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Review

High-performance liquid chromatography-based assays of enzyme activities

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Abstract

Interest in using HPLC to assay enzymatic reactions continues to grow as evidenced by the more than 100 papers published during the early 1990s. HPLC can be used for any enzymatic assay that requires separation of substrates and products before quantifying the extent of the reaction. The popularity of HPLC-based assays is due to several reasons: (1) HPLC offers unsurpassed precision, specificity, sensitivity, and reproducibility. (2) Powerful microcomputers and user-friendly software automate the running of samples and collection and processing of data. (3) Current columns, especially C_{18} packings, separate a very wide variety of samples, and (4) A variety of on-line detectors provide a means to detect virtually any compound. This review surveys recent papers on the development of HPLC-based assays for enzymes that degrade or otherwise modify macromolecules. Methods for assaying enzymes involved in metabolic pathways are also reviewed. Work by the authors in developing HPLC-based assays for mitochondrial enzymes that use GTP/GDP and other nucleotides that cannot be or are not easily assayed by enzyme-coupled assays is discussed. These enzymes include nucleoside diphosphate kinase, succinate thiokinase, and GTP–AMP phosphotransferase. The assays are suitable for determining the submitochondrial compartmentation of enzyme activities. Finally, current and anticipated trends in HPLC technology, including new column packings and the trend toward smaller columns that give faster separations, are reviewed in relation to enzyme assays.

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1. Introduction

The first description of a system for high-performance liquid chromatography (HPLC) was published by Horváth and Lipsky in *Nature* in 1966 [1]. In September of that same year, Horváth described his new system at a specially arranged discussion of liquid chromatography at the Sixth International Symposium on Gas Chromatography and Associated Techniques held in Rome [2]. In the following 27 years, the equipment for HPLC has become a part of the standard equipment of many research, clinical, and quality control laboratories. The complexity of biological systems has provided a strong impetus for applying HPLC to problems in biochemistry and medicine, and early applications were described by Brown [3] and Krstulovic and Brown [4].

In retrospect, it is not surprising that investigators rapidly extended the applications of HPLC from detection and quantitation of the myriad of compounds found in biological systems to the study of the enzymes involved in their metabolism. The first papers on the use of HPLC in the assay of enzymes appeared in the late 1970s. The number of enzymes now assayable by HPLC totals several hundred. The rationale and strategies for using HPLC are described in detail in a book by Rossomando [5] and in several shorter works [6–11]. The scope of this review will be restricted to papers published in the 1990s and will include some of our work on using HPLC to study the activities and compartmentation of kinases and acyl-CoA synthetases in mitochondria [12].

2. General comments on the use of HPLC-based enzyme assays

2.1. When to use HPLC in assaying an enzyme

The essence of any enzyme is its ability to catalyze the chemical conversion of a substrate to a product. Enzymologists have been extraordinarily clever in devising ways to follow an enzymatic reaction in a crude system [13]. Whenever a product of the enzymic reaction has an absorption spectrum distinctly different from that of the substrates, the progress of the reaction can be followed continuously by monitoring the absorbance at a convenient wavelength. Early development of stable and sensitive spectrophotometers allowed this approach in the study of dehydrogenases. By using coupling enzymes in which dehydrogenases played the role of indicator reactions, continuous spectrophotometric assays were soon extended to those kinases that use ATP or ADP as substrates. Thus the rate of ADP synthesis was followed by the pyruvate kinase/lactate dehydrogenase coupled enzyme system in which the rate of NADH oxidation was the indicator reaction. In like manner, the rate of ATP formation was followed by the hexokinase/glucose-6-phosphate dehydrogenase system in which NADPH formation was followed. Such assays were a boon to the early development of enzymology because they carried the prime advantage of monitoring the reaction continuously. One of the major disadvantages was a lack of specificity because changes in absorption at 340 nm could occur for reasons other than the primary reaction under study.

For many enzymatic reactions, however, the product (or substrate) cannot be reliably quantitated while in the reaction mixture and therefore the reaction cannot be monitored continuously. Such a circumstance requires that a discontinuous assay be used in which the reaction is allowed to proceed for a predetermined interval and then terminated by a suitable method. A subsequent procedure that results in a colorimetric reaction, for example, may allow quantitation of a reaction component in the terminated reaction mixture. However, it is often desirable, if not mandatory, to separate reactants and products before quantifying the extent of the reaction. In earlier times, bioscientists used techniques such as electrophoresis or one of several chromatographic techniques (thin layer, paper, or liquid) to achieve the separation. Many of these assays have since been converted to HPLC-based assays. For any reaction in which it is desirable to separate substrates and products before quantifying the extent of reaction, HPLC

will often be the method of choice for conducting the assay.

2.2. Components of a HPLC system

The hardware of a typical HPLC system has changed little for more than a decade. Descriptions of such systems can be found in refs. 4 and 8. It should be noted that these systems were optimized for columns that are considerably larger than those likely to be used in the future. Although few reports of the applications of new columns to enzyme assays have appeared in the peer-reviewed journals, trade journals and reviews of recent meetings on applications of HPLC indicate that the field is now poised for major innovations. Columns that are shorter and designed for faster flow-rates will require the redesigning of HPLC hardware so that extra-column broadening of peaks is minimized. Thus a new generation of HPLC systems, called "micro LC" by some workers in the field, is on the horizon.

The most striking advances in HPLC during the early 1990s have been in automation, both with respect to the mechanics of running samples and the collection and processing of chromatographic data. Many of these advances are due to developments in microprocessor technology that have made powerful "personal computers" available at very low cost. Such computers can control all of the hardware components of the system, including the autosampler and any devices that may be needed to add a derivatizing agent, either pre- or post-column. Some autosamplers now available can be programmed to perform premixing operations before injecting the sample. Of special interest with regard to automation are the reports on robotic generation of samples [14] and automatic processing of data [15].

The same computer that controls hardware can be used to electronically record the data coming from the detector. Software written expressly for this purpose has rapidly improved, becoming more "user-friendly", thus making it increasingly easy to run a large number of samples automatically. The data collected by computer can be imported into spreadsheets for further analysis and the generation of secondary plots. The capability of automating sample analysis and data processing gives HPLC-based assays a decided edge over many other assay methodologies.

For more than a decade, the 250×4.6 mm I.D. column filled with 5-μm, C_{18} bonded to silica particles has been the most widely used column in analytical HPLC. The popularity of this column is due to the nearly universal role it serves in the separation of biologically-important molecules. Near universality comes about because chromatography can not only be carried out in the reversed-phase mode, which is especially useful for separation of uncharged, hydrophobic molecules, but also in the ion-pairing mode in which the retention of charged, amphipathic species is promoted by adding a large organic ion of the opposite charge to the mobile phase. Thus tetrabutylammonium ion is often used for separating anions while octyl sulfonate serves the same role for cations.

The variability in C_{18} columns, both between lots as well as manufacturers, is well-known among those who work with HPLC. This variability often makes it necessary to modify a published procedure. A nice documentation of the magnitude of column variability is found in the work of Hicks *et al.* [16] who demonstrated major differences in the columns from three different manufacturers in terms of their ability to separate uric acid and its oxidized forms. This variability was concluded to be the result of differences in the completeness of "end-capping", the process for derivatizing unreacted silanols left over after chemically bonding C_{18} groups to silica. The better-performing columns had less endcapping, thus demonstrating the role that unreacted silanols can play in some separations.

Major innovations in the columns that are used in enzyme assays may soon occur. For example, the concept of "perfusion chromatography" introduced by Regnier [17] has resulted in media with a variety of surface chemistries that are useful in the separation of enzymes, peptides, and nucleic acids. These media are

composed of wide-pore supports so that rates of intraparticle convection exceed the rate of diffusion. Separation time has been reduced to under three minutes, which is an order of magnitude less than that achieved with conventional media. Columns based on hydrophilic-interaction chromatography are discussed below.

Improvements in many methods could be achieved by using shorter columns containing packings of smaller particle size. A paper by Hopkins *et al.* [18] demonstrates what can be accomplished with regard to decreasing analysis time. They used a synthetic, fluorescein-labeled peptide as a substrate for the rhinovirus 3C protease. Only 2.5 min were required for separation of this peptide from its cleavage product on a 33 × 4.6 mm I.D. column packed with 3-μm C_{18} particles. However, a 15 × 4.6 mm I.D. column containing a 2-μm, C_8 packing allowed a useful separation in just 12 s, as demonstrated in Fig. 1. Further advances can be expected as 1-μm packings are being tested. A move toward narrower columns is also underway, driven by the need to conserve solvent and reduce waste disposal.

The popularity of C_{18} columns has strongly blunted the incentive to try other chemistries. This is particularly unfortunate in situations where a marginal separation on C_{18} is tolerated. Alpert [19] has coined the term "hydrophilic-interaction chromatography" to describe the use of a neutral, hydrophilic stationary phase with a hydrophobic mobile phase. This chromatography is in principle an example of what has long been called "normal-phase chromatography". Hydrophilic-interaction chromatography is inherently appealing because of its complementary nature to reversed-phase chromatography, which is, of course, hydrophobic-interaction chromatography. Thus a separation that is unsatisfactory with one mode of chromatography becomes a candidate for the other mode. One packing developed for hydrophilic-interaction chromatography is poly(2-hydroxyethyl aspartamide)-silica, which is typically used with a mobile phase containing a much higher concentration of organic solvent than is used for reversed-phase chromatography. Species elute in the order of increasing hydrophilic properties, and therefore in the reverse order of what is generally observed in reversed-phase chromatography. The direction of an applied gradient is from high to low percentage of organic solvent. A comparison of the two modes of chromatography in the separation of a mixture of peptides is shown in Fig. 2.

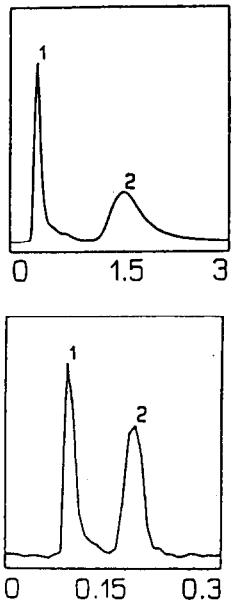

Fig. 1. Separation of a fluorescein-labeled, 14-amino acid peptide (peak 2) used as a substrate for *rhinovirus* 3C protease from its fluorescein-labeled, octapeptide product (peak 1). (Top) Separation on C_{18} column (33 × 4.6 mm I.D., 3 μm particles, flow-rate of 3 ml/min, 75 μl injected, ambient temperature). (Bottom) Separation of the same peptides on a M-pel C_8 column (15 × 4.6 mm I.D., 2 μm particles, flow-rate of 4 ml/min, 1 μl injected, 80°C). From ref. 18 with permission.

Hydrophilic-interaction chromatography is in the early stages of development and considerable experimentation will likely occur with various packings. One group [20] has shown that there may be advantages to using a mixed mode of separation in which both ion exchange and hydrophilicity will be important. A packing suitable for mixed-mode separations is silica-based polysulfoethylaspartamide. Hydrophilic-interac-

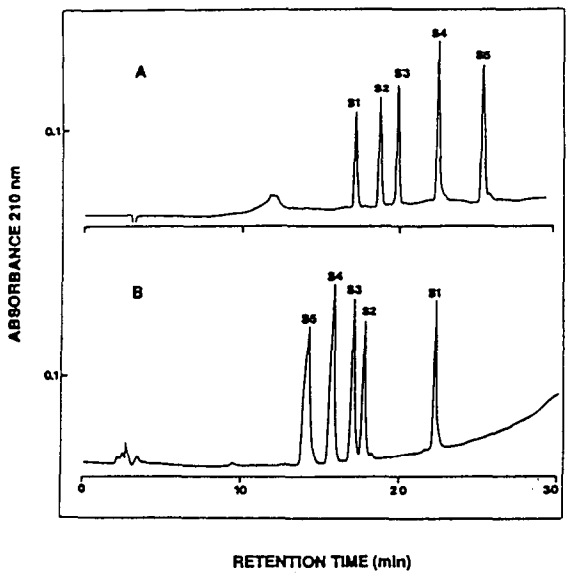

Fig. 2. Separation of a mixture of synthetic positively charged peptides by (A) hydrophobic-interaction (reversed-phase) chromatography, and (B) hydrophilic-interaction chromatography. Note that the order of peaks is reversed in (B) as compared to (A). In (A), the column was a SynChropak RP-P C_{18} (250 × 4.6 mm I.D.) column; mobile phase was a linear A–B gradient (increasing 1%B/min) where A was 0.05% aqueous TFA and B was 0.05% TFA in acetonitrile; temperature 26°C, flow-rate 1 ml/min. In (B), the column was a polyhydroxyethylaspartamide (200 × 4.6 mm I.D.) column; mobile phase was a linear A–B gradient (decreasing 1% B/min) where A was 0.2% orthophosphoric acid in acetonitrile and B was 0.2% orthophosphoric acid. Starting conditions with 85% A and 15% B; flow-rate of 1 ml/min; temperature, 26°C. From ref. 20 with permission.

tion chromatography should be applicable to separation of those substances that are poorly retained on reversed-phase columns. Thus far it has been used in the separation of charged peptides [19,20], phosphorylated peptides and amino acids [21], oligosaccharides [22], and oligonucleotides [19]. Hydrophilic-interaction chromatography has been used in the assay of ATPase activities where separation of $^{32}P_i$ from [γ-^{32}P]-ATP was required [23].

A major strength of HPLC as a tool to assay enzymes resides in the alternatives available for on-line, flow-through detection. There are at least five major alternatives in present use: spectrophotometric, fluorimetric, electrochemical, radiometric, and refractive index. Which of these detectors to use is largely dictated by the nature of the substances being separated. Capabilities to automate pre- and post-column derivitization, either chemical or enzymatic, has extended the range of reactions that can be followed. Likewise, post-column mixing of scintillation fluid with the column eluate improves the sensitivity of radiometric detection. In a number of cases, femtomol sensitivity has been attained with electrochemical, fluorimetric, and radiometric detectors. Detectors based on chemiluminescence may lower detection limits even farther, by up to two orders of magnitude. Widespread applications involving the coupling of HPLC and mass spectrometry appear likely.

3. The use of HPLC in assaying enzymes–examples from the 1990s

Approximately 100 papers on HPLC methods for the assay of enzymes have been published in the past three years. Many of these papers are indicated in Tables 1 and 2. Readers should refer to the original papers for details concerning the enzyme assays listed in the Tables. Here, some general impressions of the field obtained by reviewing these papers will be given.

3.1. Current trends and practices

Many of the methods for assaying enzymes by HPLC have been devised because of a need for increased sensitivity, specificity, versatility, or automation. Other reasons for using HPLC, based on statements by the authors in the papers reviewed, include simplification of procedures, decreased analysis time, avoidance of the expense and hazards of using isotopes, elimination of coupling enzymes, use of alternative detection methods, ability to assay turbid samples, use of alternative substrates, and reduced need to purify enzymes since secondary reactions can be detected and quantified. The power and versatility of HPLC has become increasingly important as bioscientists have increasingly turned their attention to the study of minor pathways, major

Table 1
HPLC-based assays of enzymes that modify macromolecules

Macromolecule	Substrate	Ref.
Proteins/peptides		
HIV-1 protease	Nitrophenylalanine peptides	51
HIV protease	Dansyl-septapeptide	52
Renin	N-(2-pyridyl)glycine peptide	24
Endothelin-converting enzyme	^{125}I-endothelins	53
Rhinovirus 3C protease	Fluorescein 14-mer peptide	18
Poliovirus 3C protease	16-mer peptides	54
Proline iminopeptidase	Pro-Gly-Gly	55
Prolidase	X-Pro dipeptides	56
ADP-ribosylarginine hydrolase	ADP-R-dabsyl-arginine methyl ester	57
Peptidyl α-amidating monoxygenase	Dabsyl-tripeptide	58
Transglutaminase	Benzyloxycarbonyl and dansyl deriv.	59
Protein kinase C	14-mer peptide	60
Tyrosine protein kinase	Angiotensin II	61
Myosin light chain kinase	13-mer peptides	62
Phosphotyrosine phosphatase	Dansyl-phosphopeptide	63
Polysaccharides/glycoproteins		
Glycosidases	Disaccharides	29
Sialyltransferase	Asialoglycoprotein	64
α-L-Fucosidase	*p*-Nitrophenyl-2-L-fucopyranoside	65
Mannosidase	Manβ(1-4)GlcNAc	66
Aspartylglycosylaminase	Aspartylglucosamine	67
trans-p-Coumaroyl esterase	Plant cell wall product	68
Nucleic acids		
DNA topoisomerase	pBR329 plasmid	25

pathways operating in scarce tissues or cells, modifications of macromolecules, regulatory mechanisms, and metabolism of xenobiotics. Such studies often require a discontinuous assay, and a micromethod in which the reactions are followed in microliter rather than milliliter volumes. Some enzymic reactions of current interest occur at a slow rate thus requiring either a long reaction time or a very sensitive detection system. HPLC is highly compatible with both requirements. Still another point in favor of HPLC-based assays is the capability of automating virtually all of the steps of the analysis. Although HPLC-based assays suffer the major disadvantage of being discontinuous in nature, this is offset somewhat by the capability of generating tens of samples in less than an hour, which can then be analyzed overnight. As a result it is feasible to obtain and analyze multiple samples to follow the time course of a reaction. The ability to generate multiple samples under the same conditions and within a short time is of obvious importance in the study of an unstable enzyme.

In reviewing the recent literature, current methods being used to terminate enzymic reactions were tallied. Addition of acid was used in *ca.* 60% of the papers surveyed. This method terminates most enzymic reactions by inactivating (denaturing) the enzyme and also, in many cases, by changing the pH of the mixture so that the rate of catalysis becomes insignificant. Perchloric or trichloroacetic acid was used in 64% of the protocols with other acids used including (in decreasing frequency) hydrochloric, acetic, trifluoroacetic, phosphoric, and formic acids. Other commonly used termination methods included heating (10%), cooling (6%), and addition of an

Table 2
HPLC-based assays of enzymes in metabolic pathways

Pathway	Ref.
Amino acid metabolism	
Threonine/serine dehydratase	69
Alanine/glyoxylate aminotransferase	32
Tryptophan 2,3-dioxygenase	40
Betaine:homocysteine methyl transferase	70
Methionine synthase	71
D-Amino acid oxidase	72
Aromatic L-amino acid oxidase	39
Tyrosine hydroxylase	73
Heme biosynthesis	
δ-Aminolevulinic acid synthase	74
Porphobilinogen deaminase	75
Uroporphyrinogen decarboxylase	76
Protoporphyrin oxidase/ferrocheletase	77
Purine/pyrimidine metabolism	
Dihydropyrimidine dehydrogenase	78,79
Dihydroorotate dehydrogenase	80
Guanase	81
Xanthine oxidoreductase	31
β-Ureidopropionase	82
Nucleotide metabolism	
AMP deaminase	83
cAMP phosphodiesterase	84
NAD pyrophosphorylase	85
ATPase	23
Nucleoside diphosphate kinase	12
Nucleotide sugar metabolism	
Glucose-1-P thimidylyltransferase	86
UDP-glucuronyl transferase	87,88
Glycosyltransferases	29
UDP-glucose requiring enzymes	89
Lipid metabolism	
Fatty acid ω-hydroxylase	90
Lipase	91,92
Retinal oxidase	93
Cholinesterase	94
Choline dehydrogenase	95
Acyl-CoA:alcohol transacylase	96
Steroids	
Aromatase	97
Sterol δ-14-reductase	98
5α-Steroid reductase	99
Lanosterol 14α-methyl demethylase	100
Amines	
Polyamine oxidase	101
Histamine N-methyl transferase	102
Phenylethanolamine N-methyl transferase	103
Arylamine N-acetyltransferase	37,38
Monoamine oxidases	33,35,36
Miscellaneous	
Cytochrome P450	43,104
L-Myoinositol 1-phosphate synthetase	105
Phenolsulfotransferase	106
Carbonyl reductase	107
Biocytin synthetase	108
Carboxylases	109
Phosphoribosylpyrophosphate synthetase	110
6-Pyruvoyl tetrahydropterin synthetase	75
Thiamine triphosphatase	111
Pyridoxine kinase	112
Ethoxycoumarin O-deethylase	113

organic solvent (8%) or KOH (3%). Some investigators terminated the reaction by simply injecting the sample onto the column. Additional methods reported included addition of inhibitors, precolumn derivitization (particularly when needed for detection), and dilution.

The methods used for detection in the papers surveyed were absorbance in the ultraviolet or visible range (50%), fluorescence (31%), electrochemical (13%), radiometric (10%) and refractive index (1%). The total percentage exceeds 100% because two detectors were sometimes used in series, especially in the case of radiometric detection. Radiometric techniques usually involved on-line, flow-through detectors in which scintillation fluid was mixed with the stream of post-column eluate. However, Cerkenov radiation was sometimes used for detection of ^{32}P isotopes. Diode array detectors are being increasingly used for monitoring absorbance in the UV-Vis region.

3.2. Using HPLC to assay enzymes that process macromolecules

HPLC is being increasingly used to characterize the reactions that result in the modification of

macromolecules. Recent examples are listed in Table 1. Perhaps the most active area is the study of proteases, which are involved in processing of proteins, generation of peptides with powerful biological activities, activation of proenzymes, and normal turnover and remodeling of cellular constituents. A major effort is being expended on finding inhibitors of protease activities involved in the maturation of viruses; this effort is particularly relevant to finding a means to deal with human immunodeficiency viruses (HIV).

HPLC is a particularly powerful tool in the study of proteases because of its abilities to quickly separate similar peptides and to provide accurate quantitation. As can be seen in Table 1, most enzymes involved in modification of macromolecules are assayed by using synthetic substrates that contain a suitable chromophore or fluorophore for detection. For example, an assay for renin uses a nonapeptide substrate containing N-(2-pyridyl)glycine. Both the substrate and the N-terminal fragment containing the fluorophore are detected by fluorescence [24]. The fluorophore serves to increase the sensitivity of detection while providing a relatively clean chromatogram in comparison with one that would be obtained by following UV absorbance at low wavelength, for example, 210 nm. The synthetic substrates used in most assays tend to be smaller than the natural substrates.

Few examples show the application of HPLC to the study of nucleic acids, an area which continues to be dominated by electrophoresis as the commonly-used separation technique. This could change with the advent of new chromatographic media such as those used in hydrophilic-interaction chromatography. Onishi *et al.* described the use of HPLC and a DEAE column to assay DNA topoisomerase by quantifying the amounts of the supercoiled and relaxed forms of pBR329 plasmid [25]. The two forms were resolved within 25 min by using a shallow salt gradient. Two reports describing "slalom chromatography" for the size-dependent separation of nucleic acids [26,27] have not yet resulted in an application to the enzymology of nucleic acids.

An example of the use of HPLC to study gene expression is the paper by Waldon *et al.* [28], in which they devised a HPLC-fluorescence assay for chloramphenicol acetyltransferase. This assay, designed to quantitate the activity of the keratin gene promoter to which the gene for the transferase was fused, was sufficiently sensitive to measure the enzyme activity in a single hair follicle. A similar approach can be used for assay of other promoter activities.

Willenbrock *et al.* [29] report further refinements in the use of triple-pulsed amperometry to quantitate sugars eluting from a HPLC anion-exchange column under alkaline conditions. The method should find use with a number of carbohydrate-metabolizing enzymes including glycosidases and glycosyltransferases.

3.3. Using HPLC to assay enzymes in metabolic pathways

The study of nearly all metabolic pathways has been greatly augmented by HPLC-based assays of enzymatic activities. Recent papers on enzymes metabolizing small molecules are listed in Table 2. The following paragraphs will highlight a few of these papers as well as cite other recent publications with the goal being to illustrate the power and utility of HPLC in enzyme assays.

A major strength of HPLC is that it readily lends itself to assays where the enzyme source is scarce. A paper by Li *et al.* [30] describes the assay of diphenol oxidase in mosquitoes. As little as 5 μg of hemolymph protein was needed per assay, which was important since 100 mosquitoes were required to obtain 25 μl of hemolymph. Femtomol sensitivity was attained for the assay of xanthine oxidoreductase so that the enzyme could be assayed in as little as 20 ng of rat heart [31]. An assay for alanine:glyoxylate aminotransferase could be carried out with 10 μg of liver [32]. With tissues that require homogenization, the minimum amount of tissue required is often determined by the methods used for processing the tissue rather than the amount needed for assays by HPLC [33].

A HPLC based assay for α-ketoglutarate dehydrogenase detected the succinyl-CoA formed

directly and could be used to determine enzyme activity in crude homogenates. The sensitivity of the assay makes it feasible to screen for deficiency of this important Krebs cycle enzyme in human platelets as an alternative to liver or heart [34].

The importance of arylamines and their metabolizing enzymes in medicine underlies continued attention to the development of better assays [35–38]. The paper by Freeman *et al.* [35] is exemplary in terms of the thoroughness of the work characterizing and optimizing assays for monoamine oxidases A and B. They describe conditions under which both activities can be assayed simultaneously, and yet only two minutes are required for the HPLC separation, as shown in Fig. 3. HPLC readily lends itself to the use of multiple substrates in a single assay. In another example of using dual substrates in a single assay, 3,4 dihydroxyphenylalanine and 5 hydroxytryptophan were found to be metabolized in constant ratio in a number of rat tissues. The authors concluded that one enzyme, L-amino acid decarboxylase, metabolized both substrates [39].

Studies of metabolic activities in liver slices were very important in elucidating major metabolic pathways. The use of slices fell out of fashion once techniques were developed for homogenizing tissues and isolating subcellular organelles. However, Seifert [40] recently used HPLC to assay tryptophan 2,3-dioxygenase in liver slices. Accurate assay of this enzyme requires measurement of both N-formyl-L-kynurenine and its hydrolysis product, L-kynurenine. The use of slices eliminated the need to add methemoglobin or ascorbic acid to activate the dioxygenase, perhaps because of the greater structural integrity of slices as compared to homogenates.

Chromatographic techniques have long been used to identify and quantitate intermediates in metabolic pathways. An extension of such studies can allow, in principle, determination of the activities of some of the enzymes involved. Recent examples where HPLC was used to assess one or more enzyme activities in a metabolic pathway follow.

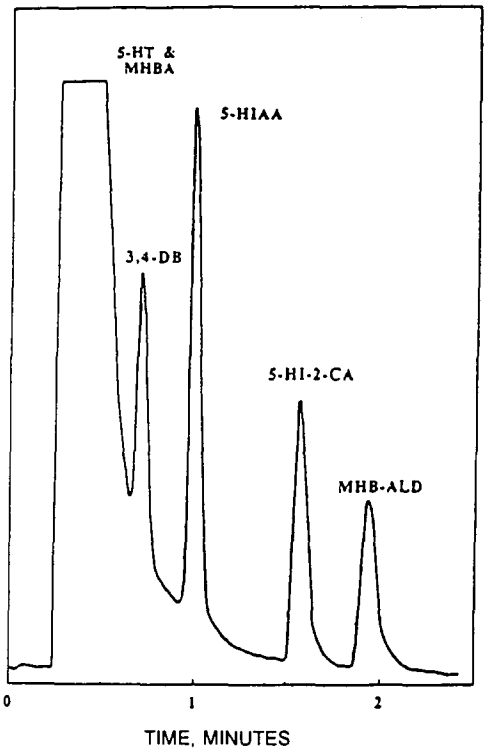

Fig. 3. Dual assay for monoamine oxidase A and monoamine oxidase B. Substrates (5-HT and MHBA) were separated from products (5-HIAA and MHB-ALD) and internal standards (3,4-DB and 5-HI-1-CA) on a 33 × 4.6 mm I.D., 3 μm reversed-phase ODS cartridge from Perkin-Elmer. A 5-μl volume was injected and the flow-rate was 1.5 ml/min. The mobile phase contained 0.10 *M* citric acid, 0.50 m*M* tetrapentylammonium chloride, 50 μM EDTA and 12.5% acetonitrile. From ref. 35 with permission.

Tordjman *et al.* [41] devised a method for separating 18 metabolites of arachidonic acid in a single run requiring 90 min. This method was used to determine the relative effects of drugs on the cyclooxygenase and lipoxygenase pathways within cells and tissues. Ziegler *et al.* [42] used HPLC to evaluate the metabolism of nucleosides by isolated mitochondria and found that they are degraded rather than converted to nucleotides by phosphorylation. HPLC is an excellent tool for elucidating the metabolism of pharmacologically-active compounds, a recent example being that of a dihydropyridine derivative [43]. In the β-oxidation of long-chain fatty acids, intermediates are generally thought to remain bound to the

oxidative enzymes as they are progressively shortened. Broadway *et al.* [44] used HPLC to show that significant concentrations of acyl-CoA intermediates accumulated during oxidation of fatty acids by a *Corynebacterium* sp., thus bringing into question whether tight channelling occurs in that species. Problems with heme arise in a number of medically important ways, both inherited and environmental. Several papers dealt with measurement of specific enzymes in heme biosynthesis (see Table 2).

4. Application of HPLC to the study of GTP/GDP-utilizing kinases and acyl-CoA synthetases

A very early paper by Horváth *et al.* [45], described the separation of nucleotides. Some of the earliest papers on using HPLC in enzyme assays involved nucleotide metabolism. Yet, HPLC-based assays have not been widely adopted for assaying kinases, probably because "coupled enzyme" assays were firmly entrenched by the time HPLC became available. Although lacking the continuity inherent in coupled enzyme assays, HPLC-based assays offer more direct visualization of the reaction and better monitoring of the secondary reactions that may occur. In addition, coupled enzyme assays are of no or limited utility when non-adenine nucleotides are to be quantitated, the principle exception being the ability to follow GDP formation by the pyruvate kinase/lactate dehydrogenase system. Here we will describe some of our experiences in using HPLC-based assays for enzymes found in mitochondria that use non-adenine nucleotides.

HPLC-based assays of enzymes were first used in our laboratory in work with the mitochondrial and cytosolic isozymes of phosphoenolpyruvate carboxykinase in rabbit liver [46,47]. This enzyme in animal species uses GTP/GDP. The enzyme is readily reversible and its activity can be followed by measuring either the GTP or GDP produced. HPLC-based assays were used to study the apparent activation of the purified isozymes of phosphoenolpyruvate carboxykinase by ferrous iron, an ion whose autoxidation leads to spectrophotometric changes that interfere with coupled enzyme assays. The HPLC-based assays permitted the effects of metal activation on both directions of catalysis to be studied under very similar conditions since no coupling enzymes were used.

The authors have since developed HPLC-based assays for several enzymes that are usable with a variety of preparations ranging from intact mitochondria to mitochondria that have been partially disrupted by various means including digitonin treatment and degradation by trypsin. These enzymes include nucleoside diphosphate kinase, succinate thiokinase, adenylate kinase, GTP–AMP phosphotransferase, and several phosphatase activities including nucleoside diphosphatase and ATPase. The capability of using HPLC-based assays with a variety of mitochondrial preparations has allowed us to study the submitochondrial compartmentation of selected enzymes.

Nucleoside diphosphate kinase provides an excellent example of a kinase that is conveniently assayed by HPLC. It is a relatively nonspecific enzyme catalyzing the transfer of a phosphoryl group from a nucleoside triphosphate to a nucleoside diphosphate: $N_1TP + N_2DP \leftrightarrow N_1DP + N_2TP$. In most cellular compartments, this enzyme serves the general role of using ATP to phosphorylate various nucleoside diphosphates, thereby forming the nucleotides needed for DNA and RNA synthesis and various metabolic reactions. However, a somewhat different role has long been assumed for nucleoside diphosphate kinase in mitochondrial matrix. It has become textbook dogma that nucleoside diphosphate kinase uses GTP produced by succinate thiokinase in the Krebs cycle to phosphorylate ADP. This explanation seems too simple because it provides no rationale for why the specificity of succinate thiokinase switched from ATP/ADP to GTP/GDP in later evolving species. Thus it seems likely that the matrix form of nucleoside diphosphate kinase serves a more complicated role than one of merely equilibrating the guanine and adenine nucleotide pools. The HPLC-based assay described below is being used to study the

nucleotide specificity and kinetic properties of the matrix isoform of nucleoside diphosphate kinase present in pigeon liver. Prior studies of mitochondrial isoforms have likely involved an isoform located either on the outer membrane or in the intermembrane space.

Interest in nucleoside diphosphate kinase has been revived by the discovery that the enzyme is associated with G-proteins [48], and the finding that the product of oncogene nm23 has nucleoside diphosphate kinase activity [49]. An HPLC-based assay for nucleoside diphosphate kinase that replaces older radiometric assays should be useful in a number of areas.

The HPLC assay developed for nucleoside diphosphate kinase uses ion-pairing, reversed-phase chromatography on C_{18} to resolve any two pairs of nucleoside di- and triphosphates. The nucleotides are eluted isocratically in a mobile phase containing phosphate, acetate, tetrabutylammonium ion, and acetonitrile. The pH of the mobile phase can be varied from 4 to 5 to optimize separation of particular pairs of nucleotides [12]. An example of the resolution that can be easily achieved for guanine and adenine nucleotides is shown in Fig. 4. The enzymatic reaction is terminated by lowering the pH to 3 or less with either formic or hydrochloric acid. Aliquots of the terminated reaction are injected directly into the HPLC system without centrifuging the samples to remove protein. Even without removing protein, the analytical column can be used for many months, if the guard column is repacked after *ca.* 300 analyses.

For routine assays of NDPK, UDP and ATP are used as substrates [12]. The formation of UTP is unlikely to occur by any other reaction. Because of the interference by ATPase and other phosphatases, quantitation is based on the percent conversion of UDP to UTP formed rather than the conversion of ATP to ADP These interfering activities are obviously a problem in any other assay method, but HPLC offers a more convenient way to evaluate the seriousness of the problem. The relatively high concentrations of both substrates that are present throughout the assay tend to saturate the enzymes responsible for secondary reactions and thereby protect the product (UTP) from degradation.

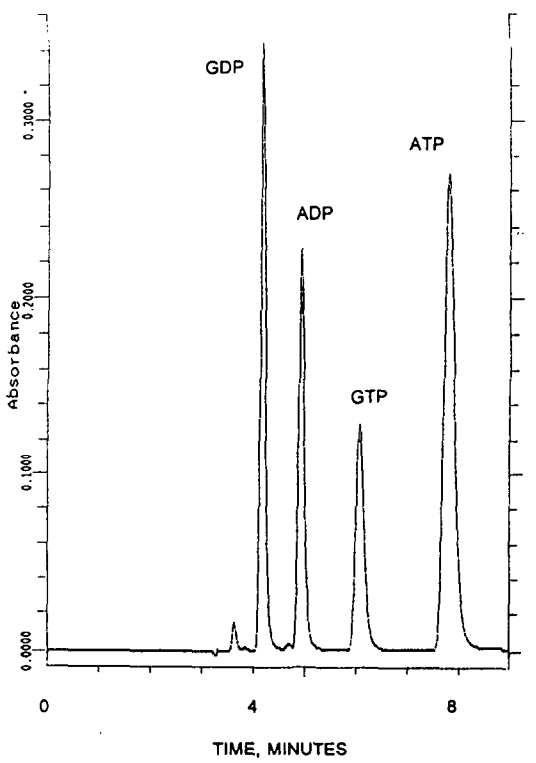

Fig. 4. Separation of a mixture of adenine and guanine nucleotides on a Beckman 250 × 4.6 mm I.D., 5-μm C_{18} Ultrasphere IP column. The mobile phase consisted of 100 m*M* phosphate (Na^+), 20 m*M* acetic acid, 5 m*M* tetrabutylammonium hydroxide, 10% acetonitrile, pH adjusted to 5.0 with NaOH. The flow-rate was 1.0. ml/min.

HPLC-based assays of nucleoside diphosphate kinase have been particularly useful in studying the compartmentation of this enzyme in mitochondria from heart and liver of pigeon, rat, and rabbit (Lambeth and Muhonen, unpublished). Advantages of HPLC-based assays include avoidance of problems associated with light-scattering, more direct accounting of such interfering activities as adenylate kinase, ATPase, and nucleoside diphosphatase since all nucleotides involved in the reaction are quantitated, and the ability to use the same assay procedure with any of several combinations of substrates. When assaying unpurified preparations, substrates can

be chosen with the objective being to minimize interferences by secondary reactions. HPLC-based assays carried out with mitochondria in the presence and absence of CHAPS (a detergent used in sufficient concentration to disperse all membranes) provide information on the membrane barriers that prevent added substrates from reaching the enzyme. Our results indicate that compartmentation of nucleoside diphosphate kinase is highly variable, depending on tissue and species. For example, this enzyme shows little if any latency in pigeon heart mitochondria where it is presumably located outside the inner membrane. In contrast, the activity is highly latent in pigeon liver mitochondria, which indicates that it is located in the matrix.

Succinate thiokinase is another enzyme for which assay by HPLC is particularly advantageous. Depending on the species, this enzyme may synthesize either GTP or ATP as the result of its participation in the Krebs cycle: succinyl-CoA + GDP + $P_i \rightarrow$ succinate + GTP + CoA. The same HPLC-based assay can be used to study an enzyme of either specificity [12]. The enzyme reaction is reversible and can be studied in either direction by HPLC simply by changing the initial substrates. Although GDP synthesis could in principle be followed by the pyruvate kinase/lactate dehydrogenase coupling system, this assay is unsatisfactory for crude systems that have a high GTPase activity. The most commonly used assay, which follows absorbance changes at 235 nm [50], lacks specificity, is relatively insensitive, and is troubled by high background absorbance.

The HPLC-based assay developed for succinate thiokinase involves an isocratic separation of CoA, succinyl-CoA, GDP and GTP on a C_{18} column [12]. The succinate thiokinase reaction is quantified by measuring the amount of CoA converted to succinyl-CoA. Because low pH stabilizes succinyl-CoA to non-enzymic hydrolysis, the mobile phase is adjusted to pH 4 and contains phosphate, tetrabutylammonium ion, and acetonitrile. Inclusion of thiodiglycol in the assay prevents the accumulation of the disulfide form of CoA (mention of this was omitted in our prior publication [12]). Fig. 5 shows that baseline

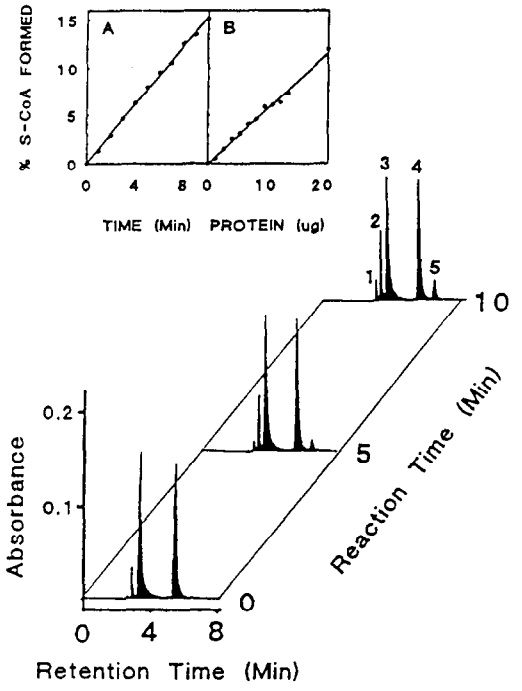

Fig. 5. HPLC-based assay for succinyl-CoA synthetase in an extract from rabbit liver mitochondria. The separation was carried out on the same column used in Fig. 4, using the same mobile phase except the pH was 4.0 and the percent acetonitrile was 17.5%. Peaks: 1 = GMP; 2 = GDP; 3 = GTP; 4 = CoA; and 5 = succinyl-CoA. The insets show the ranges in which succinyl-CoA formation was linear with time and protein added. From ref. 12 with permission.

resolution of reaction components can be achieved within eight min, and that the reaction is linear with time and with protein concentration. The HPLC-based assay is being used in the study of the nucleotide specificity of succinate thiokinases in tissues from various species (unpublished work) and is also being used in our efforts to isolate an ATP-specific form from pigeon tissues.

The assay for succinate thiokinase has been modified to separate CoA from acyl-CoA derivatives of various chain lengths. Because increasing the chain-lengths of fatty acids increases retention of the corresponding acyl-CoAs on C_{18} columns, higher concentrations of acetonitrile are required for elution. To obtain a satisfactory

run time, a linear gradient in acetonitrile is necessary when quantitation is desired for both CoA and a medium-chain acyl-CoA. Otherwise, a concentration of acetonitrile that elutes acyl-CoA within an acceptable time will cause CoA to elute in the void volume. The separation of CoA and several short-chain acyl-CoAs shown in Fig. 6 can readily be incorporated into a HPLC-based assay for an acyl-CoA synthetase. The authors have used such assays to survey the levels and nucleotide specificity (ATP *vs.* GTP) of acyl-CoA synthetases in mitochondrial matrix of various tissues and species.

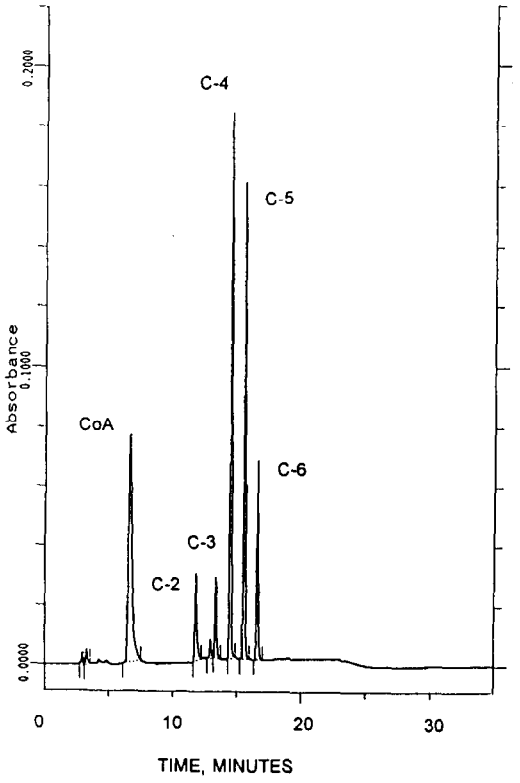

Fig. 6. HPLC separation of CoA and a series of short-chain fatty acyl-CoA esters on a Beckman 250 × 4.6 mm I.D., 5-μm C_{18} Ultrasphere IP column. The mobile phase (flow-rate 1 ml/min) consisted of 100 m*M* phosphate (Na^+), 20 m*M* acetic acid, 5 m*M* tetrabutylammonium hydroxide, and percent acetonitrile as follows: 15% for 2 min, linear gradient from 15 to 45% in 15 min, hold at 45% for 3 min, return to 15% in 1 min, hold at 15% for 14 min before injecting the next sample.

5. Concluding comments

The large number of papers in the literature describing HPLC-based enzyme assays serve as a useful guide to the development of an assay for almost any other enzyme that might be encountered. Many of the recent papers describe HPLC-based methods designed to replace older methods based on other techniques. Other papers reflect the growing need for methods for following the metabolism of macromolecules, especially proteins and glycoproteins. HPLC has become increasingly popular because of its precision, sensitivity, specificity, reproducibility, and capability of being automated. The future of HPLC as a method for assaying enzymes appears very bright as the field seems poised to absorb a number of new innovations. These include modifications of traditional packings used in reversed-phase chromatography, especially smaller diameter packings in shorter columns. Packings suitable for hydrophilic-interaction chromatography may find their niche by offering an alternative to ion-pair reversed-phase and ion-exchange chromatography. The continuing trend toward miniaturization will likely result in the redesign of HPLC systems. The end result will be assays carried out in shorter times and with smaller samples injected onto columns. Finally, detectors will continue to undergo modification or refinement.

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