Journal of Chromatography, 492 (1989) 345–359
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4769

REVIEW

IMMOBILIZED ENZYME REACTORS FOR DETECTION SYSTEMS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received December 23rd, 1988, revised manuscript received February 9th, 1989)

CONTENTS

LIST OF ABBREVIATIONS

AAO	Amino acid oxidase
Ach	Acetylcholine

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AchE	Acetylcholine esterase
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ch	Choline
ChO	Choline oxidase
CK	Creatine kinase
CPG	Controlled-pore glass
ED	Electrochemical detection
FL	Fluorimetric (detection)
G6PD	Glucose-6-phosphate dehydrogenase
HK	Hexokinase
HPLC	High-performance liquid chromatography
HSD	Hydroxysteroid dehydrogenase
IMER	Immobilized-enzyme reactor
NAD	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
NADP	Nicotinamide adenine dinucleotide phosphate, oxidized
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
PMS	Phenazine methosulphate, oxidized
PMSH	Phenazine methosulphate, reduced
POD	Peroxidase
UV	Ultraviolet

1 INTRODUCTION

Since the first success by Kirkland in 1969 [1a], high-performance liquid chromatography (HPLC) has been extensively developed HPLC is a highly selective, rapid, reproducible, non-destructive and automatable method Another advantage is that it can be applied to a variety of compounds lacking volatility and thermal stability However, the sensitivity of the detection system is not always satisfactory The common HPLC methods involve the use of refractive index, ultraviolet (UV), fluorimetric (FL) or electrochemical (ED) detection The response of the refractive index detector is not satisfactory and the other detectors are applied to the compounds having a chromophore, fluorophore or electrophore, respectively

In recent years, pre- and post-column labelling methods have been developed to extend the applicability of these detectors Among these methods, enzyme reactors using immobilized enzymes (IMERs) are noteworthy because of their high sensitivity, selectivity and simplicity A compound that is unresponsive to the detector is enzymatically converted to a highly responsive com-

pound The high specificity of enzymes also gives high selectivity for the determination of trace components in complex matrices This review deals with the application of IMERs to detection systems in HPLC.

2 IMMOBILIZATION OF ENZYME

Analytical methods using various enzymes have been summarized in books edited by Bergmeyer [1b,2] The methods have been widely applied to the determination of the substrate, product, activator, inhibitor or enzymic activity The enzymes most commonly used are oxidoreductases, whose activities are easily detectable, and in combination with these enzymes other enzymes are also used Enzymes are usually used in solutions and hence are unstable towards heat or organic solvents, and cannot be used repeatedly The purpose in immobilizing enzymes is to overcome these problems and to take advantage of the particular characteristics

A variety of support materials have been developed for enzyme immobilization Among the factors to be considered are the capacity of the carrier to bind enzyme and the mechanical and chemical stability of the support In general, Sephadex, cellulose, polyacrylamide, nylon, ceramics, glass beads and silica gel are chemically modified and then used as a support Immobilization methods can be divided into two groups, that is, physical and chemical methods The former method involves the physical entrapment of an enzyme in the spaces of a polymer matrix This immobilization method is easy but the durability of the immobilized enzyme is not satisfactory In contrast, the latter method involves the immobilization of enzyme by the formation of covalent bonds, which provides a stable immobilized enzyme Most of the IMERs used for HPLC detection systems are obtained by this method. However, sometimes the enzymic activity is depressed by the formation of covalent bonds, and therefore the method is not always satisfactory The details have been reviewed in several reports [3–7]

Immobilized enzymes are advantageous in the following respects repeated use of a single batch of enzyme, facile removal of the enzyme from the reaction mixture, and increased stability against heat, organic solvents and pH In HPLC the organic modifier in the mobile phase exerts a considerable influence on the activity and stability of the enzyme The flow-rate also affects the conversion efficiency for the substrate Band broadening due to non-specific binding to the enzyme has sometimes been observed These drawbacks often limit the application of IMERs

3 APPLICATION TO THE ANALYSIS OF VARIOUS COMPOUNDS

3 1 Steroids

Since the late 1970s, IMERs have been applied to the analyses of steroids and many HPLC–IMER systems have been developed This section deals with

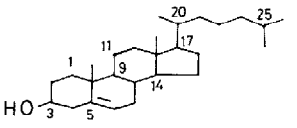
the application of the system to the determination of steroids in biological fluids

3 1 1 Sterols

Cholesterol itself has an absorption maximum of moderate molar absorptivity at 202 nm, but it is not convenient to select wavelengths lower than 210–212 nm because of low lamp intensity and solvent absorption Ogren et al [8] developed a post-column reactor containing immobilized cholesterol oxidase, which is capable of converting cholesterol to cholest-4-en-3-one, having an absorption maximum at 241 nm (Fig 1) The enzyme was immobilized on silanized porous glass with glutaraldehyde as the coupling reagent Ethanol–water (4 1) was selected as the mobile phase Ethanol contents higher than 30% depressed the activity of the IMER rapidly and irreversibly and dilution with buffer was therefore necessary to bring the ethanol content below 20% prior to the enzymic reaction The sensitivity was improved with IMER (detection UV, 241 nm) by 3 6–4 4 fold over that without the IMER at 211 nm The conversion efficiency was 72 and 60% for cholesterol and 7α-hydroxycholesterol, respectively If the amount of enzyme in the IMER was increased so that the conversion efficiency became close to 100%, the sensitivity could be increased 6–7 5 fold, but the system would be much more complicated and the peak broadening in the IMER might not be negligible

3 1 2 Bile acids

In recent years, considerable attention has been focused on the determination of bile acids in biological fluids in connection with hepatobiliary diseases Five main bile acids are present principally in free and glycine- and taurine-conjugated forms HPLC is a promising method for the simultaneous determination of these fifteen polar compounds Bile acid has a chromophore in the 17β-side-chain which is detectable at 205 nm but not so responsive to a UV detector Therefore, pre- or post-column labelling methods have been devel-



Cholesterol

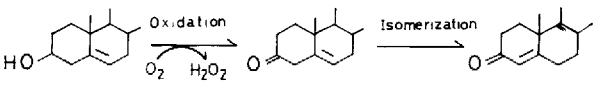


Fig 1 Reaction scheme for cholesterol oxidase

oped [9] Enzymic conversion followed by FL detection of reduced co-factor (NADH, excitation at 340 nm, emission at 457 nm) has been widely used for the analysis of bile acids.

Baba and co-workers [10–12] proposed an HPLC method for the analysis of individual bile acids using 3α-hydroxysteroid dehydrogenase (3α-HSD) together with NAD in the mobile phase However, this assay procedure consumes considerable amounts of expensive enzyme and NAD Okuyama et al [13] and Arisue et al [14] applied immobilized 3α-HSD, covalently bound to aminopropyl-CPG (controlled-pore glass), to an HPLC detection system A modified system has been developed and is currently commercially available as a bile acid analyser, the detection limits of bile acids being 10–12 pmol [15,16] Despite further improvements, satisfactory reliability and reproducibility are still serious unsolved problems with this technique, because of the unstable enzyme activity.

An HPLC method using an immobilized 3α-HSD column and ED was developed by Kamada et al [17] Bile acids in the eluate reacted with NAD in the IMER to yield NADH, which in turn was oxidized on a glassy carbon electrode after mixing with oxidized phenazine methosulphate (PMS) used as a mediator (applied potential +0 1 V) The sequential reactions took place as shown in Fig 2 NADH itself is responsive to ED using a glassy carbon working electrode, where a high applied potential (+1 0 V) is necessary and unfavourable for selective and sensitive detection A 20-pmol amount of each bile acid could be determined at the highest sensitivity of the detector These methods have inevitable disadvantages· depression of the enzymic activity of the IMER under the conditions for the separation of the fifteen bile acids, durability of the IMER, and economic problems due to the constant flow of expensive NAD solution Another problem with these systems is the insufficient sensitivity for the determination of bile acids in blood

An attempt to overcome these problems was made by Kawasaki et al [18] Employing an IMER, bile acids were converted into 3-keto bile acids, which were extracted with a Sep-Pak C_{18} cartridge (Millipore, Waters, Milford, MA, U S A), labelled with dansylhydrazine and separated by HPLC The method is highly sensitive (detection limit 0 5–1 0 pmol), but the system is off-line and

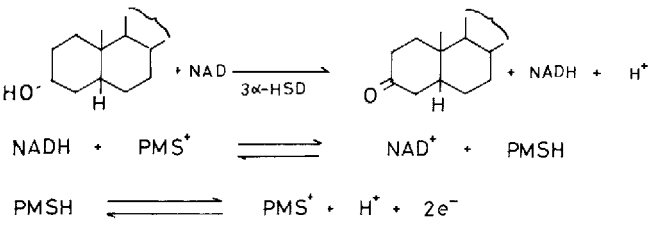


Fig 2 Reaction scheme for 3α-HSD oxidation and subsequent ED

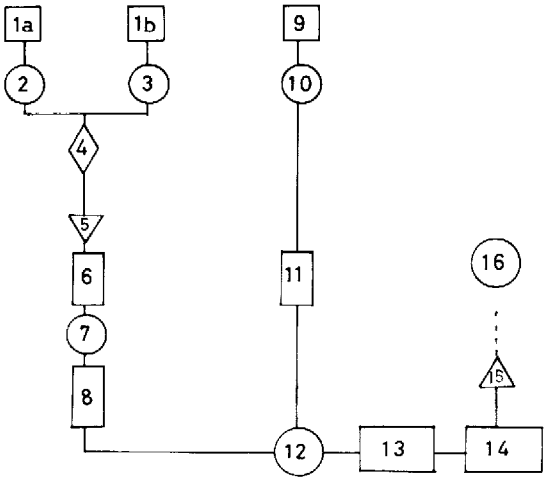


Fig 3 Schematic diagram of HPLC–IMER and off-line FL detection 1a, b = Solvent reservoirs, 2, 3, 10 = pumps, 4 = gradient elution controller, 5 = mixer, 6, 11 = pre-columns, 7 = injector, 8 = column, 9 = NAD reservoir, 12 = T-connector, 13 = IMER, 14 = FL detector, 15 = fraction collector, 16 = spectrofluorimeter for off-line detection

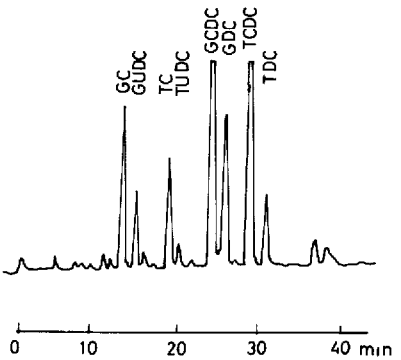


Fig 4 Chromatogram of bile acids in serum from a patient with liver cirrhosis GC = Glycocholic acid, GUDC = glycoursodeoxycholic acid, TC = taurocholic acid, TUDC = tauroursodeoxycholic acid, GCDC = glycochenodeoxycholic acid, GDC = glycodeoxycholic acid, TCDC = taurochenodeoxycholic acid, TDC = taurodeoxycholic acid

derivatization into dansylhydrazone sometimes provides two peaks corresponding to *E* and *Z* isomers

Recently, an HPLC assay system for glycine- and taurine-conjugated bile acids in serum has been developed, using a post-column reaction with immobilized 3α-HSD and off-line FL detection [19] (Fig 3) Fractions of the eluate containing each bile acid conjugate were collected and quantified with an FL detector after renewed fluorescent labelling with a commercially available en-

zyme kit (Sterognost-3α Flu®, Nyegaard, Oslo, Norway), the detection limit being 0 2 μM with reliable reproducibility A typical chromatogram is shown in Fig 4

A method with off-line FL determination for serum bile acid conjugates is too tedious for routine assays However, the separate determination of the glycine and taurine conjugates of major bile acids in serum offers the possibility of investigating serum bile acid profiles for limited purposes, e g , monitoring during gallstone dissolution therapy [19]

3 1 3 Androgens

Wu et al [20] investigated the use of an immobilized enzyme, covalently bound to aminopropyl-CPG, for the analysis of Δ^5-3β-hydroxysteroid sulphates, which possess no significant chromophore A microcolumn of immobilized 3β,17β-HSD was prepared and used for detection in HPLC The NADH produced from Δ^5-3β-hydroxysteroids by this IMER was monitored fluorimetrically Androgen conjugates in serum were extracted with ethanol followed by enzymic hydrolysis with sulphatase, which was then subjected to the HPLC–IMER system described above 5-Androstene-3β,17β-diol and dehydroepiandrosterone sulphates were detected with a detection limit of 3–10 ng A clinical trial demonstrated that this HPLC–IMER system is superior to the soluble-enzyme method, giving reliable and reproducible results at a low cost The stability of the immobilized enzyme was assessed by duplicated assays of a standard solution of dehydroepiandrosterone When the immobilized enzyme column was washed with NAD solution for 20 min before storing at 4 °C, the IMER retained 80% or more of its initial activity for one month even when five serum samples were assayed every day If methanol is used as a polar component of the gradient mixture in this method, its maximum concentration should be kept below 60%

Recently, a similar post-column IMER containing either 3α- or 3β-HSD immobilized on glass beads was prepared by Lam et al [21] In the assay of epimeric hydroxysteroids (androstane or pregnane), 3α-hydroxysteroids were selectively detected with the 3α-HSD whereas 3β-hydroxysteroids were not When the FL detector was used, picomole amounts of steroids could easily be distinguished from noise

3 1 4 Oestrogens

Oestrogens are found in free, glucuronide and sulphate forms in biological fluids It is generally accepted that the status of the foeto-placental unit is closely reflected by the amounts of oestrogens in biological fluids HPLC with UV detection (280 nm) is widely used for the analysis of oestrogens, but is not satisfactory with respect to sensitivity (detection limit 0 1 nmol) and selectivity Monitoring of the fluorescence inherent to oestrogens is a promising method However, sufficient sensitivity cannot be obtained because of the

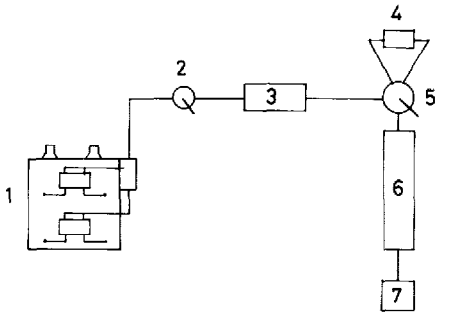


Fig 5 Schematic diagram of the combined IMER–HPLC system 1 = Pump, 2 = injector, 3 = pre-column, 4 = IMER, 5 = switching valve, 6 = analytical column, 7 = FL detector

proximity of the excitation and emission wavelengths ED in HPLC is promising for the trace analysis of electrochemically active compounds such as phenols, catechols and aromatic amines (detection limit 1–5 pmol) Recently, we developed derivatization reagents having ferrocene as an electrophore for the determination of such compounds by HPLC–ED [22,23]

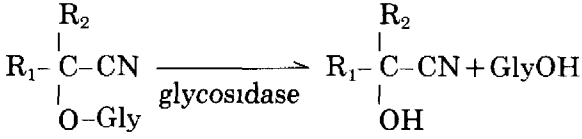
Bowers and Johnson [24–26] proposed a new system in which urinary oestriol conjugates were hydrolysed on-line with β-glucuronidase immobilized on CPG and deconjugated oestriol was separated and determined by HPLC with FL detection (Fig. 5) The chromatographic analysis was accomplished by the use of column switching, separating the analyte simultaneously from several samples Methanol was used for elution of the oestriol from the IMER. The effect of methanol content on the enzyme activity has been shown to be negligible at levels up to ca. 15% The method was applied to the determination of oestriol glucuronides in pregnancy urine and the result obtained was in good accord with that given by radioimmunoassay The method is applicable to other oestrogen conjugates, although the information on the conjugated form is lost by enzymic hydrolysis prior to HPLC analysis.

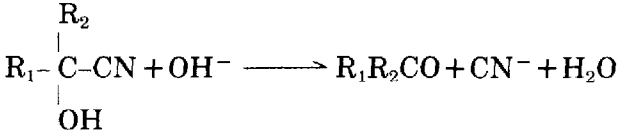
Dalgaard et al [27] used β-glucuronidase (from bovine liver) immobilized on agarose beads as a post-column IMER in HPLC to determine oestrone glucuronide Electrochemical oxidation on a glassy carbon electrode was used for detection and proved to be highly sensitive (detection limit 13 pmol at a signal-to-noise ratio of 3) Application to other oestrogen glucuronides has not been reported, however, owing to the non-specific binding of oestrogens to the IMER

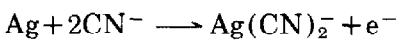
3 2 Conjugates of various compounds

The method developed by Dalgaard et al [27] was also applied to the determination of *o*- and *p*-nitrophenyl glucuronide and phenolphthalein glucuronide The method proved to be useful for the identification and sensitive determination of phenolic glycosides They also used the IMER as a post-column

reactor in the HPLC of cyanogen glycosides [28,29] The addition of sodium hydroxide to the flow stream after the IMER provided cyanide ion, which was detected at a silver electrode by amperometric measurement at 0 0 V vs Ag/AgCl, as shown below The selective detection of cyanide permits measurements in a complex matrix with detection limits in the low picomole range







A similar system was applied to the determination of glucuronide conjugates of an antihypertensive agent, fenoldopam, having a catechol structure [30] These conjugates, which are electrochemically active at +1 0 V but not at +0 5 V vs Ag/AgCl, were separated by HPLC and passed through the IMER The glucuronides were enzymatically converted to the more electrochemically active aglycone, fenoldopam (Fig 6)

Elchisak [31] developed a post-column reactor which was involved in hydrolysis of catecholamine sulphates with perchloric acid followed by ED. However, the system is very complicated and not so useful Boppana et al [32] developed an HPLC–IMER system in which sulphatase and β-glucuronidase were immobilized They used the system for the determination of xenobiotic conjugates (sulphates and glucuronides) and obtained satisfactory results

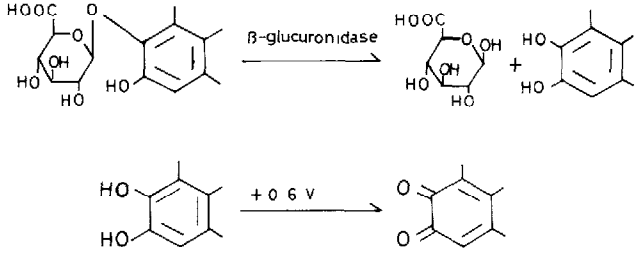


Fig 6 Schematic representation of reactions occurring in a post-column β-glucuronidase reactor and at a thin-layer glassy carbon electrode in an electrochemical detector

3 3 *Acetylcholine and choline*

Acetylcholine (Ach) is one of the neurotransmitters in the central nervous system and exists with choline (Ch) in biological fluids Numerous methods for the separation and determination of these two compounds have been developed However, they are all unsatisfactory with respect to simplicity. The use of HPLC has not hitherto been attempted for this purpose because Ach and Ch are unresponsive to the detectors Recently, a sensitive and selective method for the simultaneous determination of Ach and Ch has been reported [33] The two were separated on a reversed-phase column, passed through a column of immobilized acetylcholine esterase (AchE) and choline oxidase (ChO) on silica gel The resulting hydrogen peroxide was detected by ED using a platinum working electrode (applied potential +0 5 V) A highly sensitive analysis was attained with a quantitation limit of 5 pmol within 7 min Kaneda and co-workers [34,35] applied the method to the assay of enzymic activities of Ch/Ach transferase and AchE They also developed a simple method for the simultaneous determination of Ach, Ch, noradrenaline and serotonin in brain tissues by HPLC with ED The detection system consisted of two electrochemical cells aligned in series a glassy carbon electrode for catecholamines and serotonin and a platinum electrode for Ach and Ch. A column of boronic acid gel was placed just ahead of the IMER to remove catecholamines, which caused interfering responses on the platinum electrode [36].

The HPLC–IMER–ED method was applied to the determination of Ach and Ch in brain tissues by Fujimori and Yamamoto [37] Damsma and Flentge [38] also applied the method to the determination of Ach in plasma or blood cells, but were unsuccessful

Yao et al [39] presented a method for the determination of Ach and Ch by HPLC using an enzyme electrode for ED The enzyme electrode was prepared by cross-linking of AchE and ChO with bovine serum albumin (BSA) or AchE, ChO and peroxidase (POD) with BSA using glutaraldehyde on a platinum electrode The AchE–ChO and AchE–ChO–POD electrodes were based on the

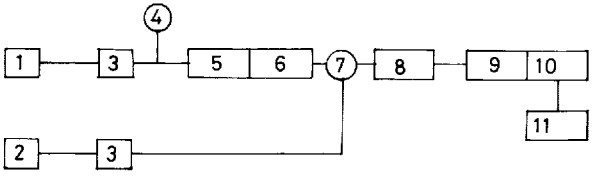


Fig 7 Schematic diagram of HPLC-IMER-chemiluminescence detection 1 = Solvent, 2 = acetonitrile containing crown ether, 3 = pump, 4 = injector, 5 = cation exchanger, 6 = IMER, 7 = vortex-type mixing tee, 8 = mixer, 9 = solid bis(2,4,6-trichlorophenyl) oxalate, 10 = immobilized fluorophore, 11 = photomultiplier

amperometric detection of hydrogen peroxide and hexacyanoferrate(III) formed from hexacyanoferrate(II) by the enzymic reactions shown below

$$\text{Ach} + H_2O \xrightarrow{\text{AchE}} \text{Ch} + CH_3COOH$$

$$\text{Ch} + 2O_2 + H_2O \xrightarrow{\text{ChO}} \text{betaine} + 2H_2O_2$$

$$H_2O_2 + 2[Fe(CN)_6]^{4-} + 2H^+ \xrightarrow{\text{POD}} 2[Fe(CN)_6]^{3-} + 2H_2O$$

$$H_2O_2 \xrightarrow{+0.5\ \text{V vs Ag/AgCl}} O_2 + 2H^+ + 2e^-$$

$$[Fe(CN)_6]^{3-} + e^- \xrightarrow{0.0\ \text{V vs Ag/AgCl}} [Fe(CN)_6]^{4-}$$

The method was highly sensitive and specific to Ach and Ch The detection limit was 1 5 pmol for Ch and 2 9 pmol for Ach They also used the chemiluminescence reaction to detect hydrogen peroxide formed enzymatically [40] Bis(2,4,6-trichlorophenyl) oxalate was reacted with hydrogen peroxide to give dioxetane, which in turn served to excite perylene, exhibiting chemiluminescence The detection limit (1 pmol) obtainable is higher than that by the enzyme electrode Recently, Van Zoonen et al [41] improved the method, the hydrogen peroxide formed being quantified by means of a solid-state peroxalate chemiluminescence detector based on an immobilized fluorophore (3-aminofluoranthene) and addition of oxalate from a solid bed (Fig 7) The reproducibility of the method was satisfactory with a relative standard deviation of 3 4–3 7% The detection limits are at the sub-picomole level and a linear relationship is observed over a range of at least three orders of magnitude No matrix effects were observed in measurements on urine and serum specimens

3 4 Amino acids

Kiba and Kaneko [42] prepared IMER in which L-amino acid oxidase (L-AAO)–POD was immobilized on aminopropyl-CPG beads After the separation of amino acids by reversed-phase HPLC, each amino acid was passed through the IMER and detected fluorimetrically L-AAO catalyses the deamination of the L-amino acid to give hydrogen peroxide, which is determined fluorimetrically by the POD–homovanillic acid system

$$\text{L-amino acid} + O_2 + H_2O \rightarrow \text{2-keto acid} + NH_3 + H_2O_2$$

The method is less sensitive (detection limit 0 25–0 5 nmol) and less selective than previous derivatization methods

Conversion of L-amino acids by an IMER (L-AAO), which would cause a change in the ionic strength of an HPLC eluent, could be utilized for the con-

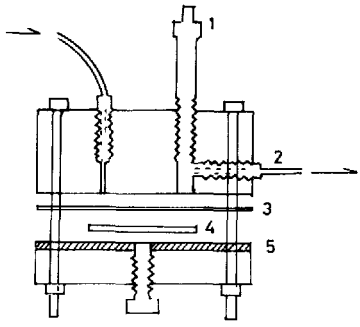


Fig 8 Schematic configuration of the enzyme electrode as an HPLC detector 1 = Ag/AgCl reference electrode, 2 = auxiliary electrode, 3 = spacer, 4 = enzyme-modified platinum sheet, 5 = silicone rubber

ductimetric detection of L-amino acids [43] The method was much less sensitive but its principle indicated the possible use of other selective enzymes for the development of a conductimetric HPLC detector

Jansen et al [44] described a stereoselective HPLC–IMER (L-AAO) system for the detection of eight L-amino acids (Cys, Met, Ile, Leu, His, Tyr, Phe and Trp) The combined use of the selectivities of the enzyme and peroxyoxalate chemiluminescence detection provided an extremely selective detection system The detection system afforded linear responses over a range of two orders of magnitude and detection limits at the $(0\,35\text{–}3\,0)\cdot10^{-6}$ *M* level The method was used for the determination of selected L-amino acids in urine and beer

Two enzyme electrodes based on immobilized L- and D-AAO, specifically responsive to L- and D-amino acids, respectively, have been developed (Fig 8) They are used for ED in HPLC, by dividing the effluent into two and detecting the D-amino acid in one line and the L-enantiomer in the other The detection limit for some amino acids (Met, Tyr, Leu and Phe) is ca 2 pmol The procedure is useful for the specific detection of L- and D-amino acids without a complicated pretreatment The electrode retains almost the original activity even after repeated use for one month [45]

3 5 Other compounds

HPLC–IMER involving immobilized hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PD) has been used for the determination of creatine kinase (CK) isoenzyme activity The isoenzymes in human serum were separated on an anion-exchange column by the gradient elution method and reduced nicotinamide adenine dinucleotide phosphate (NADPH) formed in the reactor was monitored by FL detection [46–49] The reactor was found to have

high activity for adenosine triphosphate (ATP) and the detection limit was 15 pmol [48,49] The enzymic reactions are as follows [50]

creatine phosphate + ADP $\xrightarrow{CK}$ creatine + ATP

ATP + glucose $\xrightarrow{HK}$ ADP + glucose-6-phosphate

glucose-6-phosphate + NADP $\xrightarrow{G6PD}$ glucono-δ-lactone 6-phosphate + NADPH

The determination of urea in serum and urine is an important routine test in clinical laboratories These measurements are especially important for estimating the nitrogen balance in hospitalized patients who are malnourished The blood level value is used as an indicator of renal function Jansen and coworkers [51,52] developed a method for the determination of urea and ammonia based on ion-pair HPLC with on-line post-column derivatization on an IMER In the IMER, urea was quantitatively converted into ammonia, which in turn was determined fluorimetrically by reaction with *o*-phthalaldehyde The method was sensitive, highly specific and practicable, the detection limits being 0 4 and 0 3 ng for urea and ammonia, respectively

Creatinine in urine was determined together with sodium, ammonium and potassium ions by ion chromatography with an immobilized creatinine deiminase reactor [53] Hydrolysis of creatinine by the IMER gave ammonium ion, which was then separated and detected by ion chromatography. The analytical values of creatinine were obtained from the difference between the peak heights of ammonium ion with and without the IMER. The method proved to be very effective for the simultaneous determination of creatinine and inorganic cations in human urine

Meek and Nicoletti [54] reported an HPLC detection system based on inositol bis- and trisphosphates and other organic phosphates After separation of the compounds by anion-exchange chromatography, the column effluent was first passed through a column of immobilized alkaline phosphatase for hydrolysis of the phosphate bond, then the system was continuously mixed with molybdate solution to allow the detection of the inorganic phosphate formed The enzyme was immobilized simply by adsorbing it on a moderately hydrophobic support The limit of detection is below 1 nmol

4 SUMMARY

The applications of immobilized-enzyme reactors (IMERs) to detection systems in high-performance liquid chromatography (HPLC) have been reviewed A substrate unresponsive to the detector is enzymatically converted to a highly responsive product The main analytes detected by this system are various steroids, conjugates of various compounds, acetycholine, choline and

amino acids The repetitive use of a single batch of expensive enzyme has economic advantages On the other hand, the organic modifier in the mobile phase exerts a considerable influence on the activity and stability of the IMER Usually the activities of the IMER decrease after two or three months of ordinary use These problems need to be overcome in the future

HPLC–IMER systems are being used in the field of clinical chemistry, and further applications to other fields are expected to be developed

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