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Review

Determination of enzyme activity by high-performance liquid chromatography

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Abstract

The application of high-performance liquid chromatography (HPLC) in the study of enzymatic reactions is reviewed. The rationale for using HPLC is given and whether the components of the reaction mixture should be derivatized prior to or after HPLC. An alphabetical list of enzymes assayed by HPLC is given. Substrate and product are included as well the derivatization reagent, detection method and biological matrix. Specific examples of these assays in a complex biological matrix *viz.* faeces are given. Future prospects are the detection of new enzymes using synthetic substrates and implementation of mass spectrometry to elucidate enzyme specificities.

Contents

List of abbreviations

o-Aminobenzald.	Reaction of *o*-aminobenzaldehyde resulting in dihydroquinozolinium
benz	Benzoin reaction with the guanidino moiety resulting in fluorescent derivatives, 2-substituted amino-4,5-diphenylimidazoles
Dns	Dansyl chloride
DTNB	Ellmans' reagent

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ED	Electrochemical detection
FL	Fluorescence detection
fluoresca	fluorescamine
FMOC	9-Fluorenylmethyl chloroformate
OPDA	1,2-*o*-Phenylenediamine
OPA	*o*-Phtalaldehyde
PITC	Phenylisothiocyanate
post	Postcolumn derivatization
pre	Precolumn derivatization
pyrr	δ-Aminolevulinic acid conversion to 2-methyl-3-carbethoxy-4-(3-propionic acid) pyrrole
RC	Radiochemical detection
RF	Refractive index

1. Introduction

Most biological reactions are dependent on enzyme catalysis. Reactant(s), called substrate(s) in the case of enzymatic catalysis, are converted to product(s) via the formation of one or more intermediate enzyme–substrate complex(es) in which a transition state is facilitated, thus lowering the kinetic barrier for the formation of product.

In its most simple form:

Enzyme + substrate ⇌ enzyme–substrate complex ⇌ enzyme + product(s), or:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P \qquad (1)$$

The enzyme–substrate complex becomes saturated in practically all enzymatic reactions. Therefore, the velocity of an enzyme-catalyzed reaction often depends more on the concentration of the enzyme than on the concentration of substrate(s).

The rate constants k in eq. (1) correlate with the disappearance rate of substrate and appearance rate of product. Instead of k_2, sometimes k_{cat} is used, especially in more complex reactions.

Soon after mixing enzyme and substrate, usually a steady state is reached in which the concentration of enzyme–substrate complex (and other possible intermediates) and the overall reaction rate are nearly constant while the rate constant k_{-2} is still practically zero. Theoretically, the initial velocity of a reaction under these conditions approaches first order kinetics with respect to substrate concentration. This is described by the Michaelis–Menten equation [1],

$$v = \frac{V_{max} s}{K_m + s} \text{ or } K_m = s\frac{V_{max} - 1}{v} \qquad (2)$$

in which v is the initial rate of product formation, s is the substrate concentration, and V_{max} is the maximal initial reaction rate at a given concentration of enzyme. K_m or the Michaelis constant is an important characteristic of an enzyme–substrate combination under specific circumstances. Its value depends on temperature, pH, and other reaction conditions like buffer composition, *etc.* K_m is a complex constant composed of the reaction rate constants from eq. (1):

$$K_m = \frac{k_{-1} + k_2}{k_1} \qquad (3)$$

The K_m values for most enzyme–substrate complexes range from 10^{-1} to 10^{-6} M, the turnover number per molecule of enzyme–substrate complex usually is in the range between 10^1 and 10^4 s^{-1}.

The steady-state approach to enzymatic reactions is an idealized one and will often suffer from oversimplification. Several pitfalls and remedies in kinetic enzyme studies have been clearly depicted by K.F. Tipton [2] and should be kept in mind when determining V_{max} and K_m by any method.

When eq. (2) applies, V_{max} and K_m can be estimated with graphic methods from measurements of initial reaction rates at different substrate concentrations, *e.g.* with the well-known Lineweaver–Burk plot, which is the least appropriate one, however. Although visual graphic methods have become outdated with the advent of suitable statistical software, they are still useful for revealing irregularities in the data points obtained [3].

If possible with the enzyme–substrate combi-

nation studied, the most convenient way to determine v will be direct and continuous measurement of product or substrate concentration. Such 'real-time' assays include continuous monitoring of pH, absorbance, fluorescence, electrochemical titration, volumetric gas development, and many others. Continuous methods may be indirect, *e.g.* when the reaction product is converted to a secondary product in a relatively fast reaction by additional reagent or enzyme. This approach is preferred when the secondary product is more readily detectable than the primary one. Examples are the detection of liberated free sulfhydryl groups with Ellman's reagent (DTNB) or coupled assays like those involving a dehydrogenase as the second enzyme and NADPH/$NADP^+$ [2].

Discontinuous methods involve stopping the reaction at fixed times, whether or not after sampling the reaction mixture. Afterwards the samples can be assayed in many different ways. Although this approach usually is more laborious and produces less data points for the initial reaction rate than continuous methods do, these drawbacks may be more than compensated for by the possibilities it offers for highly specific or sensitive measurement of the reaction course.

Separation of substrates and products by HPLC, the subject of this review, is one of the promising examples of discontinuous enzyme assay methods.

2. Enzymes in medicine

Already in 1908 amylase activity was determined in urine as an indicator of acute pancreatitis. Nowadays clinical chemistry laboratories routinely determine a number of enzymatic activities as diagnostic markers. For example, to diagnose heart attacks at least 100 million creatine kinase measurements are performed worldwide [4]. As a consequence of certain diseases, intracellular enzymes may leak into the general circulation. The presence in serum and the concentration of such enzymes may be related to the tissue of origin and therefore indicative of a certain disease state. The tissue distribution of a number of clinically important enzymes is given in Table 1.

Table 1
Principal tissue localization of some diagnostically important enzymes

Enzyme	Main localization
Acid phosphatase	Prostrate erythrocytes, lysosomes
Aldolase	Skeletal muscle, heart
Alkaline phosphatase	Bone (osteoblasts), intestinal mucosa, liver, placenta, kidney
Amylase	Pancreas, saliva
Arginase	Liver
Acetylcholinesterase	Brain, nervous tissue, erythrocytes
Alanine aminopeptidase	Kidney, intestine
Alanine transaminase	Liver, skeletal muscle, heart
Aspartate transaminase	Heart, liver, skeletal muscle, kidney, brain
Creatine kinase	Skeletal muscle, heart, brain
Glucose-6-phosphatase	Liver
Isocitrate dehydrogenase	Liver
Glutamate dehydrogenase	Liver
Lactate dehydrogenase	Heart, liver, skeletal muscle, kidney, erythrocytes, pancreas, lung
5′-Nucleotidase	Hepatobiliary tract, pancreas
Ornithine carbamoyltransferase	Liver
Trypsin(-ogen)	Pancreas

Data from J.H. Wilkinson, *The Principles and Practice of Diagnostic Enzymology*, Edward Arnold, London, 1976.

3. Advantages of HPLC enzyme assays

All enzyme HPLC assays do have the same basic design. A design including precolumn derivatization is shown in Fig. 1.

Enzyme activities can be determined in different ways. As mentioned in the introduction, continuous spectrophotometric monitoring is a simple way of determination of a particular enzyme activity. However, the biological matrix in which the enzyme is present may disturb spectrophotometric detection. During a simple enzymatic reaction the amount of substrate decreases and product is formed. In principle, HPLC offers the possibility of monitoring of substrate and product. A prerequisite is separation of substrate and product. Separation can be obtained by chromatography and because of its high resolution, reversed-phase HPLC is the method of choice. The procedure in which the reaction components are labeled after the enzymatic reaction and prior to separation and detection is designated as *precolumn derivatization*. An attractive variant of precolumn derivatization is labeling of the substrate molecule prior to the enzymatic reaction. However this has to be done at a position which does not interfere with the formation of the enzyme–substrate complex, to allow undisturbed reaction. When the reaction components are first separated by HPLC and then labeled, it is designated as *postcolumn derivatization*.

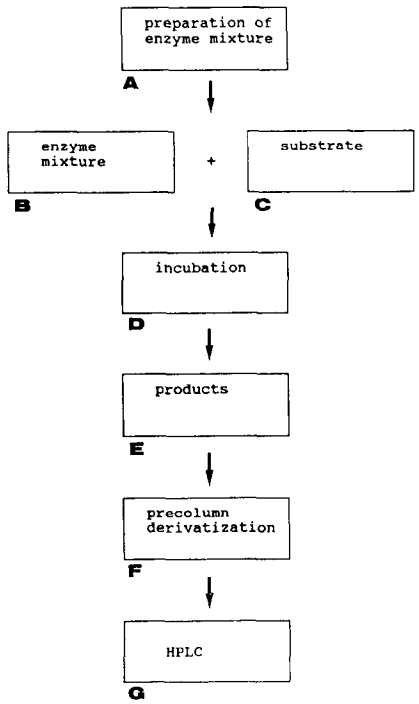

Fig. 1. (A) Homogenisation of enzyme-containing material and centrifugation; (B) the supernatant of A is adjusted to the pH required for enzymatic reaction; (C) enzyme is added to the substrate; at suitable time intervals, samples are withdrawn and the reaction is terminated (*e.g.* by inactivation of enzyme), or alternatively, many similar incubation mixtures are prepared; substrate and enzyme are added at $t = 0$ and the reaction is terminated at the desired time; (D) incubation; (E) the substrate is converted into products; (F) products are labeled with a tag; (G) HPLC and detection.

There is no requirement for labeling or pre- or postcolumn derivatization [5], when the components of an enzymatic reaction can be separated by HPLC and subsequently detected at a suitable wavelength. However, one of the advantages of pre- and postcolumn derivatization is that the reaction components can be detected at much higher sensitivity than without derivatization. An advantage of precolumn derivatization is that extraction, purification and chromatographic properties of the compounds can be manipulated. Derivatization of rather similar reaction components, *e.g.* amino acids, may result in compounds that are more easily separated than the unlabeled compounds. A disadvantage is that degradation may occur during derivatization and that reactions may not always be complete. This results in additional peaks in the elution profile and in inaccurate determination of the enzyme activity.

Postcolumn derivatization is always carried out on-line and has the advantage that the elution profile will not contain additional peaks due to degradation. The reaction components are separated in their original form, derivatized and detected. Disadvantages are the extra-column bandbroadening due to the volume of the postcolumn reactor and the presence of an excess of the derivatization reagent which may interfere with the detection.

A choice of reagents is available for pre- and postcolumn derivatization [6]. Some of the re-

agents particularly important to this review are those reacting with amines producing fluorescent derivatives which are then separated by reversed-phase HPLC. They generally result in considerably lower detection limits than without derivatization. Frequently used are dansyl-Cl [7,8], *o*-phtalaldehyde (OPA) [9,10], 9-fluorenylmethyl chloroformate (FMOC) [11] and phenylisothiocyanate (PITC) [12]. The reagents used in the studies mentioned in this review are listed in Table 2.

Whether derivatization and which type of derivatization is necessary, depends not only on the reaction components but also on the biological matrix in which the reaction takes place. The possibility to assay an enzyme in a complex biological matrix is one of the main advantages of an HPLC enzyme assay. Separation mode and other conditions can be manipulated easily to allow detection of substrate and product peaks without interference of the background resulting from the biological matrix. In a number of cases, pretreatment of the samples is still necessary. Often, precipitation of protein originating from the biological matrix by the organic component of the elution buffer suffices as precleaning procedure.

When a suitable separation can be designed, it is also possible to determine several enzymatic activities simultaneously. The peak heights of the reaction components are indicative of these activities.

More detailed information on HPLC in enzymatic analysis can be found in refs. 13–16. An excellent review on the application of HPLC to the study of biogenic amine-related enzymes was given by Nagatsu [17].

In the following paragraphs, enzymatic activities determined by HPLC without derivatization and those determined after pre- and post-column derivatization will be summarized. The derivatization procedures as well as the biological matrices in which the enzymes were determined will be indicated. No specific mention of the mode of HPLC will be given. In the majority of the assays, reversed-phase HPLC was the preferred method. Specific examples will be given from the literature and our own research with an extremely complex biological matrix, *i.e.* faecal material. Finally, future prospects in using HPLC enzyme assays will be given.

4. Enzymatic activities determined by HPLC

Enzymes involved in cleavage of antibiotics, proteins and peptides, in the metabolism of amino acids, carbohydrates, catecholamines, heme, polycyclic hydrocarbons, pterins, purines and steroids are listed in Table 2. Whether post- or precolumn derivatization or no derivatization at all was used is indicated. The table contains the name of the enzyme, the substrate and the product. In addition, the biological matrix and the detection wavelength or derivatization reagent is given. In more than 50% of the assays direct detection at various wavelengths or direct fluorescence detection was used. This suggests that a satisfactory level of sensitivity was obtained without derivatization. Precolumn derivatization was used in a quarter of the assays cited and it is the most popular derivatization method, probably because of the advantages mentioned in paragraph 3. Postcolumn derivatization is not very popular and was used in only four assays. Direct detection at a particular wavelength at which background absorbance is at its minimum and the absorbance of substrate and products at its maximum is the easiest way to perform an HPLC enzyme assay. Direct fluorescence detection may enhance the sensitivity of the assay. Precolumn derivatization is not only aiming at enhancement of sensitivity, but also provides substrate and products with a fluorescent tag which often facilitates separation by reversed-phase HPLC. An excellent example of this is the labeling of amino acids by 9-fluorenylmethylchloroformate (FMOC). The resulting FMOC-amino acids can then be separated by reversed-phase HPLC [11]. Postcolumn derivatization generally requires more effort. After separation of substrate and product, an on- or off-line derivatization procedure has to be carried out. A reason to use postcolumn derivatization, may be the availability of an

Table 2
Enzymes assayed by HPLC

Enzyme	Substrate	Product	Biological matrix	Detection	Ref.
Acetanilide 4-hydroxylase (EC 1.14)	Acetanilide	4-Hydroxyacetanilide	Mouse liver	RC/254 nm	18
ACV synthetase (EC 6.3.2)	L-α-Aminoadipic acid	L-α-Aminoadipyl-L-cysteinyl-D-valine	Mycelium extract	Pre/OPA/FL	19
Adenosine deaminase (EC 3.5.4.4)	Adenosine	Inosine	Erythrocyte lysate	254 nm	20,21
Adenylyl cyclase (adenylate cyclase, EC 4.6.1.1)	Formycin ATP	Cyclic formycin AMP	Rat osteosarcoma cells	FL	22
Adenylate kinase (myokinase, EC 2.7.4.3)	Formycin adenosine ATP + AMP	Formycin AMP 2ADP	Mouse liver Fungal cells	FL RC 254 nm	23 24
Adenylosuccinate synthetase (EC 6.3.4.4)	IMP + aspartic acid	Adenylosuccinate	Fungal cells	254 nm	25
Adenosine 3′-phosphate 5′-sulfophosphate sulfotransferase (EC 2.8.2)	Adenosine 3′-phosphate 5′-sulfophosphate (PAPS)	PAP	Plants	RC/254 nm	26
Alkaline phosphatase (EC 3.1.3.1)	AMP Formycin 5′-monophosphate	Adenosine Formycin A	Calf intestinal mucosa	254 nm FL	27 28
δ-Aminolevulinate dehydrase (EC 4.2.1.24)	2 × δ-Aminolevulinic acid	Porphyrobilinogen	Blood lysate	240 nm	29
δ-Aminolevulinic acid synthetase (EC 2.3.1.37)	Gly + succinyl-CoA	δ-Aminolevulinic acid	Bone marrow cells	Pre/RC/pyrr/278 nm	30
Aminopeptidase (EC 3.4.11)	Enkephalins, β-endorphins	Tyr-Gly-Gly, Tyr-Gly, Tyr	Serum, rat brain	ED/205 nm	31
AMP-Deaminase (EC 3.5.4.4)	AMP or formycin 5′-AMP	IMP or formycin 5′-IMP	Fungal cells	254 or 295 nm	25
α-Amylase (EC 3.2.1.1)	Maltoheptose	Smaller oligosaccharides	Human pancreas	RF	32
Angiotensin-converting enzyme (EC 3.4.15.1)	Hyp-His-Leu ANG I ANG II	Hippuric acid ANG II His-Leu	Blood, lung, kidney etc. Serum Rat lung	228 nm Pre/benz/FL Pre/fluoresca/FL	33 34 35
Aromatic L-amino acid decarboxylase (EC 4.1.1.28)	L-DOPA	Dopamine	ED		36
Aryl alkylamine N-acetyltransferase (EC 2.3.1.87)	Tryptamine	N-acetyltryptamine	Retinal/pineal gland	FL	37
Aryl hydrocarbon hydroxylase (EC 1.14.14.2)	Benzo[*a*]pyrene	Detoxified benzo[a]pyrene	Rat microsomes	FL	38
Arylsulfatase B (EC 3.1.6.1)	UDP-galNAc-4-sulfate	UDP-galNAc + sulfate	Human liver	262 nm	39
β-Aspartylpeptidase (EC 3.4.13.10)	β-Aspartylpeptides	Asp and other amino acids	Faeces	Pre/FMOC/FL Pre/PITC/254 nm	40,41 42

cont.

Table 2 *(continued)*

Enzyme	Substrate	Product	Biological matrix	Detection	Ref.
Asparagine synthetase (EC 6.3.1.1)	Asp + Gln + ATP	Asn + Glu + AMP	Bovine pancreas	Pre/OPA/FL	43
ATP pyrophosphohydrolase (EC 3.6.1.8)	ATP	AMP	Fungal cells	254 nm	24
Biotinidase (EC 3.5.1.12)	Biotinyl-6-aminoquinoline	6-Aminoquinoline	Milk, serum	FL	44
Ceruloplasmin	Biogenic amines e.g. adrenaline	Oxidized biogenic amines (aminochromes)	Commercial enzyme or serum	300 nm	45
Collagenase (EC 3.4.24.3)	DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg	DNP-Pro-Gln-Gly	Tadpole skin	206 or 365 nm	46
Creatine kinase (EC 2.7.3.2)	Phosphocreatine + ADP	Creatine + ATP	Commercial enzyme	254 nm	47
Cyclic nucleotide phosphodiesterase (EC 3.1.4.17)	Cyclic formycin monophosphate	Formycin AMP	Fungal cells	FL	25
	2',3'-cyclic AMP	2'-AMP	Rat cerebral tissue	254 nm	48
	2',3'-cyclic CMP	2'-CMP			
Cytidine monophosphate-sialic acid synthetase (EC 2.7.7.43)	N-Acetylneuraminic acid (NANA) + CTP	CMP-NANA	Calf brain	270 nm	49
Diamine oxidase (EC 1.4.3.6)	Putrescine	2,2'-dihydroxy-3,3'-dimethoxybiphenyl-5,5'-diacetic acid	Pea seedlings	FL/254 nm	50
Diaminopimelate decarboxylase (EC 4.1.1.20)	*meso*-DAP	L-Lysine		Pre/OPA/FL	51
Diaminopimelate epimerase (EC 5.1.1.7)	*meso*-DAP	LL-DAP		Pre/OPA/FL	51
Dihydrofolate reductase (EC 1.5.1.3)	H_2-Biopterin	H_4-Biopterin	Rat brain	FL	52
Dihydroorotase (EC 3.5.2.3.)	^{14}C Carbamyl-aspartate	^{14}C Dihydroorotate	Rat liver	RC	53
Dinucleoside polyphosphate pyrophosphohydrolase (EC 3.1.4)	Diadenine nucleotides	Adenine nucleotides	Fungal cells	254 nm	54
Dipeptidase (EC 3.4.13.11)	Hippurylhistidylleucine (Hip-His-Leu)	Hippuric acid	Rat blood, lung, kidney	228 nm	55
Dopamine β-hydroxylase (EC 1.14.17.1)	Dopamine	Noradrenaline		ED/280 nm	56,57
	Tyramine	Octopamine		Post/OPA/FL	17
Folic acid cleaving enzyme	Folic acid	Pterin-6-aldehyde + *p*-aminobenzoyl-glutamate	Fungal cells	254 nm/RC	58
β-Galactosidase (EC 3.2.1.23)	1-O-Gal-2-N-Dns-sphingosine	N-Dns-Sphingosine	Rat brain	Pre/Dns/FL	59
Galactosyltransferase (EC 2.4.1.22)	UDP-Gal	UDP	Commercial; human serum	260 nm	60
Glutamate synthase (EC 1.4.7.1)	L-Glutamine	L-Glutamic acid	Bacteria, rice leaves	Pre/OPA/FL	61
Glutaminyl cyclase	Q-L-Y-E-N-K-Dns-OH	< E-L-Y-E-N-K-Dns-OH	Bovine pituitary	Pre/FL	62

cont.

Table 2 *(continued)*

Enzyme	Substrate	Product	Biological matrix	Detection	Ref.
Glutathione S-transferase (EC 2.5.1.15)	Styrene oxide	Conjugates of styrene oxide and reduced glutathione	Rat lung, liver	254 nm	63
Guanosine triphosphate cyclohydrolase I (EC 3.5.4.16)	GTP	D-*erythro*-dihydroneopterin triphosphate	Rat liver	254, 278 nm/FL	64
Hepatic microsomal testosterone hydroxylase (EC 4.1.99)	Testosterone	Hydroxylated testosterone products	Rat liver microsomes	240 nm	65,66
Hexokinase (EC 2.7.1.1)	ATP	ADP	Commercial hexokinase	254 nm	67
Histamine N-methyltransferase (EC 2.1.1.8)	Histamine	N^{τ}-methylhistamine	Rat kidney	Post/OPA/FL	68
11-β-Hydroxylase and 18-hydroxylase (EC 1.14.99)	11-Deoxycorticosterone	Corticosterone, 18-OH-11-deoxycorticosterone	Rat adrenal cortex	254 nm	69
Δ^5-3β-hydroxysteroid dehydrogenase (EC 1.1.1.145)	Pregnenolone	Progesterone	Rat ovary	240 nm	70
25-Hydroxyvitamin D_3-1α-hydroxylase	25-Hydroxyvitamin D_3	1,25-Dihydroxy-vitamin D_3	Chicken kidney, liver	254 nm	71
Hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8)	Hypoxanthine + P-ribosylpyrophosphate	IMP + pyrophosphate	Fungal cells	254 nm	25,72
β-Lactamase (EC 3.5.2.6)	β-Lactam antibiotics	Antibiotic open-ring form	Faeces	Several wavelengths	73–76
Lactose-lysine β-galactosidase (EC 3.2.1)	Lactose-lysine	Fructose-lysine	Mouse intestine	Post/OPA/FL	77
Luteinizing hormone-releasing hormone peptidase (EC 3.4.4)	Luteinizing hormone-releasing hormone ($LHRH_{1-10}$)	$LHRH_{1\text{-}5,6\text{-}10,1\text{-}3}$	Rat hypothalamus	210 nm	78
$C_{17\text{-}20}$ Lyase	^{3}H-17α-Hydroxypregnenolone	Dihydroepiandrosterone	Human testes	RC	79
Lysosomal enzymes *e.g.* glucosaminidase (EC 3.2.1.30)	Several compounds *e.g.* N-acetyl-glucosamines conjugated with 4-methylumbelliferone	4-Methylumbelliferone	Urine	Pre/FL	80
Methionine synthase (EC 2.1.1.13)	N^5-Methyltetrahydrofolate	Methionine	Rat liver cell extract, Mouse embryo fibroblasts	Pre/OPA/FL	81
NAD Glycohydrolase (EC 3.2.2.5)	NAD^+	Nicotinamide	Fungal cells	259 nm	82
Nicotinate phosphoribosyltransferase (EC 2.4.2.11)	5-Phosphoribo-α-D-pyrophosphate + nicotinic acid	Nicotinate mononucleotide + pyrophosphate	Yeast	254 nm	83
Nucleoside phosphorylase (EC 2.4.2.1)	Inosine + phosphate	Hypoxanthine + ribose-1-phosphate	Erythrocyte lysate	254 nm	84
5′-Nucleotidase (EC 3.1.3.5)	Nucleoside monophosphate	Nucleoside	Erythrocyte lysate	254 nm	85
Ornithine aminotransferase (EC 2.6.1.13)	L-Ornithine, 2-oxoglutarate	Glutamate and ^{1}-pyrroline-5-carboxylic acid	Rat liver	Pre/O-aminobenzald 254 nm	86

cont.

Table 2 *(continued)*

Enzyme	Substrate	Product	Biological matrix	Detection	Ref.
Ornithine decarboxylase (EC 4.1.1.17)	Ornithine	putrescine	Rat intestinal mucosa	Pre/fluoresca/FL	87
Oxalate oxidase (EC 1.2.3.4)	Oxalic acid	2,2′-Dihydroxy-3,3′-dimethoxybiphenyl-5,5′-diacetic acid	Barley seedlings	254 nm	88
Papain esterase (EC 3.4.22.2)	N-Benzoyl-L-arginine ethyl ester (BAEE)	Benzoylarginine	Plants	254 nm	78
Phenylethanolamine N-methyltransferase (EC 2.1.1.28)	Noradrenaline (norepinephrine)	Adrenaline (epinephrine)	Rat hypothalamus	ED Post/trihydroxyindol Pre/DPED/FL	89 90 91
Plasma carboxy-peptidase (kininase I, bradykinine destroying enzyme) (EC 3.4.17.1)	Hippuryllysine (Hip-Lys)	Hippuric acid	Plasma	230 nm	92
Sialidase (neuraminidase, EC 3.2.1.18)	PA-Sialyllactose (fluorescent)	PA-Lactose	Urine	FL	93
Spermidine synthetase	Putrescine	Spermidine	Mouse brain	RC/254 nm	94
Sulfotransferase (EC 2.8.2)	β-Naphthol	β-Naphthol sulfate	Mouse hepatic cells	235 nm	95
Taurodeoxycholate hydrolase (EC 3.5.1.24)	Taurodeoxycholate	Taurine	Faeces	Pre/FMOC/FL	40
Threonine/serine dehydratase (EC 4.2.1.16)	Threonine, serine	2-Keto acids	Plant extract	Pre/OPDA	96
Trypsin (EC 3.4.22.4)	N-Benzoyl-L-arginine ethyl ester (BAEE)	Benzoylarginine	Commercial trypsin	254 nm	97
Tryptophanase (EC 4.2.1.e)	Tryptophan	Indole	Bacterial cells	FL	98
Tryptophan hydroxylase (EC 1.14.16.4)	L-Tryptophan	L-5-Hydroxy-tryptophan		FL	99
Tyrosine hydroxylase (EC 1.14.16.2)	L-Tyrosine	L-DOPA	Brain tissue	ED FL Pre/DPED/FL	100 101 102
β-Ureidopropionase (EC 3.5.1.6)	N-Carbamoyl-β-alanine	β-Alanine + CO_2 + NH_3		Pre/PITC/245 nm	103
Uridine diphosphate glucuronosyltransferase (EC 2.4.1.17)	Uridine diphosphate glucuronic acid + 4-nitrophenol	4-Nitrophenol glucuronide	Rat liver	300 nm	104
	+ α-Naphthol	α-Naphtol glucuronide	Mouse hepatic microsomes	240 nm	95
Urokinase (EC 3.4.99.26)	Gly-Arg-4-nitroanilide	4-Nitroaniline	Commercial urokinase	230 nm	105
Uroporphyrinogen decarboxylase (4.1.1.37)	Uroporphyrinogen	Coproporphyrinogen	Mouse liver	Light oxidation/400 nm	106
Xanthine oxidase (EC 1.2.3.2)	6-(3-Methylbut-2-enylamino)purine (IPA)	8-Hydroxy IPA	plant tissue	275 nm	107

adequate separation method for the unlabeled components of the reaction mixture.

5. Selected biomedical applications

5.1. Inactivation of antibiotics by faecal enzymes; no derivatization

Examples of enzymatic activities present in a complex biological matrix *viz.* faecal material, will be presented to illustrate the subject of this paragraph.

Faecal material from various animal sources, *e.g.* experimental animals, is a complex mixture of many compounds. Among these are polypeptides of different sizes and pigments. The presence of these substances results in high backgrounds in a wide wavelength-range. This may interfere with a detection wavelength that could be normally used to detect the pure components of an enzymatic incubation mixture after HPLC. This is even more difficult when human faecal material is used, since intra- and interindividual differences are generally large. We have studied the inactivation of ß-lactam antibiotics by ß-lactamase-activity in human faeces.

The normal flora of the digestive tract protects the host against colonization by potentially pathogenic bacteria from the environment. Especially in immunocompromised patients infections by potentially pathogenic (endogenous) Gram-negative bacteria should be avoided. To this end, a selective non-absorbable ß-lactam antibiotic can be applied. Such an antibiotic selectively eliminates Gram-negative organisms from the intestinal tract whilst the normal flora (>99% anaerobic bacteria) remains intact. The normal flora provides a protective barrier against colonization by potentially pathogenic bacteria [108]. The suitability of the ß-lactam antibiotic aztreonam for this purpose was investigated [109]. It was found that Gram-negative bacteria remained present in the faeces of a few volunteers taking aztreonam orally and that despite the non-absorbable properties of aztreonam, it was not present in the faeces of these volunteers.

This prompted us to investigate the possible presence of β-lactam-inactivating enzymes in faeces [73]. Aztreonam was used as substrate and incubated with faecal enzyme preparations. The enzyme preparations were crude mixtures, *i.e.* a 1:3 diluted suspension of faecal material without solids. Despite the high background from the faecal material it was possible to design a reversed-phase HPLC separation in which most of the faecal background material was eluted before the aztreonam peak which was eluted at 9 min (see Fig. 2d). The absorbance was monitored at 293 nm. Peak heights were proportional to concentration and the percentage inactivation was calculated from the decreases in peak height by comparison with the peak height of aztreonam in the incubation mixture without faecal enzyme. Aztreonam was inactivated for 50% in 4 h in the faeces of 2 volunteers. The enzyme activity could be inhibited by clavulanate, a well-known β-lactamase inhibitor indicating that the inactivating enzyme was a β-lactamase. This was further supported by the appearance of a peak after aztreonam cleavage with an elution position identical to that of the open-ring form of aztreonam (Fig. 2c, 5 min). Such enzymes have

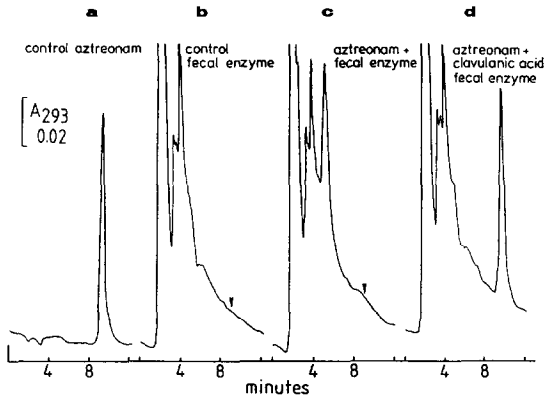

Fig. 2. Enzyme HPLC assay of aztreonam incubated for 20 h with a faecal enzyme preparation from a healthy volunteer. (a) Control aztreonam; (b) control faecal enzyme preparation; (c) aztreonam incubated overnight with faecal enzyme preparation; (d) aztreonam incubated overnight with faecal enzyme and 400 μmol clavulanate. The arrow indicates the elution time of aztreonam (reproduced with permission from ref. 73).

been reported to be produced by anaerobic bacteria [110] and this β-lactamase activity was present, independent of aztreonam treatment [74].

This HPLC enzyme assay shows that it is possible to determine enzyme activity in a complex biological matrix. Despite the high background, substrate (aztreonam) and product (open ring form) could be detected. These studies were extended to β-lactam antibiotics with other properties, *e.g.* the absorbable amoxycillin which is frequently applied to treat infections. Although a large fraction is absorbed in the upper intestinal tract, the remainder of this broad-spectrum antibiotic may eliminate the protective anaerobic flora in the lower intestinal tract, thereby lowering the level of colonization-resistance. However, β-lactamase activity similar to the one mentioned above is frequently present in the lower intestines. Surplus amoxycillin is then inactivated and the protective barrier against colonization by potentially pathogenic microorganisms remains intact. An average of 49% inactivation of amoxycillin in 20 h was found [75]. This antibiotic is also administered together with the β-lactamase inhibitor clavulanate in a ratio 5:1 (Augmentin). The advantage of the enzyme assay by HPLC is that not only the substrate can be seen as a peak but also the inhibitor which has a structure similar to the antibiotic. In the same study Augmentin was used as substrate and it was shown that clavulanate itself was barely if at all inactivated by faecal enzyme preparations.

In addition it was found that the 49% inactivation of amoxycillin was reduced to an average of 20%. The effect was most pronounced with the faecal enzyme preparations from those volunteers who showed the highest percentage of amoxycillin inactivation, *e.g.* a decrease from 65 to 10% inactivation. These findings may have implications for the oral application of either amoxycillin or Augmentin (amoxycillin/clavulanate) for treatment of infections. The combination has the advantage of ß-lactamase resistance but its use may result in a substantial reduction of the protective bacterial flora. Amoxycillin is less ß-lactamase-resistant and is likely to be inactivated by ß-lactamase activity as soon as it arrives in the lower intestinal tract. As a result, the protective bacterial flora will remain largely intact.

5.2. Examples of HPLC enzyme assays using precolumn derivatization

5.2.1. Methionine synthase in liver and cell extracts

N^5-Methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase) plays an important role in metabolic regulation and the enzyme is also involved in several processes related to human disease. It catalyzes transfer of a methyl group from N^5-methyltetrahydrofolate to homocysteine resulting in methionine and tetrahydrofolate. Assays of this enzyme are generally based on measurement of the formation of [^{14}C]methionine from N^5-[*methyl*-^{14}C]methyltetrahydrofolate after separation of substrate and product by anion-exchange chromatography. A reducing system is required and the incubation is often performed in an anaerobic atmosphere. Garris *et al.* [82] developed a nonradioactive assay based on precolumn derivatization of the product methionine with *o*-phtalaldehyde followed by reversed-phase HPLC and fluorescence detection. The assay requires reducing conditions which were provided by 2-mercaptoethanol which is already present in the *o*-phtalaldehyde derivatization reagent mixture. Anaerobic conditions were not necessary when the reaction mixtures were covered with an oil layer of bis(3,5,5-trimethylcyclohexyl)phtalate. Almost complete conversion of methionine was obtained by derivatization. The reaction was terminated by adding perchloric acid, followed by neutralization with $KOH/KHCO_3$. The *o*-phtalaldehyde reagent contains 0.1 *M* sodium borate, pH 9.5, which results in a pH of *ca.* 9 in the derivatization mixture. At low pH, fluorescence of *o*-phtalaldehyde adducts decreases. Norvaline was added to the incubation mixture as internal standard to correct for error due to inaccurate pH adjustment. Fig. 3 shows the elution profile when methionine synthase was assayed in Hl-60 cells. This nonradioactive assay

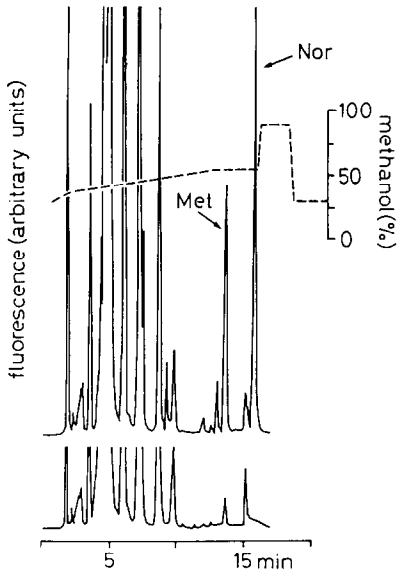

Fig. 3. Extracts from Hl-60 cells (4.4 mg/ml) were assayed for methionine synthase for 120 min; the incubation mixture was deproteinized with acid, neutralized and derivatized with *o*-phtalaldehyde. The sample was subjected to reversed-phase HPLC on an ODS Hypersil column equilibrated with 30% methanol in 50 m*M* sodium phosphate, pH 5.0. The column was eluted with a methanol gradient. The flow-rate was 2 ml/min. The retention times of the *o*-phtalaldehyde adducts of methionine (Met) and norvaline (Nor) were 13.6 and 15.7 min, respectively. The lower trace represents the corresponding assay blank (reproduced from ref. 81 with permission).

was evaluated by comparison with a conventional radioactive assay. They gave comparable results. However the nonradioactive method has the advantage that the unstable and expensive substrate N^5-[*methyl*-^{14}C]methyltetrahydrofolate is not required.

5.2.2. Threonine dehydratase in plants extracts

Threonine dehydratase catalyzes the production of 2-keto-butyrate from threonine. It is a key enzyme in plants in the biosynthesis of isoleucine. Threonine dehydratase is commonly assayed by derivatization of the ketoacid products with 2,4-dinitrophenylhydrazine (DNPH). The resulting hydrazones are colored under alkaline conditions. Other enzymes present in a plant extract may interfere with this assay and the total color yield will originate from the activity of more than one enzyme. Therefore, Singh *et al.* [97] developed an HPLC assay that allowed identification of the reaction products. Incubation of a crude enzyme preparation extracted from tomato plants with threonine was followed by derivatization of the resulting ketoacids with 1,2-*o*-phenylenediamine (OPDA). Derivatized ketoacids were extracted with ethylacetate. The ethylacetate was dried and subjected to reversed-phase HPLC. The absorbance was monitored at 254 nm. For fluorometric analysis, the excitation and emission wavelengths were 340 and 410 nm, respectively. Fluorescence detection gives a 40-fold higher sensitivity compared to UV detection. When DNPH was used as derivatizing agent 10 nmol of keto acid was the lowest amount that could be detected. OPDA derivatization with fluorescence detection is 1000 times more sensitive. These results clearly illustrate the advantage of this HPLC assay over a conventional threonine dehydratase assay.

5.2.3. ß-Aspartylpeptidase and conjugated bile acid hydrolase activity in the intestinal tract; precolumn derivatization

ß-Aspartylpeptidase is an example of a bacterial enzyme that was discovered *via* its substrate which accumulated in the intestinal tract in a bacteria-free environment. The dipeptide ß-aspartylglycine (substrate) was found to be present in faecal supernatants of germ-free mice and antibiotic-treated patients [111–113]. This dipeptide was shown to be a useful indicator of a substantial decrease in the total number of bacteria in the intestinal tract [113,114]. ß-Asp and ß-Asn present in the amino acid sequence of a protein cyclize to the cyclic imide and are converted to ß-Asp. An example of such a protein is bovine pancreatic RNase which contains an Asn–Gly sequence that is converted to a ß-Asp–Gly sequence. Extensive degradation of RNase by proteolytic enzymes (non-bacterial) has been shown to result in a mixture of free amino acids and ß-Asp peptides [115]. ß-Asp peptides accumulate in the intestinal tract as a result of host-derived proteolytic enzymes and the *absence* of a special type of bacterial enzymes, *i.e.* ß-aspartylpeptidases. The specific

peptide bond of β-aspartylpeptides can only be cleaved by these bacterial enzymes. This particular enzymatic activity might be a useful indicator of a largely intact intestinal flora, provided that it is produced by various strains of bacteria. β-Aspartylpeptidase activity was assessed by an HPLC enzyme assay in strains of anaerobic bacteria isolated from human faeces. In 12 of 14 strains this activity was present, which indicates that its absence indeed reflects a substantial disturbance of the anaerobic bacterial flora [41]. The results of this study indicate that β-aspartylpeptidase activity in faeces might be used as an indicator of the level of colonization-resistance, *i.e.* the presence of a barrier against colonization of potentially pathogenic microorganisms from the environment. One study in which it was used to determine the level of colonization resistance will be given as an example [40]. In addition, the usefulness of a second enzymatic activity, taurodeoxycholate hydrolase, was investigated. Bile acid hydrolases are produced by many bacterial species [116–118].

Faecal suspensions from 8 human volunteers and from 10 patients treated with non-absorbable broad-spectrum antibiotics were prepared by homogenization. Supernatants were centrifuged and half of the resulting supernatants was dialyzed against demineralized water for the deconjugation assay and the other half against phosphate buffered saline (PBS) pH 7.2 for the ß-aspartylpeptidase assay. The retentates were used as enzyme preparations. Taurodeoxycholate and ß-aspartylalanine were incubated with these enzyme preparations and the reaction was terminated by adding FMOC-reagent (see below).

For precolumn derivatization, FMOC-Cl in acetonitrile (3.85 mg/ml) was added to the incubation mixtures, followed by sodium borate, pH 8.5. This mixture was subjected to reversed-phase HPLC. In the deconjugation assay the column was eluted with 35% acetonitrile in 0.1% TFA. In the ß-aspartylpeptidase assay, 50% instead of 35% acetonitrile was used. Detection is possible by fluorescence but in this study the absorbance was monitored at 254 nm.

The amino group of taurine is available for derivatization after cleavage by a bacterial hydrolase. Therefore the presence of an FMOC-taurine peak indicates deconjugation. An average of 1.5 nmol (median 1.6) of taurine was liberated by faecal enzyme preparations from the volunteers which were expected to have a normal intestinal flora and an average of 0.1 nmol (median 0) was liberated by those from antibiotic-treated patients.

The disappearance of the substrate ß-Asp–Ala was used to measure ß-aspartylpeptidase activity. The average activity (expressed as percentage degradation per 20 h) in faecal enzyme preparations of healthy volunteers was 53% (median 57) and in those of antibiotic-treated patients 2.2% (median 2.5). These results show that an enzyme HPLC-assay can be used to determine bacterial enzymatic activity in faeces and therewith provides the possibility to monitor the level of colonization resistance during treatment with antibiotics.

5.3. Bacterial β-galactosidase in the intestinal tract; postcolumn derivatization

The presence of amino acids and peptides in faeces in complete absence of intestinal microflora was studied with high-voltage paper electrophoresis. Two substances were found that appeared to be identical to the spots observed by Ersser *et al.* [119] in the faecal contents of a germ-free infant and several newborn infants. These two substances were identified as ε-N-1-(1-deoxylactulosyl)-L-lysine (lactose–lysine) and ε-N-1-(1-deoxyfructosyl)-L-lysine (fructose–lysine), which are products of the Maillard reaction [120]. This reaction takes place between a reducing sugar and the amino group of an amino acid. When protein is heated in the presence of reducing sugars, *e.g.* during milk processing, the free ε-amino group of lysine in proteins may react resulting in a glycosylated protein. Erbersdobler *et al.* found that fructose–lysine was only degraded by microorganisms [121,122]. This prompted us to study the bacterial enzymatic activity (β-galactosidase) which cleaves lactose–lysine into β-galactose and fructose–lysine. This activity might be used as a marker for the

presence of bacteria in the intestinal tract. Since lactose–lysine and its degradation product fructose–lysine could not be separated by reversed-phase HPLC, ion-exchange HPLC on a Durrum DC6A resin was used for separation. Postcolumn detection was performed with *o*-phtalaldehyde [123]. The ß-galactosidase activity in different parts of the intestinal tract of germ-free and control mice was investigated and it was shown that this activity was only present in the cecum and colon of control mice [77].

6. Conclusion and future prospects

During a simple enzymatic reaction the amount of substrate decreases and product is formed. In principle, HPLC offers the possibility of monitoring of substrate and product. A prerequisite is separation of substrate and product. Because of its high resolution, reversed-phase HPLC is often chosen as the mode of separation. There is no requirement for labeling or pre- or postcolumn derivatization, when the reaction components can be detected at a suitable wavelength. However, when they have similar chromatographic properties or do not absorb light at convenient wavelengths, or when the sensitivity has to be increased, it is advantageous to label the reaction components after the enzymatic reaction to facilitate subsequent separation and detection. Substrate and products can be distinguished quantitatively in crude mixtures. This is not only true for the primary enzymatic reaction but secondary reactions can also be accounted for. Determination of enzymatic activities by HPLC is therefore an excellent way to study multienzyme systems [15].

HPLC enzyme assays also provide the possibility to characterize postulated enzymes which have not been isolated previously. This was shown by Rossomando and Hadjimichael [124] in a chemotaxis study. They postulated the presence of a phosphoamidase in cells that might cleave a phosphoamide bond in a chemoattractant. They synthesized a substrate, hippuryllysyl(N-ϵ-5′-phospho)adenosine. As a result of phosphoamidase activity, the products hippuryllysine and AMP were separated and quantitated with HPLC.

Developments in mass spectrometry methodologies allow structural characterization of products generated during an enzymatic reaction [125]. Exact mass determination of proteins and peptides is possible by electrospray mass spectrometry (ES–MS) under mild conditions [126,127] and in addition to that, plasma desorption mass spectrometry, which is based on differcnt principles of ionization and analysis from fast atom bombardment mass spectrometry, may give structural information on fragmented peptides. The application of these mass spectrometry techniques is relatively easy when a protein substrate of known amino acid sequence is cleaved into peptides. Since the masses of the amino acids and other structural properties are known, it is possible to determine the specificity of that particular proteolytic enzyme. It will be more difficult with a non-protein substrate, *e.g.* a cephalosporin antibiotic. Cleavage by a β-lactamase present in a faecal enzyme preparation of the β-lactam ring resulting in the open-ring form of the antibiotic is one of the expected enzymatic reactions to happen. Further degradation to smaller fragments often occurs but it is difficult to predict the nature of the enzymatic reactivity responsible for this. Characterization of the products by mass spectrometry could be very helpful in the elucidation of the specificity of the secondary enzymatic reactions.

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