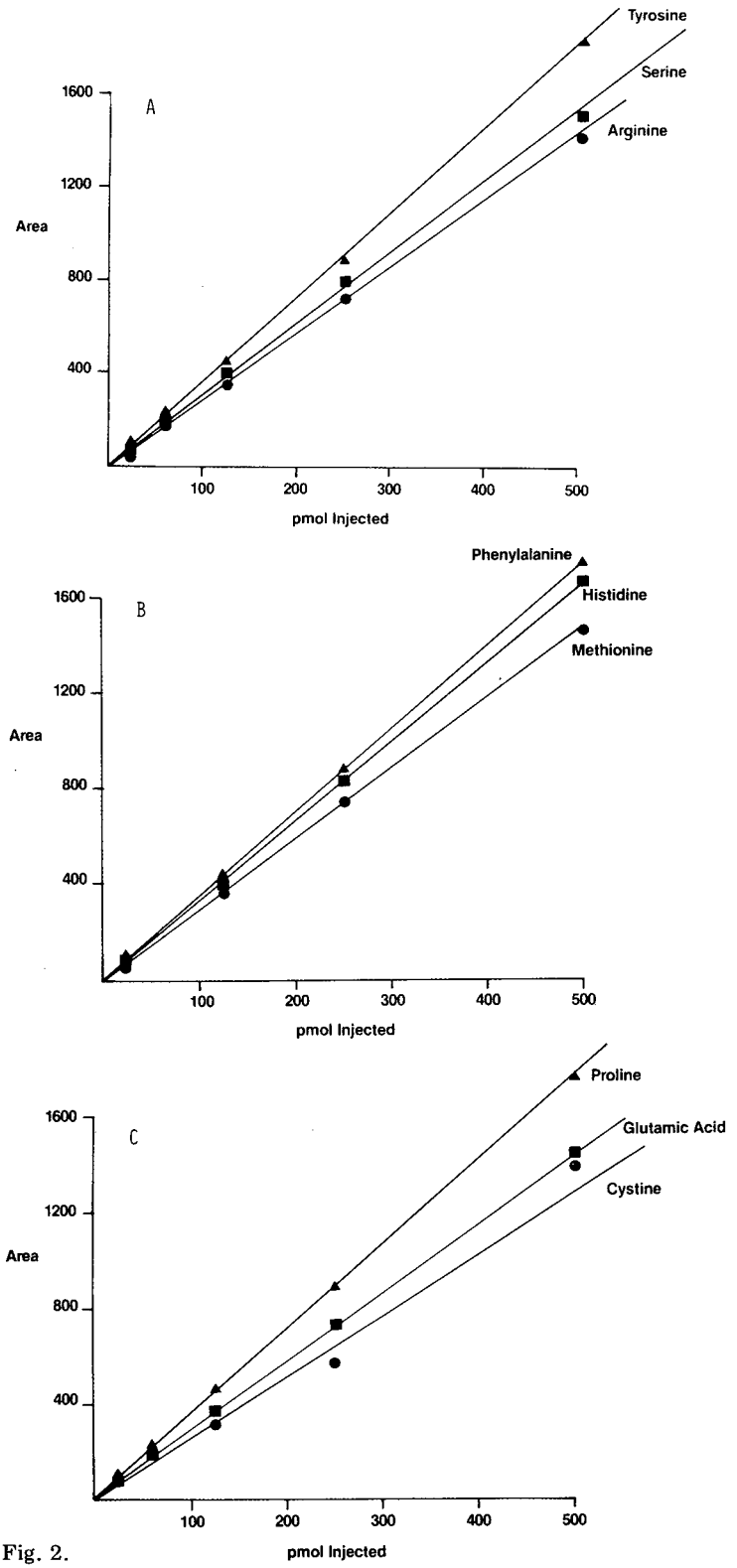
Tyrosine
Serine
Arginine
A
1600
1200
800
400
Area
100
200
300
400
500
pmol Injected
Phenylalanine
Histidine
Methionine
B
1600
1200
800
400
Area
100
200
300
400
500
pmol Injected
Proline
Glutamic Acid
Cystine
C
1600
1200
800
400
Area
100
200
300
400
500
pmol Injected

Fig. 2.

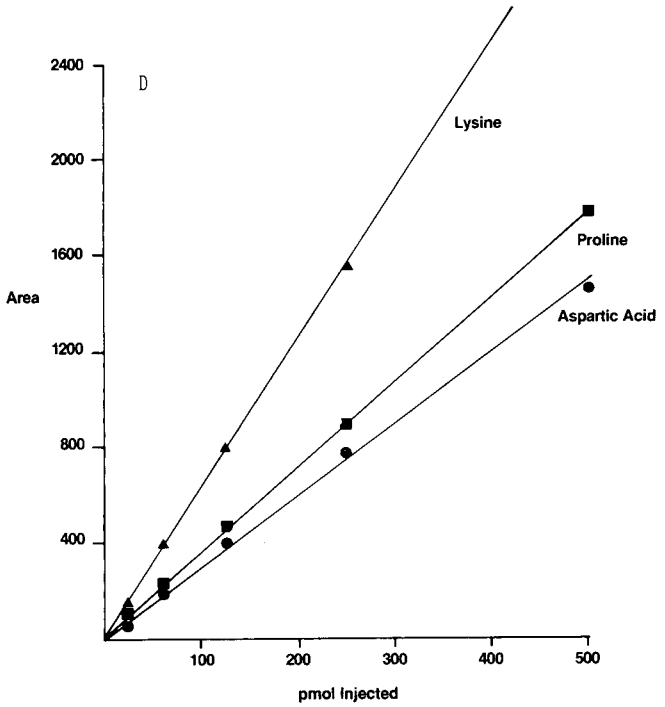

Fig. 2. Linearity of response. See text for details.

TABLE I

REPRODUCIBILITY OF METHODOLOGY

Amino acid	Percent relative standard deviation (n = 8)		
	Retention time	Peak area	
		Uncorrected	Normalized
1. Aspartic acid	0.50	1.86	0.97
2. Glutamic acid	0.45	1.90	1.05
3. Serine	0.41	1.89	0.85
4. Glycine	0.37	1.44	1.48
5. Histidine	0.19	1.82	1.15
6. Arginine	0.23	1.44	1.22
7. Threonine	0.13	2.55	1.56
8. Alanine	0.21	2.07	0.96
9. Proline	0.16	2.39	1.46
11. Tyrosine	0.37	2.15	1.78
12. Valine	0.22	2.03	0.95
13. Methionine	0.22	1.84	0.87
14. Cystine	0.14	4.25	3.85
15. Isoleucine	0.07	2.38	1.23
16. Leucine	0.08	2.04	1.07
17. Phenylalanine	0.11	2.31	1.55
18. Lysine	0.06	4.76	4.60
Average	0.23	2.30	1.48

percentage relative standard deviation of ± 0.23. The largest percentage relative standard deviation occurred for the early eluting peak aspartic acid; however, This was a reflection of a retention time of 1.9 with a variation of less than ± 0.01 min. The average percentage relative standard deviation in peak area was ± 1.48.

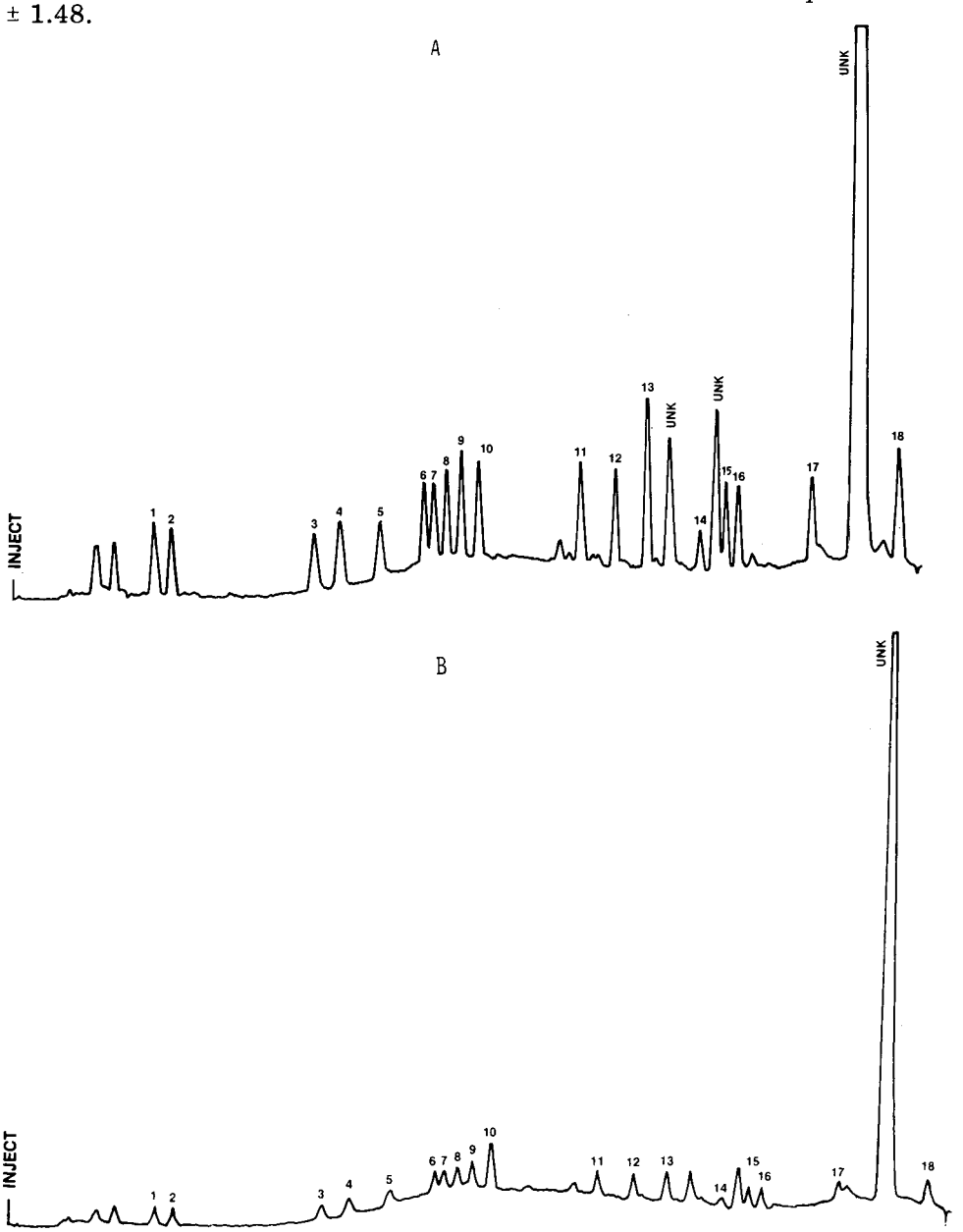

Fig. 3. High-sensitivity amino acid analysis. (A) Amino acid standards at 4 pmol, (B) amino acid standards at 1 pmol. Conditions as in Fig. 1 except gradient is 10% B to 51% B in 10 min on curve 5 and the detector was at 0.005 a.u.f.s. For peak identification see Table I: 10 = ammonia; UNK = unknown.

Detection limits

Fig. 3 shows the analysis of the standard amino acid mixture (Pierce H) at 4- and 1-pmol levels. By defining the detection limit as having a signal-to-noise ratio of 5, the detectable limit for most of the amino acid derivatives can be considered to be 1 pmol. However, because of background amino acids being contributed from solvents, chemicals, glassware, and pipet tips, quantitation of real samples at these levels (hydrolysis and derivatization included) is not practical. It has been the authors' experience that starting with greater than 500 ng of protein before hydrolysis is best suited for the amino acid analysis of proteins. This range is readily handled by the system described earlier using the ultraviolet detector at 0.005 a.u.f.s. With more sophisticated data devices the level of detectability should be much lower.

High sensitivity analysis

The feasibility of using PITC derivatization for the analysis of samples in the nanogram range was investigated. The results show a favorable comparison between compositional analyses performed on 100 ng and 5 μg of oxidized bovine insulin B chain. The 100-ng sample amount was equivalent to approx. 33 pmol of sample being hydrolyzed and 3.3 pmol of the sample injected. Chromatography of 10% of the hydrolyzed samples (10 ng and 500 ng, respectively) gave the results calculated in Table II. As is evident, there is slight loss in reliability at this extremely low level, especially for threonine, proline, phenylalanine and leucine. The results for these amino acids may be high owing to contaminating levels that are evident in blank samples. Nonetheless, it should be emphasized that the low-nanogram-level accuracy demonstrated is sufficient for compositional analysis.

TABLE II

HIGH-SENSITIVITY ANALYSIS OF INSULIN B CHAIN

Amino acid	Calculated ratio of amino acids		Theoretical composition
	10 ng injected*	500 ng injected*	
Aspartic acid	1.2	1.1	1
Glutamic acid	2.4	3.2	3
Serine	1.2	1.0	1
Glycine	3.2	3.0	3
Histidine	1.6	1.9	2
Arginine	1.3	1.0	1
Threonine	1.3	0.9	1
Alanine	2.1	2.0	2
Proline	1.3	1.0	1
Tyrosine	1.5	2.0	2
Valine	2.5	2.9	3
Methionine	0	0	0
Isoleucine	0	0	0
Leucine	2.0	4.0	4
Phenylalanine	2.1	2.9	3
Lysine	0.8	1.0	1

*The injected amount was 10% of total hydrolyzed peptide.

Comparison to ion-exchange analysis

Amino acid analysis is a major analytical need in numerous scientific studies. A new procedure for amino acid analysis must be equivalent or better in speed, sensitivity, precision, and ease of operation when compared to present ion-

TABLE III

AMINO ACID ANALYSIS OF TRYPSIN (0.2 mg HYDROLYSATE)

Amino acid	Mol percent	
	Pico-Tag	Ion-exchange
Aspartic acid	9.6	10.2
Glutamic acid	8.1	8.7
Serine	11.9	10.6
Glycine	15.6	15.3
Histidine	1.7	1.7
Arginine	2.1	2.3
Threonine	4.3	4.4
Alanine	7.7	8.4
Proline	5.7	6.1
Tyrosine	3.3	3.3
Valine	5.1	5.4
Methionine	0.9	0.9
Cystine	4.8	4.0
Isoleucine	5.4	5.0
Leucine	7.3	7.4
Phenylalanine	1.8	1.7
Lysine	4.6	4.5

TABLE IV

AMINO ACID ANALYSIS OF HUMAN INSULIN

Amino acid	Known	Calculated residues per mol*	
		Pico-Tag	Ion-exchange
Aspartic acid	3	3.0	2.9
Glutamic acid	7	6.9	6.8
Serine	3	2.8	2.8
Glycine	4	4.0	4.0
Histidine	2	1.8	1.8
Arginine	1	1.1	1.1
Threonine	3	3.0	2.8
Alanine	1	1.2	1.1
Proline	1	1.0	1.0
Tyrosine	4	4.0	3.7
Valine	4	3.3	3.0
Methionine			
Cystine	6	5.0	4.9
Isoleucine	2	1.3	1.2
Leucine	6	6.2	5.9
Phenylalanine	3	3.1	2.8
Lysine	1	1.1	1.0

*Based on 4.0 residues per mol glycine.

TABLE V

AMINO ACID ANALYSIS OF OXYTOCIN

Amino acid	Known	Calculated residues per mol (Pico-Tag)
Aspartic acid	1	0.8
Glutamic acid	1	1.0
Serine		
Glycine	1	1.0
Histidine		
Arginine		
Threonine		
Alanine		
Proline	1	1.0
Tyrosine	1	0.9
Valine		
Methionine		
Cystine	2	1.5
Isoleucine	1	0.9
Leucine	1	1.0
Phenylalanine		
Lysine		

exchange analyzers. Clearly the new procedure described here exceeds these needs. However, it is also essential that equivalent results be obtained with the new method compared to the traditional method. In Tables III—V the results of the new derivative procedure is compared to the results of ion-exchange analysis [18]. In all cases, the results compare favorably suggesting that the PITC amino acid methodology described in this communication provides an alternative to current ion-exchange analyzers.

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HIGH-SENSITIVITY PHENYLTHIOHYDANTOIN AMINO ACID ANALYSIS USING CONVENTIONAL AND MICROBORE CHROMATOGRAPHY

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SUMMARY

Reversed-phase microbore high-performance liquid chromatography was investigated for high-sensitivity analysis of phenylthiohydantoin (PTH) amino acids. A mixed nitrile alkylsilane bonded phase was developed and ternary gradient elution conditions were devised for resolution of the common PTH amino acids. Elution conditions were developed with a conventional 150 × 4.6 mm I.D. column and transferred to a 150 × 1 mm I.D. microbore column. The performance of these columns was evaluated in terms of PTH amino acid resolution, enhanced sample detectability, and retention time precision. For this work a general purpose high-performance liquid chromatograph was modified to reduce extra column band broadening and a preformed gradient elution technique was developed to achieve rapid analysis times at microbore flow-rates. The microbore high-performance liquid chromatographic system is useful for high-sensitivity analysis of PTH amino acids in micro-sequencing applications.

INTRODUCTION

Trends in biomedical research have required the generation of protein sequence information from increasingly limited amounts of material. Recent advances in protein microsequencing technology have enabled acquisition of reliable sequence data from sub-nanomole quantities of polypeptides, and sequencing at the low picomole level is considered to be within the capability of existing microsequencing methods and instrumentation. It is anticipated that refinements in Edman-based approaches in the near future will permit sub-picomole sequencing of polypeptides eluted from two-dimensional gels [1].

The excellent resolution and reproducibility of reversed-phase chromatography, coupled with the high sensitivity of ultraviolet absorbance detectors have made high-performance liquid chromatography (HPLC) the method of choice for identification of the phenylthiohydantoin (PTH) amino acids generated by Edman degradation [2]. While the performance of existing HPLC

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instrumentation permits analysis of PTH amino acids (PTHAA) at the 5—10 pmol level, reduction of detection limits to subpicomole levels will require improvements in detection methods. Sequence information has been obtained at the 100-fmol level using radioactively labelled peptides [1]. Such improvements could encompass the use of modified phenylisothiocyanate (PITC) reagents with enhanced detectability [3] or the use of laser-based optical detectors with enhanced sensitivity [4]. An alternative approach is the use of microbore HPLC columns to achieve increased solute detectability by reducing peak dilution. Since peak volume is proportional to column cross-sectional area, reduction of column internal diameter from 4.6 mm to 1 mm can produce up to a twenty-fold increase in peak concentration for the same sample mass.

Microbore HPLC has several advantages compared to other means of enhancing detectability. First, many commercial HPLC systems are compatible with microbore columns or can be modified with relative ease for microbore chromatography. Secondly, microbore columns are typically packed with 4—10 μm microparticulate silica-based bonded-phase materials and therefore exhibit selectivity and efficiency comparable to that of the conventional 4 mm and 4.6 mm I.D. reversed-phase columns currently used for PTH amino acid analysis. Thirdly, microbore chromatography can be used as a complementary means of sensitivity enhancement in conjunction with improved detector design and derivatization chemistries.

We have investigated the utility of microbore HPLC for high-sensitivty analysis of PTH amino acids in microsequencing. This report will cover three aspects of this investigation: (a) development of a specific reversed-phase support and elution conditions for rapid resolution of the common PTH amino acids, (b) configuration of a general-purpose high-performance liquid chromatograph for operation with 1 mm I.D. columns, and (c) application of a preformed ternary gradient elution technique permitting reproducible chromatography of PTH amino acids at microbore flow-rates.

MATERIALS AND METHODS

Instrumentation included a Varian 5500 liquid chromatograph equipped with a UV-200 detector (4.5 μl flow cell, 4-mm path length) or, as noted, an optional 0.5-μl (2-mm pathlength) flow cell. A Rheodyne Model 7125 (10-μl loop) or automated 7410 (1-μl loop) injection valve was used. Data acquisition, reduction and automatic sampling were accomplished using a Varian Vista CDS 402 data system and 8085 autosampler.

Standards were prepared in methanol, stored at -20°C, and diluted daily to appropriate concentrations with the starting mobile phase.

Individual PTH amino acid standards were obtained from Sigma (St. Louis, MO, U.S.A.) and a premixed PTH standard was obtained from Pierce (Rockford, IL, U.S.A.).

Mobile phases for HPLC of PTH amino acids included methanol and acetonitrile (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). Buffers were prepared using HPLC-grade sodium acetate and potassium dihydrogen phosphate (Fisher Scientific, Pittsburgh, PA, U.S.A.). HPLC-grade water was generated using Hydro Service's (Varian, Sunnyvale, CA, U.S.A.) water purification system.

The CN (150 × 4 mm, particle size 5 μm) and C_{18} (150 × 4.6 mm, particle size 5 μm) columns were custom synthesized and the PTHAA column, a mixed nitrile and alkyl silane bonded phase (150 × 4.6 mm and 150 × 1 mm, particle size 4 μm) was purchased from Varian.

A 2 cm × 2 mm guard column (Upchurch Scientific, Oak Harbor, WA, U.S.A.) was packed with Vydac 40-μm pellicular C_{18} packing. Addition of the guard column had little effect on overall resolution using the 4.6 mm I.D. column. No guard column was used with the 1.0 mm I.D. column.

RESULTS AND DISCUSSION

Stationary phase optimizations

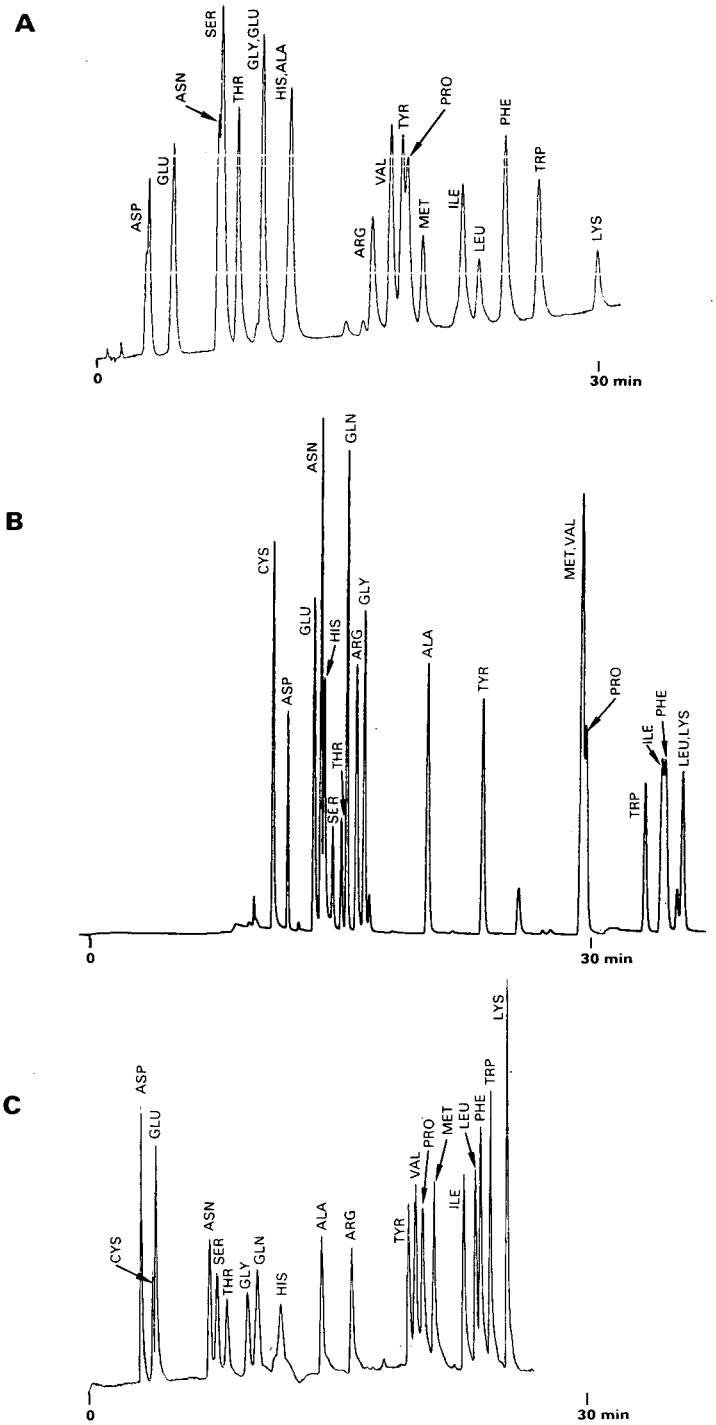
The first step in developing any separation is selection of the proper column. Previous work in our laboratory as well as other published material showed that either a CN (nitrile) column or C_{18} column could be used for PTH amino acid separations. Inspection of the literature reveals many methodologies using both types of column [2, 5—10]. Although columns from various manufacturers differ widely, most, including the MicroPak CN and MicroPak C_{18}, offer better selectivity for different regions of the PTH separation. An example of this can be seen in Fig. 1. Upon inspection, it is apparent the CN column is a better column for resolving the later eluting peaks Met, Ile, and Tyr, but a C_{18} column can best resolve the early eluting components between Cys and Met. Clearly, a stationary phase combining the best selectivity of these two phases would be ideal for PTH amino acid separations.

Experiments using a C_{18} column in series with a CN column were not successful and therefore a stationary phase was synthesized that had both CN and alkyl character. The relative molar ratios of CN and alkyl carbon were adjusted in order to obtain a column with maximum selectivity for the separation of PTH amino acids, herein referred to as a PTHAA column.

Mobile phase optimization of PTH separation

The mobile phase used with the PTHAA column was adjusted empirically to improve selectivity. Experiments with pH and the ionic strength of the buffer showed that the PTH derivatives of aspartic acid, cysteic acid, glutamic acid, histidine and arginine were the most sensitive to changes in these mobile phase parameters. Increase in ionic strength will decrease the retention time of His and Arg. An increase in pH in the range 2.0—6.5 will decrease the retention of the acidic PTH amino acids Asp, Glu and Cys and increase the retention times of Arg and His. Increasing the gradient slope will, paradoxically, increase the resolution of Pro, Val, Tyr and Met.

Simple linear gradients from buffer—methanol (90:10) to 60% methanol were compared to gradients in which acetonitrile had been substituted for methanol. Such experiments showed that use of methanol provided maximum selectivity for separation of the early eluting PTH amino acids between Asp and Arg but resulted in poor resolution of the more hydrophobic PTH amino acids between Tyr and Lys. Using acetonitrile had the opposite effect. In such a circumstance ternary gradients can be exploited to maximize PTH separation. Fig. 2A is a separation using a ternary gradient such that methanol is the principal strong solvent in the beginning of the chromatogram and acetonitrile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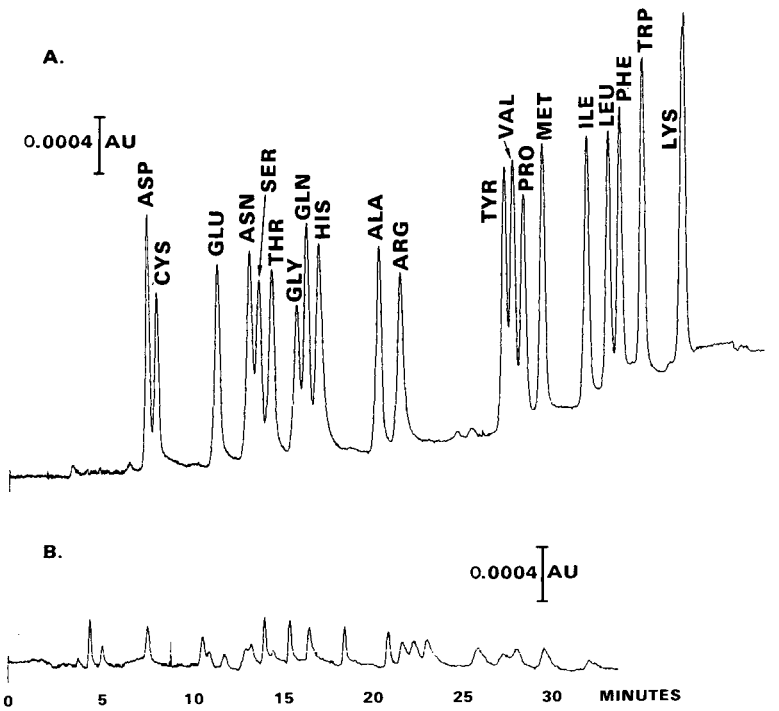

Fig. 2. Comparison of a 10-pmol injection of PTH amino acid using (A) a 15 cm × 1 mm I.D. Microbore-1 PTHAA-4 column and (B) a 15 cm × 4.6 mm I.D. MicroPak PTHAA-4 column. (A) Gradient conditions for the microbore column can be found in the text and in the legend to Fig. 3B. Solvent A: 0.01 *M* sodium acetate, pH 5.0; solvent B: methanol; solvent C: acetonitrile. Temperature 30°C; flow-rate 50 μl/min; 1-μl injection; detection at 270 nm with a UV-200 detector and 0.5-μl flow cell. (B) Solvent A: 0.01 *M* potassium dihydrogen phosphate, pH 5.0; solvent B: methanol; solvent C: acetonitrile. Gradient conditions for the conventional column: t = 0 min, A = 92%, C = 8%; t = 4 min, A = 85%, B = 15%; t = 8.0 min, A = 85%, B = 10%, C = 5%; t = 8.1 min, A = 85%, C = 15%; t = 12.0 min, A = 70%, C = 30%; t = 30 min, A = 40%, B = 60%. Temperature 30°C; detection at 270 nm with a UV-200 detector and 4.5-μl flow cell. Same attenuation on both columns.

Fig. 1. PTH separation on three different columns. (A) Column type CN, 5 μm particle size, 15 cm × 4.0 mm I.D.; flow-rate 1.3 ml/min; temperature 30°C. Solvent A: 0.05 *M* potassium dihydrogen phosphate, pH 6.5; solvent B: acetonitrile. Solvent program: t = 0 min, A = 95%; t = 10 min, A = 75%; t = 27 min, A = 40%. (B) Column type C_{18}, 5 μm particle size, 15 cm × 4.6 mm I.D.; flow-rate 1.0 ml/min. Solvent A: 0.05 *M* potassium dihydrogen phosphate, pH 5.0; solvent B: acetonitrile. Solvent program: t = 0 min, A = 95%; t = 10 min, A = 75%; t = 27 min, A = 60%; t = 37 min, A = 50%. (C) Column type PTHAA, 4 μm particle size, 15 cm × 4.6 mm I.D.; flow-rate 1.3 ml/min; temperature 30°C. Solvent A: 0.05 *M* sodium acetate, pH 6.0; solvent B: methanol; solvent C: acetonitrile. Solvent program: t = 0 min, A = 85%, B = 10%, C = 5%; t = 8.0 min, A = 85%, B = 10%, C = 5%; t = 8.1 min, A = 85%, C = 15%; t = 30 min, A = 40%, C = 60%. Injection of 10 μl on all columns.

at the end. Varying the relative ratios of methanol/acetonitrile allows one to optimize resolution and compensate for changes in selectivity that may occur over the lifetime of the column. Increases in ionic strength will decrease the retention time of His and Arg. Decreasing the starting solvent strength will increase the resolution of the early eluting PTHs between Asp and Arg.

The column temperature was thermostated at 30°C to improve retention time reproducibility. Experiments at higher temperatures (40—60°C) did not improve the resolution of the solutes.

Microbore chromatography

Once the mobile phase conditions had been selected on a conventional

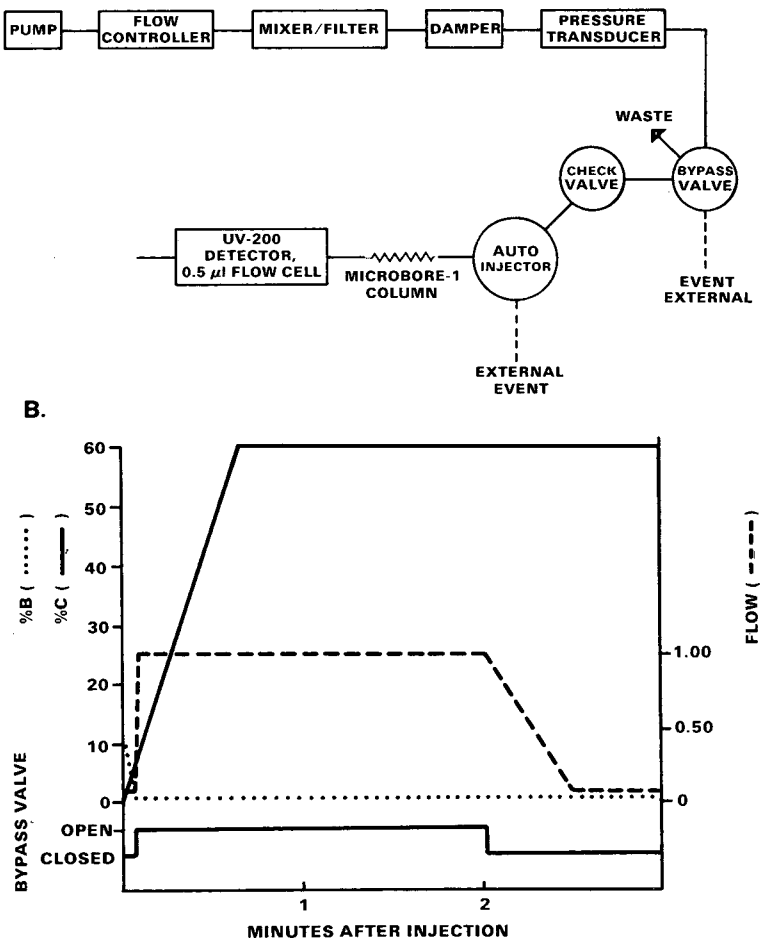

Fig. 3. (A) Scheme of high-performance liquid chromatograph components for microbore chromatography; external events are relays controlled by the instrument microprocessor. (B) Timing diagram used in preforming a microbore gradient. Injection was made at time 0. Solvent A = 0.01 *M* sodium acetate, pH = 5.0; solvent B = methanol; solvent C = acetonitrile. Flow-rate in ml/min. Bypass valve opened 0.1 min after injection, shown here slightly later for clarity.

15 cm × 4.6 mm I.D. column, it was necessary to devise a method to generate a mobile phase gradient using the 1 mm I.D. microbore column at a flow-rate of 50 μl/ml. The LC 5500 chromatograph is capable of forming gradients at microbore flow-rates since solvent proportioning is accomplished during the fill stroke of the single-piston pump and is therefore independent of flow-rate. However, the volume between the pump and injector is about 2 ml so the time delay between gradient formation at the pump delivery to the column top is excessive at 50 μl/min. This time delay can be overcome by using a preformed gradient technique. A variation on a technique outlined by Katz and Scott was explored [11]. Fig. 3 represents a diagram of the system and a listing of the timed events required to carry out such a preformed gradient for PTH amino acid separation. Valve switching is controlled by the instrument microprocessor to insure reproducibility. The injection is made at time 0. At 0.01 min an automatic bypass valve is opened, and the microbore gradient is formed at 1 ml/min over a 0.60-min period. This gradient is then pumped through the hydraulic volume in the chromatograph, and at 2.0 min the bypass valve is closed. At this time the system is pressurized as the flow-rate is gradually reduced over 0.5 min to 50 μl/min. The time at which the bypass valve closes determines when the gradient reaches the column head and may be varied in order to control the initial isocratic portion of the mobile phase program. A check valve is placed in the system between the bypass valve and the injector to minimize rapid column pressure drops when the bypass valve is opened. Gradient profiles were calculated and checked by doping the final solvent with 0.1% acetone. Preformed microbore gradient profiles generated upon running undoped to doped solvents were compared to direct gradients and adjusted to achieve congruency with the direct-gradient slope.

Once the preformed gradient profile has been programmed into the LC 5500 chromatograph, operation is no different than in a conventional mode, i.e. the injector is turned to the inject position and the program started.

Fig. 2 shows a comparison of 10-pmol injections on a conventional 4.6 mm I.D. column versus a 1.0 mm I.D. column. Note the selectivity in both systems is very similar, but the peak signals with microbore are greater.

Sensitivity

The primary reason for investigating microbore chromatography of PTH amino acids was to determine the actual increase in sensitivity measured as signal-to-noise ratio (S/N) gained by reducing the column internal diameter from 4.6 to 1.0 mm.

Detection levels and sensitivity in chromatography are related to a large number of variables including: injection volume, column diameter, path length (UV detectors), noise level, wavelength, peak volume, k', column efficiency and extra column band broadening.

Extra column band broadening can significantly decrease the observed column efficiency resulting in decreased S/N. Reducing the flow-cell volumes without increasing noise is a necessary prerequisite when operating with 1 mm I.D. columns. Substituting a 1.0 mm I.D. column for a 4.6 mm I.D. column without consideration of extra column effects can lead to a decrease in sensitivity and severe losses in resolution. Methods for measuring and quantifying such effects have been outlined by Kok et al. [12].

The major features of the microbore HPLC system used in this study include (1) a 1-μl injector (2) 0.13 mm I.D. connecting tubing and (3) a 0.5-μl flow cell.

For the system tested, the theoretical increase in S/N should be about 10.5 × [i.e. (4.6 mm I.D./1.0 mm I.D.)^2 divided by 2 to account for the different flow cell path lengths of the 0.5- and 4.5-μl flow cells]. This factor assumes the noise level is the same for both flow cells. This was verified experimentally. In a parallel experiment, an isocratic elution of PTH alanine was performed using the 4.5-μl, 4.0-mm path length, flow cell and a 1-μl injector on both 15 cm × 4.6 mm I.D. and 15 cm × 1.0 mm I.D. columns (Fig. 4). The flow-rate was adjusted to give the same retention time for PTH alanine on both columns. This experiment is in agreement with the gradient results in that a 8 × improvement in S/N is observed when a correction for differences in path length is made.

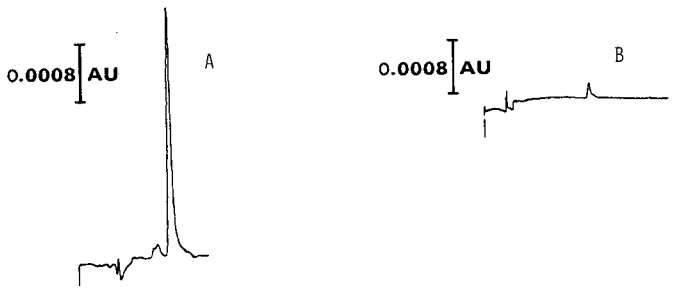

Fig. 4. Isocratic elution of PTH alanine using a 4.5-μl flow cell and 1-μl injection for both columns. All conditions except flow-rate and column diameter were identical, 2.4 pmol per injection. (A) Microbore column, 15 cm × 1.0 mm I.D., flow-rate 0.06 ml/min, S/N = 82. (B) Conventional column, 15 cm × 4.6 mm I.D., flow-rate 1.0 ml/min, S/N = 5.

Precision

Precision data for both microbore and conventional gradient systems are exhibited in Table I. As expected, the relative standard deviations are higher for preformed gradients but nevertheless adequate for PTH amino acid identification. The PTH amino acids were chosen to represent acidic, basic and neutral PTH derivatives.

TABLE I

RETENTION TIME PRECISION IN PTH AMINO ACID ANALYSIS, NINE CONSECUTIVE RUNS

Retention time (R.T.) and standard deviation (σ) in min, and relative standard deviation (R.S.D.) in percent.

Column (PTHAA)	Glu			Arg			Lys		
	R.T.	σ	R.S.D.	R.T.	σ	R.S.D.	R.T.	σ	R.S.D.
Conventional, 4.6 mm I.D.	3.81	0.02	0.5	15.27	0.06	0.4	25.70	0.03	0.1
Microbore, 1 mm I.D.	9.66	0.11	1.1	17.82	0.11	0.6	32.58	0.38	1.1

CONCLUSIONS

The use of a mixed stationary phase offers high selectivity for separation of PTH amino acids. Microbore HPLC on 1.0 mm I.D. columns offers a significant

improvement in sensitivity. This places the minimum detectable quantity of PTH derivatives in the range 0.5—1.0 pmol.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF FATTY ACIDS AS PENTAFLUOROBENZYL ESTERS

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SUMMARY

Using ultraviolet detection (254 nm), pentafluorobenzyl esters have been shown to be suitable derivatives for the semi-preparative separation of fatty acids by number of double bonds on silica columns and by chain length on reversed-phase columns. The two chromatographic systems are entirely complementary in that a critical pair in one of the two systems can be completely separated in the other system, thus allowing the isolation of any given fatty acid from a complex mixture following two sequential injections. The complete separation of pentafluorobenzyl *cis*-9,10-methylene-hexadecanoate and pentafluorobenzyl heptadec-10-enoate in both systems has also been achieved.

INTRODUCTION

Methods for the analytical separation of fatty acids as ultraviolet (UV) absorbing acyl esters in reversed-phase systems have been given in the literature [1, 2] as have methods for the preparative separation of various unsaturated methyl esters on silver-saturated ion-exchange resins [3]. However, there seems to be a dearth of methods for the convenient isolation of fatty acids from complex mixtures for, for example, liquid scintillation counting or for further identification. The analytical methods cannot be conveniently scaled up since critical pairs such as arachidic and erucic [1] or palmitoleic and arachidonic [2], or even palmitic and oleic acids [2] on the preparative scale would not be sufficiently separated to give clean isolations. On the other hand, the preparative method cannot be readily scaled down since the resolution and sensitivity is relatively low. In attempting to find a suitable semi-preparative method we initially turned our attention to normal-phase separations on two types of columns containing Ag^+. The results, particularly for polyunsaturated fatty acids, were not encouraging so we decided to investigate normal-phase separa-

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tions on silica. According to Schwarzenbach [4] separation, by number of double bonds, of underivatised fatty acids can be made on silica provided the ionisation of the carboxyl group is suppressed. We surmised that a similar separation could be made if the fatty acids were to be esterified such that the ester had no additional functional groups to interact with the silica. Since we wished to use a fixed-wavelength detector set at 254 nm, if possible, and since we were also aware of the hydrophobic properties of polyfluorocarbon compounds we decided to investigate pentafluorobenzyl (PFB) esters initially. These proved to be eminently suitable so this paper details the separations of the PFB esters of the various types of fatty acids in both normal- and reversed-phase systems.

EXPERIMENTAL

The high-performance liquid chromatographic (HPLC) equipment was from Waters Assoc. (Milford, MA. U.S.A.): M45 or M6000A pump, U6K injector and M440 detector with a filter for 254 nm detection. Samples for normal-phase separations were dissolved in benzene and for reversed-phase separations were dissolved in isopropanol. Two HPLC columns containing silver were investigated for their ability to separate fatty acid benzyl esters on the basis of the number of double bonds that they contained. First, silver nitrate coated silica (10 g per 100 g) was prepared from LC Porasil A (Waters Assoc.) essentially as described by Battaglia and Fröhlich [5]. This was packed into a 600 mm × 9 mm column (particle size 37—75 μm) using a column packer of the Micromeritics type and the Waters M6000A pump. Secondly, silver aluminosilicate was prepared according to the procedure of Lam and Grushka [6] and packed into a 300 mm × 3.9 mm column as above.

Benzyl esters of stearic (18:0), oleic (18:1), linoleic (18:2) and α-linolenic (18:3) acids were prepared from O-benzyl-N,N′-diisopropyl isourea as described by Knapp and Krueger [7]. This reagent was prepared from diisopropylcarbodiimide [8].

PFB esters were synthesised from 2,3,4,5,6-pentafluorobenzyl bromide (Fluka, Buchs, Switzerland) by a procedure based on the method of Ehrsson [9]. Up to 1 mg of fatty acid sample was dissolved in 1 ml of dichloromethane and 1 ml of a solution containing 0.1 mmol tetrabutylammonium hydrogen sulphate (Fluka) and 0.2 mmol sodium hydroxide was added. Pentafluorobenzyl bromide (20 μl) was then added and the mixture shaken vigorously at room temperature for 30 min. The dichloromethane phase was then evaporated. Ehrsson [9] has shown that the esterification is quantitative for octanoic and longer acids under similar conditions. The residue was taken up in hexane and loaded onto a silica Sep-Pak (20 × 10 mm plastic columns, Waters Assoc.) and the PFB esters were eluted with dichloromethane—hexane (3:17).

Four sets of standard PFB esters were prepared: (a) PFB 18:0; (b) an approximately equimolar mixture of PFB 18:0, PFB 18:1, PFB 18:2, PFB 18:3; (c) an approximately equimolar mixture of the straight-chain fatty acid PFB esters: C_2, C_3, C_4, C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16} and C_{18}; (d) a mixture containing two parts PFB *trans*-18:1 and one part PFB *cis*-18:1. Mixture d was prepared as described above while the other three standards required a scaling up of the quantities. The first three standards were therefore purified by

application to a Merck (Darmstadt, F.R.G.) Lobar Lichroprep® silica gel 60 (particle size 40—63 μm) column (size A, 240 mm × 10 mm) followed by elution with dichloromethane—hexane mixtures and detection at 254 nm with a Pye Unicam SP-6 spectrophotometer (Cambridge, U.K.), rather than on Sep-Pak as described above.

Separations of PFB esters were carried out by normal phase on a μPorasil semi-preparative (300 mm × 7.8 mm; particle size 10 μm) column (Waters Assoc.) using dry dichloromethane—hexane half saturated with water (3:17 or 1:9) and by reversed phase on a C_{18} μBondapak semi-preparative (300 mm × 7.8 mm; particle size 10 μm) column (Waters Assoc.) with methanol—water (19:1).

PFB esters were collected from HPLC separations and identified by comparison with standards by gas chromatography (GC) (EGSS-X on Chromosorb W-HP, 100—120 mesh columns: 1.5 m × 4 mm at 180°C and a flow-rate of 20 ml/min; 5.5 m × 4 mm at 200°C and a flow-rate of 20 ml/min. Effluent gas was divided: 24 parts to a flame-ionization detector and 1 part to an electron-capture detector, Pye GCV). In some cases these identifications were confirmed by gas chromatography—mass spectrometry (GC—MS). A Model 3200 chemical-ionization (CI) system was interfaced to an Incos 2300 data system both from Finnigan-MAT (San Jose, CA, U.S.A.). GC carrier gas (flow-rate 20 ml/min) and CI reagent gas (source pressure 0.8 Torr) was methane. The ion source temperature was 130°C, the column was glass, 1.8 m × 2 mm, 3% OV-17, Gas Chrom Q, 100—120 mesh, programmed from 200°C at 10°C/min to 300°C.

Methyl *cis*-9,10-methylenehexadecanoate (Me 16:CH_2) and methyl *cis*-9,10-methyleneoctadecanoate (Me 18:CH_2) were purchased from Applied Science Labs. (State College, PA, U.S.A.). Methyl heptadec-10-enoate (Me 17:1) and methylnonadec-10-enoate (Me 19:1) were purchased from Nu Check Prep (Elysian, MN, U.S.A.). Each methyl ester was converted into the free acid by saponification with potassium hydroxide in methanol (200 g/l) for 40 min at 80°C and PFB esters synthesized as above.

RESULTS AND DISCUSSION

Initially the silver nitrate column separated benzyl (Φ) esters by number of double bonds, but this selectivity was lost, presumably by the leaching of silver nitrate from the column. Although this problem can be overcome [10] we felt we would be better served to take a new approach since silver nitrate columns are not particularly suitable for use with gradient elution [10] and the large proportion of saturates in our samples was likely to swamp the relatively small proportion of monounsaturates. The separation of the type of samples we are dealing with has been achieved on the preparative scale on silver-saturated ion-exchange resins [3] but this took much longer, some 26 h, and was on a larger scale then we required. Although separations of Φ 18:0, Φ 18:1, Φ 18:2 and Φ 18:3 were obtained on the silver aluminosilicate column, the peak shapes were poor and double peaks were sometimes seen. Presumably this behaviour was related, at least in part, to the destruction of the silica in the preparation of the silver aluminosilicate.

Using the reversed-phase column with methanol—water (93:7), on a preparative scale Φ 18:1 elutes just after Φ 16:0. The resolution (where resolution is

defined as the difference in elution volumes of the two peaks divided by the average peak width measured at their bases) is 0.37. With 10^{-2} *M* Ag^+ (as silver nitrate) [11] added to the mobile phase, Φ 18:1 elutes just before Φ 16:0 with a resolution of 0.38. Thus, in both systems Φ 16:0 and Φ 18:1 are too close to give a satisfactory isolation of either compound.

The PFB 18:0 standard was purified by HPLC on the μPorasil column which afforded a sample for a UV spectrum; the wavelength of maximum absorption (hexane) being 263 nm and with log ϵ = 2.78. PFB esters do not have a high molar absorptivity at 254 nm (log ϵ in hexane is 2.67). Thus 1 μmol with a retention of 15 min gives 0.113 a.u.f.s. However, this is quite adequate for semi-preparative work particularly since the calibration graph is linear. These derivatives have the additional advantage that aliquots can be taken directly from fractions collected from the chromatograph for analysis or identification by GC or by GC—MS.

Fig. 1A shows the separation of the PFB esters of 18:0, 18:1, 18:2 and 18:3 on the μPorasil column. Baseline separation of these compounds is obtained in less than 20 min. The peaks that elute prior to PFB 18:0 (Fig. 1A) appear to be butylated hydroxytoluene, excess pentafluorobenzyl bromide and perhaps a

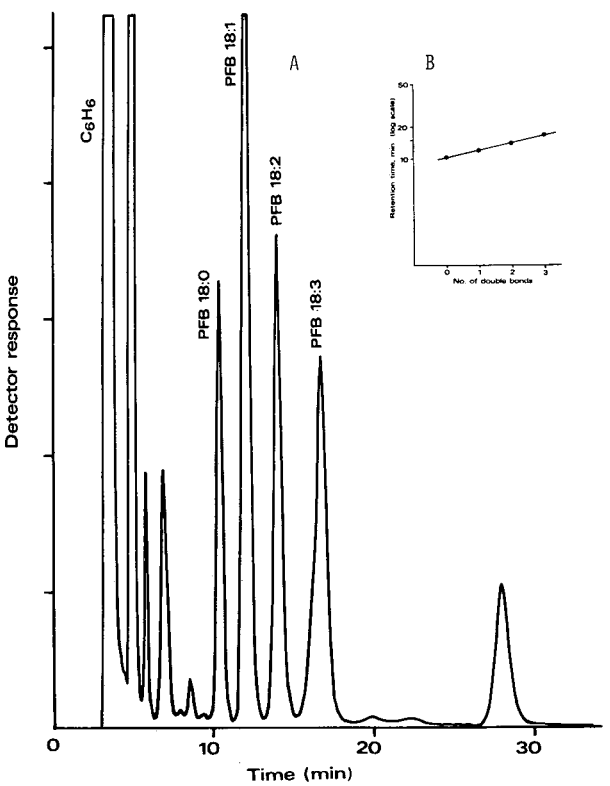


Fig. 1. (A) Normal-phase semi-preparative HPLC separation of PFB 18:0, PFB 18:1, PFB 18:2 and PFB 18:3, approx. 0.1 mg each. (B) Log plot of retention data. μPorasil semi-preparative column. Solvent: dry dichloromethane—hexane half saturated with water (3:17); flow-rate: 4 ml/min; UV detection at 254 nm; 0.1 a.u.f.s.

dimer formed from pentafluorobenzyl bromide during the reaction period. Since both unsaturated and shorter PFB esters elute after PFB 18:0, these impurities do not affect the desired separation. Also included is a plot of log retention time against number of double bonds (Fig. 1B). The resulting straight line suggests that retention due to a double bond is independent of a neighboring double bond, even though the two or three double bonds are only separated by single methylene groups. Further, the abscissa could perhaps be better labelled as number of pairs of electrons, the resulting data points therefore corresponding to 1, 2, 3 and 4 pairs. This would suggest that the π electrons in the carbonyl group of the ester linkage make a similar contribution to adsorption to the π electrons of the double bonds, but that the PFB group makes no contribution.

The separation of the straight-chain saturated PFB esters is shown in Fig. 2A. Although the longer-chain esters elute very close together there is some separation which increases as chain lengths shorten, suggesting that this method could be used for separating short-chain fatty acids (say $\leqslant C_6$). Fig. 2B shows a plot of log (retention time) against log (number of carbon atoms). Except for PFB acetate, the plot forms two straight lines with an abrupt change of slope at C_{10}.

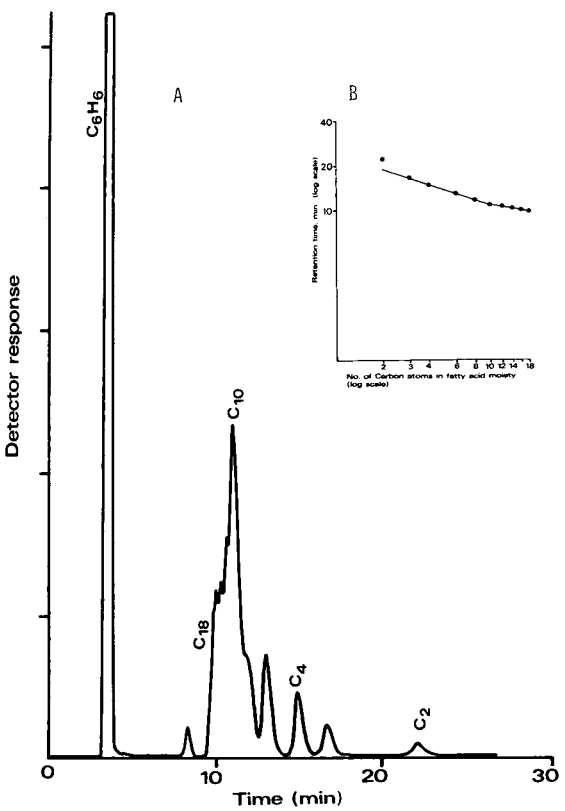


Fig. 2. (A) Normal-phase semi-preparative HPLC separation of the PFB esters of the straight-chain saturated fatty acids: C_2, C_3, C_4, C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18}, approx. 2.5 mg total. (B) Log/log plot of retention data. Conditions as for Fig. 1; 1.0 a.u.f.s.

Since the proportion of water or other hydrogen bonding compounds in the eluting solvent in these normal-phase systems is of over-riding importance in the determination of retention times, we feel that the interactions occurring at the silica gel surface are best described in terms of Scott's [12] model for that surface. This implies that the surface of the column packing is covered with a bilayer of water surmounted by a monolayer of dichloromethane. Displacement of the dichloromethane by the carbonyl of PFB esters or by double bonds would then lead to separation by double-bond number. Further, the relative displacement of dichloromethane by the carbonyl group in the homologous saturated PFB esters might well be reduced with increasing chain length due to the inductive effect. One would surmise that the increase in the inductive effect for each additional carbon atom would be logarithmic and Fig. 2B suggests that this is indeed so, up to C_{10}. The change in slope beyond C_{10} suggests that some additional interaction between the PFB esters and the silica gel surface comes into play. Does this imply that molecules longer than C_{10} can loop around so that the terminal portion of the alkyl chain can also independently displace a dichloromethane molecule? We feel that this system could provide some interesting data for those who are interested in the physical chemistry of interactions at the silica gel surface.

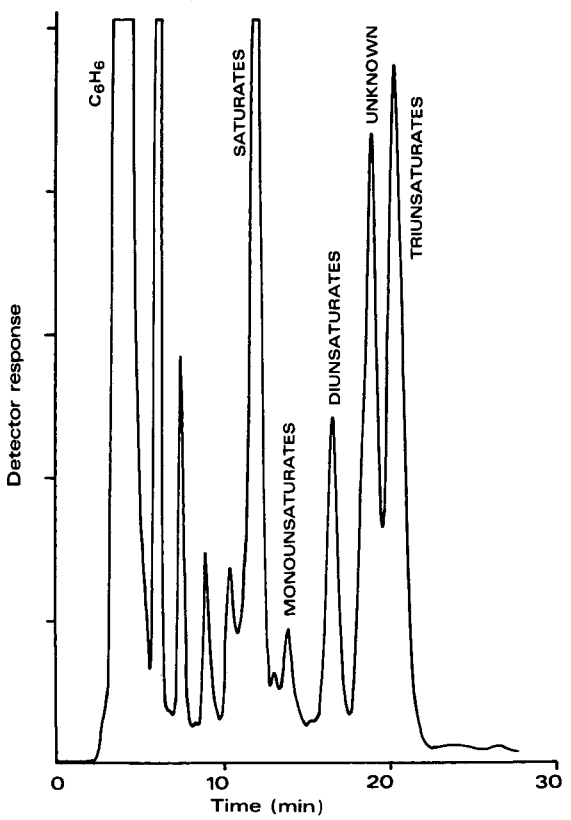


Fig. 3. Normal-phase HPLC separation of the PFB esters from a barley leaf extract. Total injected equivalent to approx. 1.8 g fresh weight leaves. PFB esters were synthesised from a saponified extract using the method given in the text. Conditions as for Fig. 1.

Fig. 3 shows the separation of the PFB esters obtained from a barley leaf extract. The four peaks containing saturates, monounsaturates, diunsaturates and triunsaturates are well separated from each other and can be isolated and separately applied to a reversed-phase column for separation on the basis of chain length. Such a separation, for the saturates isolated as in Fig. 3, is shown in Fig. 4. Taken together Figs. 3 and 4 give a typical pattern for fatty acids from barley leaves [13].

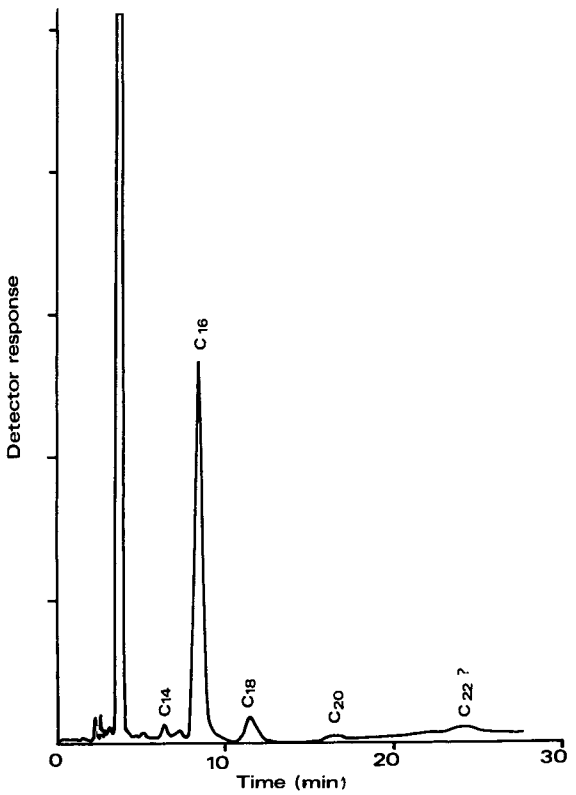
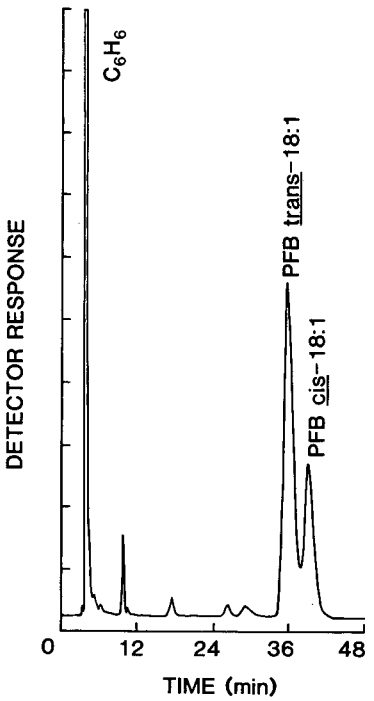


Fig. 4. Reversed-phase separation of the saturated fraction of the PFB esters from a barley leaf extract. C_{18} μBondapak semi-preparative column; solvent: methanol—water (19:1); total injected equivalent to approx. 0.2 g fresh weight leaves; other conditions as for Fig. 1.

We have also demonstrated (Fig. 5) that PFB *trans*-18:1 elutes prior to PFB *cis*-18:1. The resolution of 1.25, calculated from Fig. 5, is not sufficient to be of great practical value but it does emphasise the point that separations in the normal-phase system depend primarily on interactions of the π-electrons with the silica gel surface and are only slightly influenced by steric effects.

The chromatographic separation of cyclopropanoid fatty acids from their monounsaturated isomers in biological extracts is a difficult problem [14] and to our knowledge has not previously been achieved. Fig. 6 shows the separation of the PFB esters of *cis*-9,10-methylene hexadecanoic (16:CH_2) and heptadec-10-enoic (17:1) acids, using a normal-phase system. A similar resolution can be obtained using a reversed-phase column with methanol—water (19:1).



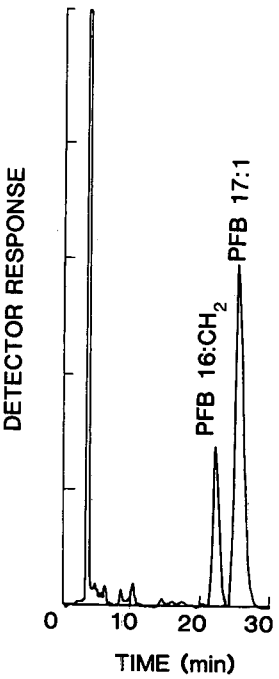


Fig. 5. Normal-phase HPLC separation of PFB *cis*-18:1 and PFB *trans*-18:1. Solvent: dry dichloromethane—hexane half saturated with water (7:93); approx. 0.38 mg PFB *trans*-18:CH_2, 0.22 mg PFB *cis*-18:1; conditions as for Fig. 1; 0.1 a.u.f.s.

Fig. 6. Normal-phase HPLC separation of PFB 16:CH_2 and PFB 17:1. Solvent: dry dichloromethane—hexane half saturated with water (1:9); approx. 0.17 mg PFB 16-CH_2, 0.65 mg PFB 17:1; conditions as for Fig. 1; 0.2 a.u.f.s.

This raises the proposition that it is not so much the chromatographic system that is responsible for the resolution in the separations described here, but the choice of derivative. That is if, for example, a separation on number of double bonds is required it is important that no other functional group in the esters to be used dominates either their adsorptive properties or their distribution coefficients. The result illustrated in Fig. 6 was duplicated with the PFB esters of 19:1 and 18:CH_2. In both cases each fraction was collected and subjected to methane CI GC—MS. The CI mass spectra of each isomeric pair were virtually identical with only minor variations in the intensity of the ions recorded. A similar result was found with the electron-impact (EI) and CI mass spectra of the isomeric methyl ester derivatives [14].

Overall we therefore feel that PFB esters are well suited for semi-preparative isolations of fatty acids. The resolution in the normal-phase separations reported here seems to be primarily due to a lack of any interaction between the PFB group and the stationary phase. Thus the strength of the interaction between the stationary phase and a PFB ester is determined by the structure of the acyl chain, other than a small contribution from the carbonyl group. It appears to us that this is not true for methyl esters and particularly for the highly UV absorbing esters used in the analytical separation [1, 2] of fatty acids. In these

reversed-phase systems, although several esters have been tried: phenacyl [1], *p*-bromophenacyl, *p*-nitrophenacyl, *p*-chlorophenacyl and 2-napthacyl [2], with some large columns: 900 mm × 6.4 mm C_{18} μBondapak [1] or two 300 mm × 3.9 mm C_{18} μBondapak in series or two 300 mm × 3.9 mm fatty acid analysis in series [2], the nett result is to only slightly change the relative retention times of the various fatty acids. Thus in these methods it has taken 3—4 h to resolve some critical pairs, and even these conditions would not have allowed clean isolation of some of the individual fatty acids. However, PFB esters used with a combination of a normal-phase followed by a reversed-phase column (only one of each) can afford the isolation of any desired fatty acid in 20 min on each column. Thus, in order to completely resolve all critical pairs, some sacrifice in sensitivity has to be made so that the complementary normal- and reversed-phase systems can both be utilised. Finally, since the esterifying methyl group of methyl esters appears to have some interaction with the silica in normal-phase separations, it may be of advantage to use PFB esters in the separation of geometrical and positional isomers of fatty acids on silver nitrate impregnated silica [5].

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MICROBIAL ACID METABOLITES

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SUMMARY

The use of high-performance liquid chromatography with a cation-exchange column and effluent monitoring at 210 nm has been evaluated for the profiling of selected microbial metabolites including aliphatic, dicarboxylic, and phenolic acids, as an adjunct to the identification of selected bacteria, detection of bacterial metabolites in foods, and the monitoring of industrial microbial fermentations. Advantages of the technique include the simultaneous profiling of different classes of organic acids without derivatization. Most applications require only qualitative or semi-quantitative data. For others, data are given on the day-to-day reproducibility for several acids.

INTRODUCTION

The profiling of C_2—C_7 fatty acids, and several dicarboxylic and keto acids, present as metabolites in spent cultures of bacteria, is a valuable adjunct to other procedures for the presumptive identification of anaerobic bacteria. Comprehensive procedures for the profiling of the metabolites use gas chromatography (GC) for the analytical separation [1—4]. The procedures require separate manipulation of the sample for different classes of the acids. The C_2—C_7 fatty acids may be chromatographed directly from the acidified culture media or after a one-stage solvent extraction. Most other acids require derivatization and extraction of the derivatives before GC. Metabolites other than acids have been successfully profiled by GC [4]. These include alcohols, amines, hydroxy acids and nitrosoamines. However, as other classes of compounds are included, sample manipulation becomes more demanding.

Developments in high-performance liquid chromatography (HPLC), especially column and detector technology, have shown that it is feasible to use HPLC as an alternative to GC for the determination of the acid metabolites. Earlier HPLC studies for the separation of carboxylic acids included those using detection based on pH [5], ion exchange with chemical detection [6], and detection

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using conductivity [7]. More recently separations of carboxylic acids on cation-exchange resin columns used ion-exclusion chromatography (IEC) coupled with ultraviolet (UV) monitoring of the effluent [8—11] or conductometry [12]. Mobile phases for IEC have been water [13], dilute mineral acid [8, 10], dilute mineral acid modified with acetonitrile [11], and aqueous *n*-butyric acid [12]. The use of HPLC is attractive because, unlike GC, several classes of acids, including phenolic acids, may be determined within one analysis. Extraction, but no derivatization, of the acids is required. Procedures have been reported for the analysis of bacterial metabolites using a cation-exchange resin column with 220-nm [9] or 210-nm [10, 11] monitoring of the eluent. The bacterial metabolites in buttermilk [9], *Clostridia* cultures [10] and in cultures of a group of clinically significant bacteria [11] have been successfully analyzed.

In the two latter reports [10, 11] the object has been to aid in identifying bacteria chiefly for clinical purposes. Other important applications of the procedures are to monitor bacterial fermentations in general. Types of sample may include food products, such as cultured buttermilk [9], wines [12], starter cultures, food contaminated with bacteria, liquid from waste digesters, and body fluids. For these applications qualitative data are often sufficient.

For supplementary identification purposes, semi-quantitative results are adequate. However, HPLC can provide excellent quantitative data under controlled conditions. In this study, we present the results of evaluating HPLC procedures of Guerrant et al. [11], with minor differences, on a wide variety of sample types. These include the acid products of bacteria, including several *Salmonellae*, *Clostridia*, and *Lactobacilli*. Differentiation into hetero- and homofermentative types of *Lactobacilli* was evaluated. Fermentations of heterogeneous mixtures of bacteria present in wine-making, an artificial gut and a fruit waste digester were monitored. Generally, comparing the acid profiles of samples was most useful. Both semi-quantitative and quantitative data, including reproducibility, are presented.

EXPERIMENTAL

Materials

Acetonitrile (Acetonitrile-190, Ajax Chemicals, Sydney, Australia) was HPLC grade. Water was from a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). Analytical reagent grade sulphuric acid and diethyl ether were used. Reference carboxylic acids were obtained either from Aldrich (Milwaukee, WI, U.S.A.) or from Sigma (St. Louis, MO, U.S.A.) as free acids or alkaline salts. Standard acids were prepared first as individual stock solutions in 0.003 mol/l sulphuric acid then combined to give a diluted working reference standard. Aliquots of the working standard were dispensed into 1-ml vials (Hypo-Vials, Pierce, Rockford, IL, U.S.A.). Standards were stored at 4°C when not in use. The acids used were 99+% pure, except for lactic, pyruvic, oxaloacetic, and the aromatic acids (approx. 95% pure). The acids and concentrations of the working standard are given in Table I.

TABLE I

CONCENTRATIONS OF ACIDS IN THE WORKING STANDARD, THEIR MEAN (n=6) RETENTION TIMES (t_R) AND RESPONSE FACTORS (RF) RELATIVE TO n-VALERIC = 1.000

Elution order	Organic acid	Mol/l	t_R (min) (S.E.M.)*	RF
1	Oxalic	0.002	8.1 (0.04)	2.352
2	Oxaloacetic	0.0001	9.2 (0.06)	0.074
3	Pyruvic	0.004	11.0 (0.07)	2.532
4	Succinic	0.001	13.4 (0.04)	0.074
5	Lactic	0.01	15.0 (0.05)	10.210
6	Formic	0.02	17.0 (0.08)	1.164
7	Acetic	0.02	17.3 (0.06)	0.751
8	Propionic	0.02	19.1 (0.10)	0.806
9	Isobutyric	0.02	21.0 (0.09)	1.154
10	n-Butyric	0.02	23.9 (0.25)	0.841
11	Isovaleric	0.02	25.7 (0.20)	0.999
12	n-Valeric	0.02	28.9 (0.18)	1.000
13	Caproic	0.02	30.9 (0.08)	0.481
14	2-Methylvaleric	0.005	34.0 (0.24)	0.051
15	p-Hydroxyphenylacetic	0.0001	38.6 (0.32)	9.140
16	Phenylacetic	0.002	43.2 (0.22)	10.647
17	Heptanoic	0.02	50.9 (0.25)	0.376
18	3-Phenylpropionic	0.0001	60.2 (0.49)	0.224

*S.E.M. = standard error of the mean, given between parentheses.

Apparatus

The liquid chromatographic equipment (Perkin-Elmer, Norwalk, CT, U.S.A.) consisted of a Sigma 3B pump module, an LC 100 column oven, an LC 75 detector, and a Sigma 15 Data Station. The column, 300 × 7.8 mm (9 μm particle size), Aminex HPX-87H (Bio-Rad Labs., Richmond, CA, U.S.A.) was obtained prepacked. The injection system was a Rheodyne Model 7105 injector valve (Rheodyne, Berkeley, CA, U.S.A.).

HPLC conditions

The detector was operated at 210 nm at full scale sensitivity of 0.1 absorbance unit. The mobile phase was 0.003 mol/l sulphuric acid—acetonitrile (90:10, v/v). The column was operated at 50°C and a mobile phase flow-rate of 0.6 ml/min. Before use the new column was conditioned under temperature and flow conditions with the mobile phase for one day to remove high UV-absorbing residues from the column to ensure a stable baseline. Flow-rate through the column during use increased from a starting pressure of approx. 5.0 MPa to 7.0 MPa after about fifty separations. At 8.0 MPa, flow-rate through the column was reversed for 16 h or until the pressure was reduced close to 5.0 MPa. The column was then returned to its original flow direction and analyses continued. A volume of 3.5 l of the mobile phase was prepared. The column effluent was collected into a clean container, protected from dust

contamination and excessive evaporation, and recycled as a batch when about 3 l were collected. It was possible to repeat the recycling about five times before fresh mobile phase was prepared.

Bacteria

Clinical isolates of *Clostridium perfringens* (FRR B179) and *Clostridium difficile* (FRR B180) were each grown in 20 ml cooked-meat medium (CMM) [1] and in peptone-yeast extract glucose (PYG) broth [1] for four days at 37°C. *Salmonella infantis* (FRR B278), *Salmonella sofia* (FRR B279), *Salmonella typhimurium* (FRR B277), *Streptococcus faecalis* (FRR B343), *Staphylococcus aureus* (FRR B343) and *Staphylococcus epidermidis* (FRR B342), *Lactobacillus casei* (FRR B738) and *Lactobacillus fermentum* (FRR B737) obtained from food industry sources, were individually grown in PYG anaerobically for four days at 37°C. Quantities of sterile media were incubated and tested for low-residual acids. Acetic acid is often present to excess as a result of pH adjustment. Such media should not be used.

Samples from mixed microbial fermentations

Samples included waste digester and artificial gut liquids, food products including soy sauce, and wines.

Sample pretreatment

Bacterial cultures were centrifuged at 10,000 *g* to remove particulate matter. Aliquots of 1 ml were transferred to 5-ml tapered, capped centrifuge tubes and 0.25-ml of 9.0 mol/l sulphuric acid added with 0.6 g sodium chloride, 5 ml diethyl ether and 25 µl acetonitrile. The mixture was mixed with a vortex for 1 min and centrifuged at 1500 *g* for 5 min. Using a 5-ml variable-pipettor set to 4.5 ml the diethyl ether phase was transferred to a clean tube. To the diethyl ether was added 0.25 ml of 0.1 mol/l sodium hydroxide, the contents mixed cautiously using a vortex mixer and centrifuged at 1500 *g* for 5 min. The diethyl ether was discarded and 25 µl acetonitrile were added. Residual diethyl ether was allowed to evaporate from the open tube. The residue was mixed and 20-µl aliquots used for chromatography. Less liquid samples were first homogenized on a weight/volume basis with water, then extracted. Samples may be stored in a capped tube at 10°C for several months if taken to the step before adding the final 25 µl acetonitrile. Minor losses only may occur in the ketoacids.

Food products that have resulted from microbial fermentations, including some dairy products, sauces and wines, were processed similarly. Where quality control requirements only need be met, extraction may not be necessary providing that a clarified sample is available. Wines were profiled successfully without extraction. There is less control over peak identification, but a reproducible fingerprint is obtainable. Where extraction was not used, or where the product such as soy sauce was a concentrate, dilution with water was used. Wines were diluted × 10 and soy sauce × 50.

External standard. The reference standard was extracted by the same sample pretreatment as above for the quantifying of acids in test samples.

Calculations

Chromatograms were evaluated by use of an external standard. Identification of peaks was assumed by coincidence of retention times with the standard. To assess peak profiles of the extracts of bacterial isolates as an adjunct to identification, semi-quantitative assessment was made by comparison of peak areas of test samples with those of the standard and recording the areas as larger, equal to, or smaller than, those of the standard. Quantitative assessment was required for assignment of *Lactobacillus* spp. to hetero- or homofermentative groups based on the relative amounts of acetic and lactic acids produced [14]. For this and similar requirements, sterile media containing a range of concentrations of the acids of interest were extracted and chromatographed. Peak areas were plotted for the extracted standards and concentrations of acids in test samples were calculated from the standard plot.

Extraction recoveries

The ratios of the peak areas of the extracted standards to the peak areas of unextracted standards were expressed as percentage recoveries.

Reproducibility studies

For within-day reproducibility of the method, six extracts from a single culture of *C. perfringens* were chromatographed. To assess day-to-day reproducibility, cultures of *C. perfringens*, were grown on each of four days to give a total of four cultures. As each incubation was completed it was processed.

RESULTS AND DISCUSSION

A chromatogram of the working standard is given in Fig. 1. Baseline separation is adequate for most of the acids. The chromatogram shows several peaks additional to those labelled. Several of these were related to impurities in the

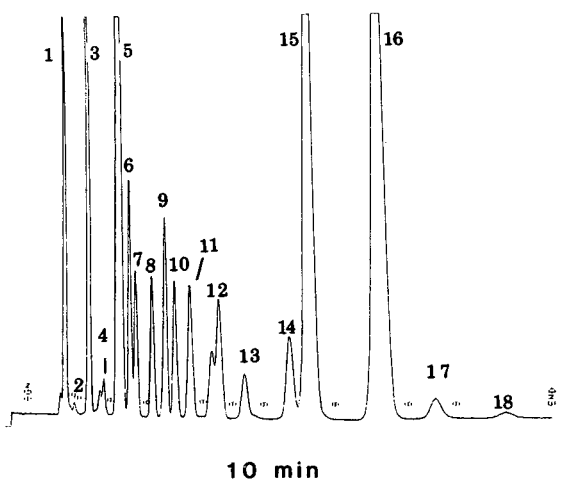

Fig. 1. Chromatogram of a standard solution of eighteen acids. The key to the numbered peaks and the concentrations used are given in Table I. The peak eluting on the leading edge of peak 12 was attributed to an impurity in the 3-phenylpropionic acid.

acids used for the standard. The peak eluting on the leading edge of peak 12 (Fig. 1) was a component of the 3-phenylpropionic acid standard.

Resolution is partly dependent on temperature and concentration of acetonitrile. Adjustment of these may be necessary to optimize conditions for a new column or to modify the resolution required for specific groups of the acids. The mean retention time (n=6) and the detector response relative to n-valeric acid (1.00) for each of the acids under the recommended conditions is given in Table I. The response of the detector was much greater for several acids such as the phenolic acids. Where large quantities of any acids are present in a sample, it may for some extracts be necessary to attenuate detector response in order to evaluate the peaks correctly.

TABLE II

RECOVERIES OF A MIXTURE OF SELECTED ACIDS IN 1 ml WATER EXTRACTED INTO 5 ml DIETHYL ETHER

Results were obtained from five extractions.

Acid	Concentration (mmol/ml)	Recovery (%) (S.E.M.)*
Pyruvic	0.004	22.4 (1.10)
Lactic	0.010	24.0 (0.91)
Acetic	0.020	41.2 (0.98)
Propionic	0.020	55.2 (0.76)
Isobutyric	0.020	69.0 (0.08)
n-Butyric	0.020	61.4 (0.72)
Isovaleric	0.020	72.1 (0.90)
n-Valeric	0.020	63.3 (0.71)
Caproic	0.020	54.0 (0.84)
Heptanoic	0.020	49.6 (0.69)
Phenylacetic	0.002	50.2 (0.89)

*S.E.M. = standard error of the mean, given between parentheses.

TABLE III

REPRODUCIBILITY DATA FOR SELECTED ACIDS PRESENT IN CULTURES OF *CLOSTRIDIUM PERFRINGENS*, TYPE *C*

Within-day data were from six aliquots of a single culture. Day-to-day data were from four individual cultures started on consecutive days and processed as each incubation was completed.

Acid	Concentration (μmol/ml of culture)					
	Within-day (n=6)			Day-to-day (n=4)		
	Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)
Acetic	8.5	0.42	4.9	8.3	0.51	6.2
Propionic	0.8	0.05	5.8	0.7	0.05	7.3
n-Butyric	3.3	0.11	3.4	3.5	0.18	5.2
Isovaleric	0.6	0.03	5.1	0.7	0.05	7.1
n-Valeric	0.7	0.03	4.7	0.6	0.03	4.1
Phenylacetic	0.1	0.006	6.1	0.1	0.008	7.8

Extraction recoveries were comparable to those reported by Guerrant et al. [11] for formic, propionic, fumaric and lactic acids allowing for the smaller volume of diethyl ether taken for the back-extraction step. Recoveries of these and other acids studied by us are given in Table II.

The within-day reproducibility (coefficient of variation, C.V.%) of the individual acids tested ranged from 3.4% to 6.1%. The day-to-day reproducibility (C.V.%) of the cultures of *C. perfringens* was calculated to be 4.1—7.8%. The data are given in Table III. The data show that for most quantitative applications, reproducibility is adequate. Caution would be needed where grossly different ratios of acids with close retention times are present or where unidentified components are present.

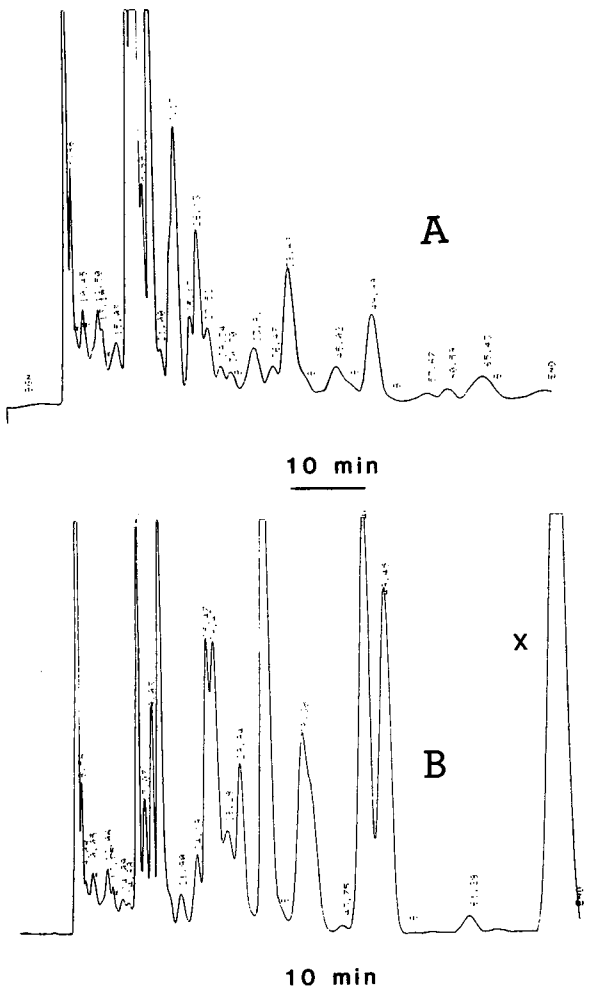

Fig. 2. The acid profile of *Clostridium perfringens* (A); and *C. difficile* (B) grown in cooked-meat medium. An important distinction is the large final peak (X) present in the *C. difficile* chromatogram. The peak retention time fits that of *p*-cresol, produced by this microorganism.

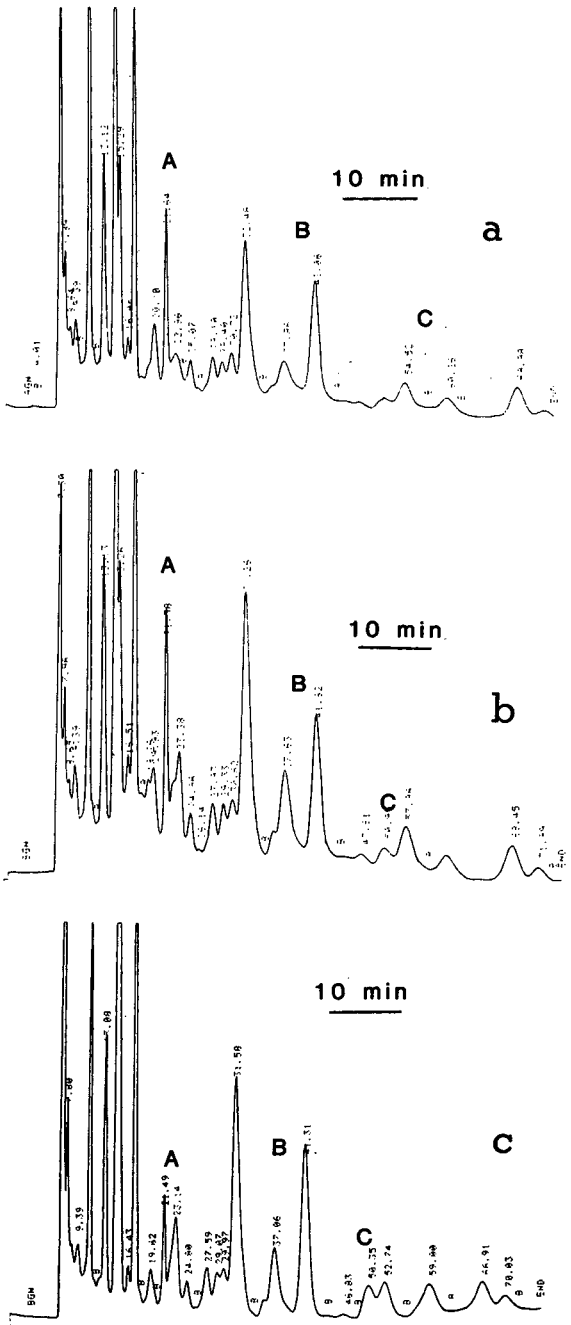

Fig. 3. Chromatograms of extracts of cultures of *Salmonella sofia* (a); *S. infantis* (b); and *S. typhimurium* (c) grown anaerobically in PYG medium. Close similarities exist between the chromatograms but differences may be noted between the peak groupings about A, B and C.

The acid profiles of bacterial cultures showed that considerable differences existed between genera and between the species tested. Fig. 2 illustrates chromatograms obtained from the cultures in CMM of *C. perfringens* (Fig. 2A) and *C. difficile* (Fig. 2B) showing major differences in quantities of several of the acids. The chromatogram of the *C. difficile* extract shows a large peak (X) which corresponds, as noted by Guerrant et al. [11] to the retention time of *p*-cresol, a metabolite expected in *C. difficile* cultures. No significant advantage to interpretation of the chromatograms was given by using CMM rather than PYG. A greater quantity of *p*-cresol was obtained with CMM for *C. difficile* cultures. Fig. 3 shows the chromatograms of extracts from the *Salmonellae*, showing close similarity within the group but useful differences were noted for the peak groups A, B, and C. Fig. 4 shows the different profiles obtained for *L. casei* and *L. fermentum*. The differences were considered to be sufficiently marked to be useful as an adjunct to other identification procedures.

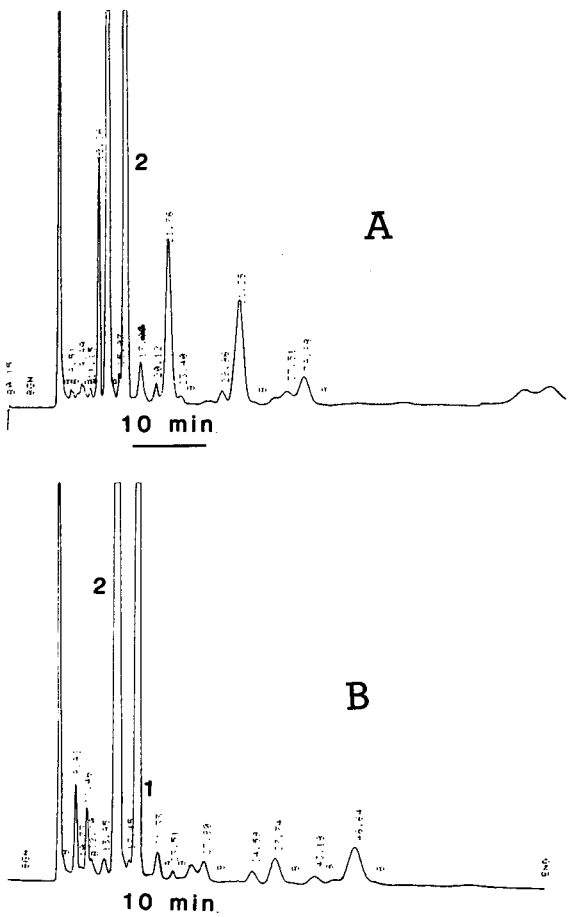

Fig. 4. Chromatograms of extracts from *Lactobacillus casei* (A); and *L. fermentum* (B). The two profiles are distinctive. Additionally, the ratios of acetic (peak 1) to lactic (peak 2) permitted assignment of *L. casei* and *L. fermentum* to homofermentative and heterofermentative, respectively.

Distinctive differences were also found for the acid profiles of the *S. faecalis*, *S. aureus* and *S. epidermidis* cultures (data not shown). Further data are needed to confirm such use for a wide range of bacterial species.

The basis for assignment of a *Lactobacillus* as hetero- or homofermentative is whether it produces acetic acid greater or less than 50% of the lactic acid produced [14]. The *L. casei* (Fig. 4A) and *L. fermentum* (Fig. 4B) were readily assignable to homo- and heterofermentative by this criterion, the values of acetic:lactic ratios being 7.2:92.8 and 68.5:31.5, respectively. The reproducibility of acetic acid was about 4.9% C.V. and that for lactic acid about 5.2% C.V. on a within-day basis. For these *Lactobacilli* the variation would not significantly effect the result. For other species where the ratio is closer interpretation may be difficult, but in such cases modification of the mobile phase may improve resolution and consequently the reproducibility.

The potential of the HPLC procedure for some food products is indicated in Fig. 5 showing chromatograms obtained for diluted wine samples, a red wine (A), a port wine (B), and one obtained for a soy sauce (C) obtained by a mixed fermentation including *Lactobacilli*, yeasts and *Fungi*. The wines were not extracted and must therefore have contained a mixture of acids, bases and neutrals. The soy sauce was extracted but contained several compounds not identifiable. However, the profile itself was considered to be a probable useful indicator of product integrity and stability. The chromatogram was generally characteristic of several samples of soy sauce of the same make. Benzoic acid is a common preservative in some food products, including some soy sauces. The peak X on the chromatogram (Fig. 5C) was provisionally assigned to benzoic acid.

The profiles obtained from the fluid of a citrus waste digester [15] were useful in indicating the healthy or otherwise status of the digester. In Fig. 6 the profiles of a 'healthy' and a 'bad' fermentation are given. A healthy digester was considered to be one producing close to theoretical quantities of methane. For a similar type of mixed fermentation sample, the procedure was used successfully with the supernatant of an artificial gut. The aim in this case was to study changes in metabolism of a mixed culture.

From these applications, useful information involving relatively little effort was obtained both on cultures of pure bacterial isolates and of mixed microbial populations. Every effort was made to ensure that the same batch of media was used for any series of samples. It would be advisable to check media of different batches against standard bacterial isolates to ensure similarity of acid profiles. Different makes of media of the same type would be expected to give different profiles, although this was not checked. Where use of different media is unavoidable, the re-running of standard bacterial isolates is essential.

The use of retention time is not specific for the identification of analytes. Because of this, the complexity of the sample matrices presents limitations to the use of the procedures for quantitation. The extraction procedure and the type of column packing does improve selectivity for the acid metabolites but there will be other acids and compounds present some of which may interfere because of coincidence with the retention times of the analytes. The procedure has greatest value used qualitatively, such as for the development of profiles of microbial metabolites for quality control purposes and semi-quantitatively as an aid to bacterial identification.

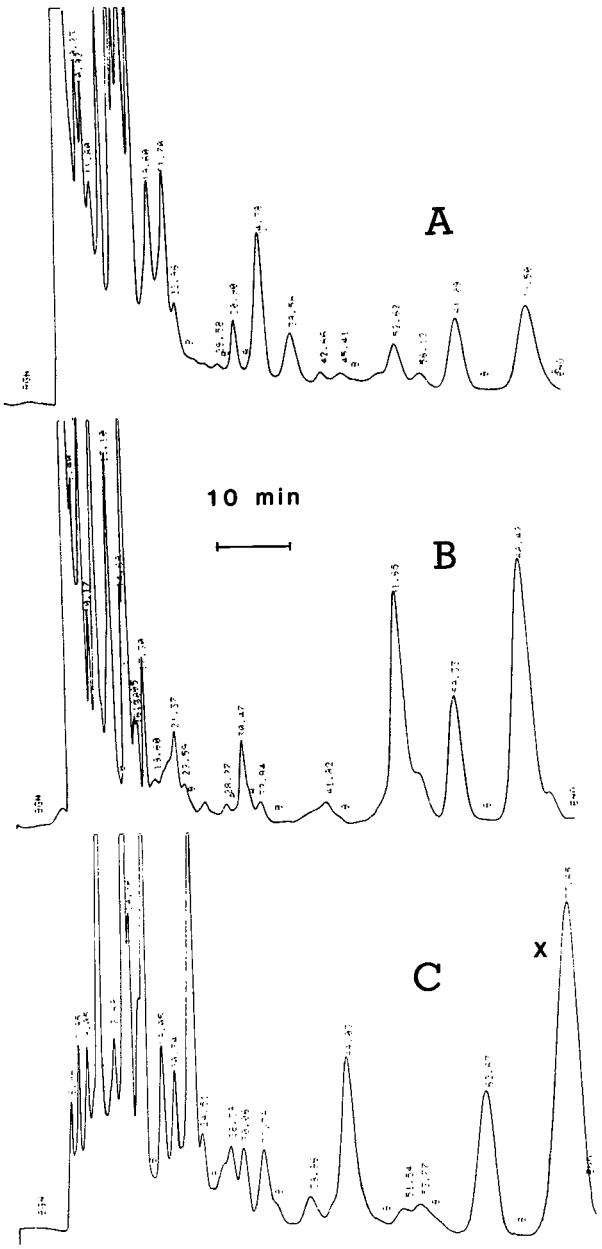

Fig. 5. Chromatograms of a red wine (A), a port wine (B) and a soy sauce (C). The wines were not extracted, being chromatographed directly as a 1:10 dilution in water. The soy sauce was extracted after dilution 1:50 with water. The wine sample would contain bases and considerable quantities of neutral compounds as well as the acids. The chromatograms show that relatively uncluttered profiles are obtainable both from the unextracted wine samples and a complex sample such as soy sauce. The final peak (X) of C was designated benzoic acid because of identical retention time and because benzoic acid is used as a preservative in some soy sauces.

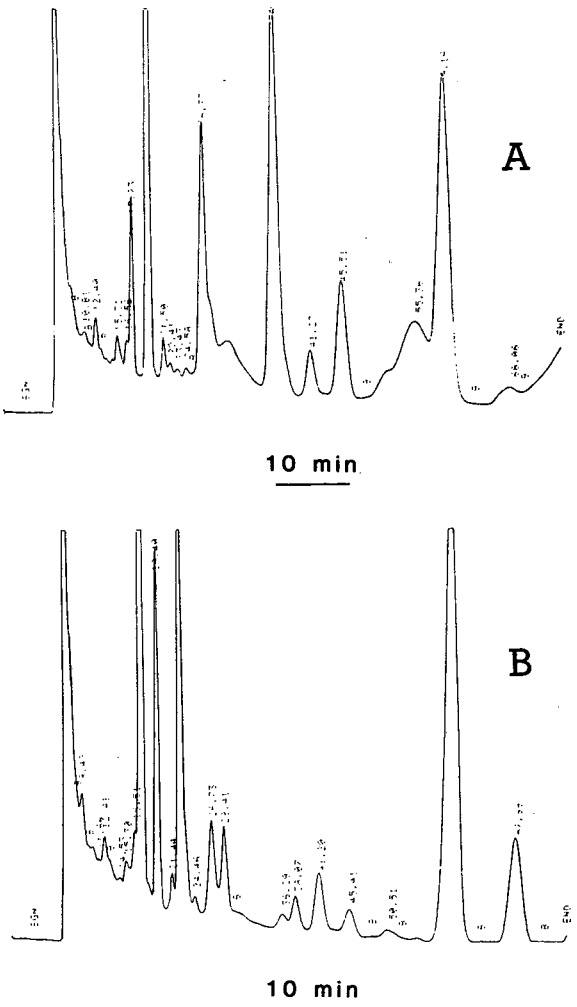

Fig. 6. Chromatograms of an extract of fluid from a fruit waste digester. (A) Profile obtained from a healthy digester. (B) Profile from a digester that was not operating efficiently. The distribution of peaks shows that metabolites are more uniformly spread throughout A, compared with B.

The column used for this report has been in use for greater than 800 analyses and about 100 separations of standards. This was without use of a guard column. The use of a guard column may be advisable, but can introduce minor losses of resolution. To maintain maximum efficiency of the analytical column every effort was made to ensure that the injected solution was particle-free. Additionally, the column was back-flushed weekly and when not in use, a flow-rate of 0.1 ml/min was maintained.

The column temperature of 50°C was chosen to give adequate resolution and to permit a higher flow-rate before back-pressure in the system became too high.

In our hands the extension of the procedures of Guerrant et al. [11] has

proved useful for a wide range of studies involving microbial fermentations. Although as presently recommended the procedure does not analyze important metabolites such as alcohols, acetone, acetaldehyde and diacetyl, a large amount of information was obtainable from a single analysis.

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ESTIMATION OF AMNIOTIC FLUID PHOSPHOLIPIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We have developed a high-performance liquid chromatographic (HPLC) method for the analyses of surface-active amniotic fluid phospholipids, lecithin (L), sphingomyelin (S), phosphatidyl glycerol (PG), phosphatidyl inositol (PI), phosphatidyl ethanolamine (PE), and phosphatidyl serine (PS), which are important in the prediction of fetal lung maturity. The method incorporates an internal standard in the amniotic fluid extract, and utilizes a 10-μl aliquot of a 2:1 chloroform—methanol extract of amniotic fluid injected onto a 5-μm DIOL or CN HPLC column, and a variable-wavelength detector set at 203 nm.

Amniotic fluid phospholipid estimations were determined on 40 amniotic fluid samples by the HPLC method and by the routine thin-layer chromatographic (TLC) method. Good agreement was observed between the two methods for the L/S ratio, PG, and PI (r_{PG} 0.94, r_{PI} 0.95, $r_{L/S}$ 0.97).

The advantages of the HPLC procedure include: (i) Selective separation for PG, PI, PS, and PE, as well as L and S at the same time. (ii) The internal standard allows individual concentration of phospholipids to be estimated. (iii) The procedure is rapid: 16 min for a single assay compared with 50 min for the standard TLC procedure.

INTRODUCTION

Immaturity of the fetal lung leading to respiratory distress syndrome (RDS) is the principal cause of death in the premature neonate. RDS is primarily due to a lack of pulmonary surfactant. Recent advances indicate that surfactants appear in the amniotic fluid (AF) during gestation and their quantity and pattern are determined by their production in the lung. Surfactant enables a low, stable surface tension of the air—water interface within the alveoli to be maintained, it decreases the amount of pressure needed to distend the lung and prevent alveolar collapse. Therefore, in the clinical situation, rapid analysis of amniotic fluid phospholipids for the detection of lung maturity is important in the management of the premature infant.

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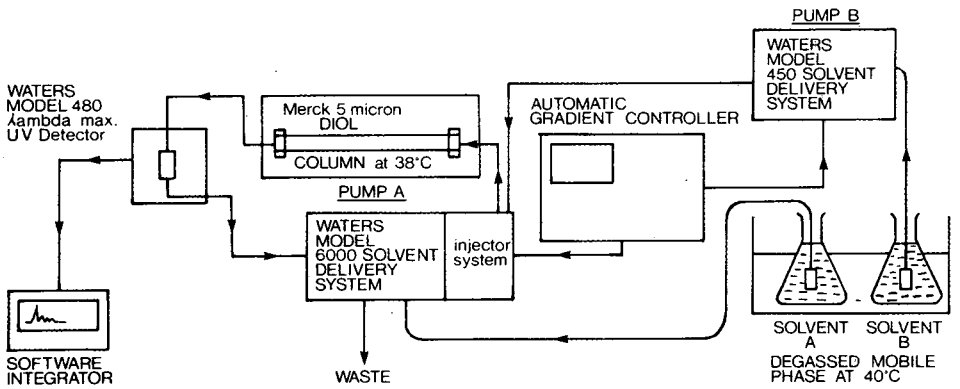


Fig. 1. A line diagram of the HPLC apparatus used for the analyses of the amniotic fluid phospholipids.

Thin-layer chromatography (TLC) was first established by Gluck et al. in 1971 [1] for the separation and measurement of the predominant amniotic fluid phospholipids lecithin (L) and sphingomyelin (S) as the L/S ratio and later by Hallman et al. in 1976 [2] for the estimation of two other important phospholipids, phosphatidyl glycerol (PG) and phosphatidyl inositol (PI) by two-dimensional TLC. However, TLC is a relatively laborious procedure and gives limited information. High-performance liquid chromatography (HPLC) is a much faster procedure than TLC but its application to lung maturity studies of amniotic fluid phospholipids has to date resulted in very limited progress. The present paper describes the development of an HPLC procedure for separation and quantitation of all the amniotic fluid phospholipids of interest in the study of fetal lung maturity, namely L, S, PG, PI, phosphatidyl serine (PS), and phosphatidyl ethanolamine (PE).

MATERIALS AND METHODS

A line diagram of the current HPLC apparatus is shown in Fig. 1. Apart from an ultraviolet (UV) detector, it comprises the following solvent delivery system: a Waters Model 6000A pump and a Waters M45 pump with a U6K injection system and a Waters automatic gradient controller for gradient elution. The column used is a 5-μm DIOL 12.5 cm × 4.6 mm and is upplied by Merck—BDH (Australia). A Merck 3 cm × 4.6 mm guard column packed with 5-μm silica Si60 was fitted between the analytical column and the precolumn filter and injection system. The column and guard column were maintained at an oven temperature of 38°C while the solvents were kept at 40°C in a water bath. All solvents were HPLC grade and, prior to use, were filtered through a 0.2-μm Durapore filter and degassed by sonication for 0.5 h. Regeneration of the column was carried out periodically in accordance with the Merck literature on column care.

The choice of detectors is a major problem with the HPLC separation of phospholipids. Of the common detectors available, the differential refractometer is probably the most suitable, since it allows the use of solvents such as chloroform and ammonium hydroxide which are commonly used in TLC sep-

arations of phospholipids; its major drawback is that it is less sensitive than most other detectors. Fluorescent detectors require derivatisation of the phospholipids and, for this reason, were not considered. The detector used in our system was a Waters Lambda Max-480 UV detector (190—380 nm). The direct detection of phospholipids at low UV wavelength according to Geurts van Kessel et al. [3] is dependent not only on the degree of unsaturation of the fatty acid side-chains of the phospholipids, but also on the functional groups such as carbonyl, carboxyl, phosphate, amino and quaternary ammonium of each molecule. This accounts for the difference in response to UV absorption for the individual phospholipids.

Standards

Table I shows the concentration of a typical working calibration standard used in routine analyses. The seven phospholipids, PG, PI, PS, PE, L, S, and the internal standard (IS) γ-capryloyl lysolecithin were of the highest purity available from either Calbiochem—Behring or PL-Biochemicals. Each individual standard was made up to 1 μg/μl in a 2:1 (v/v) chloroform—methanol solvent. In order to prepare a composite working standard, aliquots of each individual standard were mixed together and dried under nitrogen in a water bath at 50°C, then reconstituted in 1.0 ml of 2:1 chloroform—methanol mixture. The working standard (2 μl) was injected onto the column at the beginning of each run and then after every third sample. The working standard phospholipids were stored at −10°C when not in use.

TABLE I

TYPICAL WORKING CALIBRATION STANDARD

A typical calibration standard of phospholipids found in amniotic fluid which includes the internal standard γ-capryloyl lysolecithin: 2 μl of this standard are injected initially in the column and then after every third specimen.

Phospholipid	Concentration (μmol/l)
Phosphatidyl glycerol	1.92
Phosphatidyl inositol	2.26
Phosphatidyl ethanolamine	0.29
Phosphatidyl serine	2.56
Lecithin	2.66
Sphingomyelin	0.70
γ-Capryloyl lysolecithin	39.50

Extraction of phospholipids from amniotic fluid

Fig. 2 shows a flow diagram of the extraction procedure for the phospholipids.

Duplicates of 1.5 ml of centrifuged amniotic fluid were extracted with an equal volume of methanol, followed by vortexing for 30 sec, then addition of twice the volume of chloroform and then vortexed again for 30 sec. After centrifugation for 10 min at 1500 *g*, the supernatant aqueous methanol layer was aspirated to waste. The lower chloroform layer was withdrawn and evaporated to dryness under nitrogen on a water bath at 50°C. The lipid residue was ace-

tone-fractionated, using ice cold anhydrous acetone according to the well established procedure of Gluck et al. [1].

The acetone precipitate after thorough drying was taken up in 20 μl of 2:1 chloroform—methanol mixture containing 22.5 μmol/l internal standard γ-capryloyl lysolecithin. Of this 10 μl were injected onto the HPLC column. For the present study, the second replicate was taken up in 10 μl of chloroform and spotted on a prepared TLC plate.

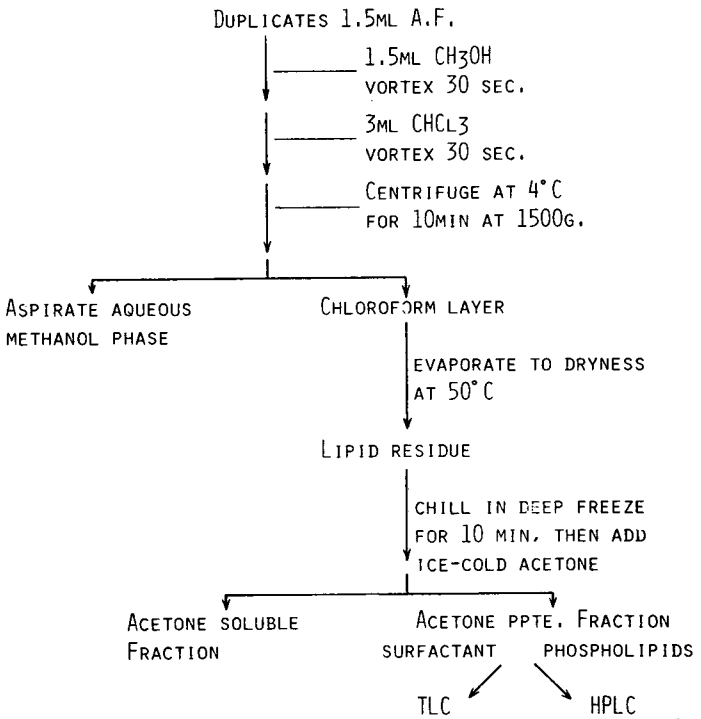


Fig. 2. Flow diagram for the extraction procedure of the phospholipids from amniotic fluid.

HPLC procedure

The HPLC system was set up as follows for gradient elution of phospholipids. Initial conditions consisted of solvent A (acetonitrile 100%) 88% and solvent B (a mixture of acetonitrile—water in the ratio 3.5:1) 12%. A linear gradient was run from 4.20 to 12.20 min when the final conditions were 25% solvent A and 75% solvent B. The flow-rate was constant at 2.0 ml/min and the maximum allowable back-pressure on the column was 14 MPa. The column effluent was monitored at 203 nm and the detector was set at 0.02 a.u.f.s. deflection. Quantitation was by integration of peak areas using the Waters Data Module and a Hewlett-Packard 85 computer.

To determine the best system for the separation of amniotic fluid phospholipids, several columns and solvent systems were explored using an isocratic system. The best separation achieved by this procedure was with a Waters μPorasil 60 A°GPC column using hexane—isopropanol—water (6:8:1.15) as the mobile phase, illustrated by Figs. 3 and 4. While this gave adequate separation

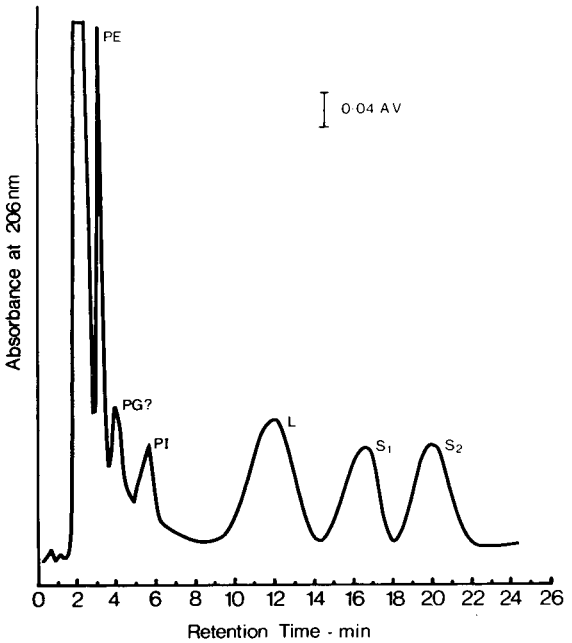


Fig. 3. Separation of amniotic fluid phospholipids before lung maturity using an isocratic system. Mobile phase: *n*-hexane—2-propanol—water (6:8:1.15, v/v/v). Note the broad peaks of both lecithin (L) and sphingomyelin which is split into two peaks (S_1 and S_2). PE = Phosphatidyl ethanolamine; PG = phosphatidyl glycerol; PI = phosphatidyl inositol; PS = phosphatidyl serine.

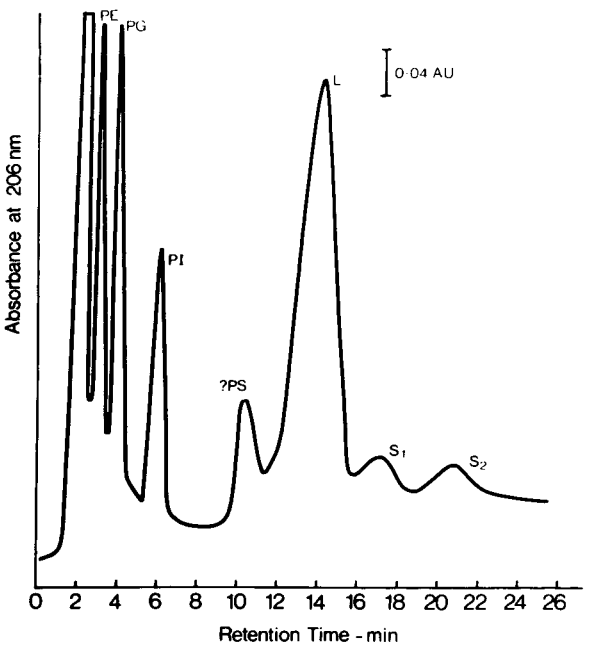


Fig. 4. Typical isocratic separation of amniotic fluid phospholipid observed after lung maturity is attained. Mobile phase: *n*-hexane—2-propanol—water (6:8:1.15, v/v/v). The large peak due to the lecithin (L) and the very flat broad peaks of sphingomyelin (S_1 and S_2) cannot be resolved by the integrator to give an accurate estimation of lecithin and sphingomyelin. For peak identification, see Fig. 3.

of the phospholipids, the lecithin and two sphingomyelin peaks (S_1 and S_2) were too broad to be adequately quantitated by the integrator.

Gradient elution using the DIOL column and the conditions already described allowed the lecithin and sphingomyelin to elute much closer to the earlier eluting phospholipid peaks, i.e. less than 16 min. In addition, PG, PI and PE all eluted much further away from the solvent front, allowing baseline resolution to occur before peak detection began. The broader peaks that were observed in the isocratic system were much sharper, and sphingomyelin eluted as a single peak as shown in Fig. 5.

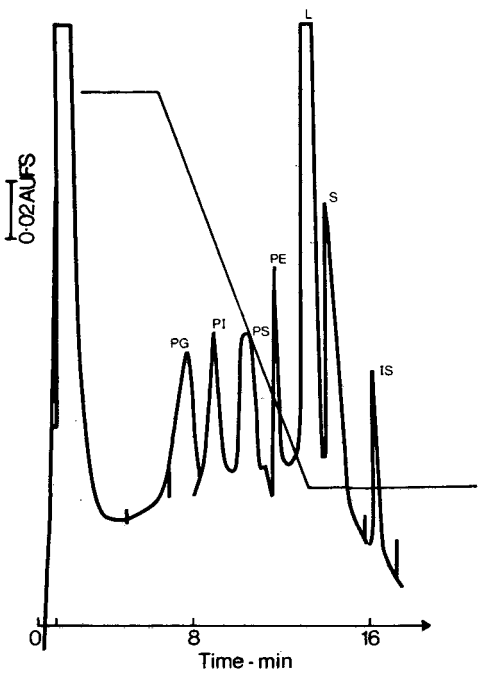


Fig. 5. Gradient elution chromatogram of a typical calibration standard containing the internal standard (I.S.), γ-capryloyl lysolecithin. All the peaks are well resolved and sphingomyelin (S) appears as a single peak. Initial conditions: 88% solvent A (100% acetonitrile) and 12% solvent B (acetonitrile—water, 3:5:1). Final conditions: 25% solvent A and 75% solvent B. Flow-rate: 2.0 ml/min. UV detector: 203 nm. For peak identification, see Fig. 3.

Fig. 6 shows a comparison of another separation procedure using a CN column instead of a DIOL column. The same aliquot of amniotic fluid was injected into the two different systems. Although initial conditions are slightly different and solvent B has much less water (5:1 acetonitrile to water) the phospholipids elute in the same order. The advantage of the CN column is that it is a more versatile column and easier to re-equilibrate than the DIOL column.

Internal standard

The reason for the use of an internal standard in the HPLC separation of amniotic fluid phospholipids is two-fold: (i) it allows the absolute concentration of individual phospholipids to be estimated, and (ii) it acts as a reference peak for all the other phospholipids. The choice of internal standard was made after

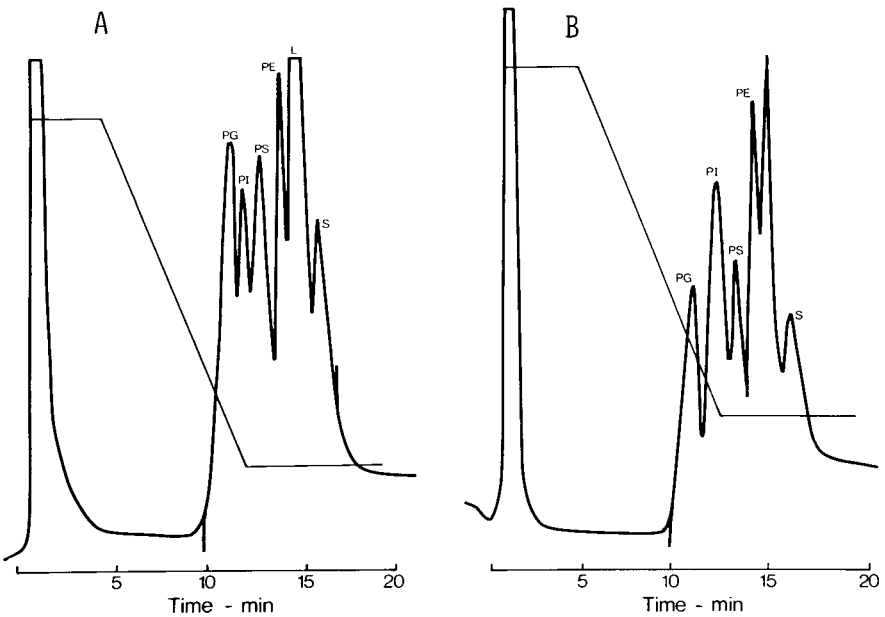


Fig. 6. Comparison of the same gradient elution procedures, with two different columns (CN and DIOL), using similar solvents but containing different concentration of water in solvent B. The phospholipids separate in the same order but are better resolved on the CN column. (A) 5-μm CN column (25 cm). Initial conditions: 90% solvent A (100% acetonitrile) and 10% solvent B (acetonitrile—water, 5:1). Final conditions: 25% solvent A and 75% solvent B. (B) 5-μm DIOL column (25 cm). Initial conditions: 88% solvent A (100% acetonitrile) and 12% solvent B (acetonitrile—water, 3.5:1). Final conditions: 25% solvent A and 75% solvent B. For peak identification, see Fig. 3.

observation that lysolecithin does not appear in physiological or pathological conditions in the acetone precipitate fraction of amniotic fluid. For this reason γ-capryloyl lysolecithin was chosen as an internal standard. Fig. 5 shows a typical calibration standard, the internal standard appears as a sharp, single peak eluting after sphingomyelin at 16 min.

For the gradient elution procedure, using individual phospholipid standards of varying concentrations, the response factor (RF) for each of the phospholipid standards was established as shown in Fig. 7. Each point represents a mean of five injections at that point. A linear relationship with widely different sensitivity was found between peak areas and concentration for each of the phospholipids. Linearity was observed over the range of working standards and these cover the physiological/pathological ranges for amniotic fluid phospholipids in the system.

Stability of standards

The stability of the prepared calibration standards is quite good, provided they are stored in a freezer when not in use. However, the quality of PS varied considerably from batch to batch. At times the sensitivity of detection was so low that very high concentrations of PS standard in excess of 40 μmol/l had to be injected onto the column before a peak could be detected. In most of the amniotic fluids analyzed, the concentration of PS was low by both HPLC and TLC.

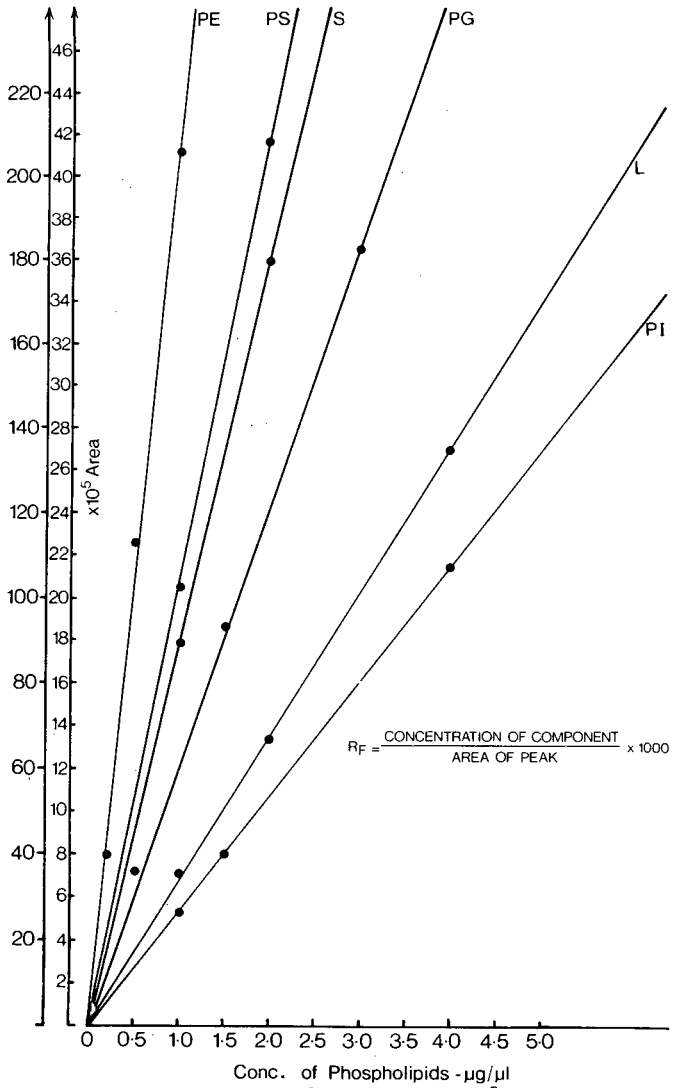


Fig. 7. Standard calibration curves of the six phospholipids showing the plot of the area $\cdot 10^5$ as measured by the integrator versus the concentration of the individual phospholipid at that point. Each point represents a mean of five injections. The formula for the calculation of the response factor (RF) of each peak is shown. For abbreviations, see Fig. 3.

TLC procedure

The TLC estimations on the amniotic fluid specimens were determined for comparison with the HPLC procedure. The TLC procedure was an adaptation of the method of Painter [4]. One-dimensional TLC was carried out using borosilicate TLC plates coated with silica gel 60 F254 and 1 g/l copper chloride utilising chloroform—ammonium hydroxide—methanol (65:3:25, v/v/v) as the mobile phase. The plates were run for 50 min, sprayed with cupric acetate—8% phosphoric acid stain, followed by charring on a hot plate and quantitation using a densitometer.

RESULTS

The sensitivity of the HPLC method was established for each phospholipid in the amniotic fluid. Sensitivity depends not only on the peak area and the concentration, but also on the baseline noise and quantity of amniotic fluid extracted. These factors must be optimized for the particular analytical column in use over the physiological/pathological concentrations of phospholipids found in amniotic fluid. Mean values for each phospholipid measured in four "immature" amniotic fluids (L/S ratio by TLC < 1.0:1) are shown in Table II.

TABLE II

SENSITIVITY OF THE HPLC PHOSPHOLIPID METHOD

The sensitivity of the HPLC method depends on the peak area, weight ratio for each phospholipid, baseline, and the quantity of amniotic fluid (AF) extracted.

Phospholipid	Towards lower limit of detection (1.5 ml of extracted AF in μmol/l)
Phosphatidyl glycerol	0.19
Phosphatidyl inositol	0.40
Phosphatidyl ethanolamine	0.01
Phosphatidyl serine	12.6
Lecithin	0.50
Sphingomyelin	0.20
γ-Capryloyl lysolecithin	19.9

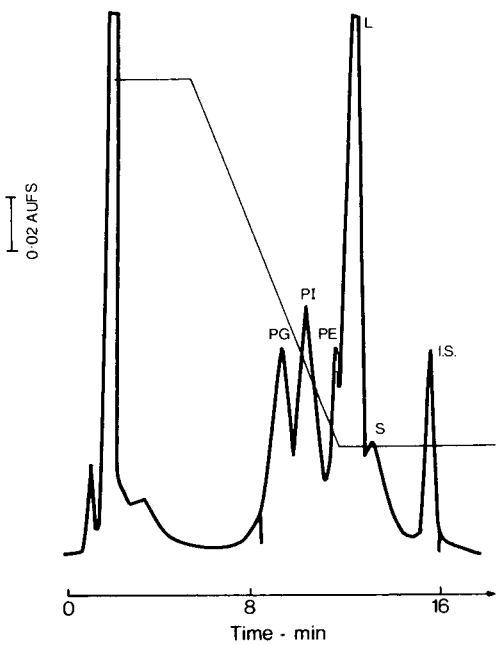
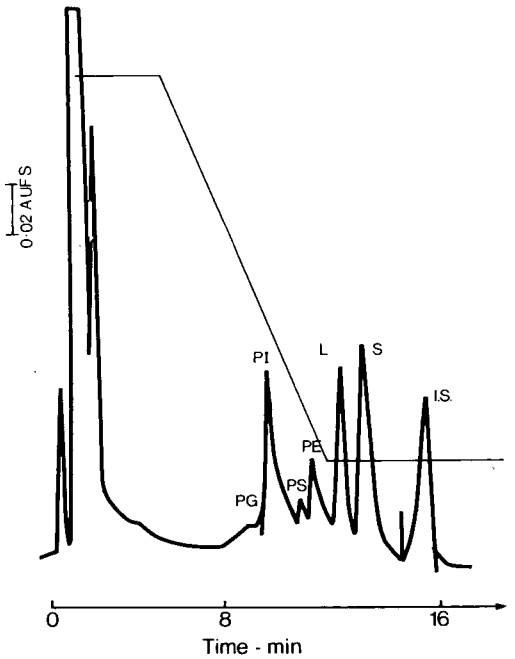


Fig. 8. Typical HPLC chromatogram of a mature amniotic fluid (L/S 3.9:1) using gradient elution. The concentration of individual phospholipids expressed as a percentage of the total phospholipid concentration are L 48.82%, S 12.48%, PG 7.11%, PI 16.26%, PE 15.31%, PS < 0.01%. Gestation 37 weeks. For abbreviations, see Fig. 3.



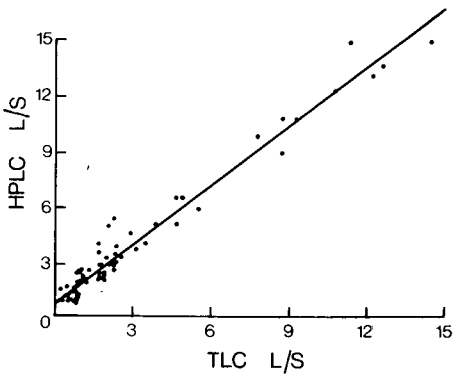
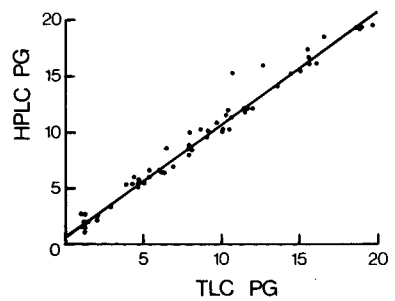


Fig. 9. HPLC trace of an immature amniotic fluid (L/S 1.2:1) using gradient elution. Phosphatidyl glycerol (PG) is not present in this trace. Initial conditions: 88% solvent A (100% acetonitrile) and 12% solvent B (acetonitrile—water, 3.5:1). Final conditions: 25% solvent A and 75% solvent B. Flow-rate: 2.0 ml/min. UV detector: 203 nm. For peak identification, see Fig. 3.

Fig. 10. Comparison of the L/S ratio by both HPLC and TLC procedures in the acetone precipitate of amniotic fluid. $Y = 1.08\ X + 0.52$; $r = 0.97$; $n = 40$.



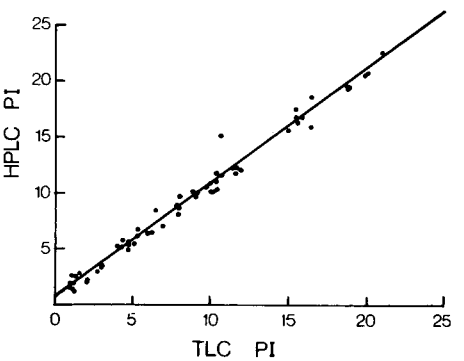


Fig. 11. Comparison of phosphatidyl glycerol (PG) by both HPLC and TLC procedures in the acetone precipitate of amniotic fluid. PG is expressed as a percentage of the total phospholipid concentration in the amniotic fluid. $Y = 1.02\ X + 0.8$; $r = 0.94$; $n = 40$.

Fig. 12. Comparison of phosphatidyl inositol (PI) by both HPLC and TLC procedures in the acetone precipitate of amniotic fluid. PI is expressed as a percentage of the total phospholipid concentration in the amniotic fluid. $Y = 1.01\ X + 0.86$; $r = 0.95$; $n = 40$.

In a preliminary patient study, 40 amniotic fluids were analysed by both the HPLC and the TLC procedures. Typical chart traces for the mature and immature amniotic fluids are shown in Figs. 8 and 9, each chromatogram being completed in 20 min running time. The retention times of the separated phospholipids are similar to the retention times observed in the standard chromatogram of phospholipids (Fig. 5). Note that interference by other peaks, which is a common problem observed in many HPLC separations of biological extracts, is minimal in this separation of the phospholipids, because of the acetone precipitation step which, as shown in Fig. 2, extracts only the surfactant phospholipids from the amniotic fluid.

Clinically the most important parameters are the L/S ratio, and proportions of PG, and PI. Figs. 10—12 illustrate the correlation coefficients and regression lines for these estimations. PG and PI are expressed as a percentage of the total phospholipid fraction estimated by both the HPLC and TLC procedures (r = 0.97 for the L/S, r = 0.94 for PG, and r = 0.95 for PI).

DISCUSSION

As can be seen from the present study from the comparison between quantitation by HPLC and quantitation by TLC, the HPLC procedure gives results which compare well with the longer established TLC technique.

Recently several authors (Paton et al. [5] and Briand et al. [6]) using similar procedures and columns but different detectors, have used HPLC to separate phospholipids in biological fluids. Paton et al. [5] stated that the HPLC method was less sensitive than their TLC method even though large volumes of amniotic fluid (5 ml) were used to overcome the lack of sensitivity of the detector. Our preliminary observations (Table II) show that the HPLC method with respect to the phospholipids PG, PI, L and S is much more sensitive. However, as mentioned previously, PS is difficult to detect, owing partly to its presence in low concentration in amniotic fluid and partly to the variability in the purity of the commercial standards. This problem was also observed by Paton et al. [5] and Briand et al. [6].

Preliminary evidence obtained from the comparison of the CN column with the DIOL column (Fig. 6) shows that it may improve the resolution, not only of PS but all the phospholipids. Further work is continuing in this area. A disadvantage of the HPLC procedure compared with TLC is the initial preparation of the solvents which require filtering and degassing for lengthy periods to remove all dissolved oxygen and other impurities which may cause variation in baseline and spurious peaks due to UV absorption at very low wavelengths.

Certain maternal conditions such as diabetes, severe hypertension, and Rhesus haemolytic disease are known to accelerate or retard lung maturity. The L/S ratio is known to be unreliable in such cases; however, several authors have stated that the presence of PG and PI, despite an immature L/S ratio, i.e. a value of less than 2.0:1, will preclude RDS from a premature neonate. In our preliminary study, all those infants who had a PG value of greater than 2.5% of the total phospholipid content by TLC did not develop RDS, irrespective of the value of the L/S ratio. It was also noted that PI was present in excess of 5% of the total phospholipid content in this study. Comparison of the two

procedures as shown in Figs. 10 and 11 of the L/S ratio and PG indicates that an L/S ratio of 2.0:1 by the TLC is associated with an HPLC L/S ratio value of 2.7:1 and a PG value of 2.5% of the total phospholipid value is associated with an HPLC PG value of 3.4%.

Although an internal standard has been used in our HPLC procedure and absolute concentrations may be estimated, to allow for the variability of total phospholipid concentration caused by differences in amniotic fluid volume, the "amniotic fluid phospholipid profile" as presented in our hospital is reported as a percentage of the total phospholipid assayed in amniotic fluid.

For all analysis runs, the concentration of each component is calculated as follows by the computing integrator:

$$\text{Concentration of phospholipid} = \frac{\text{RF} \times \text{area}}{1000} \times \frac{\text{area of I.S. in calibration standard}}{\text{area of I.S. in sample}} \times 66.7\ \mu\text{mol/l}$$

$$\text{Percentage phospholipid} = \frac{\text{concentration of individual phospholipid} \times 100}{\text{total concentration of phospholipid}}$$

An example of a typical calculation of "mature" amniotic fluid phospholipid profile is shown in Fig. 8. The total phospholipid concentration for this specimen was 149 μmol/l.

The comparison between quantitation by the TLC and HPLC procedures gave results that compare favourably with longer established TLC techniques. Although HPLC may require more expertise to set up, its advantages are that it enables a full amniotic fluid phospholipid profile to be separated in 16 min after extraction, and quantitated by an internal standard, compared to the 50 min for the same separation by TLC. As well as this, it is non-destructive and can also be used to collect the individual phospholipid fractions for further study, for example, of their fatty acid constitutents.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF APOLIPOPROTEINS IN SERUM HIGH-DENSITY LIPOPROTEINS

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SUMMARY

A simple and rapid method for apolipoprotein analysis in serum high-density lipoproteins (HDL) has been developed using high-performance liquid chromatography (HPLC) with sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulphate (SDS) as eluent. In contrast to the use of urea solution as an eluent, apolipoproteins can be analysed by applying an incubation mixture of HDL and the eluent buffer. A TSK-GEL column of G3000SW was found to be more profitable than G2000SW or G4000SW for analysis of HDL apolipoproteins. Elution patterns monitored by absorbance at 280 nm using a G3000SW column can give precise quantitative as well as qualitative information about apolipoproteins of molecular weight between 10^4 and 10^5. HPLC patterns of HDL apolipoproteins were compared between individual human subjects with various diseases. Elution profiles for lipid components in an incubation mixture were also examined.

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INTRODUCTION

Serum apolipoproteins have become the centre of intense interest during recent years in the study on lipoproteins, because they have important roles in lipoprotein structure and metabolism. Serum lipoproteins are divided into several major classes according to density: chylomicrons, $d < 1.006$; very-low-density lipoproteins (VLDL), $d < 1.006$; low-density lipoproteins, $d = 1.006$—1.063; high-density lipoproteins (HDL), $d = 1.063$—1.210; very-high-density lipoproteins, $d > 1.210$. Individual lipoprotein classes contain a number of structurally different apolipoproteins.

In HDL, apolipoprotein (apo) A-I and apo A-II are major apolipoproteins, and apo C, apo E, apo D and apo A-IV are minor ones. Separation of these apolipoproteins has been performed by gel chromatography with urea solution as an eluent on the basis of differentiation of molecular weight [1, 2]. Recently, high-performance liquid chromatography (HPLC) has been applied to the analysis of HDL apolipoproteins using an aqueous gel permeation column (TSK-GEL, G3000SW) [3, 4]. In these cases, 6 *M* urea or 6 *M* guanidinium chloride solution is used as an eluent, and an apolipoprotein fraction, which is prepared by delipidation with organic solvents (ethanol—diethyl ether) from HDL, is applied to the HPLC system. More recently, we have developed a simple and rapid method for analysis of HDL apolipoproteins [5]. With our method, delipidation with organic solvents can be eliminated by incubation of a mixed solution of HDL and an eluent buffer: 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulphate (SDS). The good reproducibility and quantitation of this method have been reported previously [5].

In this paper, separation profiles of HDL apolipoproteins are compared between SDS and urea solutions on HPLC with a gel permeation column (G3000SW). Elution patterns of apolipoproteins under SDS solution are examined using TSK-GEL columns with different pore sizes. A few examples for analysis of HDL apolipoproteins from various subjects are reported. Elution profiles of lipid components in an incubation mixture of HDL and the eluent are also examined.

EXPERIMENTAL

Apparatus

HPLC was carried out using an HLC 803D (Toyo Soda, Tokyo, Japan) equipped with a variable-wavelength absorbance detector.

Ultracentrifugation for preparation of HDL from serum was performed using an RP 65 rotor in a Hitachi 55 P-2 ultracentrifuge (Hitachi Koki Co., Tokyo, Japan).

Lyophilization for preparation of apolipoproteins from HDL was carried out using a freeze drier (EYRA FD-5, Tokyo Rikakikai Co., Tokyo, Japan).

Materials and methods

Samples. Human serum and dog serum used in the experiments were obtained from humans and dogs after 12—16 h of fasting. HDL used for HPLC analysis was prepared from serum by the sequential flotation method by

Havel et al. [6]. The $d > 1.063$ fraction was obtained as the infranate layer after centrifugation (105,000 *g* at 15°C) for 24 h at a density of 1.063. After density adjustment to 1.210 with sodium bromide and centrifugation for 44 h at 105,000 *g*, the top layer was collected as HDL.

The apolipoprotein fraction of HDL (apo HDL) was prepared from HDL by delipidation with ethanol—diethyl ether as described by Scanu and Edelstein [2]. Standard apolipoproteins, apo A-I, apo A-II, apo E and apo C, were prepared as described previously [5]. Proteins used for column calibration were high-molecular-weight and low-molecular-weight electrophoresis calibration kits (Pharmacia Fine Chemicals, Uppsala, Sweden).

Reagents. Cholesterol and choline-containing phospholipids in the effluent from the gel permeation column were detected enzymatically by commercial reagent kits: Determiner TC"555" (Kyowa Medex Co., Tokyo, Japan) for cholesterol and PL kit K"f" (Nippon Shoji Co., Osaka, Japan) for choline-containing phospholipids. All chemical reagents were of the highest grade commercially available: sodium dodecyl sulphate (SDS), Wako; tris(hydroxymethyl)-aminomethane (Tris), Nakarai Chemical Ltd; urea (ultrapure), Schwartz/Mann (Spring Valley, NY, U.S.A.).

Sample preparation for HPLC analysis. HDL (d = 1.063—1.210), 10 μl containing 20—300 μg of protein, was added to 200 μl of 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.1% SDS. The mixed solution was incubated at 60°C for 5 min and was used as a sample for HPLC analysis. Standard proteins, standard apolipoproteins and apo HDL were dissolved in the same buffer and incubated at 60°C for 5 min before HPLC analysis.

Analysis by HPLC. The separation of apolipoproteins in HDL was carried out by HPLC with aqueous gel permeation columns (TSK-GEL, Toyo Soda). HPLC conditions in this experiment were as follows. Columns: G2000SW, G3000SW, G3000SW + G3000SW, G4000SW (each column 600 × 7.5 mm I.D.). Eluents: 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.1% SDS, 6 *M* urea at pH 3.15 (HCl), 6 *M* urea containing 0.05 *M* Tris—HCl buffer (pH 7.0). Flow-rate: 0.33 ml/min. Loaded volume: 200 μl. Eluted proteins from the column were detected by A_{280}. Cholesterol [7, 8] or choline-containing phospholipids [9, 10] in the effluent from the column was monitored by A_{550} or A_{500} after on-line enzymatic reaction as previously reported.

RESULTS AND DISCUSSION

The relation between elution volume and molecular weight in 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.1% SDS was examined for various aqueous gel permeation columns (G2000SW, G3000SW and G4000SW) using standard proteins and apolipoproteins of known molecular weight. Molecular weights of these standard samples used for column calibration are listed in Table I. Other apolipoproteins contained in the HDL fraction from human serum are also presented in Table I. As shown in Fig. 1, the plot of elution volume against the logarithm of molecular weight is linear for all columns except for apo C. Several components with different molecular weights are contained in the apo C group (Table I). Since a mixture of these components is used as a standard for apo C in our experiment, the mean molecular weight of apo C is

TABLE I

MOLECULAR WEIGHTS OF STANDARD PROTEINS AND APOLIPOPROTEINS

Protein	Molecular weight	Apolipoprotein	Molecular weight
Thyroglobulin	669,000	Apo A-IV	46,000
Ferritin	440,000	Apo E	39,000
Catalase	232,000	Apo A-I	28,300
Lactate dehydrogenase	140,000	Apo D	20,000
Phosphorylase *b*	94,000	Apo A-II	17,000
Bovine serum albumin	67,000	Apo C-I	6500
Ovalbumin	43,000	Apo C-II	8800
Carbonic anhydrase	30,000	Apo $C\text{-}III_{0,1,2}$	8900
Soybean trypsin inhibitor	20,100		
α-Lactalbumin	14,400		
Cytochrome *c*	13,000		

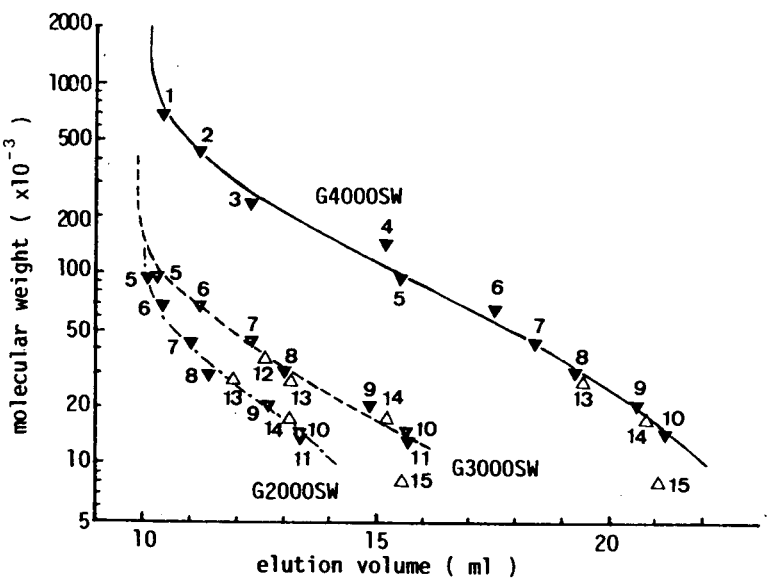

Fig. 1. Relation between elution volume and molecular weight for various gel permeation columns. Columns: G4000SW, G3000SW, G2000SW (each column 600 × 7.5 mm I.D.). Eluent: 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.1% SDS. Flow-rate: 0.33 ml/min. Sample: 1, thyroglobulin; 2, ferritin; 3, catalase; 4, lactate dehydrogenase; 5, phosphorylase *b*; 6, bovine serum albumin; 7, ovalbumin; 8, carbonic anhydrase; 9, soybean trypsin inhibitor; 10, α-lactalbumin; 11, cytochrome *c*; 12, apo E; 13, apo A-I; 14, apo A-II; 15, apo C.

estimated to be about 8400. As reported by Fish et al. [11], the linear relation between Stokes' radius and molecular weight of proteins is broken at molecular weights of less than 15,000 for gel chromatography in SDS solution. Recently, the separation range of proteins by HPLC with gel permeation columns (G2000SW, G3000SW and G4000SW) under SDS solution has been reported [12, 13], and the lower limit of molecular weight is 10,000 or 15,000. The result of our experiment clearly shows that apo C is out in the separation range

under SDS solution for all three columns. Therefore, an eluent other than SDS solution, such as urea solution, is better for the separation of apo C as discussed later.

We have developed a simple and rapid method for the analysis of apolipoproteins in HDL without delipidation by organic solvents [5]. With this method, apolipoproteins separated on the basis of differentiation of molecular weight can be detected in terms of A_{280} only by injecting a mixed solution of HDL and eluent buffer (0.1 *M* sodium phosphate buffer containing 0.1% SDS) preincubated at 60° C for 5 min. In Fig. 2, elution patterns of an incubation mixture of HDL from a normal male subject and the eluent are shown for various columns. Elution positions of apo E, apo A-I, apo A-II and apo C determined by using the standard samples are shown as arrows with numbers. For all columns, elution positions of apo E and apo A-I, apo A-II and apo C are very close to each other. As previously reported, the separation of apo E from apo A-I can be improved by column elongation (G3000SW × 2) (see Fig. 4), but the separation of apo A-II and apo C can not be improved under SDS solution for the reason mentioned above. From the calibration curves in Fig. 1, molecular weights at the void volume of the column are estimated to be 1,000,000, 200,000 and 100,000 for G4000SW, G3000SW and G2000SW, respectively. From the elution pattern of Fig. 2, it is indicated that G3000SW is preferable for the analysis of HDL apolipoproteins than the other two columns.

It has been reported that urea or guanidinium chloride solution is better than SDS solution for the separation of proteins of molecular weight less than

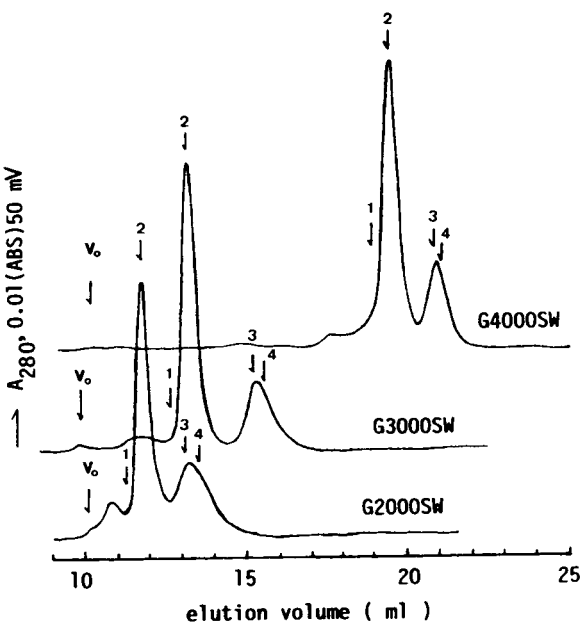

Fig. 2. Elution pattern of HDL apolipoproteins. Column: G3000SW (600 × 7.5 mm I.D.). Eluent: 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.1% SDS. Flow-rate: 0.33 ml/min. Sample: incubation mixture of HDL and the eluent. Loaded volume: 200 μl. Detector: A_{280}, 0.01 [ABS] 50 mV. Elution position: 1, apo E; 2, apo A-I; 3, apo A-II; 4, apo C.

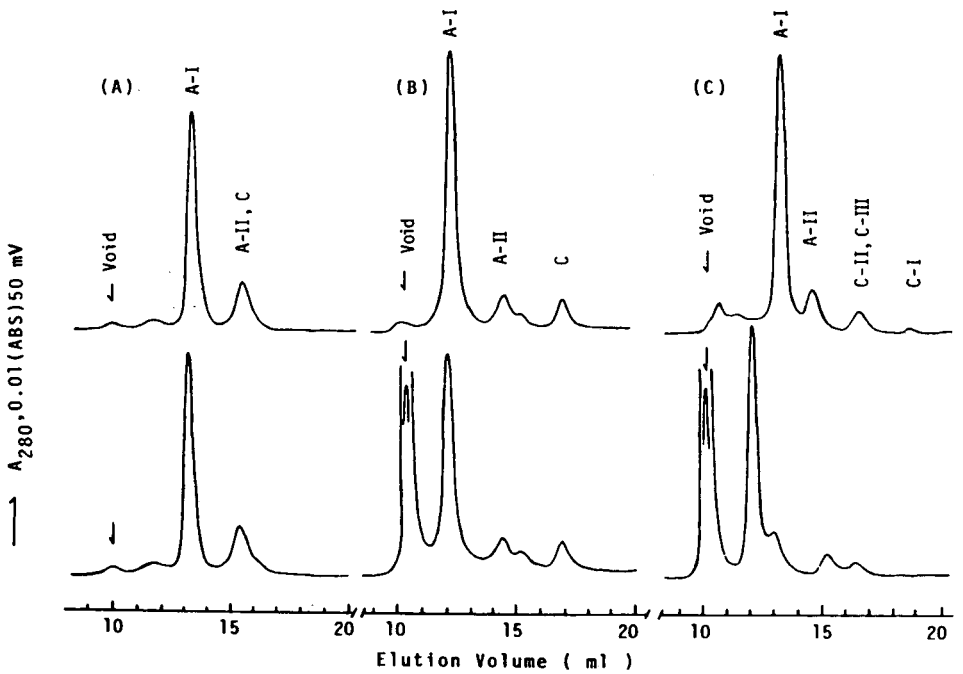

Fig. 3. Elution pattern of HDL apolipoproteins. Column: G3000SW (600 × 7.5 mm I.D.). Eluents: (A) 0.1 *M* sodium phosphate buffer (pH 7.0) + 0.1% SDS; (B) 6 *M* urea (pH 3.15 with HCl); (C) 6 *M* urea + 0.05 *M* Tris—HCl buffer (pH 7.0). Sample: apo HDL (upper traces) and an incubation mixture of HDL and the eluent (lower traces). Other HPLC conditions as in Fig. 2.

10,000 [13, 14]. In fact, apolipoproteins from HDL (apo HDL) have been analysed by HPLC using urea or guanidinium chloride solution [3, 4]. We compared the separation profiles of apo HDL using the G3000SW column between SDS and urea solutions for the same subject as in Fig. 2 (upper part of Fig. 3). In contrast to SDS solution, apo A-II and apo C can be separated by 6 *M* urea solution. Morever, apo C can be separated into large components (apo C-II + apo C-III) and a small one (apo C-I) by 6 *M* urea at pH 7.0. We also compared the elution patterns of an incubation mixture (60°C, 5 min) of HDL from the same subject and the eluent for three solvent systems: SDS, 6 *M* urea at pH 3.15, and 6 *M* urea at pH 7.0. The results are presented in the lower part of Fig. 3. In the case of SDS solution, the elution pattern as well as the elution position of each peak of the incubation mixture were very similar to those of apo HDL, and this suggests that HDL is delipidated completely by incubation with the eluent buffer. But the elution pattern of the incubation mixture was very different from that of apo HDL for the two 6 *M* urea solvent systems. In contrast to SDS solution, large peaks at the void volume were observed for the incubation mixture in both urea systems. In the case of 6 *M* urea at pH 3.15, the elution pattern except for the void peak of the incubation mixture was comparable to that of apo HDL. On the other hand, peak positions of the incubation mixture were quite different from those of apo HDL in the case of 6 *M* urea at pH 7.0. Therefore, urea solution can be used only for the

delipidated sample with organic solvents, apo HDL. This suggests that despite poor resolution of apo A-II and apo C, SDS solution is useful for apolipoprotein analysis because the experimental procedure is simplified without the delipidation by organic solvents. A detailed examination of the quantitation of apolipoproteins from the HPLC pattern monitored by A_{280} using an incubation mixture of HDL and SDS solution was reported previously [5].

Resolution of each apolipoprotein except for apo C can be improved by elongation of the G3000SW column [5]. HPLC patterns monitored by A_{280} using two G3000SW columns for an incubation mixture of HDL from various subjects and SDS solution are presented in Fig. 4. Among these subjects, hyper-

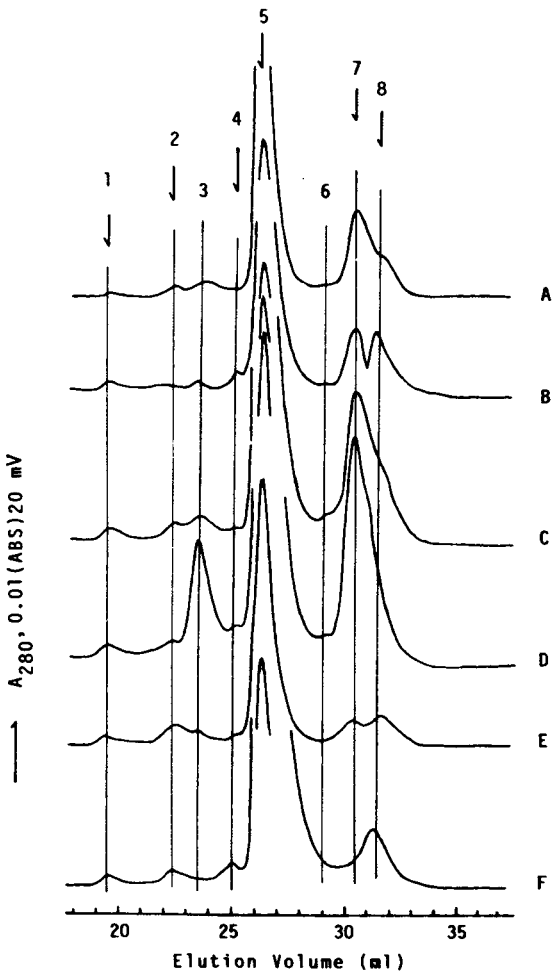

Fig. 4. Elution pattern of HDL apolipoproteins. Column: G3000SW + G3000SW (each 600 × 7.5 mm I.D.). Eluent: 0.1 *M* sodium phosphate buffer (pH 7.0) + 0.1% SDS. Sample: an incubation mixture of HDL from human serum (A—E) or dog serum (F) and the eluent. (A) normal male; (B) lecithin:cholesterol acyltransferase deficiency; (C) acute hepatitis; (D) hyper α-lipoproteinemia; (E) liver cirrhosis. Elution position: 1, void volume of the column; 2, bovine serum albumin; 3, apo A-IV (?); 4, apo E; 5, apo A-I; 6, apo D (?); 7, apo A-II; 8, apo C. Other HPLC conditions as in Fig. 2.

α-lipoproteinemia (Fig. 4D) has been confirmed to contain a large amount of apo A-IV by electrophoretic analysis on SDS-polyacrylamide gel [15]. Dog serum (Fig. 4F) is well known to lack apo A-II in HDL. A significant amount of apo E is known to be present in lecithin:cholesterol acyltransferase deficiency (Fig. 4B) compared to normal subjects. As shown in Fig. 4, as a small void peak, a large peak of apo A-I and a variable shaped peak depending on the composition of apo A-II and apo C were commonly observed for all subjects. For some subjects, a small peak of apo E was observed separately from apo A-I, and a small broad peak was observed at the elution volume of bovine serum albumin. Moreover, peaks at the elution volume corresponding to molecular weights of 46,000 and 20,000 for the calibration curve of Fig. 1 are assumed to be apo A-IV and apo D, respectively. These patterns obtained using our HPLC technique are found to give useful quantitative as well as qualitative information about apolipoproteins in HDL from a very small amount of HDL (10 μl) for a short experimental time.

Lastly, we examined the elution profiles of lipid components of HDL in our analytical system. Cholesterol or choline-containing phospholipids in the effluent from the G3000SW column was monitored by A_{550} or A_{500} by our established on-line technique using a commercial enzyme reaction kit [7—10]. Elution patterns monitored by A_{280}, cholesterol and choline-containing phospholipids for an incubation mixture of HDL and SDS solution are presented in Fig. 5. Elution patterns of both cholesterol and choline-containing phospholipids showed three peaks, the peak positions being consistent. This indicates that lipid components in HDL elute to form an aqueous mixed micelle with SDS and that there are three different sized particles depending on

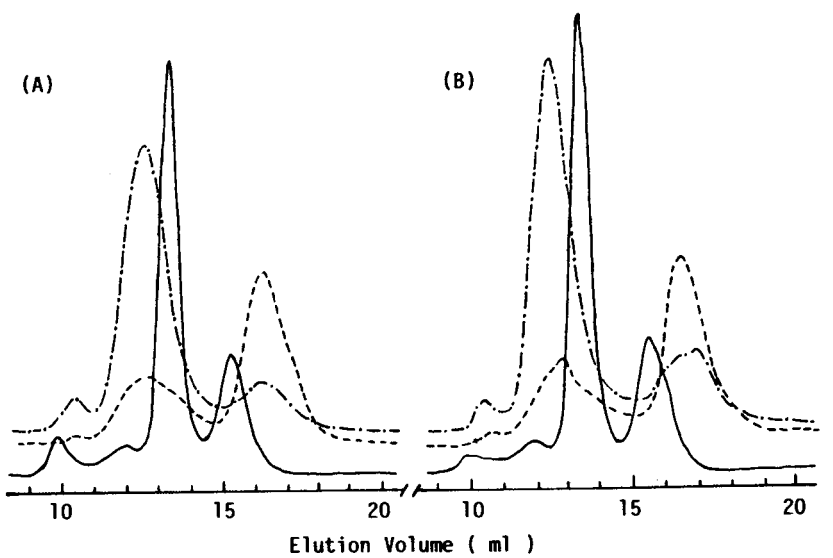

Fig. 5. Elution pattern of HDL apolipoproteins (——), cholesterol (—·—·—) and choline-containing phospholipids (— — —). Column: G3000SW (600 × 7.5 mm I.D.). Eluent: 0.1 *M* sodium phosphate buffer (pH 7.0) + 0.1% SDS. Sample (A and B): incubation mixture of human HDL and the eluent. Loaded volume: 200 μl. Detector: ——, A_{280}, 0.01 [ABS] 50 mV; —·—·—, A_{550}, 0.02 [ABS] 20 mV; — — —, A_{500}, 0.02 [ABS] 10 mV. Other HPLC conditions as in Fig. 2.

chemical composition. The size of the particles is assumed to increase with increase of the ratio of cholesterol to choline-containing phospholipids from the HPLC pattern of Fig. 5. Since these lipid micelles have no significant absorption at 280 nm, analysis of apolipoproteins by monitoring A_{280} may not be disturbed by the co-elution of lipid micelles. This is also confirmed by the similarity of HPLC patterns between the incubation mixture and apo HDL as presented in Fig. 3A.

Our analytical method for HDL apolipoproteins using SDS solution is very useful for clinical research because of the simple experimental procedure, short experimental time and small amount of sample. We are examining the application of this analytical method to other lipoprotein fractions such as VLDL.

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SOME APPLICATIONS OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO OLIGOSACCHARIDE SEPARATIONS

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SUMMARY

Oligosaccharide separations on reversed-phase high-performance liquid chromatographic columns have been examined using a range of aqueous solvents. Addition of anionic, cationic and non-ionic surfactants, tetramethyl urea and organic solvents to the mobile phase cause faster elution of oligosaccharides, and allow the separation of the larger oligomers in an acceptable time. Addition of neutral, inorganic salts increases the retention factors considerably, and allows good resolution of some compounds poorly resolved in water alone.

The mechanism operating in the separations approximates to that invoked in the solvophobic theory of reversed-phase chromatography. There is some evidence also of hydrogen bond effects. The improvements described should prove useful in the isolation and analysis of neutral oligosaccharides in general, and in structural analyses of polysaccharides in particular.

INTRODUCTION

Neutral oligosaccharides may be separated by a variety of methods [1]. Gel permeation chromatography (GPC) separates mainly on the basis of molecular size [1, 2] and sometimes on the basis of chemical structure [3, 4]. The latter observations imply some solute—matrix interactions. Some separations are excellent up to a degree of polymerization (D.P.) of 40 or more [2], but retention times of up to 20 h are involved.

High-performance liquid chromatography (HPLC) of oligosaccharides on anion-exchange resins is not often reported. Cation-exchange resins, in a number of ionic forms, e.g. calcium [5, 6] or silver [7], using water as the sole eluent have proved useful.

Polyfunctional amines added to the solvent (usually acetonitrile—water) allow the use of silica columns [8, 9]. Of the chemically bonded silica based HPLC phases, aminopropyl is the most widely used [10—12], though octadecyl-bonded silica is becoming increasingly popular [13—15]. It was with a

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view to extending the usefulness of the latter type of column that the present work was undertaken. It was observed [13] that oligosaccharides greater than about D.P. 9 were eluted as broad peaks having relatively long retention times. Large oligosaccharides were retained on the column, eventually causing deterioration of its performance and an increase in back pressure. A means of eluting higher oligomers was thus sought. Observations by ourselves confirm those of Vrátný et al. [14] for both steel and glass columns, that increased temperature reduces the retention times of oligosaccharides markedly. It is not possible, however, to heat Dextropak columns in the Radial Compression Module used in the present work. In other cases we found that resolution between closely related oligosaccharides was poor and this initiated the work on added neutral salts.

EXPERIMENTAL

The HPLC system consisted of the following Waters Assoc. equipment: U6K injector, M6000 pump, R401 refractive index detector, and Radial Compression Module RCM-100. Columns: (a) Waters Dextropak Cartridge 10 × 0.8 cm (10 μm particle size, spherical silica, 125 Å pore size, 10% loading of octadecyl silane bonded phase). (b) LiChrosorb RP-8 steel (E. Merck) 25 × 0.4 cm (10 μm particle size, LiChrosorb SI-60 porous silica; octylsilane bonded phase). Sodium dodecyl sulphate (SDS) and cetyl trimethylammonium bromide (CTAB) were Eastman products; Triton X-100 (TX) was purchased from Rohm & Haas and tetramethylurea (TMU) from Fluka. Water used as HPLC solvent was purified in a Milli-Q system (Millipore).

Coating of the column was carried out by pumping a solution of detergent (0.1%, w/v, in water) through the column at a flow-rate of 1 ml/min. The standard compound used to determine the extent of column modification was isomaltotetraose (IM_4) approx. 10 mg/ml in water.

IM_4 (10 μl) was injected periodically during the coating process. The coating was stopped at a particular IM_4 retention time by changing the solvent to water. Detergent on the column was removed completely by flushing with methanol (200 ml) when it was desired to return the column to its unmodified state. The mass of TX adsorbed was found by rotary evaporation of the methanol flushings, followed by drying of the detergent in vacuo at room temperature over phosphorus pentoxide and paraffin wax, and weighing.

RESULTS AND DISCUSSION

The standard compound used to determine the extent of column modification was IM_4. On the unmodified column used for the anionic and cationic detergents, IM_4 had a retention time of 4.9 min at a flow-rate of 2 ml/min.

Effect of cetyl trimethylammonium bromide

An 0.1% solution of CTAB was pumped through the column at a flow-rate of 2 ml/min. The extent of the modification was followed by injection of IM_4 solution periodically. The decrease in the retention time of IM_4 was linear with respect to solvent volume. As the coating of the column proceeded, the back-

pressure of the column rose from 33 bars to almost 133 bars (the limit for the Radial Compression Module). The experiment was stopped at this point (IM_4 retention time = 2.6 min). The solvent was changed to water, and the retention time of IM_4 was again checked periodically. The water removed CTAB from the column, the retention time increased continually, so the CTAB treatment was considered unsuitable for practical purposes.

Effect of sodium dodecyl sulphate

The effect of an 0.1% solution of SDS on the retention time of IM_4 was similar to that of CTAB. The rate of pressure increase was less than for CTAB, and IM_4 could be made to elute at the void of the column. The solvent was changed to water, and again detergent was removed, leading to increasing retention times for IM_4. In summary, the ionic detergents yielded columns whose elution characteristics changed continuously, and were thus unsuitable for most practical purposes. A coating material which would not be washed off with the solvent was sought.

Effect of Triton X-100

The non-ionic detergent Triton X-100 is a mixture of *p-tert.*-octylphenoxypolyethoxyethanol oligomers, with an average ethylene oxide (EO) number of approx. 9.5 [16]. Triton was used as a column modifier in the hope that the neutral detergent molecules might not be as easily removed by water as the anionic and cationic groups previously used. This appeared to be the case: TX (0.1% solution) could be coated onto a column to achieve for IM_4 any desired retention time from 4.9 min to the column void (1.3 min). When the eluting solvent was changed from TX solution to water, the retention time of IM_4 at that stage of modification remained constant, i.e. TX which was coating the C_{18} on the column was not removed. Up to 1 l of water was passed through the modified column without significantly changing the retention time of IM_4. Several experiments confirmed this. While the column was being coated with 0.1% TX, none of the detergent emerged in the eluent, as shown by freeze-drying of the collected water.

The change in IM_4 retention time brought about by TX solutions was linear with time (Fig. 1). This plot was used in subsequent coating experiments to

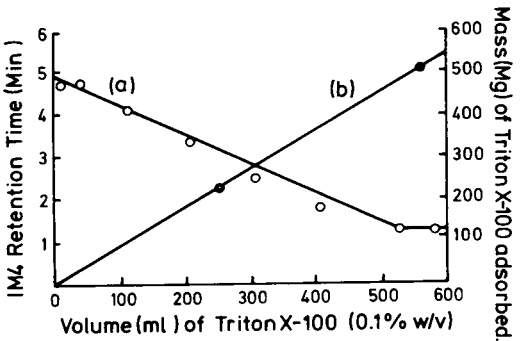

Fig. 1. (a) Isomaltotetraose (IM_4) retention time as a function of solvent volume of Triton X-100 (0.1%) passed through the column. (b) Mass of Triton X-100 adsorbed as a function of solvent volume passed through the column.

determine the volume of TX solution needed to achieve a chosen IM_4 retention time.

Experiments to determine the quantity of TX coating the C_{18} column showed that when IM_4 emerged at the column void volume, 500 ± 10 mg had been taken up. This occurred after the passage of 540 ml of 0.1% TX solution.

It was possible to absorb more TX than 500 mg. The back-pressure increased rapidly above this level so no limiting value was determined.

The coating process with TX was completely reversible. The retention time of IM_4 returned to the original value when the TX was flushed out using

TABLE I

RETENTION TIMES FOR MALTO-OLIGOSACCHARIDES ON TRITON X-100 COATED DEXTROPAK COLUMN

Extent of coating is shown by retention time of IM_4. Flow-rate: 2.0 ml/min; solvent: water.

Peak*		Retention time (min)			
		IM_4 = 4.9**	IM_4 = 4.1	IM_4 = 3.7	IM_4 = 3.0
3	i	3.0	2.9	2.6	1.9
	ii				
4	i	3.5	3.5	3.2	2.2
	ii	3.9	3.8		
5	i	4.8	4.5	4.0	2.6
	ii	5.4	4.9		
6	i	6.7	5.9	5.1	3.0
	ii	7.2	6.2		
7	i	8.6	7.1	6.0	3.8
	ii	9.1			
8	i	10.1	8.0	7.0	4.5
	ii	10.9	8.3		
9	i	12.4	9.2	8.0	5.2
	ii	14.1	10.2	8.7	
10	i	16.4	11.4	9.6	6.2
	ii	19.2	12.9	10.8	
11	i	22.6	14.6	12.2	7.6
	ii	26.5	16.6	13.6	
12	i	31.1	18.9	15.7	9.9
	ii	35.5	21.0	17.5	
13	i	41.8	23.9	19.9	12.2
	ii	47.0	26.3	22.1	
14	i	—	29.8	25.1	15.9
	ii		32.6	28.4	
15	i	—	37.2	32.0	19.4
	ii		41.5	35.5	

*Peak numbers refer to degree of polymerisation (D.P.) of the oligosaccharide. i and ii refer to the β- and α-anomers. Where only one retention time is shown, anomers were not resolved.

**Unmodified column.

methanol. The column to date has been used for several absorption/desorption cycles. (Another column whose performance in oligosaccharide separations had deteriorated slightly was actually improved after one adsorption/desorption cycle with TX, i.e. the retention time of IM_4 increased.)

Results for experiments involving the separation of oligosaccharides of the maltose series are summarized in Table I. On the unmodified column, the α and β anomers of all oligosaccharides above D.P. 3 are resolved (Fig. 2). [The data in Table I (IM_4 = 4.9 min) were not derived from Fig. 2, which was run under conditions such that a compact chromatogram resulted.] As the amount of TX was increased, the lower-molecular-weight oligosaccharides began to elute as single peaks. Finally, when IM_4 emerged at 3.0 min, all oligosaccharides eluted as single peaks (Fig. 3). The peaks are still well resolved and quantitative and qualitative analyses are possible. On the untreated column, peaks of the higher oligosaccharides are broad, resolved into anomers, and have inconveniently long retention times. The modified columns may also be used for preparative purposes. As the Triton remains on the column, freeze-drying of collected samples yields clean products. About 5 mg carbohydrate can be injected at one time.

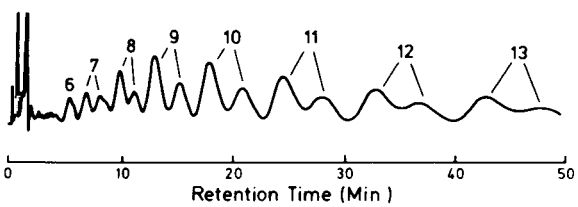

Fig. 2. Elution profile of large malto-oligosaccharides on an unmodified Dextropak column. Numbers indicate degree of polymerization (D.P.). Solvent: water; flow-rate: 2.0 ml/min.

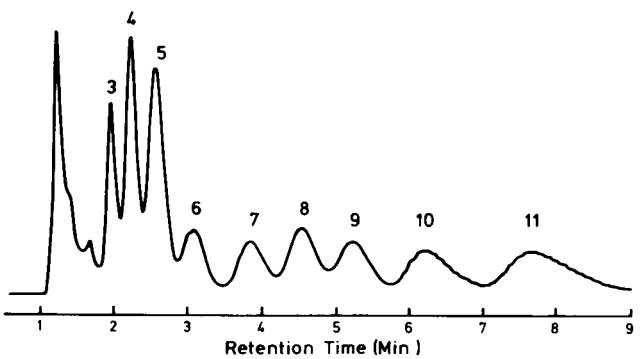

Fig. 3. Elution profile of malto-oligosaccharides on a Triton X-100 modified Dextropak column (IM_4 at 3.0 min). Numbers indicate degree of polymerization (D.P.). Solvent: water; flow-rate: 2.0 ml/min.

Similar comments may be made about the isomaltose series (Table II). Even more dramatic reductions in retention times are obtained, and resolution remains excellent (Fig. 4). Table II also shows that pumping 840 ml of water through the column does not remove the Triton X-100. In fact, a slight reduction in retention times occurs. This could be due to build up of organic compounds from the solvent.

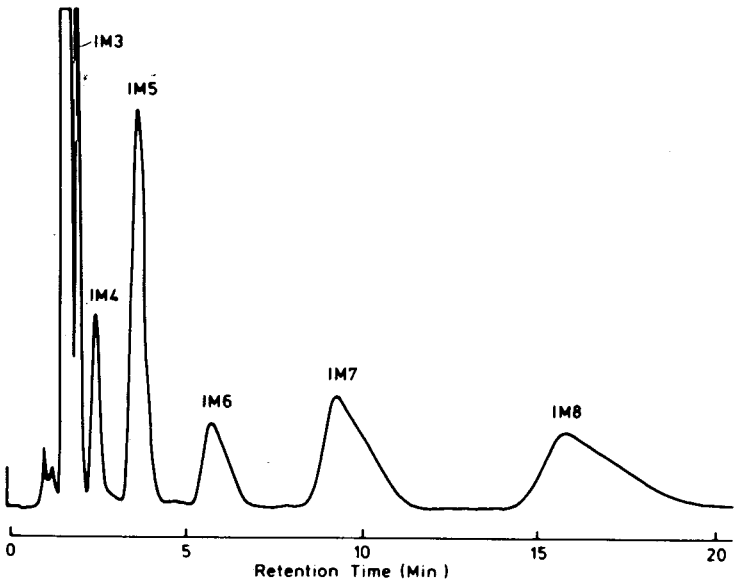

Fig. 4. Elution profile of isomalto-oligosaccharides on a Triton X-100 modified Dextropak column (IM_4 at 2.7 min). Numbers indicate degree of polymerization (D.P.). Solvent: water; flow-rate: 2.0 ml/min.

TABLE II

RETENTION TIMES FOR ISOMALTO-OLIGOSACCHARIDES ON TRITON X-100 TREATED DEXTROPAK COLUMN

Peak numbers refer to degree of polymerization (D.P.). Flow-rate: 2.0 ml/min; solvent: water.

Treatment	Retention time (min)				
	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8
1. Untreated column	5.1	9.1	17.5	29.5	66.0
2. 132 ml of 0.1% Triton X-100	3.5	5.45	9.2	15.8	28.3
3. 220 ml of 0.1% Triton X-100	2.9	4.5	7.4	12.3	21.6
4. 260 ml of 0.1% Triton X-100	2.7	3.9	6.0	9.6	16.1
5. 260 ml of 0.1% Triton X-100 + 840 ml water	2.5	3.6	5.4	8.4	14.0

The Triton modification should also be useful in reducing the retention times of other oligosaccharide series, as the column is capable of separating oligosaccharides on a structural, as well as a size, basis [1, 13]. In the case of columns used in the Radial Compression Module, increased temperatures cannot be used to decrease retention times, which is possible for steel columns [14].

Mechanism of the detergent effect

The decrease in retention times by added detergents is caused by the coating of the detergent on the C_{18} phase. As a result, the stationary phase–solute

interactions are reduced. Possible reasons for the reduced interactions include (a) reduction of the surface tension between stationary phase and the mobile phase, which affects the transfer of solute from the mobile phase to the stationary phase, and vice versa. This behaviour is characteristic of systems in which solvophobic (hydrophobic here) interactions determine the separation [17]. (b) Reduction in the size of the hydrocarbonaceous area of the stationary phase by adsorption of the detergent. This also assumes the separation mechanism to be largely hydrophobic in nature. Use of smaller alkyl chains, e.g. C_8, in reversed-phase chromatography results in shorter retention times than for C_{18} columns [18]. This effect also applies to the separation of oligosaccharides in water. The maltose oligosaccharides of D.P. 3—7 were poorly resolved on a C_8 steel column, but those of the isomaltose series were almost baseline-resolved and emerged at much earlier retention times than those on an unmodified, or even a significantly modified, column (Fig. 5 cf. Fig. 4). The Dextropak columns have a high loading of C_{18} chains (10%). Other C_{18} columns with lower loadings do not retain oligosaccharides to the same extent.

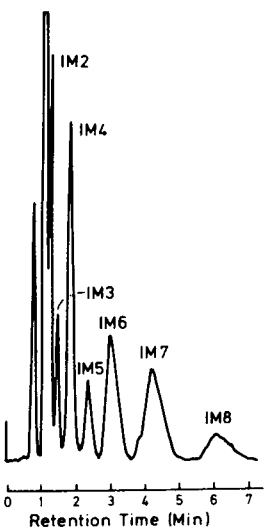

Fig. 5. Elution profile of isomalto-oligosaccharides on an unmodified steel 25 cm × 0.4 cm, octylsilane (C_8) column. Solvent: water; flow-rate: 2.0 ml/min.

Effect of mixed organic—aqueous solvents

An obvious way to reduce the retention times in reversed-phase separations is to use mixed water—organic solvents. A range of organic solvents viz. methanol, ethanol, acetonitrile, tetrahydrofuran and dimethyl formamide (DMF) were tested in amounts as low as 0.05%. Organic solvents at a concentration greater than 0.1% result in poor resolution, and up to this level they do not reduce retention times sufficiently to be useful. The results of using 0.2% methanol in water are typical (Fig. 6). The oligosaccharides elute more rapidly than in water, but are not well resolved. Use of solvents having DMF concentrations above 4% changed the separation mechanism completely. The presence of DMF reduced the solute—C_{18} and solute—silica interactions so

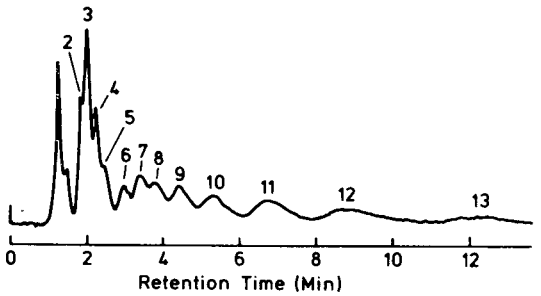

Fig. 6. Elution profile of malto-oligosaccharides on an unmodified Dextropak column. Numbers refer to degree of polymerization (D.P.). Solvent: 0.2% methanol—water; flow-rate: 2.0 ml/min.

completely that a gel permeation mode was observed, i.e. the larger oligosaccharides eluted first. Resolution in the gel permeation mode was poor, so the change in mechanism could not be put to practical use.

Effect of tetramethylurea

Tetramethylurea is reported to disrupt hydrogen bonds. It has been used in polysaccharide chemistry to improve one-step methylations by the Hakomori method, presumably by reducing hydrogen bonding between chains [19]. It was used here in an attempt to show whether hydrogen bonding to polar surface groups on the silica support was involved in the separation mechanism.

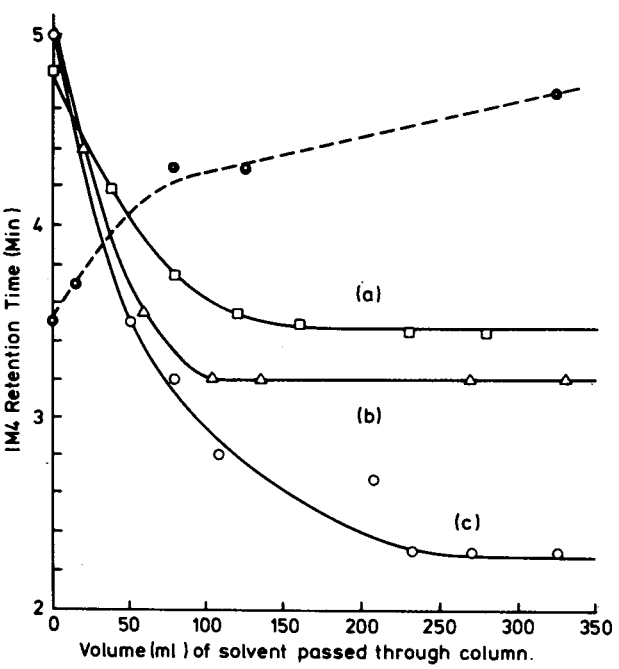

Fig. 7. Effect of tetramethyl urea (TMU) concentration on the retention time of isomaltotetraose (IM_4). (a) 0.025% TMU (□); (b) 0.05% TMU (△); (c) 0.1% (v/v) TMU in water (○); removal of 0.025% TMU with water (●). Column: Dextropak; flow-rate: 2.0 ml/min.

Results using the retention time of IM_4 as an index of column modification are shown in Fig. 7. A distinct difference between the effect of TMU and Triton is immediately evident. For the TMU, the retention time of IM_4 reaches a constant value, the magnitude of which depends on the TMU concentration in the solvent. This suggests that a dynamic equilibrium is being established, rather than a mere coating of the C_{18} as appears to be the case with detergents. A simple decrease in surface tension should have an immediate effect, as with methanol etc. The effect of TMU is reduced retention times, thus decreased hydrophobicity of the stationary phase is almost certainly involved. Involvement of hydrogen bonding in the separation mechanism has not been proven*. If it is involved, description of such hydrogen bonding by the presence of TMU would reduce retention times also, and be consistent with the above results.

TABLE III

RETENTION TIMES FOR MALTO- AND ISOMALTO-OLIGOSACCHARIDES ON A DEXTROPAK COLUMN USING 0.025% TETRAMETHYL UREA IN WATER

Flow-rate: 2.0 ml/min.

D.P.	Retention time (min)	
	Maltose series	Isomaltose series
2		2.1
3	2.8	2.6
4	3.1	3.5
5	3.7	5.3
6	4.6	8.3
7	5.4	14.3
8	6.3	—
9	7.3	—
10	8.8	—
11	10.8	—
12	14.0	—
13	18.8	—

Table III summarizes results for 0.025% TMU obtained with the maltose and isomaltose oligosaccharides. The maltose series shows single somewhat tailing peaks at this level, but well resolved. The isomaltose series in 0.025% TMU yields a chromatogram similar to that using the Triton coating in Fig. 4. The TMU-treated columns are thus useful. The major difference is that the requisite concentration of TMU must be maintained in the solvent at all times, as it is washed from the column by neat water (Fig. 7). This is a disadvantage for preparative work, but the low level of TMU present may be removed by passing the sample of oligosaccharide through a column of Sephadex G-25.

To further check for the possible involvement of hydrogen bonding, 0.1 *M* urea was used as solvent. The results (Table IV) show a large decrease in capacity factor. However, use of an ionized hydrogen bond disrupting agent, guanidine hydrochloride, up to 0.3 *M* concentration, had much less of an effect

*Editor's remark: The data presented here support the concept of tetramethyl urea as a hydrophobic bond splitting agent rather than being involved in the breakage of hydrogen bridges.

TABLE IV

RETENTION TIMES FOR MALTO-OLIGOSACCHARIDES CHROMATOGRAPHED ON A DEXTROPAK COLUMN WITH WATER, UREA AND GUANIDINE HYDROCHLORIDE SOLUTION AS SOLVENTS

Flow-rate: 2.0 ml/min; peak numbers refer to D.P.

Peak	Retention time (min)			
	Water*	0.1 *M* Urea	0.1 *M* Guanidine·HCl	0.3 *M* Guanidine·HCl
3	4.2	1.9	2.1	2.05
4	5.2	2.25	2.6	2.5
5	6.7	2.8	3.45	3.4
6	8.8	3.5	4.55	4.45
7	10.9	4.2	5.6	5.4

*In water, for D.P. 4 and above, anomers were resolved. Only the first of these has been included. A single peak was observed for each D.P. in the other solvents.

(Table IV). The latter result suggests operation of two competing effects on the capacity factor: an increase due to the ionic strength (hence surface tension) effect, and a decrease due to the disruption of hydrogen bonding.

Effect of added salts

The foregoing column treatments were designed to reduce the retention times of larger oligosaccharides. There is also a requirement for the better resolution of smaller oligosaccharides. During studies on the branching of dextrans [20], it was found that IM_4 was not resolved from a branched oligosaccharide of the same D.P., 3^3-α-D-glucopyranosylisomaltotriose (B_4).

As the work involving TX and TMU treatment was consistent with the solvophobic theory of reversed-phase chromatography, it was decided to utilize

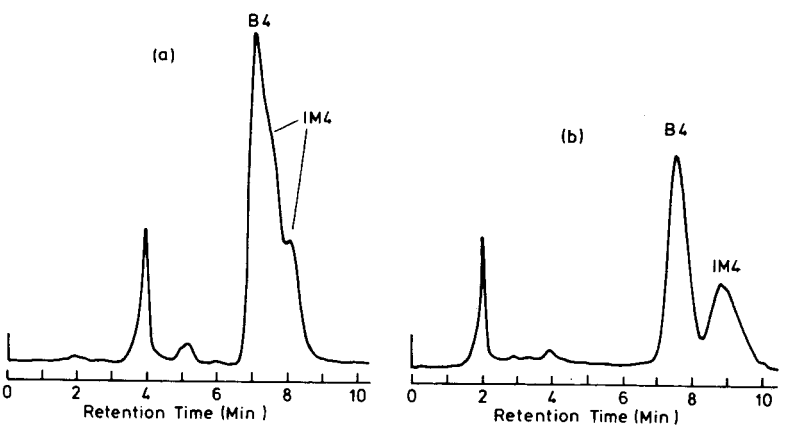

Fig. 8. Effect of the addition of ammonium sulphate on the HPLC separation of isomaltotetraose (IM_4) and 3^3-α-D-glucosylisomaltotriose (B_4). (a) Flow-rate: 1 ml/min; solvent: water. (b) Flow-rate: 2 ml/min; solvent: 1 *M* ammonium sulphate solution. Column: Dextropak.

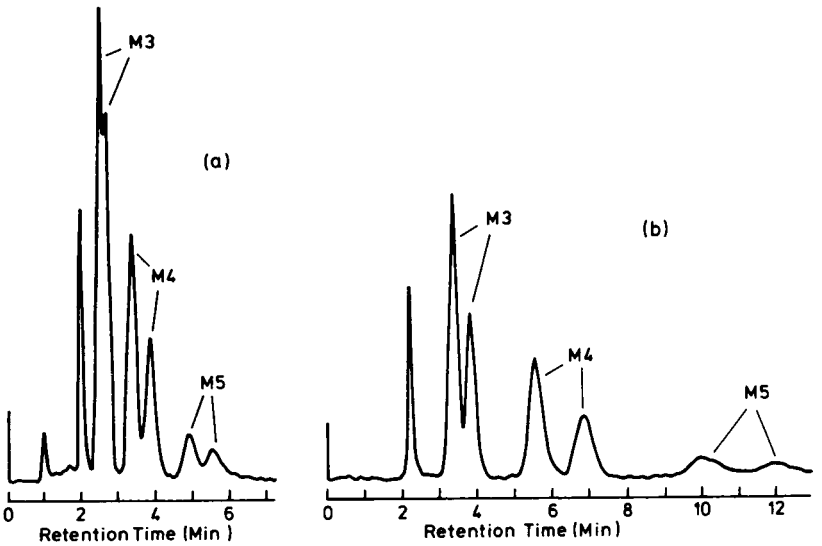

Fig. 9. Effect of the addition of ammonium sulphate on the HPLC separation of D-malto-oligosaccharides. (a) Solvent: water. (b) Solvent: 1 *M* ammonium sulphate solution. Column: Dextropak; flow-rate: 2 ml/min. Peak numbers refer to degree of polymerization (D.P.). Note resolution into α- and β-anomers.

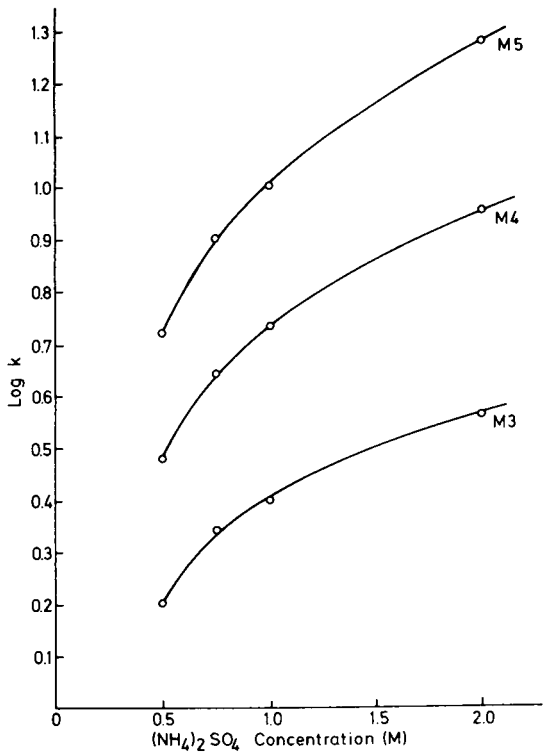

Fig. 10. The dependence of the retention factor, k, for malto-oligosaccharides M_3, M_4 and M_5 on the concentration of ammonium sulphate in the solvent. Data are for one anomer only. Column: Dextropak; flow-rate: 2 ml/min.

another prediction of that theory in order to increase the retention times of, and perhaps enhance the resolution between, IM_4 and B_4. Solvophobic theory predicts, inter alia, an increase in the retention factor of neutral solutes with an increase in ionic strength [17], the effect being due to an increase in surface tension of the solvent. The effect on the retention and resolution of IM_4 and B_4 (Fig. 8) when 1 *M* ammonium sulphate solution is used as solvent instead of water, is quite dramatic. The effect on the maltose series of oligosaccharides (Fig. 9) and other oligosaccharide separations indicate that the effect is general, and should prove extremely useful in oligosaccharide separations.

The solvophobic theory predicts that for a neutral solute, the logarithm of its capacity factor, k, should increase linearly with salt concentration. This seems to be almost so for ammonium sulphate and sodium sulphate up to 1 *M* concentration, but a curved plot is more closely followed over the range 0.5–2 *M*. Fig. 10 illustrates this point for the maltose series in ammonium sulphate solution. Similar curved plots are obtained with the isomaltose series and with sodium sulphate solution as solvent. The reason for the departure from linearity is not known. The increase surface tension of ammonium sulphate solutions is linear with concentration up to 4 *M* [21]. Perhaps as suggested above, there are hydrogen bonding or other contributions to the retention mechanism which are affected by ionic strength.

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MEASUREMENT OF URINARY VANILMANDELIC ACID AND HOMOVANILLIC ACID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION FOLLOWING EXTRACTION BY ION-EXCHANGE AND ION-MODERATED PARTITION

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SUMMARY

An improved protocol has been developed to isolate homovanillic acid (HVA) and vanilmandelic acid (VMA) from urine with strong anion-exchange resin. The sample is diluted with acetate buffer and passed through a disposable column. HVA, uric acid, and many hydrophobic organic acids are removed with 1.0 *M* acetic acid—ethanol. Then VMA is eluted with 0.5 *M* phosphoric acid. Two isocratic mobile phases allow rapid high-performance liquid chromatographic measurement of VMA (5 min) and HVA (8 mins) on a 5-μm ODS column. Selective conditions were developed with dual-electrode coulometric detection to permit specific measurement of VMA, HVA, and internal standards, with less than 5% between-run variation.

INTRODUCTION

Homovanillic acid (HVA) and vanilmandelic acid (VMA) are commonly measured in urine for the differential diagnosis of neuroblastoma, pheochromocytoma, and related tumors [1, 2]. Recently, VMA has been studied in evaluations of psychiatric patients [3], and HVA has been used to monitor chronic lead exposure [4] and response to medication during the treatment of Parkinson's disease [5, 6]. Although many techniques have been employed to separate and quantify these compounds, the use of high-performance liquid chromatography (HPLC) with reversed-phase columns has been increasingly common since the demonstration of this technique by Molnár and Horváth [7, 8].

The analysis of urinary phenolic acids by HPLC is complicated by the large number of compounds which are commonly observed in urine. Gradient

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elutions are often employed to improve resolution [9, 10]; isocratic separations have also been reported [11—13], but in both cases the analysis time is typically 30—60 min. For the analysis of individual compounds such as VMA and HVA, these procedures are quite time-consuming. Electrochemical detection [10, 14—16] and ion-pair chromatography [16] have been employed to shorten and simplify the HPLC analysis, and ethyl acetate [9] or diethyl ether [13] extraction has been used to reduce interferences. However, the chromatograms produced by these procedures show many peaks, indicating a lack of selectivity. Improvements in both the urine clean-up and the chromatographic system are needed to produce more selective methods.

The separation of VMA and HVA from other organic acids using an anion-exchange resin and salt gradients was first demonstrated by Weise et al. in 1961 [17]. The protocol was adapted to small disposable columns [18] and has been used in combination with the Pisano reaction [19] as a colorimetric test for VMA in urine. More recently, anion-exchange pre-treatment was combined with HPLC, in conjunction with electrochemical detection [16] or post-column derivatization [20].

The use of organic solvents to elute phenolic acids from resins was first demonstrated by Shelley and Umburger in 1959 [21]. Hydroxybenzoic acids were eluted from anion-exchange resin using 15% acetic acid in methanol [22]. The separation of HVA from other organic acids in brain tissue using acetic acid in ethanol was demonstrated by Shibuya et al. [23]. Since elution is not due to a change in ionic strength but rather the hydrophobic interaction between the eluting solvent and an anion which is electrostatically attached to the resin, this type of separation is termed "ion-moderated partition" (IMP) [24].

The purpose of this investigation was to develop an improved ion-exchange sample preparation to simplify HPLC analysis of urinary VMA and HVA. We used ion-moderated partition to first remove the hydrophobic compounds which are strongly retained by reversed-phase HPLC; this fraction contained HVA with a high degree of recovery. VMA was then eluted with dilute phosphoric acid. Finally, isocratic chromatographic separations were developed for analysis of each of these compounds using highly specific electrochemical conditions.

EXPERIMENTAL

Materials

1-, 3-, and 7-methyluric acids and 1,7-dimethyluric acid were purchased from Fluka Chemicals (New York, NY, U.S.A.). Iso-HVA was kindly supplied by the Research Labs. Hoffman-LaRoche (Nutley, NJ, U.S.A.). 5-Hydroxymethyl-2-furoic acid was synthesized from the corresponding aldehyde (furfural) in ethanolic sodium hydroxide (Cannizaro reaction). VMA, iso-VMA, HVA and all other organic acids were purchased from Sigma (St. Louis, MO, U.S.A.) or Aldrich (Milwaukee, WI, U.S.A.).

HPLC reagent-grade alcohol (ethanol) and 2-propanol were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). The other reagents and solutions used were reagent grade. Prepacked strong anion-exchange columns for VMA/HVA analysis were from Bio-Rad Labs. (Richmond, CA, U.S.A.) and pH paper, range 4.5—7.0, was from E. Merck (Rahway, NJ, U.S.A.).

Iso-VMA (450 mg/l) was prepared in 0.05 *M* hydrochloric acid and stored up to three months at 4°C. A stock solution of iso-vanillic acid (iso-VA) was prepared in 50% ethanol (10 mg/ml) and diluted to 500 mg/l with water for each run. Solutions of 1 mg/ml of VMA and HVA in 0.05 *M* hydrochloric acid were used to prepare a urine-based calibrator, which was subjected to the same clean-up as unknown urines.

HPLC instrumentation

The chromatographic system (Bio-Rad Labs.) was composed of a Model 1330 dual-piston pump, a Model 1305A variable-wavelength ultraviolet (UV) detector, a Model 450 column heater, a Model AS-48 automatic sampler with a 20-μl sample loop, and a Model 1322 dual-pen recorder. The column effluent was also monitored with a dual-electrode electrochemical ESA detector (Model 5100A Coulochem™ with a 5010 ESA analytical cell, Environmental Sciences Assoc., Bedford, MA, U.S.A.). A 150 × 4.6 mm, 5-μm ODS column with a 30 × 2.1 mm guard cartridge (Bio-Rad Labs.) was used for both analyses.

Mobile phase preparation

For VMA analysis, 0.05 *M* phosphate buffer, pH 3.00, was prepared from 2 *M* phosphoric acid and 1 *M* potassium dihydrogen phosphate, and 16 ml ethanol were added per 1000 ml buffer. The mixture, prepared daily, was filtered under vacuum through a 0.22-μm membrane (GVWP, Millipore, Bedford, MA, U.S.A.). For HVA analysis, 0.05 *M* phosphate buffer, pH 2.30, was prepared; 100 ml ethanol and 20 ml 2-propanol were added per 1000 ml buffer and the mixture was filtered as above.

Urine collection and clean-up procedure

Urines (24-h) were collected in plastic containers with 10 ml of 6 *M* hydrochloric acid as a preservative. Samples were stored at 4°C up to one week and at −20°C for longer periods. Urines were centrifuged at 2000 *g* to remove particulates.

The prepacked anion-exchange column was inverted to resuspend the resin, opened, and allowed to drain. Then 15 ml of 0.08 *M* sodium acetate buffer, pH 6.10, were used to prerinse the column. Urine (3.0 ml) was combined with 0.2 ml of iso-VMA, 0.2 ml iso-VA, 5 ml acetate buffer, and 0.1 ml of 0.5 *M* sodium hydroxide. The pH, which should be between 5.0 and 7.0, was verified with pH paper. Further pH adjustment with 0.5 *M* sodium hydroxide or 2 *M* acetic acid was performed if required. This mixture was poured onto the column and allowed to drain, then the column was rinsed again with 5 ml acetate buffer. HVA was eluted with 12 ml of 1.0 *M* acetic acid—ethanol (50:50, v/v). Then VMA was eluted with 18 ml of 0.5 *M* phosphoric acid. If only VMA is to be analyzed, the same procedure is followed, except the iso-VA is omitted and only 9 ml of acetic acid—ethanol are required.

HPLC operating instructions

For both analyses, the guard cartridge and analytical column were maintained at 35°C with a flow-rate of 1.5—2.0 ml/min. For VMA analysis, the first potential of the ESA cell was set at 0.18 V and the second potential

was at 0.30 V. For HVA, the first potential was set at 0.20 V and the second potential was set at 0.46 V. Equilibration from one mobile phase to the other required less than 10 min. The HPLC system was allowed to run overnight with the mobile phase flowing at 0.1 ml/min. Once a week, the system was rinsed with 30 ml distilled and filtered water, followed by 30 ml of 50% ethanol. The electrochemical cell was disconnected during the ethanol wash. No routine maintenance was required for the ESA detector, and one analytical column with two guard columns was sufficient for at least 600 analyses.

UV measurement of VMA was performed at 280 nm with 0.005 absorbance units full scale (a.u.f.s.).

Recovery studies

Urine samples were spiked with 10 μg/ml VMA and 20 μg/ml HVA to determine recovery. Absolute recovery was calculated against aqueous standards diluted with mobile phase. Analytical recovery was determined by comparison of VMA/iso-VMA and HVA/iso-VA ratios in the spiked urines to the aqueous standards.

Elution profiles for VMA and iso-VMA were prepared after a 9-ml alcohol wash by collecting successive 2-ml fractions of 0.5 *M* phosphoric acid.

Elution profiles for VMA, HVA, iso-VMA, iso-HVA, uric acid, 1-methyluric acid, 1,3-dimethyluric acid, *p*-hydroxyphenylacetic acid (*p*-OH-PAC), 5-hydroxyindole-3-acetic acid (5-HIAA), indole-3-acetic acid (IAA), and caffeic acid were prepared by spiking normal urines with 40 μg/ml of each compound. Ten columns were prepared and compounds were eluted with acetic acid—ethanol in amounts from 2—20 ml. This approach was required, as the collection of successive 2-ml applications of reagent to the same column gave substantially different results.

For comparison of different eluents, a normal urine was applied to the columns by the usual protocol but with no internal standards, and eluted directly with 0.5 *M* phosphoric acid or with 3 *M* sodium chloride in 0.08 *M* acetate buffer, pH 6.10.

RESULTS

The use of a two-step elution for urine yields a considerable improvement in the specificity of VMA analysis (Fig. 1a). The potentials chosen on the ESA detector enhance the analysis by removing easily oxidized compounds at the first electrode and reducing the background signal of the mobile phase. Detection at the second electrode is performed at a potential where most monohydroxyphenolic acids are not electrochemically active. Under these conditions, isocratic analysis is possible in 5 min and a very stable baseline is commonly observed.

The analysis of HVA is completed within 8 min (Fig. 1b). VA is commonly observed in small amounts between HVA and iso-VA (internal standard). By developing conditions where HVA, iso-HVA, VA and iso-VA were resolved, we were able to use iso-VA as an internal standard without interference from iso-HVA (Fig. 2). Small peaks from IAA are commonly observed at 15—16 min; injections may be made at one-half the elution time of IAA to avoid interference from this compound.

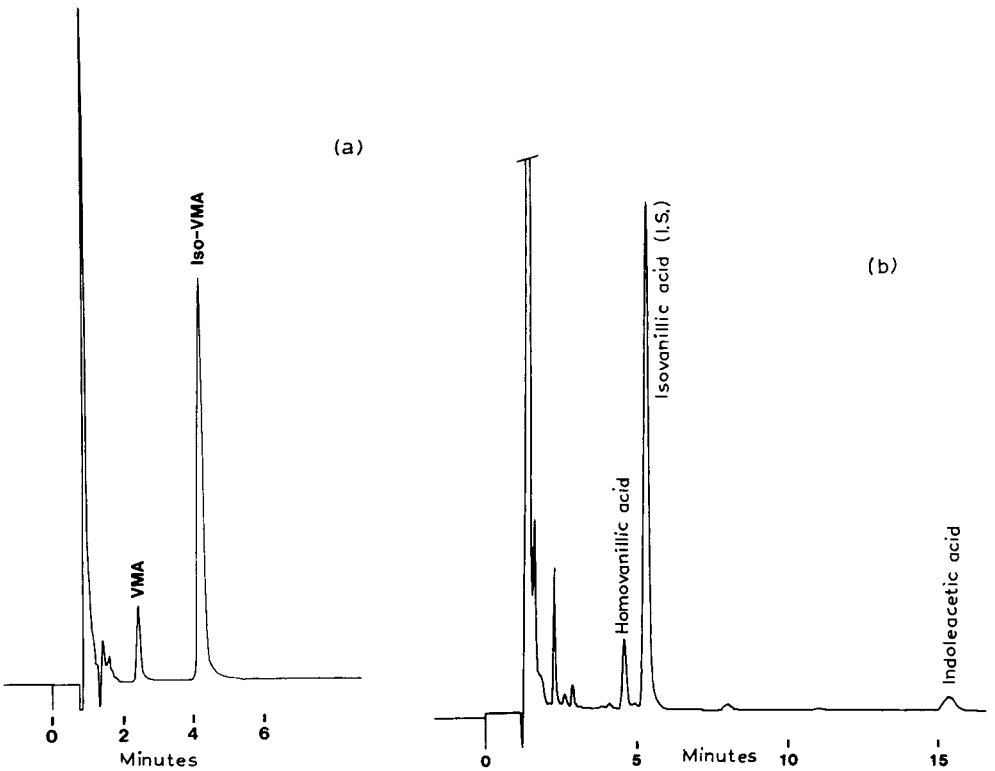

Fig. 1. (a) Chromatogram of a normal urine following pretreatment described in the text. Mobile phase: 0.05 *M* phosphate buffer, pH 3.00—1.6% ethanol. Flow-rate is 2.0 ml/min at 35°C. ESA detector gain is 10 × 1 at 0.30 V. (b) Chromatogram of a normal urine following pretreatment described in the text. Mobile phase: 0.05 *M* phosphate buffer, pH 2.30—ethanol—2-propanol (100:10:2). Flow-rate is 1.5 ml/min at 35°C. ESA detector gain is 12 × 1 at 0.46 V.

Electrochemical response is linear for HVA up to at least 40 mg/l. For VMA, we dilute samples reading higher than 20 mg/l.

The phosphoric acid eluates were also used for UV analysis of VMA (see Fig. 3). Analysis time was 15 min and hippuric acid was the last peak observed in normal urines. Results by the UV method (X) showed excellent agreement with the electrochemical method (Y); $Y = 1.002X + 0.02$, $n = 73$, $r = 0.990$, with values up to 25 mg/l. This confirms the selectivity of the HPLC conditions for VMA and iso-VMA. However, the chromatograms were more complex, and pH adjustment of the mobile phase was sometimes required to achieve a satisfactory separation of VMA from *m*-hydroxyhippuric acid and 2-furoyl chloride. Some urines from hospitalized patients contained unusual UV-absorbing peaks which eluted after hippuric acid. Also, some urines contained 5-hydroxymethyl-2-furoic acid in amounts up to 1 g/l. This compound is derived from dietary sources containing fructose, including intravenous solutions [25, 26]. Although it elutes 1 min after VMA, the very broad peak produced by high concentrations made quantitation of VMA difficult in a few cases. Satisfactory resolution could be obtained by increasing the ionic strength of the mobile phase and using a lower flow-rate for these urines.

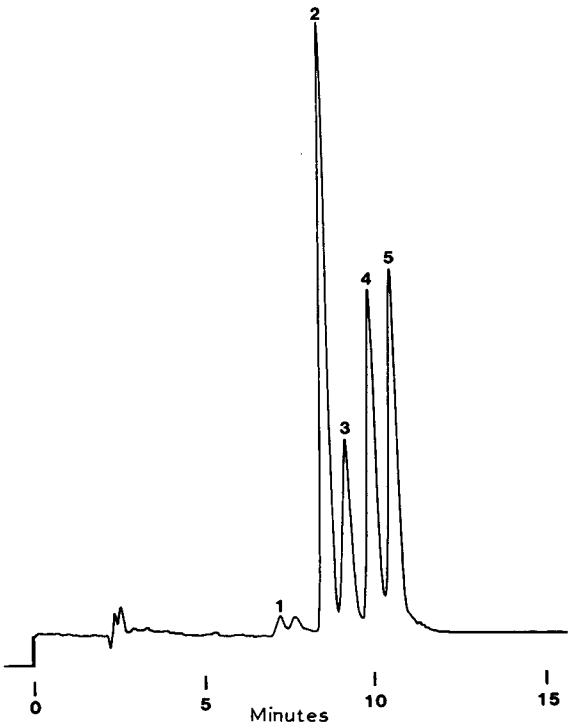

Fig. 2. Separation of HVA from related compounds. Mobile phase: 0.05 *M* phosphate buffer, pH 2.10—ethanol—2-propanol (100:10:2). Flow-rate is 0.8 ml/min at 35°C. ESA detector gain is 40 × 10 at 0.46 V. Test mix contains 1 ng each of the following compounds: *p*-hydroxyphenylacetic acid (1), homovanillic acid (2), vanillic acid (3), iso-vanillic acid (4), and iso-homovanillic acid (5).

We studied the elution profile of VMA and iso-VMA in 0.5 *M* phosphoric acid to demonstrate that both compounds eluted at the same rate and to determine the optimum elution volume. Peaks were slightly higher after 12-ml elution, but this represented only 65% of the total VMA. By using 18 ml eluent, the recovery was near 80% and further elution removed little additional VMA. Iso-VMA and VMA were present in the same ratio at all eluent volumes from 4 to 20 ml. Lower concentrations of phosphoric acid slowed the elution rate. We also found that the pH of urine at application did not affect the VMA/iso-VMA ratio. Recovery was highest when application pH was between 5 and 7.

The total capacity of the clean-up column is 1.1 mequiv., which is adequate to retain all the organic acids present in 3 ml urine. However, all urines contain chloride, and concentrations of chloride above 0.5 *M* will substantially reduce recovery and affect quantitation. Since the main source of chloride is typically the hydrochloric acid added as a preservative, over-acidification during collection must be avoided, and urines which are received with a pH less than 1.5 should be diluted before assay.

The elution profiles observed with the acetic acid—ethanol eluent varied a great deal for the compounds studied (see Fig. 4). Uric acid and methylurates were washed quickly off the column; 12 ml of eluent removed HVA, iso-HVA,

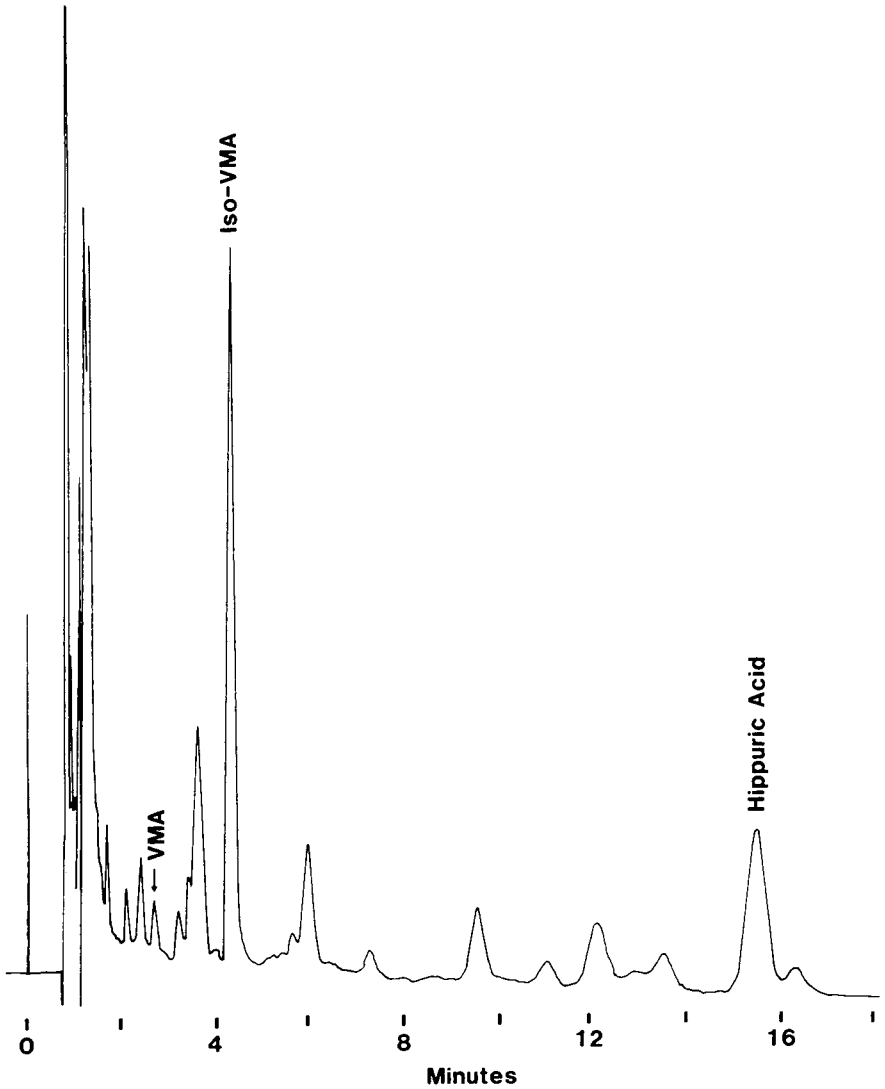

Fig. 3. Chromatogram of a normal urine measured by UV detection following pretreatment described in the text. Mobile phase: 0.01 *M* phosphate buffer, pH 3.00—1.6% ethanol. Flow-rate is 2.0 ml/min at 35°C. UV detection at 280 nm and 0.005 a.u.f.s.

VA, and iso-VA. Other compounds such as VMA, iso-VMA, and caffeic acid elute very slowly, and volumes in excess of 30 ml would be required to remove them completely. IAA and *p*-OH-PAC could be collected in 15—18 ml, but 5-HIAA eluted very slowly. Elution volumes did not correlate with pK_a values or HPLC elution order, and probably reflect a mixture of several retention mechanisms. To remove 1-methyluric acid and 7-methyluric acid, metabolites of caffeine which are found in most urines, 9 ml of acid—ethanol eluent is required. The removal of these compounds prior to VMA measurement is especially important as theophylline, a common anti-asthmatic drug, produces

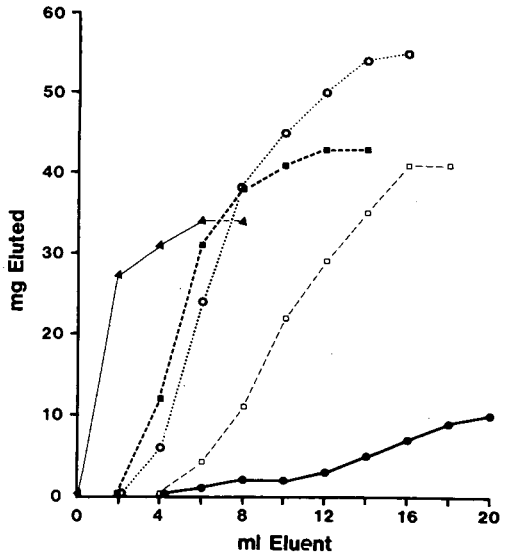

Fig. 4. Elution of different compounds from the anion-exchange column by 1.0 *M* acetic acid—ethanol (50:50). Compounds shown are homovanillic acid (■), vanilmandelic acid (●), 1,3-dimethyluric acid (▲), indole-3-acetic acid (□) and *p*-hydroxyphenylacetic acid (○). Protocol is described in Experimental.

TABLE I

RETENTION TIMES AND DETECTION CHARACTERISTICS FOR POSSIBLE INTERFERENCES IN VMA ANALYSIS

For VMA mobile phase and HPLC conditions, see Experimental. Flow-rate, 2.0 ml/min.

Compound	Chromatographic retention time (min)	Potential required for oxidation (V)
Uric acid	1.3	>0.10
p-Hydroxymandelic acid	1.8	>0.25
3-Methyluric acid	2.0	>0.05
4-Hydroxy-3-methoxymandelic acid (VMA)	2.5	>0.20
m-Hydroxymandelic acid	2.7	>0.45
m-Hydroxyhippuric acid	2.8	>0.60
7-Methyluric acid	2.8	>0.10
1-Methyluric acid	3.5	>0.05
2,5-Dihydroxyphenylacetic acid	3.6	0.01
3-Hydroxy-4-methoxymandelic acid (internal standard, I.S.)	4.0	>0.20
3-Indoxyl sulfate	4.8	>0.15
3-Hydroxyanthranilic acid	6.4	>0.05
1,3-Dimethyluric acid	6.9	>0.10
3,4-Dihydroxyphenylacetic acid	8.3	0.01
p-Hydroxybenzoic acid	9.3	>0.40
p-Hydroxyphenyllactic acid	10.0	>0.30
1,7-Dimethyluric acid	11.5	>0.20
Hydrocaffeic acid	15.5	0.01
p-Hydroxyphenylacetic acid	17.0	>0.35
5-Hydroxyindole-3-acetic acid	17.8	>0.05
m-Hydroxyphenylacetic acid	18.2	>0.45
Vanillic acid	18.5	>0.35
4-Hydroxy-3-methoxyphenyllactic acid	19.5	>0.20
Homovanillic acid	30.1	>0.20

TABLE II

RETENTION TIMES AND DETECTION CHARACTERISTICS FOR POSSIBLE INTERFERENCES IN HVA ANALYSIS

For HVA mobile phase and HPLC conditions see Experimental. The flow-rate was 1.5 ml/min.

Compound	Chromatographic retention time (min)	Potential required for oxidation (V)
p-Hydroxybenzoic acid	1.7	>0.40
p-Hydroxyphenylpyruvic acid (I)	2.0	>0.45
1,3,7-Trimethyluric acid	2.3	>0.20
3-Hydroxyanthranilic acid	2.3	>0.05
3,4-Dihydroxyphenylacetic acid	2.4	0.01
3-Indoxyl sulfate	2.5	>0.15
p-Hydroxyphenyllactic acid	2.7	>0.35
5-Hydroxyindole-3-acetic acid	2.8	>0.05
4-Hydroxy-3-methoxyphenyllactic acid	3.6	>0.20
Hydrocaffeic acid	3.7	0.01
p-Hydroxyphenylpyruvic acid (II)	3.9	>0.45
p-Hydroxyphenylacetic acid	3.9	>0.35
m-Hydroxybenzoic acid	4.1	>0.55
4-Hydroxy-3-methoxyphenylpyruvic acid (I)	4.3	>0.35
Homovanillic acid (HVA)	4.3	>0.20
Vanillic acid	4.6	>0.35
3,5-Dimethoxy-4-hydroxybenzoic acid	4.7	>0.20
Caffeic acid	4.9	0.01
m-Hydroxyphenylacetic acid	4.9	>0.45
iso-Vanillic acid (internal standard, I.S.)	5.0	>0.35
iso-Homovanillic acid	5.5	>0.20
o-Hydroxyphenylacetic acid	5.6	>0.40
4-Hydroxy-3-methoxyphenylpyruvic acid (II)	5.6	>0.35
o-Hydroxyhippuric acid	6.8	>0.40
p-Hydroxycinammic acid	9.4	>0.30
3-Indolelactic acid	9.9	>0.30
4-Hydroxy-3-methoxycinammic acid	10.6	>0.20
Indole-3-acetic acid	15.1	>0.20
3-Indolepropionic acid	35.2	>0.35

urine concentrations of 1-methyluric acid and 1,3-dimethyluric acid over 100 mg/l [27, 28]. Complete elution of HVA requires 12 ml of this eluent; a small amount of VMA and iso-VMA is also eluted. For collection of hydrophobic compounds other than HVA, different elution volumes of acetic acid—ethanol could be used.

We studied the HPLC retention time of many organic acids in the two mobile phases which were used (Tables I and II). 7-Methyluric acid, the compound which is most likely to interfere with VMA measurement, is removed by acetic acid—ethanol during sample clean-up. Although some other compounds elute just behind VMA, they cannot be oxidized at 0.30 V. Dicarboxylic acids are not eluted from the anion-exchange clean-up column and were not examined; *o*-hydroxybenzoic acids such as gentisic acid and salicylic acid are also strongly retained. For HVA analysis, only *p*-OH-PAC and VA are observed in normal urines. The split HPLC peaks from keto and enol forms of 4-hydroxy-3-methoxyphenylpyruvic acid (VPA) have been observed previously

[29]. Small amounts of this compound might coelute with HVA in patients receiving L-3,4-dihydroxyphenylalanine, although HVA levels would be extremely elevated in this case. Normal urines do not contain measurable quantities of VPA [29]. *o*-OH-PAC is normally present in trace amounts but is elevated in phenylketonuria [30]. For metabolic studies of HVA/iso-HVA ratio, the analysis could be performed at lower oxidation potentials (0.18/0.30 V) to eliminate these potential interferences, but iso-VA could no longer be used as an internal standard.

A small amount of VMA is oxidized at the first electrode of the detector, and this amount is proportional to concentration. The first electrode potential should be set to achieve maximum oxidation of 1-methyluric acid (see Fig. 5A), while VMA response is limited to less than 8% of the signal at electrode two. The second electrode potential is set so that iso-VMA reaches near 50% of its maximum peak height. For both of these compounds, and for HVA and iso-VA as well, the maximum electrode response is observed at 0.50 V (see Fig. 5). For the analysis of HVA, a slightly lower potential (0.46 V) is used to avoid interference from *m*-OH-PAC. The small peak observed from VMA at the first electrode may be used for confirmation of VMA identity in abnormal urines, since elevated specimens show a proportional increase in response at both electrodes.

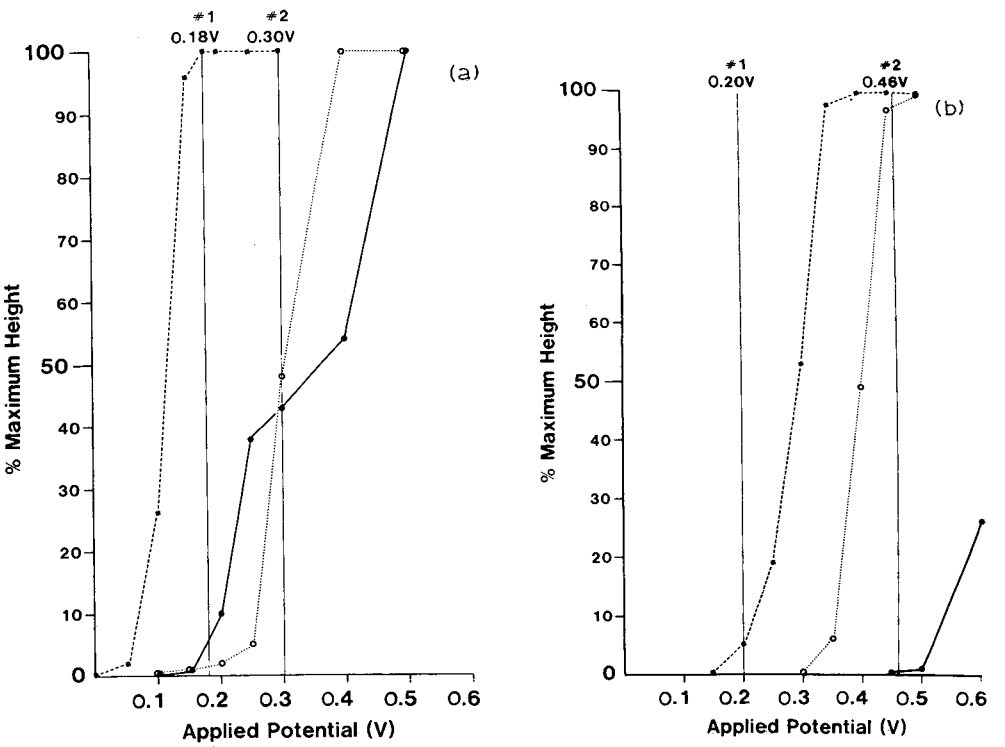

Fig. 5. Normalized response of the ESA 5010 electrochemical cell at various voltage settings. (a) Compounds shown are vanilmandelic acid (•), iso-vanilmandelic acid (○) and 1-methyluric acid (▪). (b) Compounds shown are homovanillic acid (▪), iso-vanillic acid (○) and *m*-hydroxyphenylacetic acid (•).

Elution of organic acids from anion-exchange resin is usually achieved by increasing the ionic strength of the buffer eluent without a change in pH. The elution of VMA in 0.5 *M* phosphoric acid does not suggest a traditional approach, as the eluent pH (1.2) is well below the pK_a of VMA (3.4), and less than 1% of the VMA should be ionized. We compared different eluents (Fig. 6), and the results obtained with 0.5 *M* phosphoric acid (Fig. 6b) resemble the profile obtained when 3 *M* sodium chloride in phosphate buffer, pH 6.1, was used to elute VMA (Fig. 6a). Both of these elute HVA as well, but the recovery is typically low by this approach [16]. Several minor peaks, as well as 7-methyluric acid, are eluted by 0.5 *M* phosphoric acid when the acetic acid—ethanol step is omitted. Using an amperometric detector, peaks from 1-methyluric acid and 3,4-dihydroxyphenylacetic acid would be observed in Fig. 6a and b.

The precision of both methods was adequate for clinical purposes (see Table III). For normal concentrations, within-run coefficients of variation (C.V.) were less than 2% and between-run C.V. values were less than 5%.

We measured HVA in 30 normal adults and found a mean level of 2.3 mg HVA per g creatinine with ratios ranging from 1.1 to 4.3. These results agree with previously reported findings [31, 32]. Creatinine was determined by a modified Jaffe method [33].

Analysis of HVA for neuroblastoma rarely produces borderline results, as elevations are usually 5—100 times normal concentrations [31]. With VMA, marginal elevations (two to three times of normal) may indicate pheochromocytoma [1]. For this reason, we completed a larger normal study for VMA on 24-h collections from 110 healthy adults, with ages from 16 to 70 years.

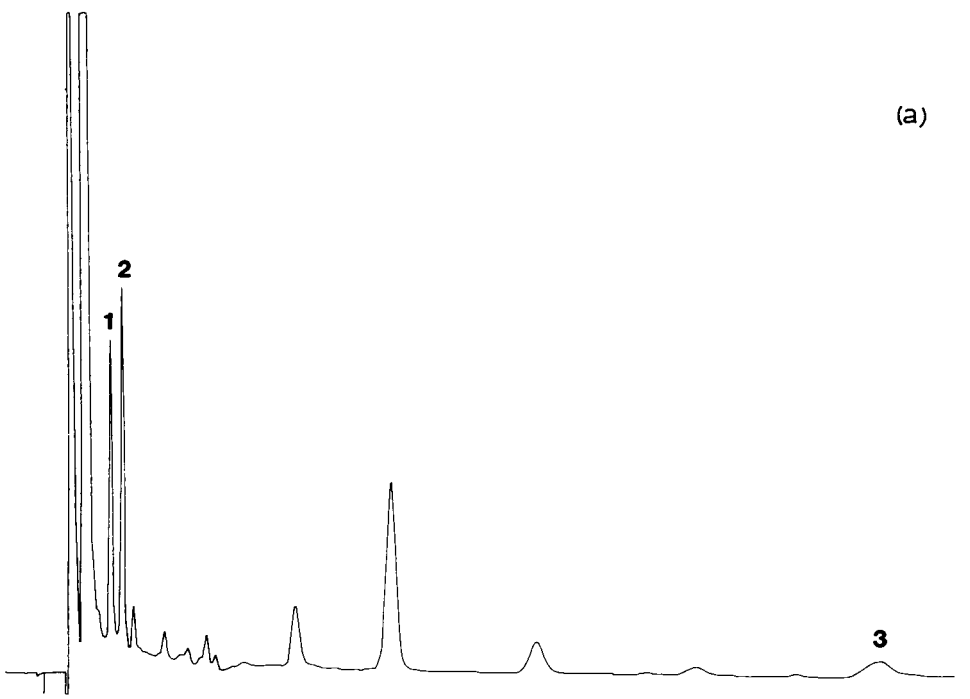

Fig. 6.

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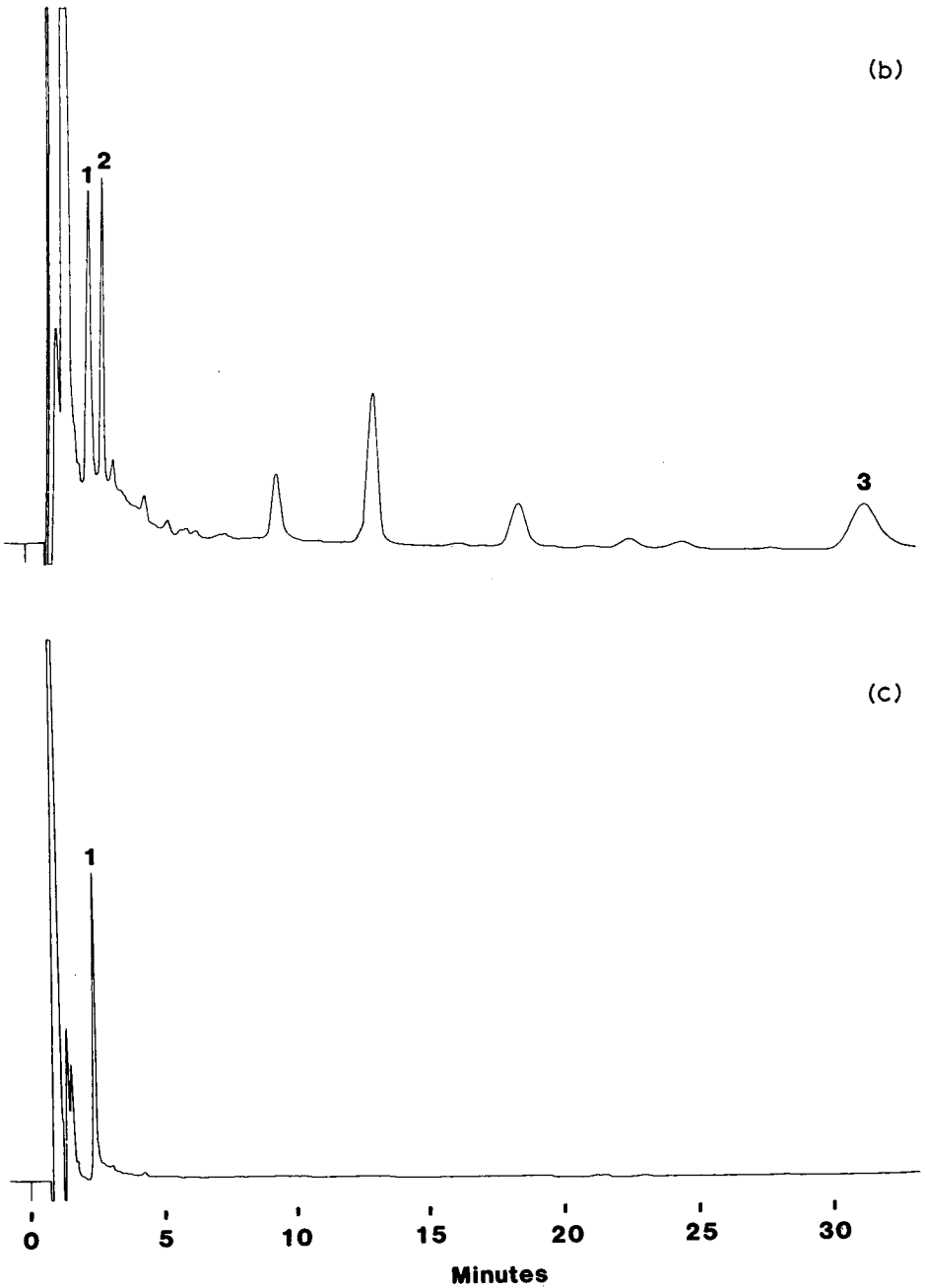

Fig. 6. Chromatograms of a normal urine after three different elution protocols: (a) 12 ml of 3.0 *M* sodium chloride in phosphate buffer, (b) 12 ml of 0.5 *M* phosphoric acid, (c) 12 ml of 0.5 *M* phosphoric acid after a prerinse with 12 ml of 1.0 *M* acetic acid—ethanol. Mobile phase and conditions are as in Fig. 1A; ESA detector gain is 40 × 1. Peaks: 1 = vanilmandelic acid; 2 = 7-methyluric acid; 3 = homovanillic acid.

TABLE III

PRECISION OF VMA AND HVA MEASUREMENT

	VMA		HVA	
	3.7 mg/l	15.0 mg/l	2.6 mg/l	15.0 mg/l
Within-run C.V. (%) (n = 6)	1.5	0.8	0.9	1.3
Between-run C.V. (%) (n = 20, 5 runs)	3.9	3.0	4.6	3.4

TABLE IV

NORMAL-RANGE STUDIES FOR URINARY VMA AND HVA

	VMA		HVA	
	mg per day	mg per g creatinine	mg per day	mg per g creatinine
Number of subjects	110	110	30	30
Range of values	1.60—7.27	1.14—5.18	0.67—7.78	1.13—4.29
95% Range (non-parametric)	1.77—6.74	1.56—4.20	—	—
Mean ± S.D.	3.86 ± 1.33	2.65 ± 0.79	3.14 ± 1.55	2.27 ± 0.78

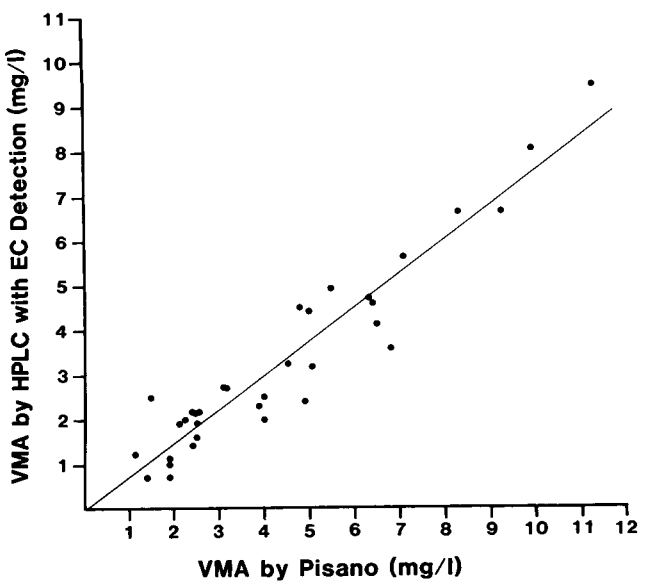

Fig. 7. Comparison of vanilmandelic acid results obtained by a Pisano [19] method (X) to results obtained by this HPLC technique (Y). For n = 34, $Y = 0.760X - 0.074$, $r = 0.9512$.

In this group we found VMA levels ranging from 1.6 to 7.3 mg per day (see Table IV). When corrected for creatinine, the ratios obtained ranged from 1.1 to 4.3 mg VMA per g creatinine with one outlier at 5.2. These results show

good agreement with the cut-off value of 4 mg VMA per g creatinine recommended by Gitlow [1]. For comparison, 34 urines assayed by a Pisano method [19] were run by this HPLC technique. Results were about 25% lower and the correlation was 0.951 (see Fig. 7).

Recovery of both compounds was reproducible. For VMA, absolute recovery was 75—85% and analytical recovery (after correction for the internal standard) was 97—103%. For HVA, absolute recovery was 90—102% and analytical recovery was 98—102%.

DISCUSSION

Many laboratories lack gradient HPLC systems; also the use of gradients with electrochemical detectors may cause baseline drift and require frequent recalibration. Specific HPLC methods have been developed using post-column reaction systems [20, 34, 35], but this apparatus is rarely found in clinical laboratories. For these reasons, we have developed two isocratic mobile phases for the HPLC analysis of VMA and HVA by electrochemical detection. The step-gradient which was used by Joseph et al. [36] for VMA/HVA analysis embodies the same approach. Other isocratic systems and ion-pairing conditions have been employed which allow simultaneous measurement of VMA and HVA, but no internal standards were used. Also, many methods do not indicate whether iso-HVA is resolved from HVA.

Recently, methods have been published which demonstrate the electrochemical measurement of VMA or HVA in urine with no clean-up step [37, 38]. Although this approach may succeed with urines from healthy persons, hospitalized patients often excrete large amounts of phenolic acids as well as basic compounds, which will decrease electrochemical response in subsequent samples. Under the best conditions, there is still some drift in response with time, which has led to the use of the standard/sample/standard sequence for quantitative analysis [39, 40]. The use of purified samples and appropriate internal standards, in combination with the high capacity of the porous graphite cell, yields conditions where drift due to sample injection is negligible in an equilibrated HPLC system.

The recovery of HVA is less than 70% when ethyl acetate followed by phosphate buffer is used for extraction [41]. Higher recoveries have been obtained using a back-extraction into potassium carbonate [5], but VMA is not stable in basic buffers [42]. An HVA recovery of 97% was recently reported from an AG® 1-X4 formate column using 4 *M* formic acid—methanol (20:80) for elution [43], so it appears that ion-moderated partition is a good choice for the extraction of HVA from urine. Iso-VMA as an internal standard has been used previously in the HPLC measurement of VMA with a post-column reaction system [20]. Endogenous levels have been quantitated by gas chromatography—mass spectrometry and typically represent 0.7% of VMA levels [44], or about 0.03 mg/l. Since the iso-VMA added represents 30 mg/l, endogenous concentrations do not affect measurement. Endogenous iso-VA has not been reported or measured to our knowledge.

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PURIFICATION OF SYNTHETIC OLIGODEOXYRIBONUCLEOTIDES BY ION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Synthetic oligodeoxyribonucleotides ranging from 11 to 37 nucleotides in length and with varying base compositions, prepared by both the phosphotriester and phosphite procedures, have been purified by ion-exchange high-performance liquid chromatography on Whatman Partisil 10/SAX columns using phosphate buffer gradients. The effects of different buffer systems on elution times and resolution have been evaluated. Oligomer composition and length had a marked effect on the resolution achieved. In general the use of formamide buffers gave the best results, particularly in the case of 2′-deoxyguanosine-rich sequences. These methods have also been successfully applied to the purification of mixtures of synthetic oligodeoxynucleotides.

INTRODUCTION

Oligonucleotides have been shown to be extremely powerful tools in the field of recombinant DNA technology. They can be used as primers [1, 2] or probes [3, 4] to isolate cDNA coding for proteins or peptides of interest. They can be used as linkers and adapters for the modification of the ends of cDNA fragments, as in the generation of "sticky ends" for ligation reactions [5—7]. Large quantitities of extremely pure synthetic oligonucleotides (a few milligrams) can provide valuable DNA structural information by either X-ray crystallography [8] or nuclear magnetic resonance analysis of the samples [9]. Oligonucleotides can also be used for in vitro site-specific mutagenesis [10] or as aids in sequencing DNA [11].

The synthetic methodologies currently available to assemble these compounds are extremely elegant, rapid techniques. This is evidenced by the total synthesis of the gene encoding α-leukocyte interferon. The synthesis of this molecule, which contains 514 base pairs, was reported by Edge et al. in 1981 [12].

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Two types of chemistry are available to perform oligonucleotide synthesis: the phosphotriester method and the phosphite procedure. Each method is capable of assembling oligonucleotides up to 30 bases in length within a few days. A major problem is the purification of the desired oligomer from the mixture of truncated failure sequences which are produced during the synthesis and are present in the crude deprotected product. Methods which have been reported for the purification of these compounds include anion-exchange high-performance liquid chromatography (HPLC) [13—16], reversed-phase HPLC [17—20], gel electrophoresis [21] and preparative thin-layer chromatography (TLC) on glass-backed silica gel plates [22].

In this paper we describe the anion-exchange HPLC purification of synthetic oligonucleotides assembled by both phosphotriester and phosphite methodologies. The purification of single oligonucleotide sequences and a series of synthetic oligonucleotide mixtures of increasing complexity (to be used as probes) is described. A comparison is also made of buffer systems containing 5% ethanol, 30% acetonitrile, 30% formamide and 60% formamide.

MATERIALS AND METHODS

Oligonucleotides synthesised by the phosphotriester method were assembled on 1% cross-linked polystyrene resin by a dimer block coupling approach [17]. The crude products were deprotected and released from the resin support (50 mg, 0.19 mmol/g starting functionality) by standard means [13] and dissolved in water (5 ml) ready for HPLC.

Oligonucleotides synthesised by the phosphite method were assembled on silical gel (Fractosil 500) by a monomer coupling approach [23]. The crude products were deprotected and released from the resin (100 mg 0.07 mmol/g starting functionality) by standard methods [18] and dissolved in water (5 ml) ready for HPLC.

The HPLC columns used were Whatman Partisil 10/SAX columns (25 × 0.46 cm, 10 μm particle size, 20,000 theoretical plates per m). Chromatography was performed on an Altex modular system (two Model 110A pumps, Model 420 system controller, a Hitachi 100-40 ultraviolet (UV) detector and Curken chart recorder). Partisil 10/SAX columns were eluted at a flow-rate of 1 ml/min with potassium dihydrogen phosphate combined with either ethanol, acetonitrile or formamide as follows: (i) 0.001—0.2 *M* KH_2PO_4 (pH 6.5, 5% ethanol) over 40 min; (ii) 0.001—0.2 *M* KH_2PO_4 (pH 6.5, 30% acetonitrile) over 40 min; (iii) 0.001—0.3 *M* KH_2PO_4 (pH 6.3, 30% formamide) over 60 min; or (iv) 0.001—0.3 *M* KH_2PO_4 (pH 6.3, 60% formamide) over 60 min.

All buffers were filtered through Millipore Type FH 0.5-μm filters prior to use. The crude oligonucleotide samples were filtered using a BioAnalytical Systems MF-1 filter kit (0.2-μm nitrocellulose filters).

Product peaks were collected from the anion-exchange HPLC runs, dialysed in Spectrapor 6 membrane tubing (obtained from Spectrum Medical Industry, molecular weight cut off 2000, four water changes) and lyophilized to dryness. The samples were then resuspended in sterile water and quantified by reading the absorbance at 260 nm. Each of the single sequences were characterised by the Maxam—Gilbert sequencing technique [24]. An example of the

characterisation technique is detailed in Fig. 2 where the sequencing data for an oligonucleotide 37 bases long are shown (space limitations prevent all sequencing data to be shown). The mixture sequences were characterised by ^{32}P-radiolabelling the 5′ end of the oligonucleotide ([γ^{32}P]ATP and T4 polynucleotide kinase) and sizing with respect to oligonucleotides of known length on 18% polyacrylamide gels in the presence of 7 *M* urea (data not shown).

RESULTS AND DISCUSSION

In the crude oligonucleotide mixture the desired product is the longest species and is usually contaminated with shorter truncated sequences. The

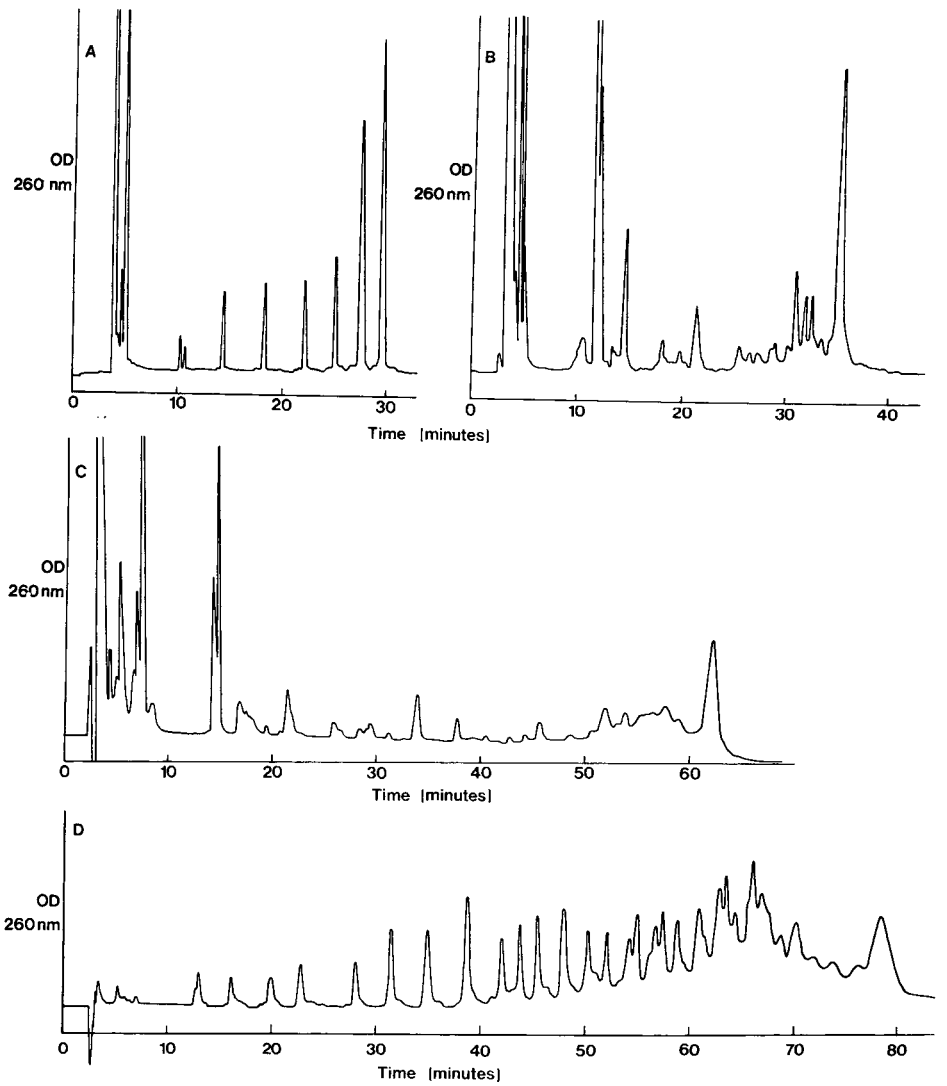

Fig. 1. Anion-exchange HPLC profiles from a Whatman Partisil 10/SAX column of (A) dT_{15} with buffer i as eluent; (B) the 11′ mer d(ATTTCAACCCA) with buffer i as eluent; (C) the 17′ mer d(ATACTGCAGCTGCTGTA) with buffer iv as eluent; and (D) the 37′ mer d(CCTCCCATTTCCCTGGATCTCACCTTCCATCTCCTCC) with buffer iv as eluent.

product, therefore, contains more negatively charged phosphate moieties than any other species in the crude product mixture and will bind most strongly to the anion-exchange column. This means that the desired product should be the last peak to elute.

The following examples illustrate the utility of anion-exchange HPLC for the purification of a range of different synthetic oligonucleotide sequences.

Purification of single sequences: dT_{15}, d(ATTTCAACCCA), d(ATACTGCAGCTGCTGTA) and d(CCTCCCATTTCCCTGGATCTCACCTTCCATCTCCTCC)

Pentadecathymidilic acid (dT_{15}) was synthesized by a phosphotriester dimer block approach and was purified by the conditions detailed for buffer i above.

The effectiveness of the chromatography method is evidenced by this synthesis. The product dT_{15} elutes as the major peak and the truncated sequences dT_{13}, dT_{11}, dT_9, dT_7, dT_5 and dT_3 are resolved into sharp peaks eluting sequentially earlier in the HPLC profile (Fig. 1A).

In other examples the 11′ mer, d(ATTTCAACCCA), and the 17′ mer, d(ATACTGCAGCTGCTGTA), eluted as the major product with very little truncated by-product (Fig. 1B and C). The 11′ mer was purified by the ethanol-based buffer i and the 17′ mer by the formamide-based buffer iv. The different properties and the advantages of each of these buffer systems is discussed in more detail below.

The power of the anion-exchange chromatography method is demonstrated by the purification of the oligonucleotide d(CCTCCCATTTCCCTGGATCTCACCTTCCATCTCCTCC) which contains 37 residues. The oligomer was purified using buffer iv and while there is evidence of a number of truncated products in the HPLC profile the product peak can be seen to be a major component of the crude mixture (Fig. 1D).

Radiolabelling the product peak from the HPLC purification with ^{32}P and analysing it on an 18% polyacrylamide gel showed the sample to be homogenous (Fig. 2A). Maxam—Gilbert sequencing analysis confirmed the structure of the 37-base oligonucleotide (Fig. 2B).

Sequences 30 bases long are now routinely synthesised and characterised in our laboratory by the methods described above.

Purification of oligonucleotide mixtures: d(TTZGTCATCTC), d(GGCTTYTTXTC), d(GGXTTYTTXTC) and d(GCXTTNGTCATXTC) (where X=T/C, Y=A/G, Z=A/C and N=T/C/A/G)

A series of oligonucleotide mixtures was synthesized for use as primers and probes. The applications of the mixtures will not be discussed in detail here, but a sample of the mixture syntheses containing a range of mixture complexities will be presented. Fig. 3 shows the HPLC profiles (buffer ii) of mixtures of two (Fig. 3A), four (Fig. 3B), eight (Fig. 3C) and sixteen (Fig. 3D) components. The oligonucleotide components, in the case of the mixtures containing two and four sequences [d(TTZGTCATCTC) and d(GGCTTYTTXTC)] could be resolved into individual sequences. This was generally true for each two-component mixture synthesized, but other four-component mixtures (data not shown) could not be totally resolved. The eight- and sixteen-com-

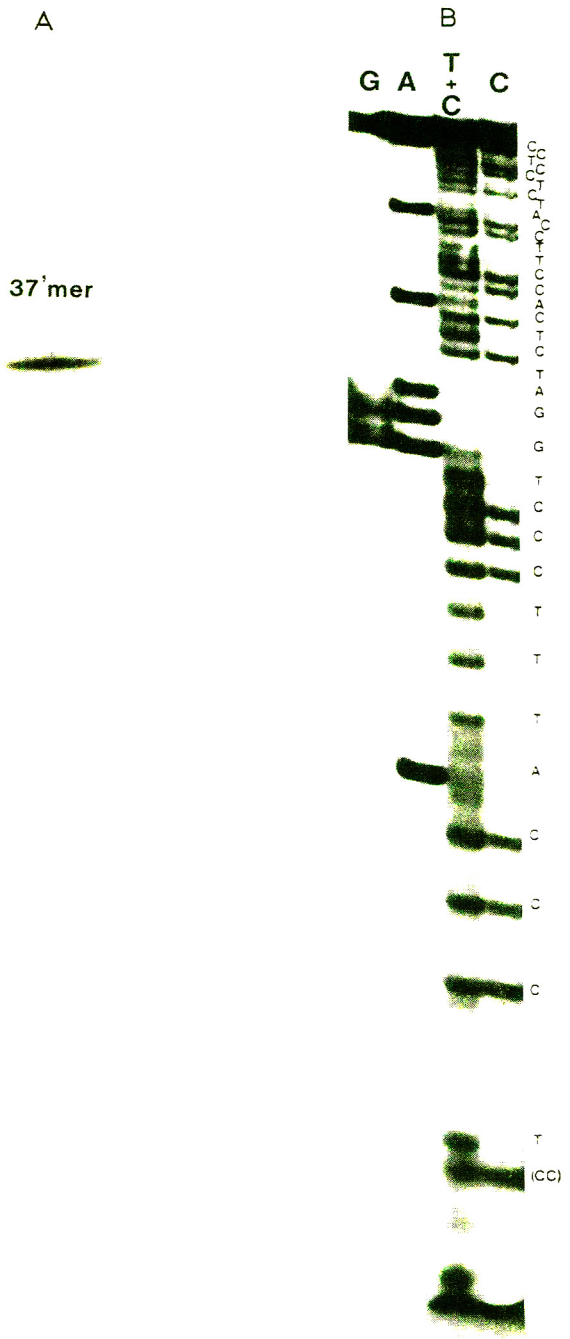

Fig. 2. (A) HPLC-purified 37′ mer radiolabelled with ^{32}P and analysed on an 18% polyacrylamide gel; (B) Maxam—Gilbert sequencing of the 37-base oligonucleotide.

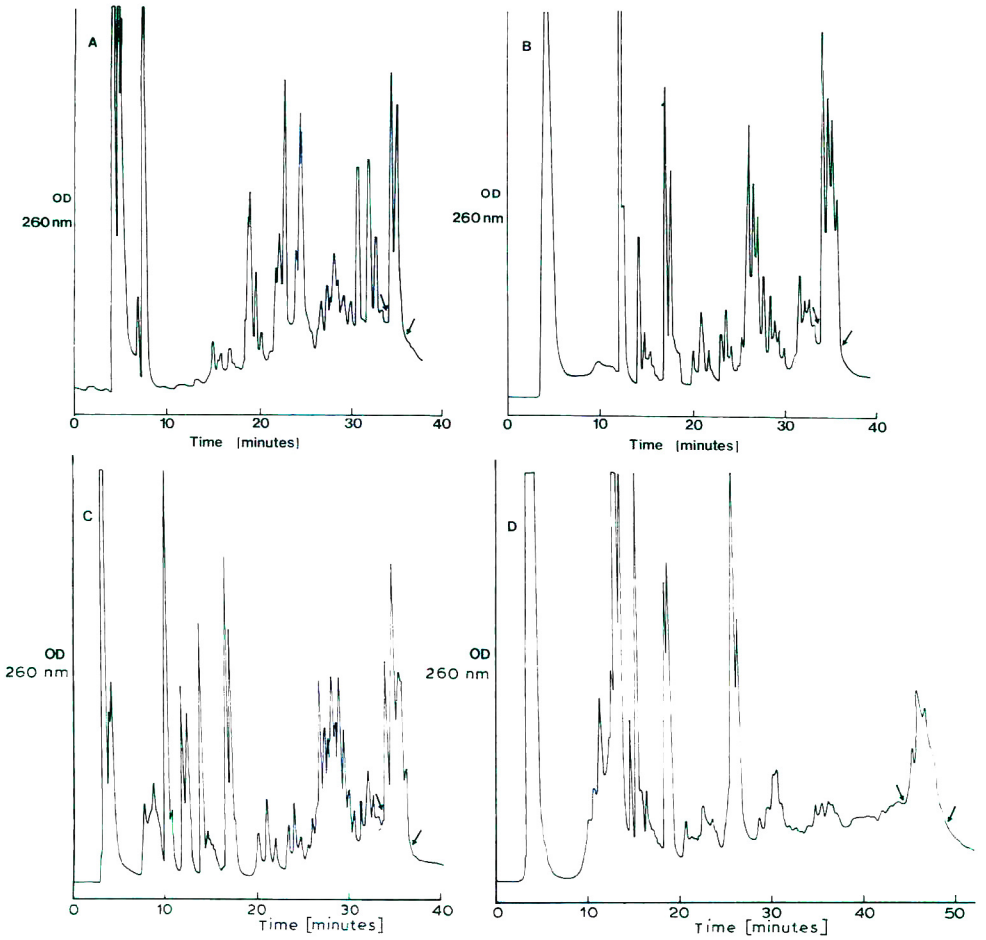

Fig. 3. Anion-exchange HPLC profiles from a Whatman Partisil 10/SAX column of (A) the two-component mixture d(TTZGTCATCTC); (B) the four-component mixture d(GGCTTYTTXTC); (C) the eight-component mixture d(GGXTTYTTXTC) and (D) the sixteen-component mixture d(GCXTTNGTCATXTC). Buffer ii was the eluting solvent. The arrows indicate the fractions collected from the column which contained the desired oligonucleotide mixtures.

ponent mixture HPLC purifications did not resolve the single sequences and hence the products were collected as a "clump" of peaks and were used experimentally in that form without further purification.

Comparison of buffer conditions: d(GTAAAACGACGGCCAGT) and d(CAGGGGTTTTGGGCCAAAG)

It was found that 30% acetonitrile buffers (buffer ii), which were used in the mixture purifications, were superior to 5% ethanol systems (buffer i), especially if the theoretical plate count of the Partisil 10/SAX column was low (in the range 16,000 to 20,000 plates per m). This, therefore, was the buffer of choice for the purification of oligonucleotides up to 15 bases long. The use of this

buffer was limited, however, as the maximum possible phosphate concentration was 0.2 *M*. HPLC profiles of sequences such as the 17′ mer d(GTAAAACGAC-GGCCAGT) when purified in buffer ii showed diminished resolution and poor peak shape (Fig. 4A). Substituting 60% formamide for 30% acetonitrile allowed higher phosphate concentrations (0.3 *M*) and the resolution was substantially improved (Fig. 4B). There was a significant difference in the yields of product obtained from 60% formamide and 30% acetonitrile buffer systems. In the example shown in Fig. 4 approx. 50% more solute product was obtained from the formamide-based system.

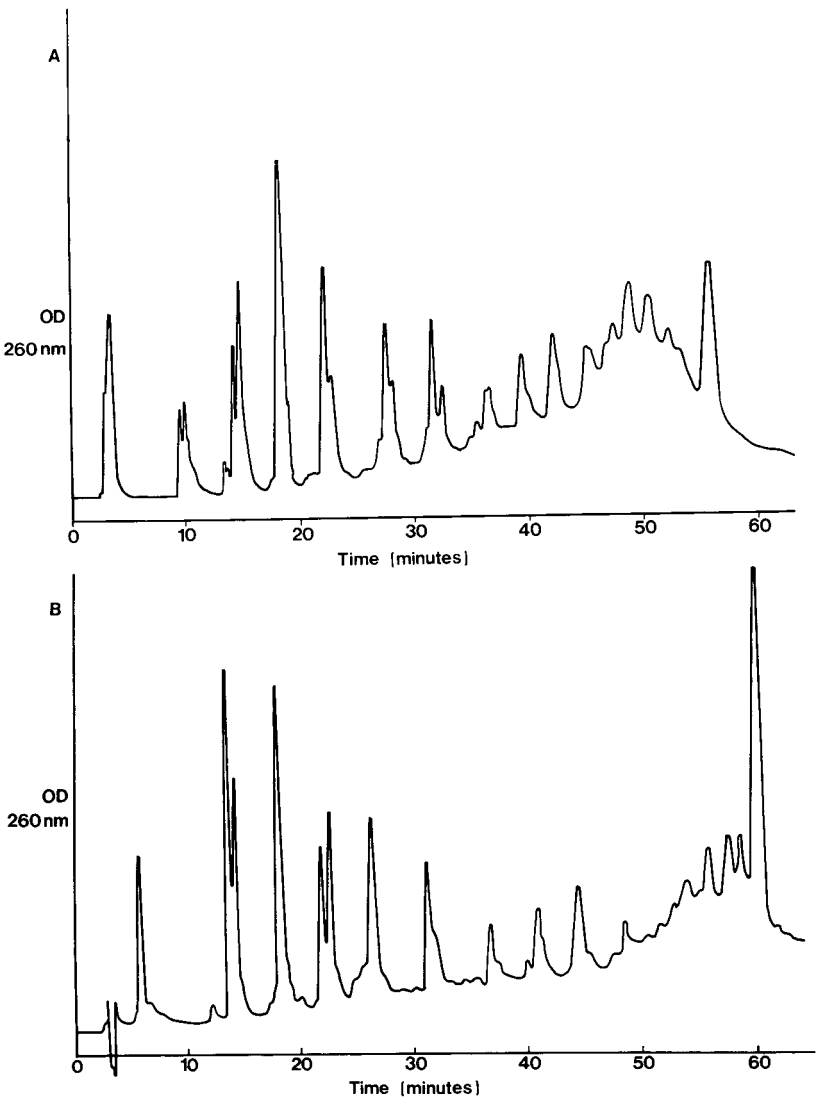

Fig. 4. Anion-exchange HPLC profiles from a Whatman Partisil 10/SAX column of the 17′ mer d(GTAAAACGACGGCCAGT) using (A) buffer ii as eluent and (B) buffer iv as eluent.

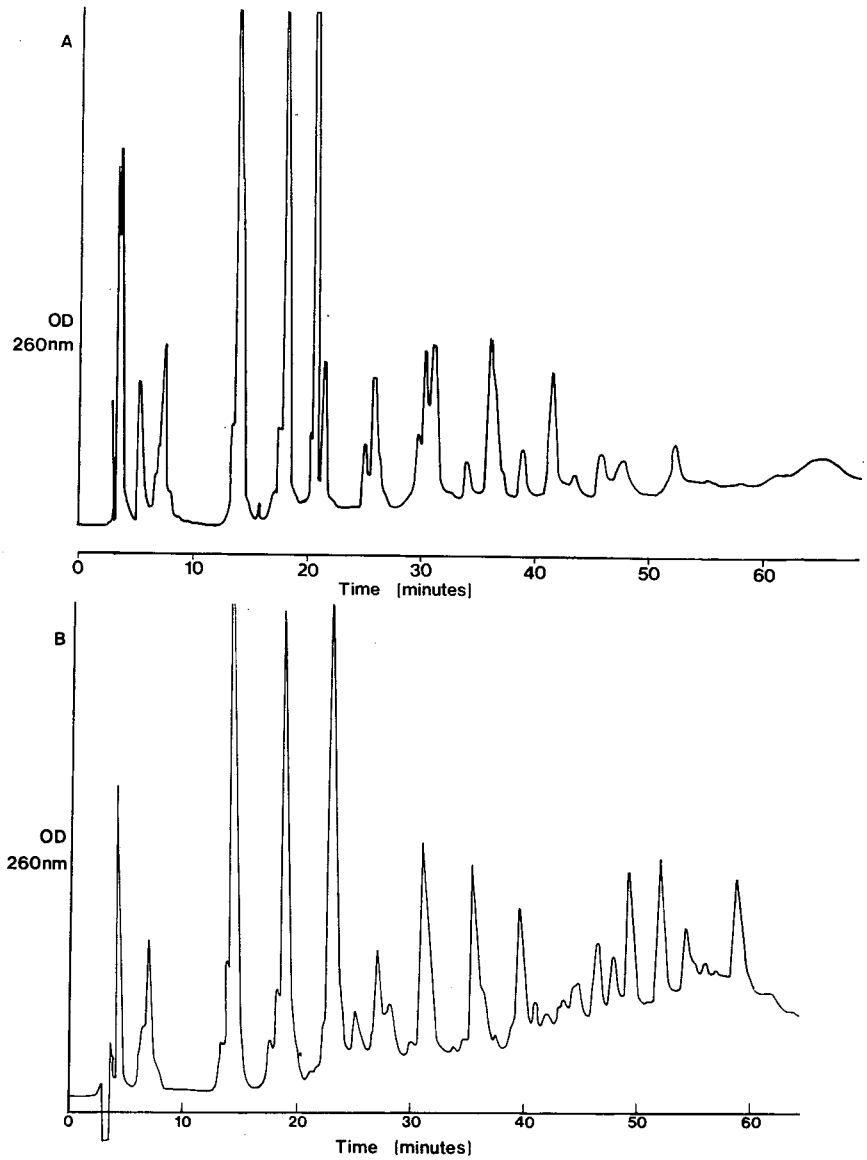

Fig. 5. Anion-exchange HPLC profiles from a Whatman Partisil 10/SAX column of the 19′ mer d(CAGGGGTTTTGGGCCAAAG) using (A) buffer iii as eluent and (B) buffer iv as eluent.

The effectiveness of differing concentrations of formamide in the HPLC eluting buffer was tested in the purification of the 2′-deoxyguanosine-rich sequence d(CAGGGGTTTTGGGCCAAAG). It is well documented that G-rich sequences, and especially those containing extended tracts of three or more consecutive G residues, are difficult to purify by anion-exchange HPLC [15, 25]. The 19′ mer is extremely G-rich and contains a tract of four consecutive 2′-deoxyguanosine residues. HPLC purifications of this oligomer in buffer iii (30% formamide) and buffer iv (60% formamide) are shown in Fig. 5. The buffer

iii HPLC profile showed a very broad product peak while the buffer iv HPLC profile showed the 19′ mer eluting as a sharp peak and to be a major component of the crude synthetic mixture. Maxam—Gilbert sequencing [24] confirmed the sequence of 19′ mer. The solute recovery yield of guanosine-rich sequences such as d(CAGGGGTTTTGGGCCAAG) was 60—70% of the expected value whereas for non-guanosine-rich sequences such as d(GTAAAACGACGGCCAGT) the value was greater than 90%.

CONCLUSIONS

For the routine purification of synthetic oligonucleotides we recommend the use of anion-exchange HPLC in 60% formamide buffer systems containing potassium phosphate. This system has been found to be superior to ethanol- and acetonitrile-based systems especially for the purification of longer nucleotide sequences (greater than 30 residues) and can be used for the purification of both single oligonucleotide sequences and oligonucleotide mixtures. One practical disadvantage of the use of formamide buffers is that the life of the Partisil 10/SAX column is generally decreased. Ethanol- or acetonitrile-based solvent systems allow a Partisil 10/SAX column lifetime of approx. four to six months. Formamide-based systems, however, reduce the column lifetime to less than two months.

The methods described here detail a rapid technique which can be used by itself or in combination with gel electrophoresis, reversed-phase HPLC and preparative TLC, for the chromatographic purification of oligonucleotides. The method is particularly useful for the purification of synthetic oligonucleotides prepared by automated solid-phase procedures.

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COMPARISON OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION AND GAS CHROMATOGRAPHY—MASS FRAGMENTOGRAPHY FOR THE ASSAY OF SALSOLINOL, DOPAMINE AND DOPAMINE METABOLITES IN FOOD AND BEVERAGE SAMPLES

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SUMMARY

High-performance liquid chromatography with electrochemical detection (HPLC—ED) and combined gas chromatography—mass spectrometry in the single-ion monitoring mode (GC—MS-SIM) have been used for the determination of salsolinol, dopamine, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylethanol and norepinephrine in a selection of food and beverage samples. The unique specificity of the SIM mode allows a simple one-step extraction to be used even for complex sample matrices. We have been able to demonstrate the quantitative and qualitative advantages offered by GC—MS over HPLC—ED by direct comparison of the chromatographic data obtained. We demonstrate that the specificity of SIM and the benefits offered by the incorporation of deuterated internal standards make GC—MS-SIM the method of choice for valid identification and precise quantitation of salsolinol, dopamine and dopamine metabolites in a complex sample matrix.

INTRODUCTION

For the analysis of biogenic amines, and biogenic amine derivatives in biological fluids, high-performance liquid chromatography coupled with electrochemical detection (HPLC—ED) has become the method of choice. Under optimal conditions this powerful combination can provide a sensitive and specific assay system.

In most assays a common structural thread, the catechol nucieus, is both the handle for extraction and the electro-oxidizable group. This means that with a combination of alumina extraction and electrochemical detection, overall speci-

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ficity is effectively determined by the complexity of the original sample matrix and the resolving power of the chromatographic step.

In practice, currently employed HPLC—ED procedures for the assay of catecholamines from complex sample matrices (e.g. urine) do not have the resolving power to allow quantitation of the analytes of interest unless there are first multi-step pretreatments to "tidy-up" samples [1]. As well as selectively removing the metabolites of interest such techniques can concentrate these analytes into as small a volume as possible. While these complex clean-up procedures are time-consuming and liable to introduce variations in recovery, particularly in multi-component assay, they are necessary in the analysis of complex sample types.

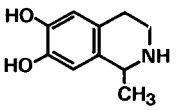

Fig. 1. Chemical structure of salsolinol (SAL).

The tetrahydroisoquinoline alkaloid, salsolinol (SAL, Fig. 1) has a direct structural link with dopamine, in particular retention of the catechol nucleus, and so SAL is also amenable to electrochemical detection. It has been suggested that SAL might be formed in vivo in mammals as a consequence of ethanol consumption; however, it is proving difficult to obtain valid analytical data that might support this proposal [2—5]. The rapid reaction that can take place bebetween dopamine in the sample and free acetaldehyde to form "artifactual" SAL under a wide range of conditions, and the low levels that might be present in biological systems, present particular problems for the detection and quantitation of this compound.

We have recently described a highly sensitive and specific combined gas chromatographic—mass spectrometric (GC—MS) method for the simultaneous measurement of SAL, dopamine (DA), 3,4-dihydroxyphenylethanol (DOPET), 3,4-dihydroxyphenylacetic acid (DOPAC) and norepinephrine (NE) which is suitable for a wide range of sample types [6]. Our continuing investigations in the area of biogenic amine metabolism have led us to examine the suitability of HPLC—ED for this same application. We were particularly concerned about the suitability of HPLC—ED to handle significantly different sample types.

Generally, researchers requiring catecholamine and mammalian alkaloid assays have exclusively used one analytical methodology and they display a very real bias when discussing the suitability of either GC/MS or HPLC—ED. This bias is sometimes evident in the views expressed about the benefits and limitations of alternative methodologies [4, 7, 8]. In this paper we directly compare the results obtained using both HPLC—ED and GC—MS assay systems when applied to catecholamine, catecholamine metabolite and salsolinol assay in a selection of food and beverage samples. Single-step alumina extraction giving high recoveries was used throughout. The results provide an opportunity for qualitative and quantitative comparison of these two techniques and discussion of their respective merits and limitations. We make particular reference to the suitability of each technique for the identification and quantitation of trace levels of salsolinol.

MATERIALS AND METHODS

Chemicals and reagents

Salsolinol·HCl was prepared via the method of King et al. [9] and recrystallized to a constant melting point. The final sample was shown to be free of impurities by GC—MS and HPLC analysis. Satisfactory elemental analysis was also obtained. The results reported in this study are uncorrected and refer to free acids and bases. Each reference compound was of the highest grade obtainable; dopamine·HCl, L-norepinephrine bitartrate and dihydroxyphenylacetic acid were all obtained from Calbiochem-Behring (Carlingford, Australia). 3,4-Dihydroxyphenylethanol was obtained from Regis (Morton Grove, IL, U.S.A.). 3,4-Dihydroxybenzylamine·HBr (DHBA), Tris and EDTA (disodium salt) were obtained from Sigma (St Louis, MO, U.S.A.). Alumina was prepared by essentially the same procedure described by Anton and Sayre [10]. Trifluoroacetic anhydride (TFAA) was obtained from Pierce (Rockford, Il, U.S.A.) and trifluoroethanol (TFE) from Sigma. All other reagents were of the highest grade obtainable.

HPLC instrumentation and instrumental conditions

HPLC determinations were performed with a Hewlett-Packard 1081 isocratic liquid chromatograph fitted with an auto-injector and auto sample changer. A BioAnalytical Systems Model LC-4 amperometric detector coupled to a Hewlett-Packard 3390A recording integrator was used for data acquisition. A glassy carbon electrode set at 0.72V vs. Ag/AgCl reference electrode was used directly after the column for detection. Detector sensitivity was set at either 5 or 10 nA full scale during these studies. Chromatography was achieved on a 250 × 4.6 mm I.D. Beckman ultrasphere ODS reversed-phase column (5 μm; Beckman, Sydney, Australia). All elutions were isocratic.

Mobile phase

The mobile phase (pH 3.0) was monochloroacetic acid buffer (0.15 *M*) containing EDTA (2.0 m*M*) and sodium octyl sulphate (75 mg/l). The mobile phase was filtered through a Millipore HA filter (0.45 μm) and degassed ultrasonically prior to use. A flow-rate of 1.5 ml/min at a temperature of 35°C was employed in all studies. The mobile phase was continuously recycled.

Extraction procedure

The extraction procedure used was alumina-based and is a slight modification of that described by BioAnalytical Systems for plasma extraction. Formic acid (0.4 *M*) was used in place of perchloric acid in the final elution step. Absolute recoveries were >70% for each analyte.

Preparation of food samples. Samples (0.2—1.0 g) were mechanically homogenized (Ultra-Turrax) in 0.1 *M* hydrochloric acid (10 ml). Samples were then centrifuged (3000 *g*, 30 min, 4°C), filtered (0.4 μm; Millipore) and portions (generally 50 μl) were extracted as described above. Sample volumes extracted were, however, adjusted to give "on-scale" chromatograms with fixed instrument parameters to allow a direct comparison.

Preparation of beverage samples. Carbonated beverages were freshly opened

and ultrasonically degassed before analysis. A portion (1—2 ml) of each beverage was extracted as previously described.

Standard curves for HPLC—ED analysis

Standard solutions containing varying known amounts of NE, DA, DOPET, DOPAC and SAL in 0.25 *M* formic acid were prepared, and a fixed (and known) concentration of DHBA was added to each of these. Samples were then extracted as described and injected into the HPLC system (10—25 μl). Standard curves were constructed by plotting the NE/DHBA, DA/DHBA, DOPET/DHBA, DOPAC/DHBA and SAL/DHBA peak height ratios against the concentration of the appropriate analyte. The standard curves were prepared by unweighted least-squares linear regression analysis from single samples and single estimations of peak heights. The lowest point on each standard curve corresponded to 5—30 pmol per sample (i.e. approx. 1—5 ng) extracted but varied slightly. Coefficients of variation (i.e. *r* values) were greater than 0.999.

Sample concentrations were calculated by standard techniques incorporating adjustment for varying recoveries using DHBA as internal standard.

GC—MS-SIM

Full details of the method used have been described elsewhere [6]. Samples were treated with a deuterated standard mixture and then extracted via the alumina technique. Formic acid in methanol (5 *M*, 1:4) was used to elute the catechols. Samples were then evaporated to dryness under nitrogen and derivatized with TFAA (200 μl) and TFE (50 μl) at 60°C for 20 min. After evaporating off the excess reagent under nitrogen the samples were reconstituted with ethyl acetate (10 μl) and a portion (1—2 μl) was injected into the GC—MS instrument.

TABLE I

RETENTION TIMES AND MAJOR IONS FOR EACH ANALYTE UNDER THE CHROMATOGRAPHIC CONDITIONS DESCRIBED

Column Type 3% OV-17; 1-m glass column. For chromatographic conditions see Materials and methods.

Analyte	Retention time* (min)	*m/z* monitored	Percentage of base peak	Group in which ion monitored
DA	2.85	328	(100%)	3
d_3-DA	2.85	331	(100%)	3
DOPAC	1.3	442	(54%)	1
d_5-DOPAC	1.3	447	(54%)	1
DOPET	1.2	328	(100%)	1
d_3-DOPET	1.2	331	(100%)	1
NE	2.1	440	(100%)	2
d_3-NE	2.1	442	(100%)	2
SAL	3.5	452	(100%)	4
d_4-SAL	3.5	456	(100%)	4
		467	(21%)	4
		471	(21%)	4

*Retention times in samples may vary because of changes in column length and temperature profile used.

A Hewlett-Packard 5993 A combined gas chromatograph—mass spectrometer was used. Glass columns, either 0.7 or 1.0 m, packed with 3% OV-17 were used. The carrier gas flow-rate (helium) was 30 ml/min. Temperatures were: column 146—200°C; injection port 222°C [6]. The ions selected for each analyte and their appropriate deuterated analogues are shown in Table I.

Peaks obtained during the GC—MS run have generally been normalized on the Y-axis to give full scale displav. This facilitates precise area calculation. The appropriate area is indicated (arbitrary units) adjacent to each peak. Two traces, (top; deuterated internal standard; bottom; endogenous) are required for each analyte. Four ions, base peak and molecular ion for deuterated standard and endogenous were monitored for SAL to obtain maximum specificity. Ions within each group are displayed in decreasing order of m/z (top to bottom).

RESULTS

Fig. 2 compares direct chromatographic data obtained via HPLC—ED and GC—MS-SIM assay for the three sample types banana pulp, beer and soy sauce.

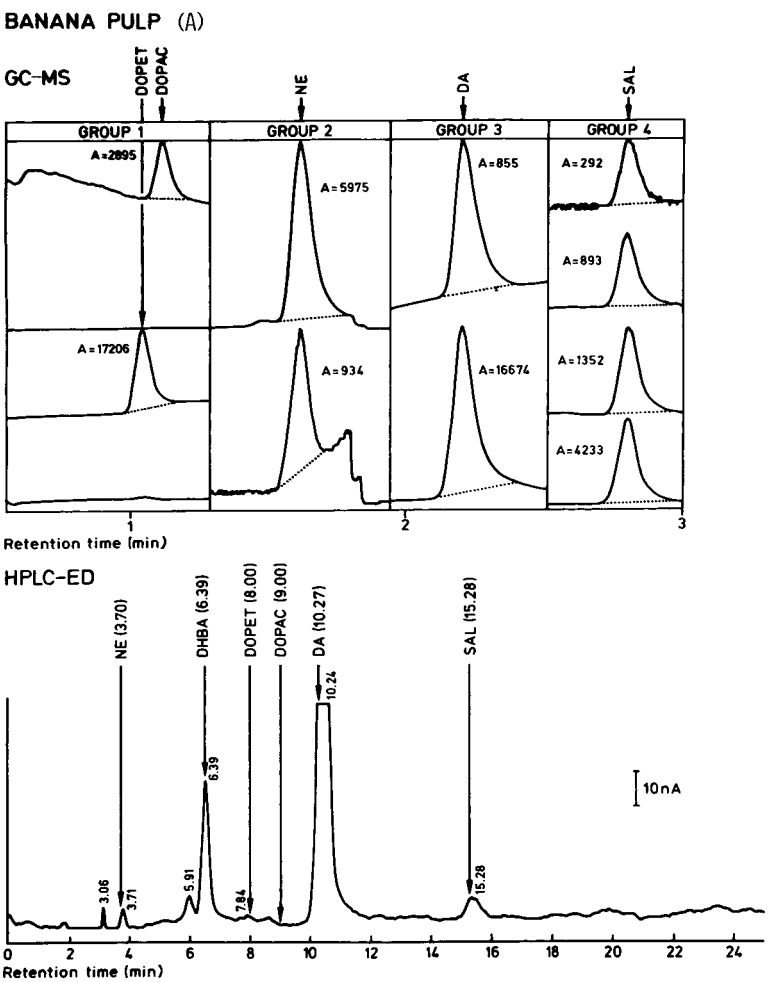

Fig. 2.

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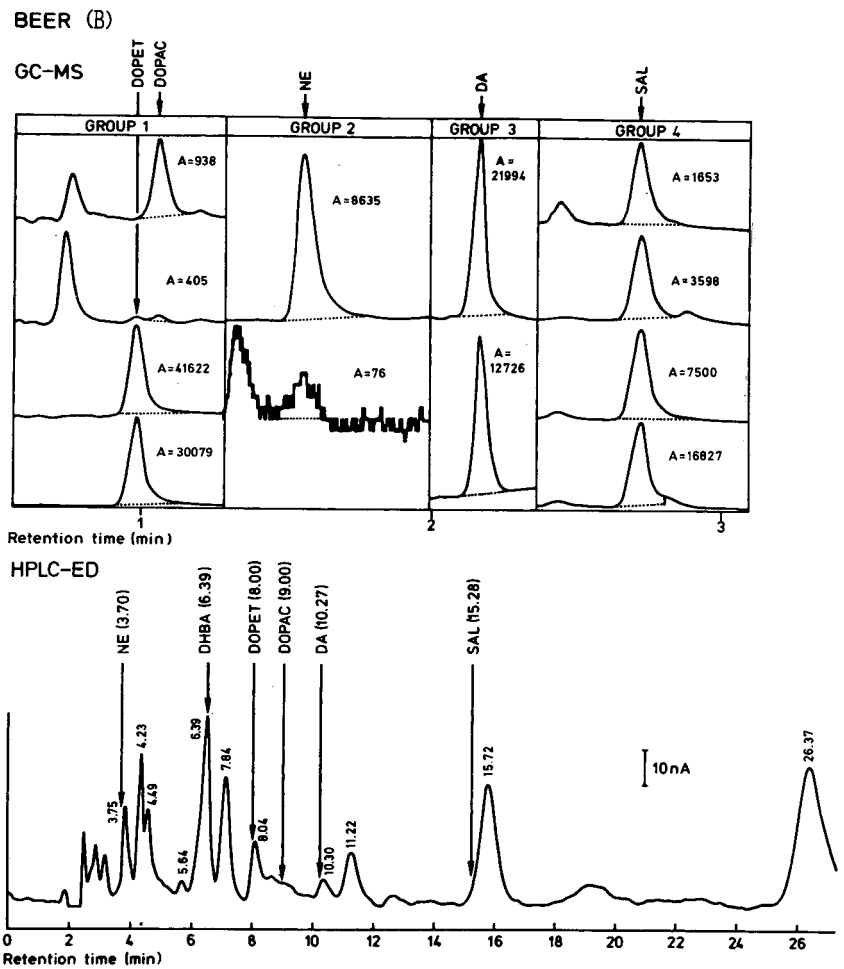
BEER (B)
GC-MS
DOPET
DOPAC
NE
DA
SAL
GROUP 1
GROUP 2
GROUP 3
GROUP 4
A=938
A=405
A=41622
A=30079
A=8635
A=76
A=21994
A=12726
A=1653
A=3598
A=7500
A=16827
Retention time (min)
HPLC-ED
NE (3.70)
DHBA (6.39)
DOPET (8.00)
DOPAC (9.00)
DA (10.27)
SAL (15.28)
3.75
4.23
4.49
5.64
6.39
7.84
8.04
10.30
11.22
15.72
26.37
10nA
Retention time (min)

Fig. 2.

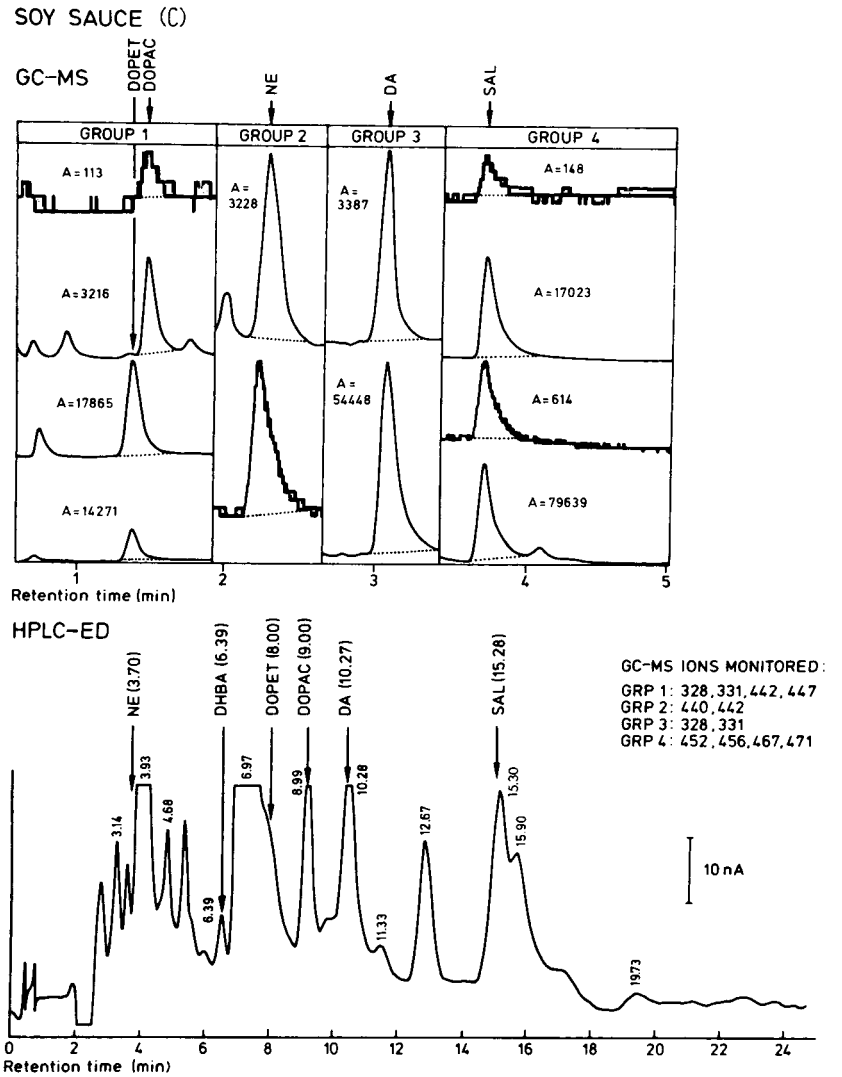

Fig. 2. Direct comparison of chromatographic data for three alumina-extracted samples. A, Banana pulp; B, beer; C, soy sauce.

The HPLC—ED trace is clearly marked in each instance with arrows indicating the retention times corresponding to the analytes of interest (based on standard samples run previously). For each sample run in the GC—MS-SIM mode arrows indicate the deuterated internal standard peaks; individual retention times may sometimes vary with minor changes in chromatographic conditions and column length. Wherever possible peaks corresponding to the retention time of the analyte of interest in the HPLC—ED trace were used to calculate original sample concentrations by reference to extracted standard samples and these results have been compared with previously obtained GC—MS results in Table II.

In only one instance (i.e. soy sauce NE) did an interfering peak in the GC—MS-SIM trace prevent accurate quantitation at the limits of sensitivity for an analyte.

TABLE II

QUANTITATIVE RESULTS VIA GC—MS AND HPLC—ED ASSAY (pmol/g)

Sample	Method	DOPET	DOPAC	NA	DA	SAL
Beer	(GC—MS)	140—210	38—58	ND*—1.5	2.5—31.8	28—75
	(HPLC—ED)	108	QI**	QI	33	QI
Soy sauce	(GC—MS)	138	$1.87 \cdot 10^3$	QI	796	$2.66 \cdot 10^3$
	(HPLC—ED)	QI	$4.45 \cdot 10^3$	QI	$2.1 \cdot 10^3$	$4.89 \cdot 10^3$
Banana pulp	(GC—MS)	$1.17 \cdot 10^3$	–	$1.02 \cdot 10^4$	$2.13 \cdot 10^5$	352***
	(HPLC—ED)	ND	–	$1.81 \cdot 10^4$	$6.63 \cdot 10^5$	$2.2 \cdot 10^5$ §
Dried banana	(GC—MS)	–	–	–	–	$1.6 \cdot 10^5$—$1.7 \cdot 10^6$
	(HPLC—ED)	–	–	–	–	$1.9 \cdot 10^5$—$1.6 \cdot 10^6$

*ND = not detected.
**QI = identification and quantitation impossible due to interfering peaks.
***Climateric pulp.
§Post-climateric pulp.

In this sample it was, however, established that NE levels were < 10 pmol/ml by direct comparison with the internal standard.

DISCUSSION

Low hardware costs, ease of automation, high sensitivity and the success of HPLC—ED in catecholamine assay to date make it an obvious choice for tetrahydroisoquinoline assay. Since the first reported application of HPLC—ED to the determination of salsolinol in biological materials by Riggin and Kissinger in 1977 [11] relatively little further work has appeared. More recent studies have dealt with the chromatographic problems associated with resolving mixtures of synthetic alkaloids or reaction mixtures into their components [4, 12, 13]. For samples of biological origin the problems are considerably more complex. The low concentrations involved, complexity of the sample matrix and, in particular, the possibility of artifactual SAL formation during sample work-up have slowed progress in this area. The alumina-based catechol extraction that forms the foundation of our GC—MS assay has proven to be a reliable, selective and efficient procedure. As well as recovery of the amines NE, DA and SAL the alumina extraction also recovers the catechol acid and neutral metabolites (e.g. DOPET and DOPAC) in a relatively "clean" medium. We found a single alumina extraction sufficed to give samples suitable for derivatization and direct GC—MS analysis in every instance. Using essentially the same extraction procedure, we found samples were not generally suitable for direct HPLC—ED analysis. The comparative chromatograms (Fig. 2) illustrate the complexity of the HPLC trace. Samples such as beer and soy sauce give complex chromatograms with an abundance of unknown peaks often with retention times corresponding to, or overlapping with, the less abundant peaks of interest. Late eluting peaks in the HPLC—ED trace in some samples (up to 2 h after injection) significantly increased the minimum time required between injections. The maximum time required for any GC—MS-SIM run was 5 min.

In the case of urinary catecholamine assay, or previously reported HPLC—ED assays for salsolinol, additional extraction or pre-treatment steps have been used to clean samples. For example cation exchange to isolate amines, or a solvent extraction step can be added to remove interfering components. However, there are disadvantages with this approach. Further sample clean-up will also reduce recoveries (and overall sensitivity) as well as resulting in a loss of some of the metabolites present that might otherwise have been measured concurrently. Complex extraction procedures also reduce the suitability of a single internal standard for multi-component analysis and therefore reduce precision.

Alternatively, attempts can be made to optimize the chromatographic conditions for each sample. However, although a set of parameters (e.g. temperature, pH, type and concentrations of ion-pairing reagent) might yield a satisfactory solution to one analytical problem, these conditions may not be optimal for a different sample type.

There is currently no simple, universally applicable extraction procedure and set of chromatographic conditions that allows complex and varied sample types to be assayed via HPLC—ED. For a fixed sample matrix (e.g. urine) a combination of extraction procedures and chromatographic conditions can be developed to assay any particular analyte. Once developed the procedure is cheap and easily automated. An important role exists for HPLC—ED in detecting and quantitating analytes via this approach in essentially "same" sample types. However, when considering the assay of salsolinol the highest priority must be given to avoiding the possibility of artifactual formation. Time-consuming and elaborate extraction procedures are far more susceptible to introducing errors of this type and must be used with caution.

The specificity associated with SIM is responsible for a significant reduction in the complexity of the data obtained when using a GC—MS system. The comparative chromatograms for each sample type aptly illustrate the advantages of this approach to complex and varied sample analysis. GC—MS-SIM allows an efficient single-step alumina extraction to be used for sample preparation. The incorporation of appropriate deuterated standards facilitates identification, adjusts for variable recovery of each analyte and allows precise quantitation. Where it is possible to use a single-extraction procedure a wider variety of analytes can be simultaneously quantitated in the one sample, recoveries are improved and most importantly, minimized sample manipulation significantly reduces the possibility of artifactual SAL formation. Judicious use of appropriate deuterated standards in GC—MS can provide the researcher with additional information not otherwise available. Suitable "tagged" dopamine can be added to samples during the extraction procedure and its conversion to tagged SAL monitored, thus providing a "built-in" check against artifactual formation for each sample type assayed.

The chromatograms included in Fig. 2 display the problems associated with determining a working limit of sensitivity in this type of study. In practice, it is the stability and level of background interference in the region of the analyte of interest that dictates the lowest level that can be detected. This is a function of the complexity of the original sample matrix, the extraction procedure used and the detection method. Sample type variations can therefore account for considerable changes in the level of sensitivity obtained. The unique specificity

of the SIM mode of detection ensures that practical sensitivity limits for samples closely approach estimates based on standard samples runs. Practical HPLC—ED detection limits are significantly higher and more variable than studies with standard samples indicate. It is only after elaborate pre-treatment steps are taken that a sufficiently stable baseline can be obtained to allow quantitation of trace components.

The same sample was extracted and used for all quantitative comparisons with the exception of banana flesh. The remarkable increase in SAL levels reported in the HPLC—ED result (post-climateric, or over-ripe flesh) compared to the GC—MS results (climateric, ripe flesh) is real and is a consequence of fruit aging. Riggin et al. [14] have observed similar increases in SAL content as the banana ripens. The poor comparison between HPLC—ED and GC—MS-SIM results for the soy sauce sample is associated with noise in the chromatogram in the region of the internal standard DHBA. Since precise quantitation of each analyte requires an accurate assessment of the DHBA peak height, background interferences in the region adversely affects measurement of all components in the sample.

The data presented illustrates that for an uncomplicated sample matrix (e.g. banana) a single-step alumina extraction combined with reversed-phase ion-pairing chromatography can provide quantitative data for a range of catechol derivatives. It was essentially this approach that was used by Riggin and co-workers to identify and quantitate levels of salsolinol in banana [14] and cocoa [15]. More complicated sample types are not amenable to this analysis unless elaborate sample pre-treatment steps are taken. For any constant sample matrix (e.g. urine, plasma or beer) it is possible to devise an extraction protocol and a set of chromatographic conditions suited to the analyte(s) of interest. In many instances considerable manipulation may be required to obtain a workable chromatographic system. However, once developed the procedure is cheap to run and easily automated. An important role exists for HPLC—ED in the analysis of samples of essentially similar or identical matrix type.

The comparative chromatograms presented illustrate the versatility and power of a GC—MS-SIM assay system for trace analysis of this type. The availability of specific ion detectors that can be directly coupled to capillary gas chromatographs is helping to offset the cost advantages that HPLC—ED has previously offered over conventional GC—MS systems. GC—MS-SIM combined with appropriate deuterated internal standards eliminates elaborate sample pre-treatment even with a complex sample matrix as well as allowing precise multi-component quantitation at low levels. The unique problems associated with salsolinol assay require elaborate precautions be taken against artifactual formation, and therefore necessitate fast, uncomplicated extraction procedures. These requirements are also best met by a combined GC—MS technique.

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ISOLATION OF TERATOGENIC ALKALOIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography was used for both analytical and preparative separations of several steroidal alkaloids which occur in extracts of *Veratrum californicum*. The inclusion of 0.1% trifluoroacetic acid in the mobile phase improved the efficiency of the chromatography and the solubility of the compounds in aqueous acetonitrile. Nuclear magnetic resonance was used to assist the identification of the isolated steroidal alkaloids. The effect of the interaction of trifluoroacetic acid with the alkaloids could be clearly seen by changes in the chemical shifts in the nuclear magnetic resonance spectra.

INTRODUCTION

High-performance liquid chromatography (HPLC) has been used for the separation of a wide range of chemical compounds. Although there have been many reports of the use of HPLC in the separation of steroids, there have been only four reports to date of the application of HPLC to the separation of steroidal alkaloids. The earliest attempt used normal-phase chromatography on Porasil A, which is a very inefficient, coarse (37—75 μm particle size) support by modern standards, to separate tomatidine, solasodine, veratramine and jervine (Fig. 1) related alkaloids [1]. Surprisingly good separations were obtained by using gradients from acetone—hexane (2:1) to 97% aqueous acetone

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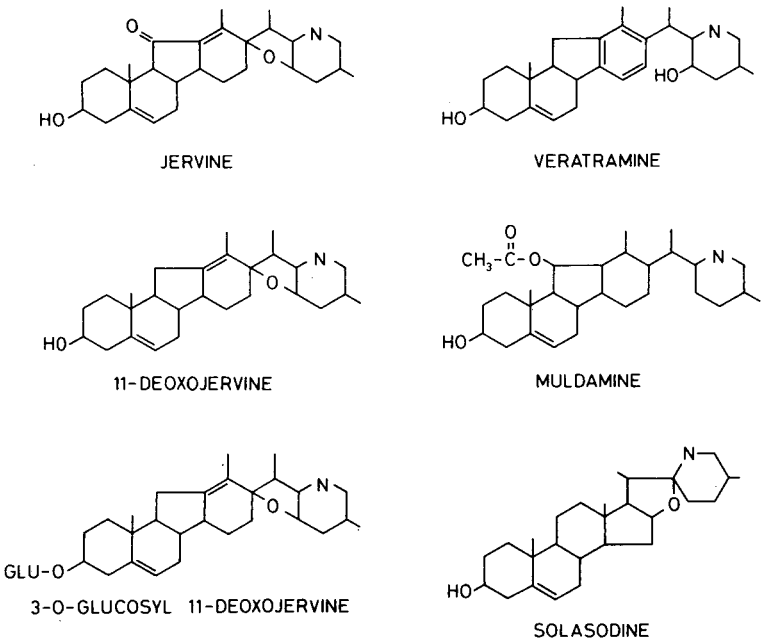


Fig. 1. Structural formulae of steroidal alkaloids.

and thin-layer chromatography as the mode of detection. Hunter et al. [1] used a 4.8 m long column of this material to prepare 1 g of tomatidine by isocratic elution. Normal-phase chromatography of these compounds was subsequently improved by Hunter et al. [2] by changing to a Zorbax-Sil (6 μm particle size) support. The first reversed-phase HPLC separation was reported for the related glycoalkaloids, α-chaconine, β-chaconine and α-solanine [3]. The alkaloids were eluted isocratically from a C_{18} μBondapak column with tetrahydrofuran—water—acetonitrile (50:30:20). A more recent report of the separation of solasodine from solasodine glycosides also used a C_{18} μBondapak column, this time with a methanol—Tris buffer, pH 7 isocratic elution system [4].

We decided to develop a method for the purification of steroidal alkaloids from extracts of the roots of the plant *Veratrum californicum*. Crude extracts of the plant roots had been demonstrated to have teratogenic activity in a number of animals, the major defect being holoprosencephaly which sometimes results in cyclopia [5]. Hence the activity was referred to as "crude cyclopamine". Three alkaloidal steroids, jervine, 11-deoxojervine and glycoside of 11-deoxojervine, all found in the crude root extract, were found to have teratogenic activity [6]. We wish to obtain the main component, 11-deoxojervine in a pure form to study its mechanism of action [7].

EXPERIMENTAL

The chromatography equipment consisted of two Waters Assoc. Model 6000A solvent delivery systems controlled by a Model 660 solvent programmer and a Waters U6J injector. Peak detection was achieved by ultraviolet (UV)

absorbance at 254 nm using a 441 nm fixed-wavelength detector or at lower wavelengths with a 481-nm variable-wavelength detector (Waters Assoc.). Flow-rate was 1.0 ml/min throughout and all separations were performed at 22°C. The following columns were tested: three 30 cm × 3.9 mm C_{18} μBondapak (10 μm particle size) from Waters Associates, Carlton, Australia; two 25 cm × 4.6 mm ODS Ultrasphere (5 μm particle size) from Beckman, Notting Hill, Australia; one 7.5 cm × 4.6 mm RPSC column (5 μm particle size) from Beckman. Acetonitrile and methanol were both HPLC grade (Waters Assoc.), trifluoroacetic acid (TFA) was purchased from BDH and was purified prior to use by making up a 1% (v/v) aqueous solution and passing it through a C_{18} Sep-Pak (Waters Assoc.) [8]. Glass-distilled water was further purified by a Milli-Q system (Millipore, Carlton, Australia) consisting of one charcoal, two ion-exchange and one Organics cartridge and a 0.45-μm Millistack filter. Precise chromatographic conditions are given in the relevant figure legends and in the text. The measured pH of the mobile phase was between 1.79 for 20% acetonitrile—0.1% TFA to 1.92 for 60% acetonitrile—0.1% TFA. Nuclear magnetic resonance (NMR) spectroscopy was performed using a Bruker 90 90-MHz spectrometer (Selby Scientific, Glen Waverley, Australia). Proton magnetic resonance data were obtained in the pulse/Fourier transform mode. NMR spectra were obtained at 300°K on steroidal alkaloids dissolved in C^2HCl_3 containing tetramethylsilane (TMS) as internal standard. Sweep width was 1202 Hz, with either 4 K or 8 K real-data points collected to give a digital resolution of 0.34 or 0.17 Hz per point. Crude cyclopamine was prepared by benzene extraction of the roots of *Veratrum californicum* which were harvested in Idaho, U.S.A. The crude extract was partially purified by liquid partition through aqueous acid and chloroform and fractional crystallization as described previously [6, 9]. All other steroidal alkaloids were prepared as described previously [5, 6, 9].

RESULTS

Initial attempts to chromatograph veratramine, jervine and 11-deoxojervine by reversed-phase HPLC were hampered by two major problems, low solubility of 11-deoxojervine and jervine in water–methanol or water–acetonitrile or water–methanol–acetonitrile mixtures, and the peaks obtained for the three compounds on all three columns were asymmetric, with severe tailing. Crabbe and Fryer [4] had tried to overcome a similar problem using a methanol–Tris buffer, pH 7.0–7.5 mobile phase. This is a non-volatile solvent system, which still does not give efficient chromatography and narrow symmetrical peaks for the non-conjugated steroidal alkaloids. We found that a water–acetonitrile–0.1% TFA mobile phase solved both problems. All three alkaloids chromatographed on all three reversed-phase columns with greatly improved peak shapes. The C_{18} μBondapak column proved to be the most efficient, typically showing 8000–10,000 plates per metre for jervine and 11-deoxojervine (Fig. 2). The ODS Ultrasphere columns were less efficient, (2000–3000 plates per metre) and the peak shapes for all three test compounds showed significant tailing. This result was found on two different ODS Ultrasphere columns and three different C_{18} μBondapak columns. All columns were

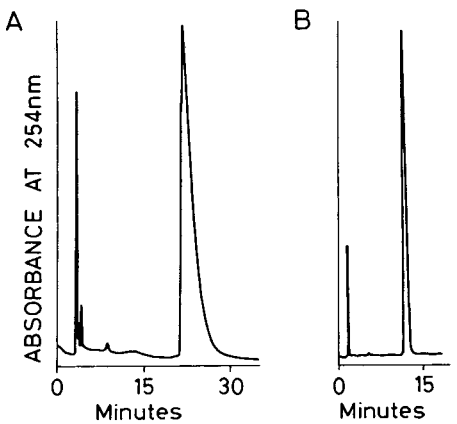


Fig. 2. Isocratic reversed-phase HPLC separation of jervine (A) without and (B) with 0.1% TFA in the mobile phase. A C_{18} μBondapak column was used for both chromatograms. (A) The mobile phase was acetonitrile—water (36:64); 20 μg of jervine were injected in 50 μl of the mobile phase; detector sensitivity was 0.05 a.u.f.s. (B) The mobile phase was acetonitrile—water—TFA (40:59.9:0.1); 20 μg of jervine were injected in 50 μl of the mobile phase; detector sensitivity was 0.1 a.u.f.s.

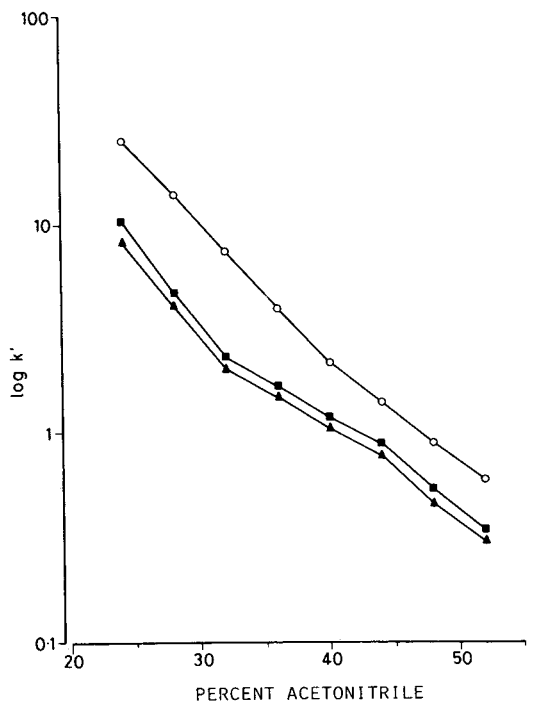
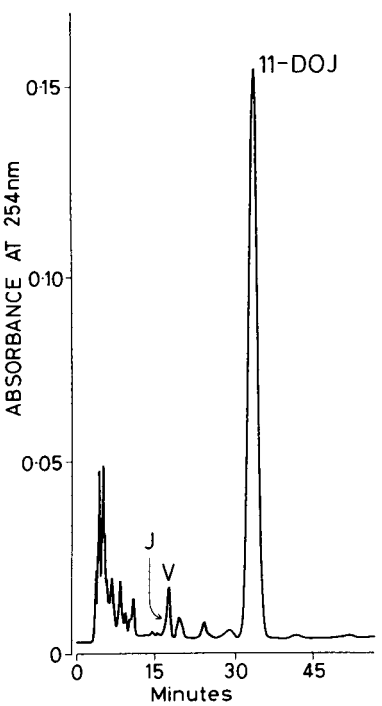


Fig. 3. Plot of log k'—acetonitrile concentration for jervine (▲), 11-deoxojervine (○) and veratramine (■). All data were obtained using the chromatographic conditions described for Figs. 4 and 5. TFA concentration was 0.1% throughout.

tested for efficiency using a standard uracil–acenaphthalene mixture, supplied with one of the C_{18} μBondapak columns by Waters Assoc. and an acetonitrile—water elution system (60:40, v/v, for the C_{18} μBondapak and 50:50, v/v, for the ODS Ultrasphere). Average efficiency for acenaphthalene on the C_{18} μBondapak columns was 16,500 plates per metre and on the ODS Ultrasphere columns was 33,800 plates per metre, under comparable conditions (flow-rate 1.0 ml/min, sample volume 10 μl, k' between 3 and 6). Isocratic elution of the steroidal alkaloids with mixtures of between acetonitrile–water–TFA (28:71.9:0.1) and acetonitrile–water–TFA (40:59.9:0.1) gave good resolution of veratramine and jervine from 11-deoxojervine (Fig. 3). Although 11-deoxojervine was only sparingly soluble (<0.1 mg/ml) in acetonitrile–water–TFA (80:19.9:0.1) and in water–TFA (99.9:0.1), it was found to be much more soluble (5–10 mg/ml) in the range acetonitrile–water–TFA (20:79.9:0.1) to acetonitrile–water–TFA (65:34.9:0.1). The reason for this effect is probably due in part to the TFA forming an ion pair with the cyclic amine function.

Two different batches of crude cyclopamine were chromatographed isocratically in the acetonitrile–water–TFA system (Figs. 4 and 5). Although



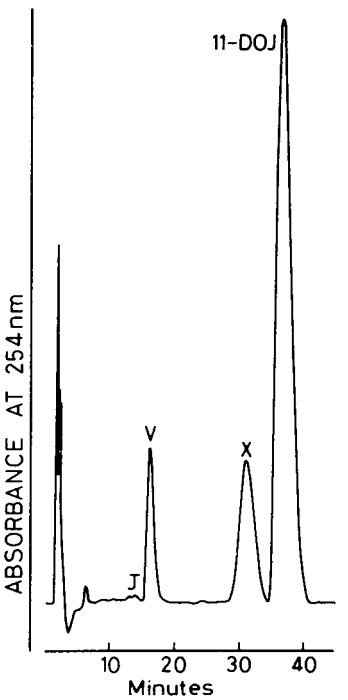


Fig. 4. Isocratic reversed-phase HPLC separation of crude cyclopamine "1981 batch". Crude cyclopamine (100 μg) was injected in 50 μl of the mobile phase, acetonitrile—water—TFA (32:67.9:0.1) onto a C_{18} μBondapak column. Detector sensitivity was 0.2 a.u.f.s. Peaks: J = jervine; V = veratramine; 11-DOJ = 11-deoxojervine.

Fig. 5. Isocratic reversed-phase HPLC separation of crude cyclopamine "1982 batch". All conditions were as described for Fig. 4, except that the mobile phase was acetonitrile—water—TFA (28:71.9:0.1). Peaks: J = jervine; V = veratramine; X = unknown; 11-DOJ = 11-deoxojervine.

the major peak of the UV-absorbing material corresponded in elution position to 11-deoxojervine in both preparations, it was clear that the pattern of the minor peaks in the two extracts was different. Each pattern was always highly reproducible and there was no evidence for degradation. The reason for this is not clear, but it may be due to seasonal variation in the composition of the roots, as one extract was made twelve months before the other. The nature of the contaminant peak X (Fig. 5) is not known. It is important to note that different isocratic conditions were used for each extract. This was to produce the optimal resolution of the 11-deoxojervine from the various minor peaks. The glucoside of jervine was not retained on the column under either of the chromatographic conditions (Figs. 4 and 5). Muldamine and solasodine were both greatly retained on the column under these conditions ($k' > 100$) and were recovered by elution with 100% methanol or 100% acetonitrile. Large-scale (50–100 mg) preparations were successfully completed by trace enrichment of a dilute solution (1–2 mg/ml) of the crude cyclopamine in acetonitrile–water–TFA (20:79.9:0.1) onto a C_{18} μBondapak column, followed by isocratic elution at acetonitrile–water–TFA (32:67.9:0.1). Gradient elution of the steroidal alkaloids was also examined. An example is shown in Fig. 6 where 3-O-acetyl jervine, which was prepared from jervine by chemical synthesis [10], was eluted in a pure form by a acetonitrile gradient. One of the contaminants

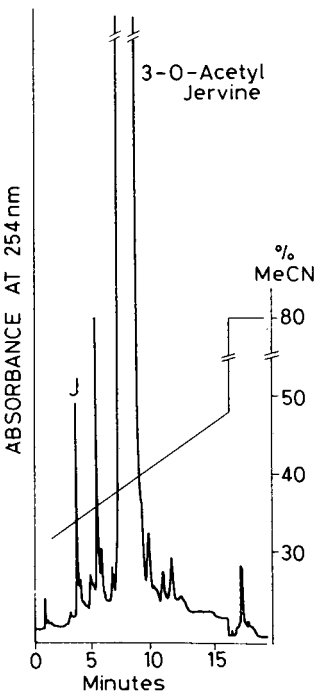


Fig. 6. Gradient chromatography of 3-O-acetyl jervine. A 450-μg amount of 3-O-acetyl jervine was injected in 450 μl of acetonitrile–water–TFA (32:67.9:0.1) onto a C_{18} μBondapak column equilibrated in the same mobile phase. The chromatogram was developed by a linear gradient from 32% to 48% acetonitrile containing 0.1% TFA throughout, over 15 min at a flow-rate of 1 ml/min. Detector sensitivity was 0.1 a.u.f.s. Peak: J = jervine.

was clearly free jervine. The identity of the 3-O-acetyl jervine was confirmed by NMR. Fig. 6 also demonstrates the efficiency of the chromatography at a moderate (450 μg) sample loading. 3-O-Acetyl jervine is of importance as it represents the only radiolabelled form of jervine (3-O[^{14}C]acetyl jervine) currently available.

In order to ensure that the material contained in the peaks obtained from the chromatograph was the desired compound, infrared and NMR spectra were run on the crude and purified alkaloids. The NMR spectra of crude cyclopamine, veratramine, and purified 11-deoxojervine are shown in Fig. 7A, B and C. The crude cyclopamine clearly had typical aromatic C—H resonances (Fig. 7A), around 7 ppm, which were similar to those seen in the NMR spectrum of

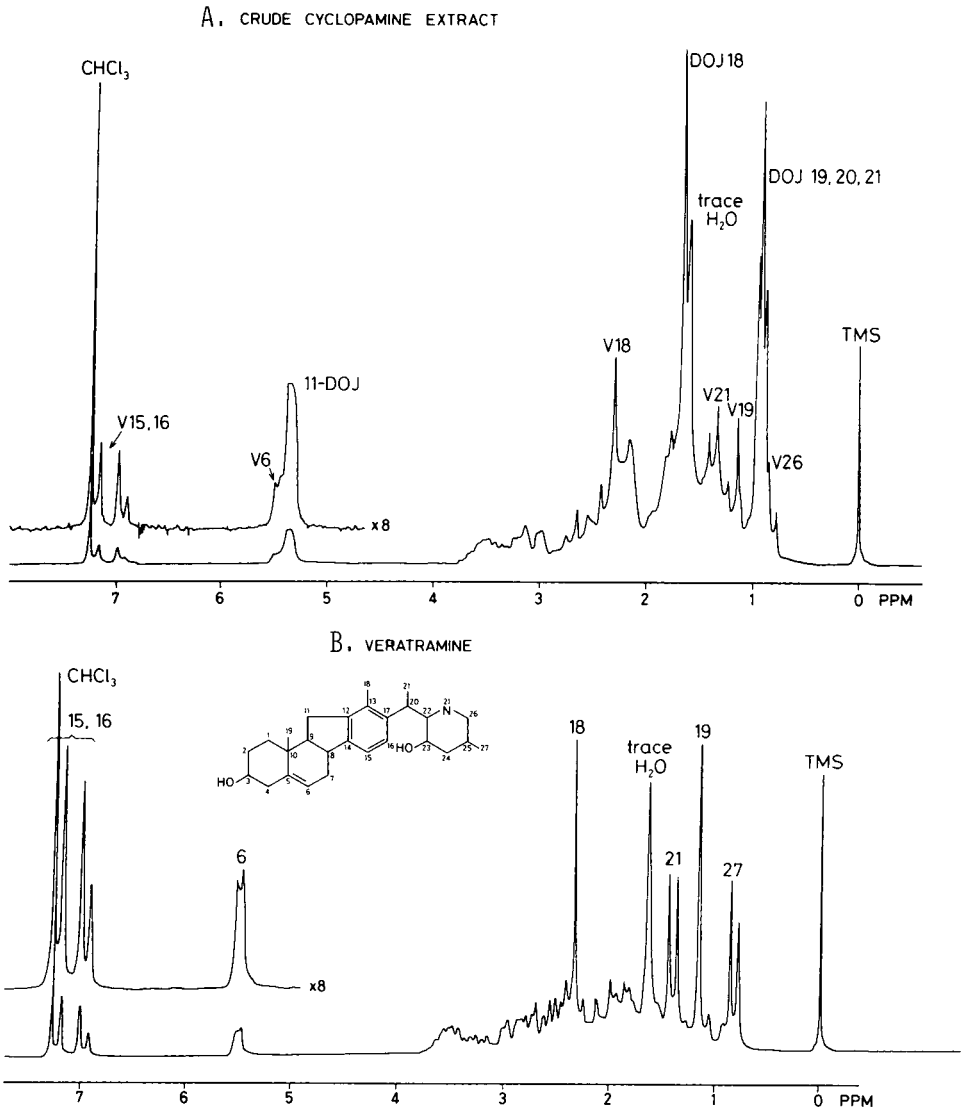


Fig. 7.

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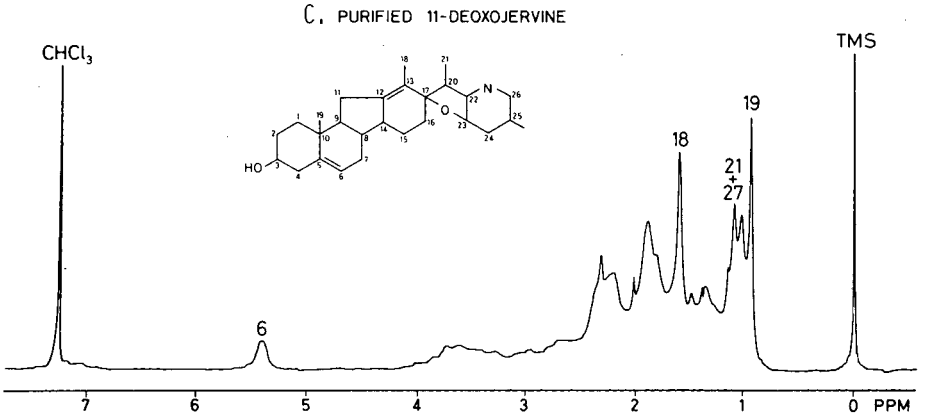

Fig. 7. Proton NMR spectra of (A) crude cyclopamine (B) veratramine and (C) reversed-phase HPLC purified 11-deoxojervine. Resonances in A which are marked with a V are due to contamination by veratramine or a closely related compound. The jervine numbering system of Brown [11] has been adopted here. Tentative assignments of the major resonances have been indicated.

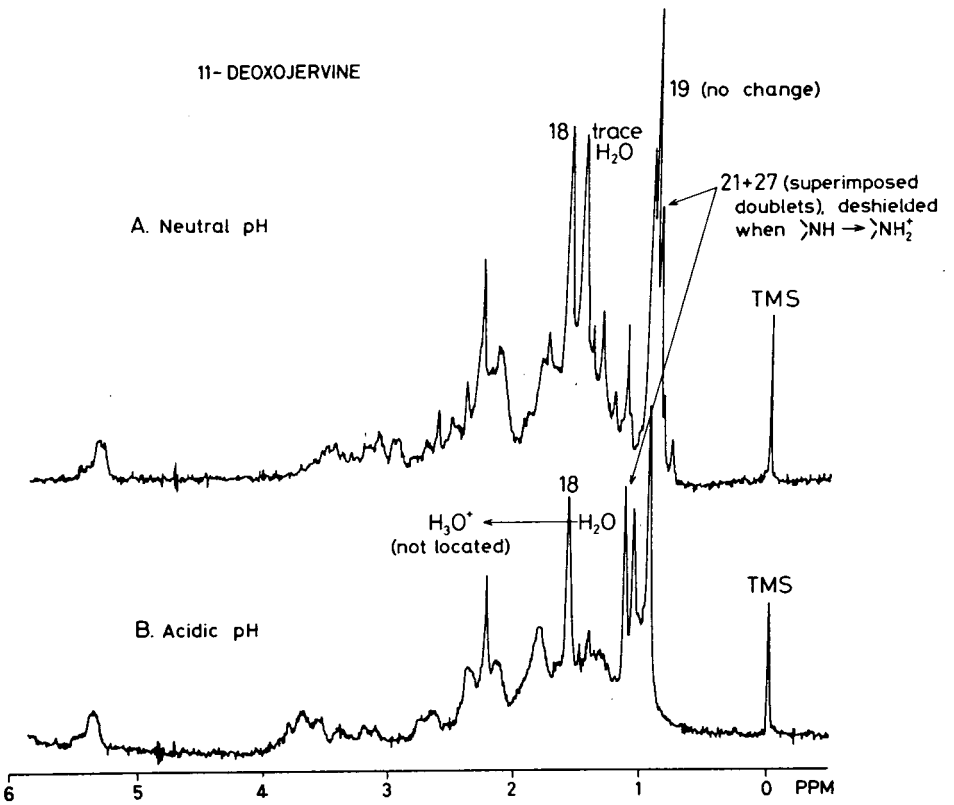

Fig. 8. Proton magnetic resonance spectra of crude cyclopamine (A) at neutral pH and (B) after acidification with 0.1% TFA. Note the pair of doublets at about 1 ppm which are dishielded by 0.176 ppm after acidification. The disappearance of the "trace H_2O" resonance in (A) is due to protonation of the H_2O, which results in a broader H_3O^+ resonance at lower field.

veratramine (Fig. 7B). The purified 11-deoxojervine no longer had the aromatic resonances. The major resonances for the veratramine and 11-deoxojervine NMR spectra were tentatively assigned from the chemical shifts and J constants. The jervine numbering system of Brown [11] has been used. There was an obvious loss of the veratramine resonances (labelled V) when the crude cyclopamine was purified to yield pure 11-deoxojervine (Fig. 7). Another change was noted in the 11-deoxojervine spectra at around 1 ppm (see Fig. 7A and C). The pair of doublet resonances owing to the C_{21} and C_{27} methyl groups had shifted downfield in the purified 11-deoxojervine compared to the crude cyclopamine. This change could be easily explained when the NMR spectra of cyclopamine before and after acidification with TFA were examined (Fig. 8). Clearly acidification had caused the shift in C_{21} and C_{27} methyl resonances. We also examined the NMR spectra of jervine, 3-O-acetyl jervine, solasodine and muldamine [12] to assist with the assignments shown in Figs. 7 and 8.

DISCUSSION

We have successfully used reversed-phase HPLC to separate steroidal alkaloids with reasonable efficiency. The inclusion of 0.1% TFA in the mobile phase gave improved peak shapes and excellent recovery of material. We now routinely use isocratic reversed-phase HPLC (Figs. 4 and 5) to prepare pure 11-deoxojervine for our biological experiments, although it is clear that gradient chromatography can also be useful in the isolation of jervine and jervine derivatives (Fig. 6). One extremely useful result of our approach has been to dramatically improve the solubility of the crude cyclopamine. Traditionally this has always been a problem in any attempt to use crude cyclopamine in aqueous physiological environment. It has routinely been administered to experimental animals as a suspension with a solid carrier such as carboxymethyl cellulose [13]. After purification of the 11-deoxojervine by reversed-phase HPLC, we concluded that the basic nitrogen atom has a TFA anion complexed to it. This is supported by the chemical shift changes shown for the C_{21} and C_{27} methyl resonances (Fig. 8). We routinely exchange this TFA ion for chloride by diluting the eluate containing 11-deoxojervine 1:1 with 0.2% (v/v) hydrochloric acid and loading the purified 11-deoxojervine into a C_{18} Sep-Pak [8]. After washing with 20 ml of aqueous 0.2% hydrochloric acid, we elute the alkaloid with 5 ml of 60% acetonitrile containing 0.2% (v/v) hydrochloric acid. This solution is diluted 1:1 with 0.2% (v/v) hydrochloric acid and is lyophilized. By this means, we obtain the pure 11-deoxojervine as a dry chloride salt which is then readily soluble in water (up to 2 mg/ml). Previous difficulties in solubility of the crude cyclopamine preparation must be put down in part to the presence of insoluble impurities, and in part to difficulty in forming the salt. In our hands, prior treatment with 0.1% TFA increased the solubility of 11-deoxojervine in 0.2% hydrochloric acid.

NMR spectroscopy was a very useful tool for the identification of the eluted alkaloids. As a general rule in HPLC, an alternative method of peak identification, other than elution volume, is mandatory. In the absence of a specific biological or immunological assay, spectroscopy seemed a reasonable

approach. Infrared spectra were generally found to be of a limited use (data not shown) although the presence of the carbonyl group in jervine gave a peak in the infrared spectrum at 1700 cm^{-1} which clearly distinguished jervine from 11-deoxojervine. We were able to obtain recognisable NMR spectra with amounts as low as 1.5 mg of steroidal alkaloid, although the spectra were much improved if 10 mg were used. The availability of a more powerful spectrometer (300 MHz) will improve the sensitivity of "detection" of the steroidal alkaloids eluted from reversed-phase HPLC by at least five-fold.

It can be seen that complete resolution was obtained between jervine and 11-deoxojervine, but that rather incomplete resolution could be obtained between jervine and veratramine (Figs. 3–5). We have found that a methanol–water–TFA mobile phase in the range 40–50% methanol will give complete resolution of jervine and veratramine [14] using isocratic elution and a C_{18} μBondapak column. The glucoside of jervine elutes just after the injection artefact in both the acetonitrile–water–TFA system and the methanol–water–TFA system. A ternary elution system comprising of aqueous TFA–methanol–acetonitrile may turn out to be the optimal isocratic system. Further studies will pursue this possibility.

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RESOLUTION OF *RS*-ABSCISIC ACID AND THE SEPARATION OF ABSCISIC ACID METABOLITES FROM PLANT TISSUE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Attempts to resolve the enantiomers of racemic abscisic acid (ABA) by high-performance liquid chromatography on a chiral stationary-phase column were unsuccessful. However, reduction of *RS*-methyl ABA (*RS*-Me-ABA) with sodium borohydride generates a new chiral centre and one of the two isomeric products, the *RS*-Me-1′,4′-*cis*-diol of ABA, was separated into its enantiomers by high-performance liquid chromatography on an optically active Pirkle column.

High-performance liquid chromatography on a μBondapak C_{18} column separated the metabolites and conjugates of [2-^{14}C]ABA fed to tomato shoots. The resolution method was used to measure the relative proportions of *R* and *S* enantiomers in the free acid liberated from conjugates of ABA.

INTRODUCTION

The plant hormone abscisic acid (ABA) occurs naturally as the (+)-*S* enantiomer but most experiments in which ABA has been fed have, perforce, used racemic, synthetic material, although differences in the physiological effects and metabolism of the natural and unnatural enantiomers have been reported [1]. The resolution of racemic ABA was first achieved by fractional crystallization of the (−)-brucine salt of ABA in methanol [2]. The fractions were further enriched by selective solubilization and microsublimation. Acetylcellulose chromatography [3] has also been used to prepare fractions slightly enriched with one or other enantiomer and optically active ABA was isolated by selective solubilization. Recently *R*- and *S*-ABA of high optical purity have been prepared by immunoaffinity chromatography [4]. However, only small amounts of ABA can be resolved and the column has a limited life. The amount of *R*- and *S*-ABA in a sample can be measured by optical rotatory dispersion and circular dichroism (CD) methods [1]. Unfortunately these methods require

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very costly equipment and relatively large quantities of sample with a high degree of ultraviolet (UV) purity. Because of the complexity of the existing methods we have developed a high-performance liquid chromatographic (HPLC) method for *RS*-ABA that completely separates the enantiomers, requires readily available equipment and can produce milligram quantities of the separate enantiomers.

The low endogenous concentrations (ppb) and the wide range of polarities of ABA and its metabolites complicate their isolation from plant tissue. Reversed-phase HPLC has been used for the purification and analysis of ABA [5—9]. We have developed a system that separates the known metabolites of ABA in tomato tissue including the recently characterized metabolites ABA 1′-glucoside (ABAGS) [10], dihydrophaseic acid 4′-glucoside (DPAGS) [11] and the 1′,4′-*trans*-diol of ABA [12].

EXPERIMENTAL

Tomato plants (*Lycopersicon esculentum* cv. Gross Lisse), 150—200 mm high, were used in feeding experiments. The plants were cut just above ground level and the shoots placed in aqueous solutions of *RS*-[2-^{14}C]ABA. A Waters HPLC system consisted of a 6000A pump, an M-45 pump, a U6K injector and a Model 660 solvent programmer. Effluent was monitored with a Model 440 absorbance detector fitted with a 254-nm filter. Solvents for HPLC were purchased from Waters Assoc. (Chippendale, Australia) or were redistilled before use. Distilled water was passed through a Milli-Q reagent water system (Millipore, Bedford, MA, U.S.A.).

HPLC resolution of RS-Me-1′,4′-cis-diol of ABA

A 10-mg amount of *RS*-ABA was methylated with ethereal diazomethane and the methyl ABA (Me-ABA) reduced with sodium borohydride in methanol—water (2:1) at 0°C for 30 min. The products were separated by HPLC on a 300 mm × 7.8 mm I.D. μBondapak C_{18} column with a mobile phase of ethanol—water—acetic acid (317:517:1) delivered at a flow-rate of 4 ml/min. Me-1′,4′-*trans*-diol of ABA, Me-ABA and Me-1′,4′-*cis*-diol of ABA had retention times of 8.2, 10 and 16 min, respectively.

RS-Me-1′,4′-*cis*-diol of ABA was loaded on a 250 mm × 4.6 mm I.D. Pirkle Type 1-A column (Regis, Morton Grove, IL, U.S.A.) and eluted with hexane—isopropanol (9:1) at a flow-rate of 1 ml/min. Peaks eluted after 21 and 23 min were the 1′*S*,4′*S*- and 1′*R*,4′*R*-*cis*-diol of ABA methyl ester, respectively. A sample of Me-1′,4′-*cis*-diol prepared from natural *S*-ABA gave one peak only (21 min). Improved resolution of *RS*-Me-*cis*-diol with baseline separation between the peaks was achieved by connecting a Pirkle Type 1-A and a Pirkle covalent *R*-phenylglycine column in series, eluting with hexane—isopropanol (97:3) at a flow-rate of 2 ml/min and recycling the eluate up to four times.

R- and *S*-methyl abscisates were regenerated by oxidation of the Me-1′,4′-*cis*-diol of ABA with a ten-fold excess of manganese dioxide. The oxidation mixture in 2 ml dry chloroform was continuously stirred at 20°C. The duration of the oxidation depended on the batch of manganese dioxide with reaction times ranging from 30 min to 72 h. The reaction mixture was loaded on a

200 × 200 mm silica gel 60 F_{254} TLC plate (E. Merck, Darmstadt, F.R.G.) and chromatographed in hexane—ethyl acetate (2:1) to separate Me-ABA from unreacted Me-*cis*-diol and manganese dioxide. ABA was released by alkaline hydrolysis in 2 *M* aqueous potassium hydroxide—ethanol (1:2) at 20°C for 30 min.

Plant feeding and extraction of metabolites

To 30 g tomato (*Lycopersicon esculentum* cv. Grosse Lisse) shoots 2.8 μCi *RS*-[2-^{14}C]ABA (25.6 mCi/mmol) were fed. After two days the plants were homogenised in acetone—acetic acid (99:1) containing 2,6-di-*tert.*-butyl-4-methylphenol (BHT) (100 mg/l). The acetone was evaporated and the aqueous residue extracted three times with diethyl ether. The aqueous phase which contained polar metabolites and conjugates of ABA was put aside for HPLC. An equal volume of water was added to the ether extract and the pH adjusted to 7.0 with saturated sodium hydrogen carbonate. This was repeated three times and the ether phase containing unlabelled neutral material was discarded. The aqueous phase was acidified with 1 *M* sulphuric acid to pH 2.5 and ABA and ether-soluble metabolites were extracted with diethyl ether (three times). The ether extract and the initial aqueous phase were combined, the diethyl ether was evaporated and the sample was concentrated and chromatographed on Sep-Pak C_{18} cartridges (Waters Assoc.).

Four Sep-Pak C_{18} cartridges were connected in series with 1 mm I.D. glass tubing. A peristaltic pump was used to maintain a flow-rate of 5.0 ml/min. The sample, which was in a volume of about 50 ml, was loaded on the Sep-Pak column and washed with 20 ml water—acetic acid (500:1). Only a small amount of radioactivity was present in this fraction and corresponds to uncharacterised polar metabolites of ABA. Then 20 ml ethanol—water—acetic acid (256:475:1) eluted abscisic acid and its metabolites from the column. Washing the column with ethanol removed less polar compounds, which contained no radioactivity, and regenerated the column.

HPLC separation of metabolites

The Sep-Pak fraction containing ABA and its metabolites was evaporated to dryness, dissolved in 50 μl ethanol—water—acetic acid (84:506:1) and loaded on a 300 mm × 7.8 mm I.D. μBondapak C_{18} column. The column was eluted at a flow-rate of 4 ml/min for 22 min with the solvent used to dissolve the sample. This was followed by a linear gradient to ethanol—water—acetic acid (158:508:1) over 15 min. After a further 20 min the column was washed with 95% ethanol. Radioactivity in fractions collected was determined by liquid scintillation counting as described elsewhere [13].

Determination of the proportion of R-[^{14}C]ABA to S-[^{14}C]ABA in ABA and its conjugates

Conjugates of ABA were hydrolysed with aqueous ammonium hydroxide (sp.gr. 0.91) at 27°C for 30 min as described by Loveys and Milborrow [10]. Amounts of 400 μg *RS*-ABA were added to the samples of [^{14}C]ABA released by hydrolysis and the samples were methylated with diazomethane. The Me-ABA was purified by HPLC on a μBondapak C_{18} column with ethanol—water—

acetic acid (256:475:1) at a flow-rate of 4 ml/min. The Me-ABA was reduced and resolved as described above. The radioactivity of the *R*- and *S-cis*-diols was determined by liquid scintillation counting.

RESULTS

Attempts to separate the enantiomers of *RS*-ABA and some of its derivatives on a Pirkle column were unsuccessful. Racemic ABA, Me-ABA, 2-*trans*-ABA, Me-2-*trans*-ABA, Me-1′,4′-*trans*-diol of ABA and 4′-O-acetyl-Me-1′4′-*trans*-diol of ABA all chromatographed as single peaks. However, the *RS*-Me-1′,4′-*cis*-diol of ABA and its 4′-O-acetyl derivative were both separated into their enantiomers by HPLC on a Pirkle Type 1-A column with a mobile phase of hexane—isopropanol (9:1) delivered at a flow-rate of 1 ml/min. A sample of Me-1′*S*, 4′*S-cis*-diol of ABA produced from natural *S*-ABA gave a single peak at 21 min and when injected with a sample of the racemic compound caused the peak at 21 min to increase in height while the peak at 23 min remained unchanged. This confirmed that separation of the enantiomers had been achieved and identified the first peak as the *S* enantiomer. The separation of the enantiomers was increased by recycling the column effluent through a Pirkle Type 1-A column and a Pirkle covalent *R*-phenylglycine column in series (Fig. 1). Baseline resolution was achieved after four cycles through the column (248 min). The Pirkle Type 1-A column was found to be more efficient than the Pirkle covalent *R*-phenylglycine column for the resolution of *RS*-Me-1′,4′-*cis*-diol of ABA and a shorter analysis time would be expected if two Type 1-A columns were used in series.

The reduction of Me-ABA to the Me-1′,4′-*cis*- and -*trans*-diols, separation of the enantiomers by HPLC, oxidation of the diols to Me-ABA and saponification to release *R*- and *S*-ABA gave an overall yield of 20% from the original

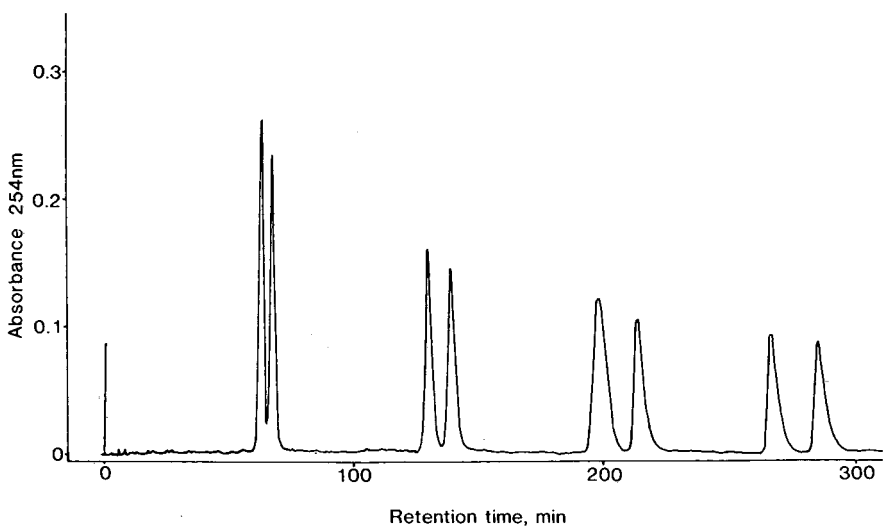


Fig. 1. Resolution of 300 μg *RS*-Me-1′,4′-*cis*-diol of ABA. Two Pirkle HPLC columns were connected in series and eluted with hexane—isopropanol (97:3) at a flow-rate of 2 ml/min and the column effluent was recycled.

ABA (i.e. 10% in each enantiomer). However, half of the ABA is reduced to the 1′,4′-*trans*-diol, which is not resolved. The yield can be increased by re-oxidizing 1′,4′-*trans*-diol to ABA and carrying out a second reduction.

HPLC separation of metabolites

The preliminary chromatography of the tomato extract on C_{18} Sep-Pak proved to be a simple, efficient (98% recovery) method of removing material that interfered with subsequent chromatographic steps. The removal of salts, polar compounds and compounds less polar than ABA made it possible to load samples more concentrated in ABA and its metabolites and facilitated the dissolution of the sample in a small volume for injection. It also removed compounds that bind irreversibly to the stationary phase of the HPLC column.

The metabolites of ABA were separated by one passage through a reversed-phase column with a gradient containing ethanol, water and acetic acid. The separation of the products formed by [2-^{14}C]ABA is shown in Fig. 2. The products were identified by comparison with the retention time of standards (Table I). A mixture of the marker compounds *p*-amino benzoic acid, *p*-hydroxybenzoic acid, benzoic acid 2-*trans*-ABA and 2-*cis*-ABA chromato-

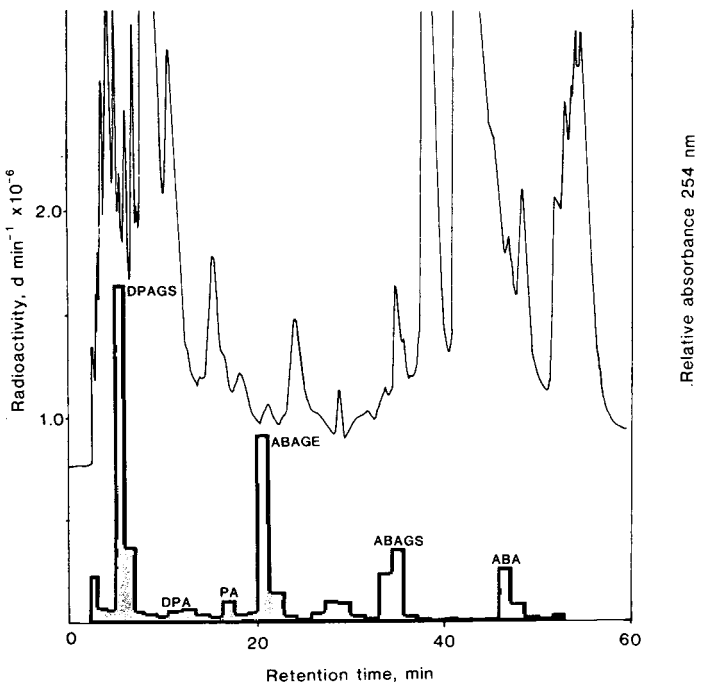


Fig. 2. HPLC separation of the products of *RS*-[2-^{14}C]ABA in tomato shoots. The sample was chromatographed on a 300 mm × 7.8 mm I.D. μBondapak C_{18} column with ethanol—water—acetic acid (84:504:1) delivered at a flow-rate of 4 ml/min for 22 min. This was followed by elution with a linear gradient to ethanol—water—acetic acid (158:508:1) over 15 min and elution with the final solvent for 20 min. The column was then washed with ethanol—water (19:1). Compounds identified were abscisic acid (ABA), ABA 1′-glucoside (ABAGS), ABA glucose ester (ABAGE), phaseic acid (PA), dihydrophaseic acid (DPA) and DPA 4′-glucoside (DPAGS).

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF STANDARD AND MARKER COMPOUNDS

HPLC on a 300 mm × 7.8 mm I.D. μBondapak C_{18} column with ethanol—water—acetic acid (84:504:1) for 22 min at a flow-rate of 4 ml/min followed by a linear gradient to ethanol—water—acetic acid (158:508:1) over 15 min.

Compound	Retention time (min)
Abscisic acid (ABA)	46.0
2-*trans*-ABA (*t*-ABA)	41.7
ABA 1′-glucoside (ABAGS)	33.0
1′,4′-*trans*-Diol of ABA	30.5
epi-Dihydrophaseic acid (*epi*-DPA)	22.7
ABA glucose ester (ABAGE)	21.0
Phaseic acid (PA)	18.0
Dihydrophaseic acid (DPA)	12.0
Dihydrophaseic acid 4′-glucoside (DPAGS)	5.3
p-Aminobenzoic acid	5.7
p-Hydroxybenzoic acid	12.3
Benzoic acid	28.0

graphed under conditions identical to those used to separate the extracts were an effective means to test the performance of the system.

Proportion of R- and S-ABA in conjugates

One of the advantages of the HPLC method of resolution is that it allows the determination of the ratio of *R*-ABA to *S*-ABA in small samples and even allows the specific activity of each enantiomer to be calculated. This is done by hydrolysing the conjugates of ABA and then the ABA released is methylated and reduced to the diols. The *cis*-diol is resolved by HPLC on the Pirkle column and the amount of radioactivity in each anantiomer determined. Conjugates of ABA formed from *S*-[2-^{14}C]ABA contain a preponderance of the *R* enantiomer (Table II). The ratio of *R*-[^{14}C]ABA to *S*-[^{14}C]ABA ranges from 5.8:1 for the glucose ester to 29:1 for ABA glucoside. The free ABA remaining also contains an excess of the *R*-[^{14}C] enantiomer.

TABLE II

PROPORTION OF *R*-[2-^{14}C]ABA AND *S*-[2-^{14}C]ABA in ABA AND ITS CONJUGATES IN EXTRACTS OF TOMATO SHOOTS FED *RS*-[2-^{14}C]ABA

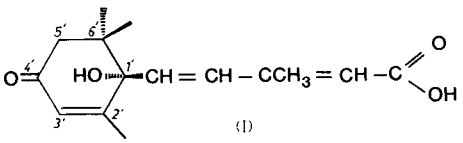
Compound*	*R*-[2-^{14}C]ABA:*S*-[2-^{14}C]ABA
ABA	6.1:1
ABAGE	5.8:1
ABAGS	29.4:1
Uncharacterised conjugate	8.3:1

*For abbreviations see Table I.

DISCUSSION

ABA is intensely optically active so it is surprising, at first sight, that in experiments in which growth is assayed the *R* and *S* enantiomers are equally or

almost equally active. It has been proposed that both *R* and *S* forms can attach to some active site(s). This could occur if the 2′-methyl group of one enantiomer takes the place occupied by a 6′-methyl of the other [14]. The molecule is not intrinsically highly asymmetric and an alternative projection of the structural formula emphasizes this (I).



(I)

Reduction of the 4′-ketone of ABA generates a second chiral centre in close proximity to the 1′ chiral centre and enables *RS*-Me-1′,4′-*cis*-diol to be separated into its enantiomers by HPLC on a column having a chiral stationary phase. It was possible to separate the enantiomers of 4′-O-(−)-camphanyl esters of both *RS*-Me-1′,4′-*cis*- and -*trans*-diols of ABA by normal-phase HPLC although yields were low. The HPLC resolution of *RS*-Me-1′,4′-*cis*-diol of ABA on a chiral-stationary phase column enables large amounts of *R*- and *S*-ABA of extremely high optical purity to be produced at much less cost than existing methods. An added advantage is that the method also allows the proportion of *R*- and *S*-ABA in a sample to be measured. If the ABA is labelled the specific activity of each enantiomer can be determined, though this is limited to samples containing an excess of 1 μg of each enantiomer. However, the proportion of labelled *R*- to *S*-ABA in very small samples of ABA may be determined by adding unlabelled ABA before reducing the sample and is only limited by the sensitivity of the method used to detect the label.

HPLC on μBondapak C_{18} column separated the products formed from [2-^{14}C]ABA by tomato shoots, including three conjugates of ABA. This procedure gives much more information about the metabolites of ABA than methods in which the level of conjugates is measured by assaying the free acids released by basic hydrolysis. Because the free acid metabolites and conjugates are separated in a single step, losses of conjugate by partitioning into solvents used to extract the free acids are avoided and conjugates that are resistant to basic hydrolysis, such as DPA 4′-glucoside [11], can also be measured.

The conjugates were not subjected to basic conditions in the extraction and chromatographic procedures so base-induced rearrangement of ABA glucose ester [15] and basic hydrolysis of conjugates were avoided. The separation of ABA glucose ester, ABA 1′-glucoside and a novel conjugate of ABA, all containing different proportions of *R*-[^{14}C]ABA and *S*-[^{14}C]ABA is evidence against rearrangement of ABA glucose ester to produce the glucoside or the other conjugate. However, stereospecific rearrangement has not been ruled out.

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CHROMBIO. 2299

Note

Simple high-performance liquid chromatographic assay for the routine monitoring of clonazepam in plasma

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The benzodiazepine derivative clonazepam is an effective oral anticonvulsant for all forms of generalized epilepsy. Used parenterally, clonazepam has been found to be most effective against status epilepticus [1]. However, clonazepam has been one of the least-monitored anticonvulsants owing to the more difficult nature of its assay. Its concentration in the plasma is in the order of 100 to 1000 times lower than the level of most other anticonvulsants, resulting in a sensitivity problem for the measurement of clonazepam by chromatographic techniques. Other anticonvulsants are often administered concomitantly with clonazepam and as these are present in higher concentrations in plasma, adequate chromatographic separation is essential.

Although clonazepam can be measured by gas—liquid chromatography (GLC) using electron-capture detection [2], it chromatographs far more readily using high-performance liquid chromatography (HPLC). However ultraviolet (UV) detection for the HPLC assay is not as sensitive as electron-capture detection used in GLC. A previously reported HPLC assay for measuring clonazepam employed the normal-phase mode [3]. We have developed an HPLC assay for clonazepam in the reversed-phase mode using hexane—ethyl acetate (90:10, v/v) as the extraction solvent. The assay produced clean chromatography with sufficient sensitivity for plasma clonazepam assays to be carried out with UV detectors that have a sensitivity limit of 0.01 absorbance units full scale (a.u.f.s.).

EXPERIMENTAL

Reagents

Pure standard samples of clonazepam (CLON), flunitrazepam (internal standard I.S.), 7-aminoclonazepam and 7-acetamidoclonazepam were obtained from Roche Products (Dee Why, Australia). Seronorm Pharmaca AED,

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which is a lyophilized reference serum of animal origin for the quality control of serum analysis of antiepileptic drugs, was obtained from Nyegaard (Oslo, Norway). The solvents hexane, ethyl acetate, acetonitrile and methanol were all of HPLC grade from Waters Assoc. (Milford, MA, U.S.A.). All other reagents were of analytical reagent grade.

Standard solutions

A stock solution of clonazepam was prepared with a concentration of 1.0 μg/ml in methanol. Aliquots of this solution were then accurately syringed into extraction tubes and carefully evaporated to dryness under a gentle stream of nitrogen. Drug-free plasma (1 ml) was added to each of these tubes to give standard plasma solutions ranging in concentration from 10 to 100 ng/ml. A solution of the internal standard, flunitrazepam, was prepared with a concentration of 5 μg/ml in methanol.

Instrumentation

Analyses were performed on a high-performance liquid chromatograph consisting of a Waters Assoc. Model 6000A solvent delivery system and a Model 450 variable-wavelength UV detector. Samples were injected by means of a WISP Model 710B autosampler. The assays were carried out in the reversed-phase mode using a Perkin-Elmer analytical C_8 (10 μm) column, 25 cm × 4.6 mm. The mobile phase consisted of acetonitrile—0.05 mol/l sodium acetate (adjusted to pH 7.5) (38:62, v/v), with a flow-rate of 1.8 ml/min. The wavelength of the UV detector was set at the λ_{max} for clonazepam of 306 nm and the range set at 0.01 a.u.f.s.

Assay procedure

To a screw-capped pyrex tube (16 × 125 mm) containing 1.0 ml of plasma sample or standard were added 0.5 ml of 1 mol/l ammonia solution (adjusted to pH 9.5 with hydrochloric acid), 20 μl of the internal standard (5.0 μg/ml flunitrazepam in methanol) and 10 ml of extraction solvent (hexane—ethyl acetate, 90:10, v/v). The tube was capped with a PTFE-lined screw cap and the contents were shaken vigorously for 1 min. After centrifugation (1 min at 1000 *g*) the organic phase (top layer) was transferred to a clean dry quick-fit test tube and the solvent removed in vacuo using a Buchi Rotovapor. The dried extract was then redissolved in 200 μl of the mobile phase and 180 μl were injected onto the column. Retention times for clonazepam and flunitrazepam were 6.5 and 7.9 min, respectively. A calibration curve was obtained from the standard solutions by plotting the ratio of the peak height of clonazepam to that of the internal standard against the concentration of clonazepam. The calibration curve obtained from three replicate determinations for the concentrations 10, 20, 40, 70 and 80 ng/ml and ten replicate determinations for the concentrations 50 and 100 ng/ml, was linear over this range of standard solution concentrations. The regression equation was $Y = 0.0102X - 0.0132$ and the correlation coefficient (r) was 0.998. The nominal therapeutic range for plasma clonazepam concentration employed by this hospital is 25—75 ng/ml. The calibration curve adequately covers this range. The precision of the assay was 2.8% (coefficient of variation, C.V.) at 50 ng/ml and 1.5% (C.V.) at 100 ng/ml

(n=10). The limit of detection was 3 ng/ml. The concentrations of clonazepam in the samples were determined from the standard calibration curve.

RESULTS AND DISCUSSION

The use of hexane—ethyl acetate (90:10) as the extraction solvent yielded clean extracts with greater than 75% recovery of clonazepam without any interference in the chromatography. Although diethyl ether gives excellent recovery as an extraction solvent, other endogenous substances are also extracted from the plasma resulting in a relatively impure extract. Dissolution of this extract with the mobile phase required a tedious filtering step using an aqueous sample clarification kit obtained from Waters Assoc. (Part Number 26865) with some loss of sample prior to injection onto the LC column. Rovei and Sanjuan [4] used chloroform as the extraction solvent to extract clonazepam from plasma that had been alkalinised with phosphate buffer (pH 9.1). However, we found the use of hexane—ethyl acetate (90:10) yielded a cleaner extract than chloroform or dichloromethane. In addition the hexane—ethyl acetate (90:10) solvent extracted a smaller quantity of the acidic anticonvulsant drugs such as phenytoin and carbamazepine.

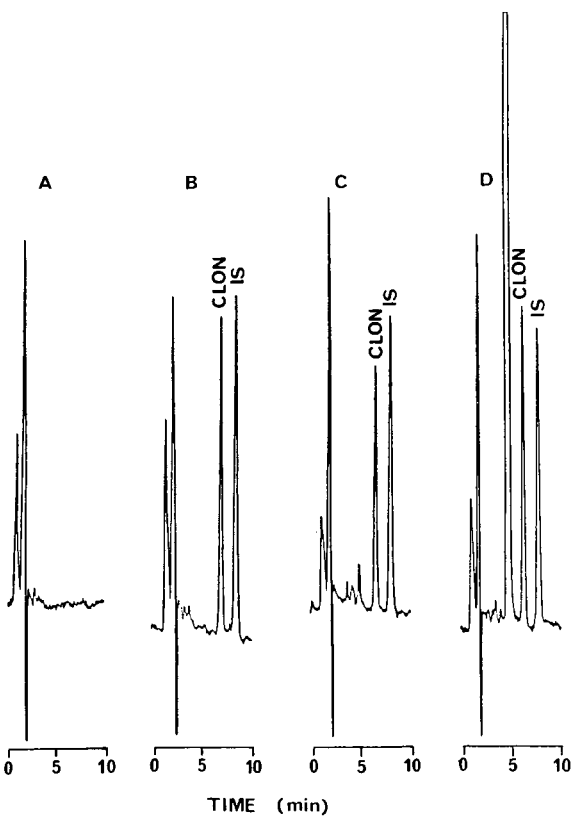

Fig. 1. Chromatograms of plasma extracts of a blank plasma (A), spiked standard (B), a typical sample (C), and an external quality control (Seronorm Pharmaca AED) (D). Peaks: CLON = clonazepam, IS = internal standard (flunitrazepam).

The chromatogram of a blank plasma extract (from a control subject who has not taken clonazepam) is shown in Fig. 1A. The chromatogram of the extract from a spiked plasma standard of clonazepam is shown in Fig. 1B, and that from a patient receiving clonazepam in Fig. 1C. Fig. 1D shows the chromatogram from an extract of an external quality control serum (Seronorm Pharmaca AED) which contains carbamazepine, clonazepam, ethosuximide, phenobarbitone, phenytoin, primidone and valproic acid. As the column slowly deteriorated after more than 500 injections, the sensitivity and resolution of clonazepam from carbamazepine was gradually reduced and the column eventually required replacement after approx. 800 injections. Thiopentone was found to co-chromatograph with clonazepam and as there are occasional clinical situations requiring simultaneous administration of thiopentone and clonazepam, e.g. status epilepticus, thiopentone interference can be eliminated by an acidic back-extraction of the thiopentone from the organic phase. None of the other commonly used anticonvulsants were found to interfere with the assay (see Table I).

TABLE I

RETENTION TIMES FOR VARIOUS OTHER DRUGS AND METABOLITES USING THE CLONAZEPAM ASSAY CONDITIONS

Drug	Retention time (min)
Valproic Acid	Approx. 2*
Ethosuximide	Approx. 2*
Primidone	2.8*
Carbamazepine epoxide	2.9
Phenobarbitone	2.9*
7-Acetamidoclonazepam	3.0
7-Aminoclonazepam	3.2
Bromazepam	4.4
Carbamazepine	4.8
Phenytoin	4.9*
Oxazepam	5.4
Lorazepam	5.8
Nitrazepam	5.8
Clonazepam	6.5
Chlordiazepoxide	6.6
N-Desmethyldiazepam (nordiazepam)	7.4
Flunitrazepam	7.9
Diazepam	10.9
Prazepam	21
Medazepam	24

*These drugs were measured at λ = 215 nm as they were not detected in our clonazepam assay at λ = 306 nm.

The main metabolites of clonazepam in plasma are 7-aminoclonazepam and to a lesser extent 7-acetamidoclonazepam [1] which had retention times of 3.0 and 3.2 min, respectively and were well resolved from clonazepam. In general the body metabolizes a drug by converting it into more polar compounds to facilitate excretion. These more polar metabolites are eluted earlier than the parent drug in the reversed-phase mode of chromatography. In our

assay these two metabolites are barely detectable because of their low absorbance at wavelength 306 nm.

The benzodiazepine chlordiazepoxide had a retention time of 6.6 min and was not able to be resolved from clonazepam under these chromatographic conditions. It is possible that on changing to a smaller-particle-size packing material (e.g. 5 μm) these two substances may be resolved. Using the same mobile phase we obtained good separation of clonazepam and chlordiazepoxide on a 5-μm C_{18} column obtained from Waters Assoc. It is unlikely that this would be of importance in clinical practice when an alternative benzodiazepine could be substituted for chlordiazepoxide. None of the other benzodiazepines tested interfered with the assay (see Table I). However, if large quantities of the diazepam metabolite, N-desmethyldiazepam (nordiazepam), were present it could interfere with the internal standard, flunitrazepam, the difference in retention time being only 0.5 min.

The described clonazepam assay has the distinct advantages of clean chromatograpy, adequate sensitivity, and routine monitoring application, thereby being a useful addition to the comprehensive monitoring of antiepileptic drugs used in clinical practice.

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Note

High-performance liquid chromatographic determination of a new anti-inflammatory agent, nabumetone, and its major metabolite in plasma using fluorimetric detection

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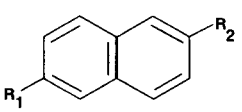
Nabumetone, 4-(6-methoxy-2-naphthyl)butan-2-one (I) (Fig. 1) is a new non-steroidal, anti-inflammatory agent that has excellent tolerance in fasting and non-fasting subjects [1]. The drug exhibits anti-inflammatory activity in acute and chronic animal models and has mild analgesic and anti-pyretic properties [2]. After oral administration of [^{14}C]nabumetone to three healthy male subjects, 80% of the radioactivity was recovered in urine and 9% in the feaces [3]. Intact nabumetone was not detected in plasma and the major labelled plasma component was 6-methoxy-2-naphthylacetic acid (II) (Fig. 1), a metabolite of nabumetone which has anti-inflammatory activity in animals [2].

This compound (II) is a more potent inhibitor of prostaglandin synthesis than nabumetone and although the mode of action of nabumetone is not known, the available evidence suggests that its activity resides in its metabolites [2].

Three other metabolites (Fig. 1), 4-(6-hydroxy-2-naphthyl)butan-2-one (III),



		R_1	R_2	RRT
4-(6-Methoxy-2-naphthyl)butan-2-one	(I)	OCH_3	$CH_2CH_2COCH_3$	1.10
4-(6-Hydroxy-2-naphthyl)butan-2-one	(III)	OH	$CH_2CH_2COCH_3$	0.57
4-(6-Hydroxy-2-naphthyl)butan-2-ol	(IV)	OH	$CH_2CH_2CHOHCH_3$	0.57
6-Methoxy-2-naphthylacetic acid	(II)	OCH_3	CH_2COOH	0.68
6-Hydroxy-2-naphthylacetic acid	(V)	OH	CH_2COOH	0.43
6-Chloro-2-naphthylacetic acid	(VI)	Cl	CH_2COOH	1.00
6-Methoxy-2-naphthoic acid		OCH_3	COOH	0.82

Fig. 1. Chemical structures and relative retention times (RRT) of nabumetone and its major metabolites. VI = internal standard.

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4-(6-hydroxy-2-naphthyl)butan-2-ol (IV) and 6-hydroxy-2-naphthylacetic acid (V) have been identified in human urine [3].

A gas chromatographic method for the determination of 6-methoxy-2-naphthylacetic acid in plasma lacks the sensitivity necessary for pharmacokinetic studies [1]. The present paper describes a high-performance liquid chromatographic assay (HPLC) with fluorescence detection, developed for the determination of nabumetone and its major metabolite (II) in plasma. This method has adequate sensitivity and specificity for single-dose pharmacokinetic studies.

MATERIALS AND METHODS

Chemicals and reagents

4-(6-Methoxy-2-naphthyl)butan-2-one (nabumetone, I, BRL 14777), 6-methoxy-2-naphthylacetic acid (II, BRL 10720), 6-chloro-2-naphthylacetic acid (VI, BRL 24333, internal standard) and other metabolites of nabumetone were obtained from Beecham Pharmaceuticals (U.K.). Stock solutions of nabumetone (10 mg per 5 ml), II (10 mg per 5 ml) and internal standard (4 mg per 10 ml) were prepared daily by dissolving the compounds in acetone.

Acetone (Hopkin and Williams, M.F.C.), hexane (Unichrom), ethyl acetate (Merck, GR), methanol (Unichrom), hydrochloric acid (Univar) and sodium acetate (Univar) were obtained from Ajax Chemicals (Sydney, Australia) and were used without further purification.

Extraction procedure

Plasma (0.5 ml) was placed in a 10-ml glass, screw-capped tube containing internal standard (1 μg for standard range 0.1—1 μg/ml and 50 μg for standard range 5—50 μg/ml). After thorough mixing on a whirlmixer, 6.0 ml of *n*-hexane—ethyl acetate (50:50) mixture and 0.7 ml of 1.5 mol/l hydrochloric acid were added. The closed tube was mechanically shaken for 30 min and then centrifuged for 10 min at 1500 *g*. The organic layer was transferred to a conical glass tube and evaporated to dryness at 37°C under a gentle stream of nitrogen. The sample residue was dissolved in mobile phase, centrifuged and 20-μl aliquots were injected into the chromatograph.

Apparatus and chromatographic conditions

Assays were carried out on a Varian 5000 liquid chromatograph (Varian, Sydney, Australia). Samples were injected with a Varian 8055 autosampler injector with a pneumatic-actuated Rheodyne 7126 injection valve (Reodyne, Berkeley, CA, U.S.A.) fitted with a 20-μl sample loop. A Schoeffel FS 970 LC fluorometer (Schoeffel, Westwood, NJ, U.S.A.) was used as the detector with the excitation monochromator set at 284 nm and the fluorescence emission at 320 nm (cut-off filter). Detector output was quantified using a 3390A integrator (Hewlett-Packard, Sydney, Australia). Calibration curves were constructed by calculating the ratio of the peak height of each compound (nabumetone or II) to that of the internal standard (VI).

Separations were performed on a 5-μm Ultrasphere ODS reversed-phase column (25 cm × 4.6 mm I.D.; Altex, Berkeley, CA, U.S.A.) with a Lichrosorb RP-8 guard column (Brownlee Labs., Santa Clara, CA, U.S.A.). The compounds

were eluted with a mobile phase of methanol in 0.05 mol/l sodium acetate buffer (pH 3.0), 70:30, v/v. The column temperature was 40°C and the flow-rate was 1 ml/min.

RESULTS AND DISCUSSION

Reversed-phase HPLC with fluorescence detection was an effective method of quantifying nabumetone and its major metabolite II in human plasma. Typical chromatograms obtained from blank plasma and plasma containing nabumetone, II and VI (internal standard) are shown in Fig. 2. Interference by endogenous substances did not occur from drug-free plasma (Fig. 2A). The three known metabolites of nabumetone (Fig. 1) were chromatographed as pure substances and did not interfere with nabumetone, II or the internal standard. Under the assay conditions described, naproxen, paracetamol, acetylsalicylic acid and salicylic acid had elution times of 7.0, 3.1, 3.1 and 3.1 min, respectively, and did not interfere with the nabumetone, II or internal standard peaks. However, acetylsalicylic acid and salicylic acid, at concentrations above 100 μg/ml, may interfere with the II peak due to the intense fluorescence of these compounds.

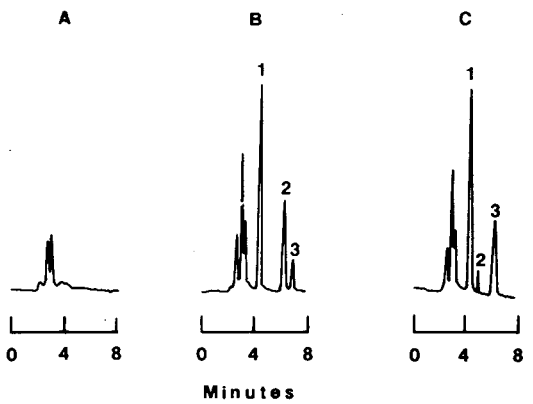

Fig. 2. Chromatograms of plasma extracts. (A) Blank human plasma; (B) human plasma containing 1 μg/ml II (1), 2 μg/ml internal standard VI (2) and 1 μg/ml nabumetone (3); (C) plasma collected 5 h after 500 mg nabumetone were administered orally to a male subject; II (1), 6-methoxy-2-naphthoic acid (2) and internal standard VI (3).

The standard curves for nabumetone and II in plasma were linear over the concentration range 0.25—50 μg/ml and 0.1—50 μg/ml, respectively. The correlation coefficient for the standard curves over this concentration range was 0.999 (n=4). The coefficient of variation was < 6% for all concentrations measured (Table I). The sensitivity of the method was approx. 0.1 μg/ml of plasma for II and 0.25 μg/ml of plasma for I. The recovery for nabumetone was 92 ± 4% (mean ± S.D.), for II, 93 ± 4% and for VI (internal standard) 90 ± 4% (Table I).

The concentration of II, was measured in plasma after a single 500-mg oral dose of nabumetone (Fig. 3). Intact drug (nabumetone) was not detected in plasma for 72 h after administration of the compound, which is in agreement with a previous study performed using [^{14}C]nabumetone [2]. After the oral

TABLE I

REPRODUCIBILITY AND RECOVERY OF STANDARDS EXTRACTED FROM HUMAN PLASMA (n=4)

Concentration (μg/ml)	Coefficient of variation (%)		Recovery (%)	
	Nabumetone	II	Nabumetone	II
0.10	N.D.*	3.8	N.D.	93
0.25	5.9	2.0	99	94
0.50	4.0	1.8	90	99
0.77	4.7	1.3	94	93
1	1.6	1.2	92	94
5	4.8	3.7	91	94
10	4.6	3.6	89	87
25	4.6	3.5	90	90
33	3.3	1.1	88	89
50	1.0	1.9	97	96

*N.D. = not detected.

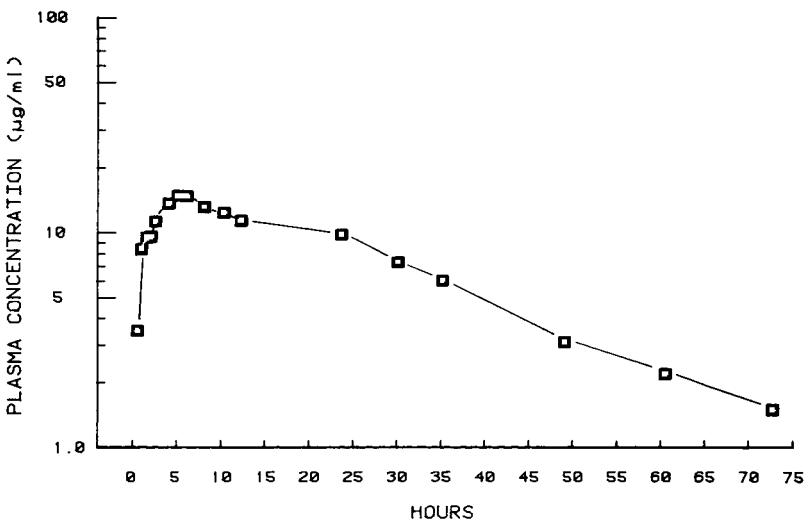

Fig. 3. Plasma concentrations of II after a single 500-mg oral dose of nabumetone.

administration of nabumetone, a peak that had not been detected in a previous study [1] appeared in plasma with a retention time of 5.2 min (Fig. 2C). This peak was identified as 6-methoxy-2-naphthoic acid, a known metabolite of nabumetone (Fig. 1), by collecting the HPLC fraction containing the unknown peak and subjecting this fraction to gas chromatographic—mass spectrometric analysis. This metabolite has little anti-inflammatory activity [4] and does not reach significant concentrations in plasma after a single oral dose.

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