HPLC IN ENZYMATIC ANALYSIS

SECOND EDITION

Edward F. Rossomando
University of Connecticut Health Center

with the collaboration of
Zdenek Deyl, Academy of Sciences of the Czech Republic
Jan Kehr, Karolinska Institute
David Lambeth, University of North Dakota
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To my wife,
Nina,
and our children,
Natasha and Michelle,
with affection;
and
to my collaborators and colleagues,
with appreciation.

CONTENTS

PREFACE

Among the various products of genes, enzymes are unique because they act as catalysts for the processes that define living systems. Given their central role, it is no wonder that advances in technologies for measuring enzymatic activities are of interest to a broad spectrum of scientists. High performance liquid chromatography (HPLC) is one such advance. In 1987 the first edition of *High Performance Liquid Chromatography in Enzymatic Analysis* was published with the following goals: to explain how enzymatic activities could be assayed by HPLC, and to serve as a reference source by cataloguing activities for which HPLC had been used. Given these goals, and the decade that has elapsed since the first edition, it is reasonable to ask whether the book has been successful.

One measure of success is anecdotal: At more than one scientific meeting, graduate students and colleagues have told me how pleased they were with the book. By following the directions provided, they have been able to set up HPLC assays, and they have found the book most useful as a reference source. A more quantitative measure would be the number of investigators who rely on HPLC to monitor enzymatic activities. If we use publications as a measure, we find that the 1987 edition reported HPLC assays of 62 activities. In contrast, the 1998 edition cites 169 activities involving assay by HPLC. Thus, 11 years brought an almost threefold increase in the number of activities assayed by HPLC.

And finally, more types of activity are being studied. The first edition listed activities in only 11 categories. This volume discusses activities in 18 categories, including enzymes for metabolism of lipids, vitamins, xenobiotics, pyrimidines, complex saccharides, and glycoproteins, and activities that modify proteins and peptides, as well the modification of nucleic acids and their expression.

Using these criteria as yardsticks, the first edition has been a success. It is now obsolete, however: Because of the increases in both number and types of activities assayed, it is no longer an accurate catalog of enzymatic activities investigated by means of the HPLC method. For this work to continue to serve as a reference source, it would need updating. While it was the obsolescence of the first edition that in part prompted the development of a second edition, there were other considerations as well. These included the introduction of high performance capillary electrophoresis (HPCE) as a method for separation, the development of microdialysis as a method for collection of samples

in situ, the application of HPLC/CE to the field of forensics, and finally the wide application of the polymerase chain reaction (PCR).

To cover these new areas in the second edition in a complete, scholarly and professional manner, I enlisted collaborators. Franco Tagliaro, Zdenek Deyl, and Ivan Mikšík not only contributed to the chapter on CE, but developed the chapter on forensics. Kathi Ulfelder also contributed to the chapter on CE and developed the chapter on PCR. Jan Kehr contributed the chapter on microdialysis. David O. Lambeth updated and extended the scope of the chapter surveying enzyme activities assayed with HPLC. I hope that this new material, by augmenting the first edition, will make this second edition of value to researchers and especially to students.

The goals of the second edition become expanded versions of those articulated in the first: to demonstrate how enzymatic activities can be assayed by HPLC/CE and microdialysis; to show how HPLC/CE can be used with PCR; and finally to provide a reference source to determine whether an HPLC assay has been developed for your activity.

No work of this scope and magnitude can be completed alone. I thank my collaborators and the investigators who have agreed to have their work cited in this volume. I also express my appreciation to Katherine O'Conner for organizing and typing sections of this edition. Finally, I thank the editors at John Wiley for working with me in the production of this second edition.

EDWARD F. ROSSOMANDO

Farmington, Connecticut
January 1998

PREFACE TO THE FIRST EDITION

The importance of the introduction of high performance liquid chromatography (hplc) to studies in the life sciences is now widely recognized. Since its introduction, this method has been rapidly accepted by biochemists and more recently by biologists and clinicians. Such rapid acceptance should not be surprising, since advances in separation and analysis have usually been readily assimilated.

It is the ability of hplc to accomplish separations completely and rapidly that led to its original application to problems in the life sciences, particularly those related to purification. An analysis of the literature revealed that this technique was used primarily for the purification of small molecules, macromolecules such as peptides and proteins, and more recently antibodies. This application to purification has all but dominated the use of the method, and there has been a plethora of books, symposia, and conferences on the use of hplc for these purposes. However, it was only a matter of time before others began to look beyond and to explore the possibilities that result from the capacity to make separations quickly and efficiently.

What emerged from these early studies was the idea that hplc might be used as a method for the analysis of enzymatic activities rather than its traditional use as a tool for separation. This change in emphasis is particularly attractive to those who wish to make use of the activity of an enzyme as an indicator of cellular function, a determinant of a given stage of differentiation (or dedifferentiation), or even as a measure of gene function. In the past, because contaminating activities led to conflicting results, tedious purification of the enzyme was often necessary to clarify the results of ambiguous activity determinations brought about in part as a consequence of methods that measure only one component of the reaction mixture. Such ambiguous results will occur much less often with the hplc method, since its ability to separate quickly a group of related compounds allows for the assay of one activity in the presence of several others. Thus, the advantage of analyzing an activity after only a minimal amount of purification is inherent to the hplc technique.

This book describes the hplc method and explains and illustrates its use. Each chapter deals with a different aspect of the method, beginning with an overview and ending with a detailed summary. Throughout, an attempt has been made to focus on questions related to the assay of the activity of an enzyme rather than its purification. More detailed discussions on the theory of hplc and on its use for purification, particularly for the purification of proteins, will be found in the references at the end of each chapter.

No task of this magnitude can be completed without the guidance, inspiration, and help of many others. I wish to thank Jessica Hodge Jahngen, who introduced me to the possibilities and potential of hplc. She and E. G. Jahngen have provided much of the work in this volume from my laboratory. More recently, data and assistance have been provided by Jane Hadjimichael, whose persistence and insistence in the final stages helped it all come together. A special note of appreciation and thanks to Vickey Shockley for organizing and typing the manuscript together with Pamala Vachon, and to Sherry Perrie for the original artwork.

My appreciation is also extended to my colleagues Edward J. Kollar and James A. Yaeger for editing the early drafts of the manuscript. More recently, editing assistance has been provided by Cynthia Beeman, Mina Mina, and David Richards. Finally, I wish to thank Phyllis Brown for encouraging me to complete this task in the way I thought best and Stanley Kudzin for his interest in the subject and for providing the opportunity to write the book. Also, this work could not have been completed without the contributions of numerous investigators who consented to have their work cited here. My thanks to them as well.

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Farmington, Connecticut
January 1986

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CHAPTER 1

Application of HPLC to the Assay of Enzymatic Activities

OVERVIEW

This chapter describes the anatomy of an enzyme assay, focusing on the significance of separation and detection in the assay procedure. A classification of the methods used in the assay of enzymatic activities is developed, using the separation step as the criterion for the grouping. Having placed the high performance liquid chromatography (HPLC) method within this classification, we then examine the question of when to use it and discuss some strategies developed for its use. The chapter also identifies and comments on the parts of the enzyme assay that will be affected by the selection of HPLC as the method of analysis.

1.1 INTRODUCTION

Increasingly, investigators in the life sciences have expressed interest in the application of HPLC to the assay of enzymatic activities. This method not only provides a method to enhance the separation of reaction components, it also allows extensive and complete analysis of the components in the reaction mixture during the reaction. In addition, it can employ sensitive detectors, and it can be used for purification.

A number of questions must first be addressed, however, concerning the biochemical reaction catalyzed by the enzyme, the assay conditions normally used for this enzyme, and the enzyme itself. This chapter explores and answers these questions.

Section 1.2 presents the anatomy of the enzymatic assay, and from a dissection of its components, it is possible to obtain an appreciation of how HPLC can be used. Section 1.3 develops a classification of enzyme methods that allows the advantages and limitations of the HPLC method to be presented fairly. Section 1.4 is devoted to criteria for the selection of HPLC as an assay

system. Wherever possible, these points are illustrated with examples taken from work carried out in the author's laboratory.

1.2 ANATOMY OF AN ENZYME ASSAY

The assay of an enzymatic activity is composed of several discrete steps or events (Fig. 1.1). The first is *preparation* of both the reaction mixture and the

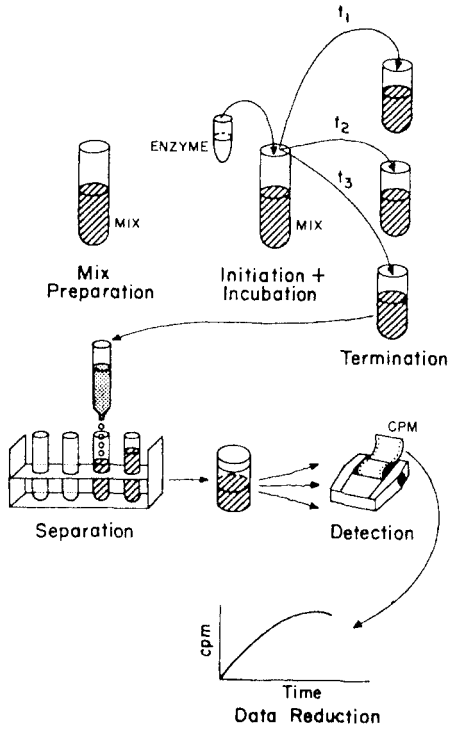

Figure 1.1 Schematic of a representative enzymatic assay. The reaction mixture is prepared (Mix Preparation) and the reaction can be started (Initiation) by the addition of the enzyme. During the reaction (Incubation), samples are removed at intervals labeled t_1, t_2, and t_3, and the reaction is stopped (Termination) by inactivating the enzyme. The incubation mixture is fractionated (the illustration shows a traditional chromatographic column), and the product is isolated from the substrate (Separation). In this assay, a radiochemical was used as the substrate and therefore the amount of product that formed is determined by its collection, the addition of scintillation fluid, and the measurement of radioactivity by scintillation counting (cpm: Detection). The progress of the reaction is given by the amount of radioactive product recovered (Data Reduction).

enzyme. The reaction mixture usually contains such components as the buffer used to establish the correct pH, the substrate, and any cofactors (e.g., metals) that may be required for catalysis. Preparation of the reaction mixtures involves mixing these ingredients in a reaction vessel such as a test tube or, for some assay methods, a cuvette. In some cases the reaction mixture is brought to the required temperature prior to initiation of the reaction. The enzyme must also be prepared. This complex topic is discussed in detail in Chapter 5.

In most cases, the second step in the assays comprises *initiation* and *incubation.* A reaction can be initiated by the addition of the enzyme preparation to the substrate in the reaction mixture, or vice versa. This step is considered the start of the reaction, and all subsequent time points are related to this time.

Many reactions require *termination,* which is the step that brings about the cessation of catalysis and thus stops the reaction. Termination may be achieved in several ways, usually via inactivation of the enzyme.

Termination is often followed by *separation* of the components in the reaction mixture. Most often separation involves isolating the substrate from the reaction product.

Detection, the fifth step, refers to that process by which the amount of product formed by the enzyme during a specific incubation interval is determined.

The last step in an assay involves *reduction* of the data. This step includes all procedures in which the data are analyzed and graphed to determine initial rates as well as kinetic constants.

Not all steps are involved in all assay methods, and in some methods one or more of the steps may be complex. The introduction of HPLC as an enzymatic assay method has improved the separation and detection steps primarily, although its use may also affect the preparation and termination steps.

1.3 CLASSIFICATION OF ENZYMATIC ASSAY METHODS

The methods in use for the assay of enzymatic activities may be divided into three groups. These will be referred to as the continuous, coupled, and discontinuous methods (see Table 1.1).

1.3.1 Continuous Methods

Continuous methods do not require a separation step prior to detection. For assays using this method, the substrate and product must differ in some property such that either one may be measured directly in the incubation solution. For example, the activity of an enzyme catalyzes the conversion of 4-nitrophenyl phosphate (4NP), a colorless compound, to 4-nitrophenol, which is yellow and has an absorption maximum at 510 nm. Since the substrate does not absorb in this region of the spectrum, the reaction can be carried out

TABLE 1.1 Classification System for Enzymatic Assay Methods

Assay Methods	Characteristics	Example
Continuous	Separation of substrate(s) from product(s) not required	$4NP \rightarrow 4N + P_i$ (colorless → yellow)
Coupled	Separation not required for detection	$PEP + ADP \rightarrow pyruvate + ATP$; $pyruvate + NADH \rightarrow lactate + NAD$
Discontinuous	System for separation of substrate(s) from product(s) required for detection	$ATP + AA \xrightarrow{enz} Enz - AA - AMP + PP_i$; $Enz - AA - AMP + tRNA \rightarrow tRNA - AA - AMP$

directly in a cuvette (Fig. 1.2), and the amount of product formed may be determined continuously by measuring the change in optical density with time at this wavelength.

1.3.2 Coupled Method

In the second category of assays, the coupled assay method, activity is measured indirectly. In this method two reactions are involved. The first is the reaction of interest, such as A → B, second, the reaction that converts B to C, might be referred to an *indicator reaction*, not only because it uses the product of the first reaction (i.e., B) as a substrate, but also because the

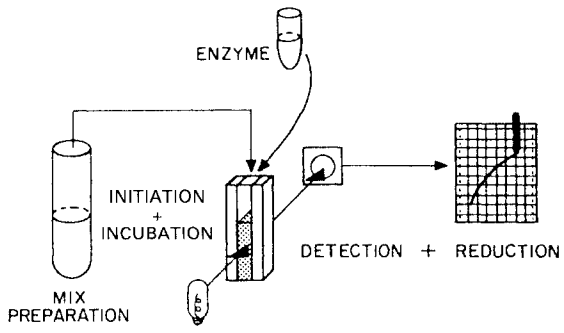

Figure 1.2 The assay of an enzymatic activity by the continuous assay method. In the illustration, the reaction mixture is transferred to a cuvette, which is shown in place in the light path of the spectrometer. The addition of the enzyme directly to the cuvette initiates the reaction. Product formation results in a change in absorbance, which is monitored continuously by the detector. This change signals a deflection on a recorder. Note that product formation requires neither termination of the reaction nor separation of the substrate from the product.

formation of C may be assayed by a continuous method—that is, without a separation step. In this way, the two reactions are coupled, the product of the first reaction, B, acting as the substrate for the second reaction.

For example, pyruvate kinase may be assayed by such a method. This enzyme catalyzes the reaction

$$\text{Phosphoenolypyruvate (PEP)} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP}$$

This, of course, is the reaction of interest that cannot be assayed directly by the continuous method. However, when a second enzyme, a dehydrogenase, such as lactate dehydrogenase, is added as the indicator together with pyruvate and NADH to the reaction mixture, a second reaction occurs and NAD forms in the cuvette as follows:

$$\text{Pyruvate} + \text{NADN} \rightarrow \text{lactate} + \text{NAD}$$

The formation of NAD may be followed in a continuous manner by the decrease in absorbance at 340 nm, and therefore the progress of the kinase reaction of interest may be followed through this coupling of the formation of pyruvate to the formation of NAD.

1.3.3 Discontinuous Method

The discontinuous method measures activity by separating the product from the substrate. Assays characteristic of this group usually require two steps, since separation often does not include detection. Thus, first, the substrate and the product are separated, and usually the amount of product formed is measured. Assays that use radiochemical substrates are included in this group, since radiochemical detectors are unable to differentiate between the radiolabel of the substrate and that of the product. Examples of enzymes whose assay methods fall into this category are legion, and these approaches characterized by a separation step.

As an illustration, consider the assay to measure the activity of the tRNA synthetases. These enzymes catalyze the covalent attachment to tRNA of an amino acid, usually radioactive as follows:

$$\text{ATP} + {}^*\text{AA} + \text{Enz} \rightarrow \text{Enz-AMP-}{}^*\text{AA} + \text{PP}_i \qquad (1)$$

$$\text{Enz-AMP-}{}^*\text{AA} + \text{RNA} \rightarrow \text{RNA-}{}^*\text{AA} + \text{AMP} \qquad (2)$$

(By convention, radioactivity is indicated by an asterisk preceding one labeled substance, here the amino acid AA.)

The activity is usually followed by measuring the amount of RNA-*AA, the product of reaction (2) formed during the incubation. Since the radiochemical detector cannot differentiate the free radioactive amino acid

used as the substrate from that bound covalently to the RNA, the free and the bound amino acids must be separated prior to the detection or quantitation step.

This separation step requires first the addition to the sample of an acid such as trichloroacetic acid (TCA), which also serves to terminate the enzymatic reaction. However, since TCA also precipitates the RNA and any radioactive amino acid covalently linked to it, the reaction product RNA-*AA will be precipitated as well. And since the precipitate can be separated from the soluble components by a sample filtration step, the separation of the bound from the free amino acid can be accomplished. As illustrated in Figure 1.3, the reaction product, which is trapped on the filter as a precipitate, can be detected by transferring the filter to a scintillation counter for quantitation and, of course, measuring the amount of product formed. Since assays of this design usually focus on one component at a time, no information is obtained about the amount of ATP, AMP, PP_i, or free amino acid during the course of the reaction.

1.3.4 HPLC as a Discontinuous Method

Within the framework of the scheme just described, the HPLC method would be classified as discontinuous, since a separation step is part of the procedure. However, because termination can be accomplished by injecting the sample directly onto the column, the HPLC detection is usually "on-line," that is, carried out continuously with separation. Thus, the separation and detection steps merge into a single operation, which for all practical considerations means that it is a "continuous" method.

In addition, unlike many other discontinuous assays that focus on only one of the components of the reaction, the HPLC assay offers the potential to monitor several. For example, consider adenosine kinase, the enzyme that uses two substrates and forms two products according to the reaction Ado + ATP $\rightarrow$ AMP + ADP. Since HPLC can readily separate all four compounds (see Fig. 1.4), and all four compounds can be detected at 254 nm, it is apparent that with the HPLC method, the level of each component can be monitored during the course of the reaction, providing a complete analysis of each "time point."

Having a complete analysis of the contents of the reaction vessel during the incubation can be helpful in another way: It provides information on what is not present as well; and since most other assay methods are designed to detect only one component, it is often difficult to account for a result that occurs unexpectedly during a study. For example, consider the results obtained during the purification of the enzyme E-1, which catalyzes the conversion of substrate A to product B. Consider also that the method used to follow activity measures only the amount of B in the incubation mixture. As illustrated in panel I of Figure 1.5, when the activity E-1 is assayed in the crude sample, the formation of substantial product (B) is observed (graph line 1).