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REVIEW

APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO ENZYME ACTIVITY DETERMINATION

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1 INTRODUCTION

Since its introduction to studies in the life sciences, high-performance liquid chromatography (HPLC) has been accepted by biochemists and more recently by biologists and clinicians as the method of choice for separation and analysis It is the ability of HPLC to accomplish separations completely and rapidly that led to its application to problems related to purification An analysis of the literature reveals that this technique was used for the purification of both small molecules and macromolecules such as peptides, proteins and nucleic acids This application 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 would begin to look beyond purification and explore the possibilities that result from the capacity of HPLC to make separations quickly and efficiently

What emerged from those early inquiries was the idea that HPLC might prove useful for the analysis of enzymatic activities especially in those cases where the enzyme had not been purified and where contaminating activities had led to conflicting results. Because the HPLC method allows for the assay of one activity in the presence of several others, it is useful for analyzing an activity after only a minimal amount of purification Thus, the HPLC technique was soon seen to be especially useful to those for whom the activity of an enzyme was an indicator of cellular function, a determinant of a given stage of differentiation, or even as a measure of gene function To date, methods have been developed for the assay of more than 100 activities, including enzymes for the metabolism of carbohydrates, steroids, purines, porphyrins, amino acids, peptides, and proteins [1] In this chapter the HPLC method for the measurement of enzymatic activities will be described and its use explained and illustrated

2 COMPARISON OF THE HPLC METHOD TO OTHERS FOR THE MEASUREMENT OF ENZYMATIC ACTIVITY

In order to compare the HPLC method with others it is useful to develop a classification of the available methods The classification scheme developed here has as its basis the inclusion of two steps, namely, separation and termination To better understand these two steps, the anatomy of a representative enzyme assay will be described

2 1 The anatomy of an enzyme assay [1]

A dissection of a representative assay reveals separate component parts (Fig 1) The first component is the preparation of the reaction mixture and the enzyme preparation The reaction mixture usually contains the buffer used to

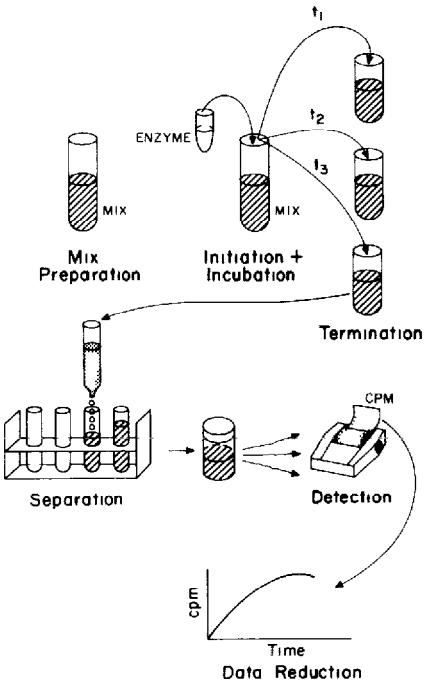

Fig 1 Schematic of a representative enzymatic assay to illustrate its component parts The reaction mixture is prepared (Mix Preparation) and the reaction can be started (Initiation) by the addition of the enzyme During the reaction (Incubation), samples are removed at intervals labeled t_1, t_2, t_3, and the reaction is stopped (Termination) by inactivating the enzyme The incubation mixture is fractionated (in the illustration a traditional chromatographic column is being used), and the product is isolated from the substrate (Separation) In the assay illustrated, a radiolabeled substrate has been used and therefore the amount of product that formed is determined by collection of fractions, the addition of scintillation fluid to each fraction, and the measurement of radioactivity in each fraction by scintillation counting (Detection) The progress of the reaction is given by the amount of radioactive product recovered (Data Reduction) (Reprinted with permission from ref 1)

establish the correct pH, the substrate, and any cofactors such as metals that may be required for catalysis Preparation of the reaction mixture involves mixing these ingredients in a reaction vessel such as a test tube or, for some assay methods, a cuvette Often, the reaction mixture is brought to a specified temperature prior to initiation of the reaction

The second component part of an assay is initiation/incubation A reaction is often started by adding the enzyme to the substrate in the reaction mixture This combination initiates the incubation phase, and all subsequent time points are referenced to this time as zero

Many reactions require a third component, namely termination, to stop the reaction Termination usually involves inactivation of the enzyme Termina-

tion can be followed by a fourth component, separation. Most often separation involves isolating the substrate from the reaction product. Detection, the fifth step, refers to that process by which the product is located, identified, and the amount formed during the incubation quantitated. Finally, 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 These six component parts of an enzyme assay are illustrated in Fig 1

2 2 *Classification of enzymatic assay methods*

Using the scheme described above as a framework, the methods for assay of activity can be divided into three classes a continuous type of assay, a coupled assay and a discontinuous assay The three methods differ on their inclusion of termination and separation steps as components of the assay Thus, whereas those assays in the first two classes require neither termination nor that the product be separated from the substrate, those in the last require both steps.

2 3 *HPLC as a discontinuous method*

Within the framework of this classification, the HPLC method would be classified as discontinuous, because both termination and separation steps are part of the procedure However, because termination can be accomplished by injecting the sample directly onto the column, both separation and termination can be performed in one operation Also note that since with the HPLC method detection is usually 'on-line', that is, carried out continuously with separation, the termination, separation and detection steps merge into a single operation

Finally it should be noted that, unlike many other discontinuous assays that focus on only one of the reaction components, usually the reaction product, the HPLC assay offers the advantage of monitoring several For example, consider adenosine kinase, the enzyme activity that uses to substrates and forms two products according to the reaction Ado + ATP → AMP + ADP Since HPLC can readily separate all four components and all four can be detected at 254 nm, the level of each can be monitored during the reaction providing a complete analysis at 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 making it possible to account for unexpected results

3 DESIGN OF THE HPLC ASSAY

In this section, a strategy will be presented for the design of an assay system By focusing on one enzymatic reaction, the steps leading to the development

TABLE 1

STEPS IN DESIGN OF AN ASSAY FOR ENZYMATIC REACTION

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1	Analyze the primary reaction
2	Analyze all secondary reactions
3	Select the method of analysis that will allow for measurement of substrates from products
4	If a discontinuous assay method is chosen, select the method for termination and separation
5	Select appropriate detection system Will it be necessary to collect fractions?

of the assay for this activity can be described. These steps are previewed in Table 1

3 1 Analysis of the primary reaction

The design of an assay system for an enzymatic activity begins with a complete analysis of the primary reaction, which, by definition, is that reaction catalyzed by the enzyme under study To being this analysis, indicate all substrates, products, and cofactors of the reaction If metals are required for catalysis, include them In the case of the metals, however, it is useful to note whether they are an integral part of the substrate, for example, when the complex Mg–ATP is the substrate, or whether they are required for some other function, such as activation of the enzyme It is also useful to indicate the pH of the reaction as well as the type and concentration of the buffer to be used The goal of this analysis is to list all the components present in the reaction mixture before the start of the reaction

To illustrate this approach, consider the assay of a pyrophosphohydrolase, an enzyme that catalyzes the reaction

$$\text{Mg–ATP} \rightarrow \text{AMP} + \text{PP}_i$$

Mg–ATP is the substrate, and AMP and pyrophosphate (PP_i) are the products Since this activity is usually assayed at a pH of 7 5 using a Tris–HCl buffer system, the reaction tube will contain ATP, Mg, and Tris–HCl as illustrated in Fig 2

3 2 Analysis of secondary reactions

The HPLC method can be used to advantage in the assay of activities in crude extracts or in preparations only partially purified Such samples will usually contain additional activities that catalyze other reactions These other reactions have been referred to as secondary reactions [1] Secondary reactions may use the same substrate as the primary reaction or they may use the

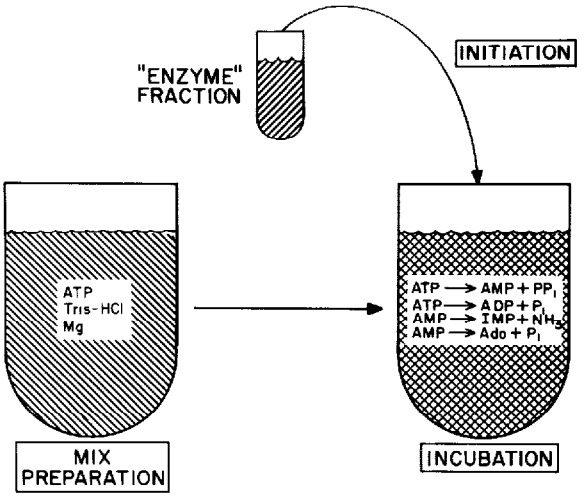

Fig 2 Overview of strategy for design of a method to determine enzymatic activity The reaction tube contains a mix preparation to measure the activity of an ATP pyrophosphohydrolase which catalyzes the formation of AMP and PP_i from ATP The mix includes the substrate, ATP, the buffer, Tris–HCl, and magnesium, a metal cofactor The addition of a sample from the 'enzyme' fraction initiates both the primary reaction and several secondary reactions as shown in the illustration (Reprinted with permission from ref 1)

product of the primary reaction In either case their presence in the preparation can affect the quantitative analysis of the enzyme under study

For example, AMP, the product of the primary reaction, may undergo secondary reactions to form adenosine and phosphate or IMP and ammonia In addition, other secondary reactions could involve ATP, an example is the degradation of ATP to ADP A summary of these secondary reactions is also given in Fig 2 in the step marked "Incubation" While these secondary reactions can be eliminated or their significance minimized, they should not be overlooked in the analysis and design of the assay system

3 3 Selection of the method of analysis

With the list of reactants, cofactors, and reaction conditions compiled, the stationary phase can be selected. Since the reactants differ in charge as well as solubility, either ion-exchange or reversed-phase stationary phases can be used However, in contrast to reversed-phase, a choice of the ion-exchange mode would require gradient elution

3 4 Modifications of reaction conditions for the HPLC assay method

Those reaction conditions acceptable for use with other assays may require some modification to be usable with the HPLC procedure For example, it may

be necessary to change the concentration of such components as hydrogen ions or metals since both might be detrimental to the operation of the column Often some changes can be made without significant loss of enzymatic activity. Often, especially with metals, substitutions can be made Also, some decrease in enzyme (protein) concentration may be required, especially when the reaction is to be terminated by injecting a sample directly from the incubation vessel onto the column Excess protein will only clog the column Note that when a sample is injected directly onto the column for analysis, it brings with it everything present in the reaction mixture Most of these problems can, if necessary, be solved by terminating the reaction and performing some clean-up operations prior to analysis

3 5 Sampling methods

Since the HPLC method is a discontinuous technique, obtaining kinetic data requires multiple samples, each one representing a single time point Reactions requiring multiple sampling can be arranged in one or two ways. In one arrangement, illustrated in Fig 3A, separate reaction mixtures are set up, each one representing a single time point In this case, the total volume required for a single reaction mixture would be the volume required for a single analysis The number of incubation tubes would be determined by the number of time points required by the experiment In the second arrangement, shown in Fig 3B, a single incubation mixture is prepared, and samples re removed from it at suitable intervals for analysis In this arrangement, the volume required for the reaction mixture would be determined as the product of the volume needed for each analysis multiplied by the total number of analyses

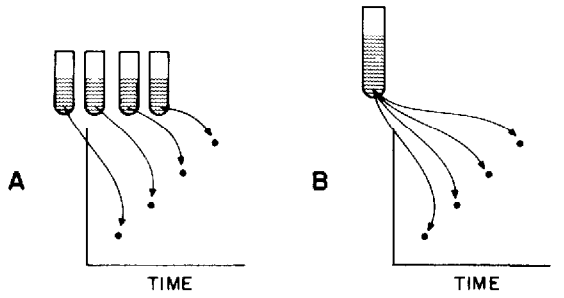

Fig 3 Representation of two procedures used to obtain multiple samples for analysis (A) In this arrangement, several identical reaction mixtures are prepared, and the enzyme will be added to each to start the reaction During the incubation each tube will be sampled only once (B) In this arrangement, only one reaction mixture is prepared and the enzyme is added to start the reaction The incubation mixture is sampled repeatedly during the course of the reaction Note that the volume of the reaction mixture in arrangement (B) is usually greater than in arrangement (A) (Reprinted with permission from ref 1)

Since with both arrangements the volume of a single analysis is the important variable, it would appear that once this value is determined the overall reaction volume can be established

3 6 Terminating the reaction

In designing the HPLC assay, it may be necessary to introduce a termination step into the protocol There are a variety of ways to accomplish this including the addition of acids, bases, or other denaturants A method that offers an alternative to these is the addition of chelators such as EDTA. This technique is suitable only for reactions in which the enzymatic activities have an absolute requirement for a metal whose removal will terminate the activities.

An alternative method we have found useful for terminating reactions is to heat the incubation mixture to a temperature that results in inactivation of the enzyme. Usually temperatures in excess of 100 °C are required. One of the techniques often used is to immerse the reaction tube in a bath of boiling water In our laboratory, this method is not employed because the incubation mixture cannot be brought to 100 °C quickly enough to effect instantaneous termination Commercially available heating blocks are also suitable

Finally, a simple device (a sand bath) has been described [1] and found to be effective in terminating reactions instantly. We filled a stainless-steel rectangular pan (ca 20.32 cm × 25.4 cm) with ca 5.08 cm of sand and placed it on a hot plate This set-up is illustrated in Fig 4A. The temperature of the sand bath is easily brought to 155 °C, and this temperature can be maintained throughout the working day without fear of evaporation There is never a problem of fitting the tubes one simply thrusts any size capped glass tube directly into the sand The insertion of an incubation tube containing as much as 500 μl of incubation mixture resulted in the temperature inside the solution reaching 100 °C 'instantly', thus terminating the reaction.

Termination of most enzymatic reactions with heat results in precipitation of any proteins present in the reaction mixture Because this precipitation is irreversible, and because when crude extracts are being assayed the amount of protein may be considerable, it is often necessary to remove the precipitate prior to sample. The precipitate can be removed either by filtration or by centrifugation as illustrated in Fig 4B Because of the small volumes usually used in the reaction mixture, volumes between 100 and 500 μl, centrifugation is difficult. Recently, filters with 'hold-up' volumes of about 50 μl have been developed that make the removal of precipitate less tedious Following the removal of the precipitate, a sample may be removed from the filtrate and analyzed

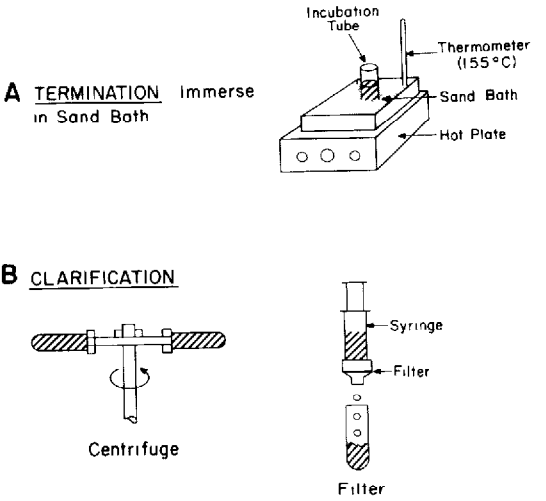

Fig 4 Preparation of a sample from a reaction mixture that contains an excess amount of protein prior to injection and analysis (A) Termination carried out by immersion of reaction sample in a sand bath maintained at 155 °C (see ref 1 for details), (B) removal of the precipitated protein by either centrifugation or filtration (Reprinted with permission from ref 1)

4 OPTIMIZING ENZYMATIC REACTIONS WITH THE HPLC METHOD

4 1 Composition of the reaction mixture

The HPLC method is particularly useful for obtaining the initial rate data necessary to the study of an enzymatic reaction To obtain initial rate data, optimal assay conditions must be established Optimization involves the determination of several variables, such as substrate concentration, pH, temperature, and enzyme concentration

Some idea of the optimal substrate concentration can be obtained from the value of the Michaelis constant (K_M), the concentration of the substrate at which the rate will be one half of its maximum rate If the K_M value can be obtained from the literature, then it is possible to begin experiments with a substrate at a concentration of two or three times the K_M value (assuming the absence of 'substrate inhibition') Such a concentration would be adequate for use in early experiments The literature can often provide information on starting values for other parameters, such as pH, any requirement for activators, and the optimal temperature for the incubation Armed with this information, a trial reaction mixture can be prepared

What remains to be determined prior to the initiation of the reaction are the amount of enzyme to be added to the reaction mixture, the time course of the reaction, and, for discontinuous assays, the time between samplings and the

volume of these samples These questions can be answered by a process of trial and error using the following scheme

First an arbitrary amount of enzyme is selected While any concentration can be used, excess protein should be avoided to eliminate clogging the column. Therefore, choose the least amount possible to start the reaction. Add this amount of enzyme to the reaction mixture to start the reaction, and the sampling (analysis) can begin at any convenient time. The chromatogram obtained from this single sample is examined for a new peak, the product Two outcomes are possible Either product is present or not If a product peak is detected, and its amount (area of the peak) is very small compared to the total amount of the substrate, then a second sample can be withdrawn from the incubation mixture and analyzed Again the areas of the product and substrate peaks should be compared. If the area of the product peak is more than 50% of that of the substrate, the reaction has progressed too far, and it is necessary to start again by preparing a new reaction mixture. Since the previous reaction progressed too fast, the reaction rate should be lowered by adding less enzyme to the new mixture. This adjustment will allow more time points to be obtained before a majority of the substrate is consumed

Alternatively, in the absence of the formation of any product, incubation should be continued and samples should be withdrawn every hour and analyzed The incubation can be continued for several hours until product is detected In the absence of any detectable product, a new reaction mixture can be prepared which contains more enzyme than the first If this does not result in the formation of detectable product, the possibility should be considered that the fraction being assayed contains no activity

4 2 Obtaining initial rate data

As a result of the preliminary trials described above, values will have been obtained for two parameters the amount of the enzyme required to form sufficient detectable product and the incubation time required to form this amount of product To obtain these values, it may be necessary to alter the reaction rate as follows if the rate of product formation is too high, that is, the reaction rate becomes non-linear to soon, then the rate should be lowered by decreasing the amount of enzyme Alternatively, if the rate of the reaction is too low, that is, if it takes all day to form product, the enzyme concentration should be increased so that a linear rate can be observed in about 2 h In the absence of termination what governs the sampling interval is the time for the HPLC analysis Clearly, it will not be possible to apply the second sample until the analysis of the first has been completed and the column readied again For example, if elution and preparation take 15 min, then it is this value that establishes the minimum sampling time In this way, the time for analysis determines in part the concentration of the enzyme used in the reaction Once a suitable concen-

tration of enzyme has been established, and three or four samples analyzed, then the quantitative data can be obtained A reaction is started, the reaction mixture sampled, chromatograms obtained, and the amount of product formed is determined directly from the chromatogram using either peak height or electronic integration of the peaks. These values should be plotted as a function of reaction time.

Next, a second and third series of reaction mixtures should be prepared, with enzyme added at concentrations of one half and twice the value used in the first These reactions are started, sampled, chromatograms obtained, and the data presented as a function of reaction time. It should be noted that at this early stage in the optimization of the assay it is advisable to continue the incubations until the rate of product formation becomes non-linear or the amount of substrate present is exhausted This prolonged incubation provides information about the extent of the primary reaction and also allows time for the formation of products of secondary reactions

4 3 Inspection of the chromatogram and quantitation of the reaction

The chromatograms obtained above can provide information on any alternative fates for the substrate. For example, assuming no secondary reactions, the amount of product formed should equal that of substrate lost. A careful inspection of the chromatograms should be sufficient to establish this point as well as to note the presence of peaks other than these two.

It is also useful at this stage to convert the amount of product formation from the 'machine units', arbitrary integration units or percent obtained, directly to units of mass Such a conversion requires a calibration curve that relates the machine units to more specific units of mass Having made the conversion, the initial rate of product formation determined above can now be plotted as a function of enzyme concentration as part of the optimization process

As a result of these procedures, a graphical representation of the rate of product formation will be obtained. Such data can be analyzed visually or be subjected to statistical analysis [2] Initial rate data can be used to produce double-reciprocal plots [1/(initial velocity) versus 1/(substrate concentration)] [3], which can in turn be used to differentiate between reaction mechanisms

4 4 Initial rate determination at low substrate concentrations

A determination of the rate of product formation becomes difficult at the lower limits of substrate concentration However, there are changes that can be made in the assay system that can solve this problem

The first change is, of course, to increase the sensitivity of the detector Most

HPLC detectors contain range switches that make this a simple matter When range switching is carried out, it is useful to determine if the calibration curves constructed at one range setting are still valid at another Next, the amount of product being detected may be increased by increasing the volume of the reaction mixture.

There have been situations in which obtaining enough reaction product requires the concentration of a large volume of reaction mixture Following concentration, the residue is resuspended in a small volume of buffer and analyzed

Finally, it is always possible to increase sensitivity by using analogues, such as radiochemicals, as substrates and determine the amount of radioactive product that has formed

4 5 Understanding and dealing with secondary reactions

When working with enzyme preparations that are only partially purified, the importance of understanding secondary reactions cannot be overemphasized This knowledge is invaluable to the interpretation of the results of enzymatic assays 'Beware of secondary reactions' is a rule that should always be kept in the forefront

How can secondary reactions be handled? Some procedures are presented in Table 2 These include purifying the activity of the primary reaction to homogeneity However, this may not always be possible or desirable Therefore some other solution must be found. The use of analogues is one such solution [4] For example, if an analogue of the substrate is used, then an analogue of the product will be formed If the latter is not a suitable substrate for the secondary enzyme, then no secondary reactions will occur Alternatively, one can try to adjust the reaction condition in such a way that the enzymes catalyzing the secondary reactions will not be active For example, if the primary reaction does not require metals but the secondary reaction does, adding a chelator will inhibit the latter

TABLE 2

BASIC RULES FOR AVOIDING SECONDARY REACTIONS

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What are they?
Secondary reactions are the result of enzymatic activities present in the sample that lead to destruction of the substrate and/or the formation of additional products, which can alter the results
Avoiding them
1 Purify the enzyme to homogeneity
2 Use analogues whose products are not substrates for secondary reactions
3 Adjust reaction conditions to minimize activity of secondary reactions

In those cases where secondary reactions make it difficult to quantitate the primary reaction, one solution is to use an analogue as the substrate The analogue should be chosen such that while it is a substrate for the primary reaction, the product will not be a substrate for the secondary reaction

Analogues can be used in another way Consider the case of developing an assay procedure for adenosine kinase, the enzyme that catalyzes the primary reaction Ado + ATP → AMP + ADP Problems will arise during the assay of this activity in crude extracts, since other enzymes may be present that can form AMP directly from ATP

Radiochemical analogues such as radiolabeled adenosine are ideal for solving this problem, because if the formation of radiolabeled AMP is monitored, it is possible to distinguish the AMP formed from adenosine form that formed from ATP, which, of course, would not be labeled

Alternatively, this same reaction can be assayed if adenosine is replaced by a fluorescent analogue [5] With such an analogue as a substrate, both it and the reaction product can again be followed with a separate detector

4 6 Quantitation

Internal standards, compounds added at any stage of the analytical procedure, can be useful in calibrating and/or calculating the effect of that procedure on the recovery of the substrate or product of the reaction The compounds chosen as internal standards should have similar detection characteristics

One more potential problem concerns the question of selecting the range of substrate concentrations to be used throughout the study Considering the sensitivity of most detectors and the apparent K_M values of most enzyme activities, the selection of the upper limit of concentration is usually not a problem A problem will develop, however, when rate determinations are made at low substrate concentrations, since at these concentrations the amount of product formed during the course of the reaction will be small and may be below the monitor's level of detection Therefore, prior to executing any experimental protocols dealing with low substrate concentrations, it is prudent to ascertain the lower limits of the detector being used in order to determine what product concentrations can be detected

5 CONCLUSION

HPLC is ideally suited to the study of many enzymes and to date the activity of over 100 has been determined with this method While appropriately classified as a discontinuous method, because the termination, separation and concentration determinations occur simultaneously, HPLC methods approach the speed and convenience of the traditional continuous methods

TABLE 3

SURVEY OF SOME REPRESENTATIVE ENZYMATIC ACTIVITIES ASSAYED BY THE HPLC METHOD

For a more complete listing of examples and summary of methods and findings see ref 1

Type of activity assayed	Ref
Catecholamine metabolism	
Tyrosine hydroxylase	6
5-Hydroxytryptophan decarboxylase	7
DOPA-decarboxylase	8,9
Proteinase	
Vertebrate collagenase	10
Papain esterase	11
Dipeptidase	12
Amino acid metabolism	
Ornithine aminotransferase	13
Tryptophanase	14
Polyamines	
Ornithine decarboxylase	15
Carbohydrate metabolism	
β-Galactosidase	16
Purine metabolism	
5′-Nucleotidase	17
Alkaline phosphatase	18
Adenosine deaminase	19
AMP deaminase	5
Creatine kinase	20
Adenylate cyclase	21
cAMP phosphodiesterase	18
Sulfur metabolism	
Adenosine 3′-phosphate 5′-sulfophosphate sulfotransferase	22

In designing an HPLC assay for an enzyme, it is necessary to select a type of HPLC system (and mobile phase) that will allow the separation of the components of the primary reaction as well as the components of any secondary reactions It is also necessary to select a detector and procedures for termination of the reaction and sampling of the incubation mixture which are appropriate to and non-destructive for all the reaction components

Optimizing an enzymatic reaction procedure with the HPLC method involves a consideration of such variables as temperature, pH and substrate concentration The choice of enzyme concentration depends in some cases on a balance between the minimum sampling interval (the time needed for analysis of a sample from the reaction mixture) and the amount of activity in the reaction mixture

Following the optimization procedure, the HPLC method can be used to obtain initial rate data and a determination of kinetic constants using traditional computational methods

6 SUMMARY

In this article some basic concepts and practical information are presented on the use of high-performance liquid chromatography (HPLC) to assay enzymatic activities A classification of enzymatic assays is developed based on the requirements for termination and separation, and the placement of the HPLC method within this classification is discussed The focus of the chapter is on developing a strategy for setting up assays, a discussion of some problems that must be avoided, and procedures to deal with these concerns Some examples have been presented in Table 3, but for a more complete listing and details on the numerous activities assayed by this method ref 1 should be consulted

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