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Development of post-column enzymic reactors with immobilized alcohol oxidase for use in the high-performance liquid chromatographic assay of alcohols with electrochemical detection[a]

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ABSTRACT

The development of a very sensitive, direct injection high-performance liquid chromatographic method, using a post-column reactor with immobilized alcohol oxidase, was undertaken with the aim of determining methanol and ethanol levels in microlitre volumes of biological samples. After reversed-phase chromatography to separate methanol and ethanol, the analytes were enzymically converted into the respective aldehydes with formation of stoichiometric amounts of hydrogen peroxide, which could be measured via electrochemical oxidation at a platinum electrode. Some problems were encountered in the development of solid-phase enzymic reactors, using a delicate enzyme, that is prone to lose activity, such as alcohol oxidase. Owing to the slightly alkaline pH required for the optimum activity of alcohol oxidase, polymeric columns seemed to be preferable for the chromatography. HEMA copolymer was chosen as the stationary phase, but the methanol and ethanol peaks eluted close together and posed severe problems of limiting post-column band spreading. Reactors based on coarse supports for enzyme immobilization gave unacceptable band spreading, causing the methanol and ethanol peaks to overlap. On the other hand high-performance liquid chromatographic packings maintained the efficiency of the chromatographic separation, quite independently of the reactor volume. Polymeric supports proved superior to silicas in maintaining the enzyme activity. However, relevant changes in the enzyme substrate specificity were observed after immobilization.

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INTRODUCTION

A rapid, specific and reliable assay for ethanol (EtOH) and methanol (MeOH) in blood and other complex matrices is of primary importance. Its main application in the biomedical field is the monitoring of blood alcohol in drivers and in cases of acute intoxication [1]. A simple and specific assay could also be of interest for other purposes, such as monitoring EtOH and especially MeOH concentrations in spirits, wines and other alcoholic beverages.

Although enzymic assays are widely used, mainly in clinical chemistry [2], it must be pointed out that they do not distinguish EtOH from MeOH. This recently caused an "epidemic" intoxication from adulterated wine in Italy and elsewhere, which was not promptly identified because of the lack of specificity of the commonly adopted assays in hospital laboratories.

Direct injection gas chromatographic (GC) methods have been reported [3] but, in order to avoid a rapid loss of column performance, at least plasma deproteinization is normally required [4,5]. The cleaner "head space" technique needs special instrumentation and larger volumes of sample [6].

This problem, in particular, hampers the enforcement in Italy of the recent law on blood alcohol limits in drivers (L. 18/3/1988, n. 111), since the Constitution of the country prohibits any physical lesion, even the drawing of blood by syringe, without the consent of the subject, which is unlikely to be obtained in such circumstances.

Reversed-phase high-performance liquid chromatography (RP-HPLC) is capable of separating short-chain aliphatic alcohols such as EtOH and MeOH, but the sensitivity and selectivity of the refractometric detector are much too poor to allow the determination of alcohol levels in blood [7].

With this background, the development of a very sensitive, direct injection HPLC method, using a post-column reactor with immobilized alcohol oxidase (AO), was undertaken in our Institute, with the aim of determining alcohol levels in microlitre volumes of biological samples, allowing the use of tears or capillary blood. These samples, in fact, are much easier to be harmlessly taken, even by unskilled personnel such as policemen. After reversed-phase chromatography to separate MeOH and EtOH, the compounds are enzymically converted into their respective aldehydes with formation of stoichiometric amounts of hydrogen peroxide, which can be measured via electrochemical oxidation. In preliminary experiments, focused on the optimization of the chromatographic separation, the method has already proved suitable for determining blood alcohol levels [8]. However, it is evident that the core of the method is the AO reactor, which has not yet been deeply investigated.

Even if the post-column addition of enzyme to the eluate from the HPLC column is in principle possible, the development of an immobilized enzyme reactor seems to be the best choice, as far as practicability and cost are concerned. Despite the widespread use of immobilized enzymes in clinical chemistry, bio-

chemistry, medicine and in some industrial fields, only recently has attention been paid to this bio-technological approach in HPLC [9,10]. Traditional immobilized enzyme reactors mostly rely on soft gels or semi-rigid coarse supports [11], which hardly meet the requirements of HPLC for high flow-rates and limited band spreading. To the best of our knowledge, the only commercially available AO reactor still uses this technology [12].

The recent availability of supports "tailored" for HPLC, allowing easy and stable protein binding, has provided workers with materials of reportedly high stability, high density of reactive groups, high permeability and ability to maintain the efficiency of the HPLC separations.

The aim of this paper is to discuss some problems encountered in the development of solid-phase enzymic reactors, using a delicate enzyme that is prone to lose activity, such as AO. Moreover, since this paper is basic to the development of an HPLC assay, some aspects of the influence of the reactor volume and of the size of the support on the post-column band spreading are briefly outlined.

EXPERIMENTAL

Reagents, standards and samples

HPLC-grade solvents, including MeOH and EtOH, and analytical-grade chemicals from Carlo Erba (Milan, Italy) were used.

Apparatus and procedures

An isocratic HPLC pump (Model 880 PU, Jasco, Tokyo, Japan), fitted with a pulse damper (BioAnalytical Systems, West Lafayette, IN, U.S.A.), a Model 7125 sample injector with a 5- or 20-μl loop (Rheodyne, Cotati, CA, U.S.A.) and an electrochemical detector (LC4B/17A, BioAnalytical Systems) with a thin-layer cell and a platinum (Pt) working electrode, were used for chromatographic as well as flow-injection studies. The applied potential was +500 mV *vs.* an Ag/AgCl reference electrode.

A Model 875 UV detector (Jasco), operated at 280 nm, was connected downstream from the column when protein binding was evaluated.

As the chromatographic column, one or, when needed, two glass cartridges (150 mm × 3 mm I.D.) in series, packed with 10-μm HEMA-S 1000® (copolymer of 2-hydroxyethylmethacrylate and ethylenedimethacrylate) (Tessek, Prague, Czechoslovakia), were used. The mobile phase was phosphate buffer (0.01–0.2 *M*, pH 7–9.5), previously filtered through a 0.45-μm Nylon 66 membrane (Alltech, Eke, Belgium). The flow-rate was 250 μl/min.

Ready-to-use cartridges of different dimensions and with different supports (silicas or polymers), some commercially available, some still experimental, kindly furnished by Tessek and Chrompack (Middelburg, The Netherlands), were used for enzyme immobilization. A commercially available reactor Model EP45, with immobilized AO from *Pichia pastoris*, was purchased from Biometra (Göt-

TABLE I

MAIN FEATURES OF THE CARTRIDGES USED FOR THE DEVELOPMENT OF THE ALCOHOL OXIDASE REACTORS

Reactor	Dimension (mm)	Volume (μl)	Packing size (μm)	Pore size (Å)	Support	Functionality	DF[a]
EP45	30 × 2.1	104	30–50	?	Polymer	Isocyanate	0.28
CHR/1	10 × 2.1	34	5	120	Silica	Aldehyde	0.84
CHR/2	10 × 3.0	70	20–40	500	Silica	Aldehyde	–
CHR/3	10 × 3.0	70	50–200	300	VADUE	Epoxy	0.26
TES/1	30 × 3.0	212	10	350	HEMA	Epoxy	0.72

[a] The resolution between MeOH and EtOH peaks was evaluated using the discrimination factor (DF) (or peak-to-valley ratio) calculated according to Schoenmakers [18].

tingen, F.R.G.). The main features of the tested reactors are summarized in Table I.

AO RL-100 (Alcohol, oxygen oxidoreductase; EC 1.1.3.13) from *P. pastoris* (Provesta, Bartlesville, OK, U.S.A.) was a generous gift from P. T. Kissinger and R. E. Shoup, BioAnalytical Systems. Its specific activity was reportedly 29.5 U/mg[a], and the protein concentration was 42 mg/ml. The main properties of the enzyme are summarized in Table II (from ref. 12) and Table III.

TABLE II

PROPERTIES OF *PICHIA PASTORIS* ALCOHOL OXIDASE

Most of these values are similar to those of other yeast alcohol oxidases.

Property	Value
Native molecular mass	630 000
Monomer molecular mass	75 000
Subunits	8
Cofactor	FAD
Temperature optimum	40°C
pH optimum	7.5–8
Specific activity	10–40 I.U./mg
K_M (pH 7.5)	0.7 m*M* (MeOH)
	9.0 m*M* (EtOH)
Inhibition by H_2O_2	>100 m*M*
Isoelectric point	5.7

[a] Unit definition: one unit will oxidize 1.0 μmol of ethanol to acetaldehyde and hydrogen peroxide per minute at pH 7.5 at 25°C.

TABLE III

SUBSTRATE SPECIFICITY OF ALCOHOL OXIDASE RL-100

According to Technical Bullettin No. 2, Provesta Corporation.

Substrate	Relative activity at pH 7.5 (%)
Methanol	100.0
Ethanol	75.4
n-Propanol	54.0
2-Propanol	1.1
n-Butanol	38.7
2-Butanol	2.3
n-Pentanol	25.1

Enzyme binding was accomplished as follows. As the coupling buffer, 0.2 *M* phosphate (pH 7.5) was used with aldehyde-silicas and 0.2 *M* phosphate (pH 9.0) with polymeric, epoxy-derivatized supports. The cartridge was conditioned with 5 ml of the coupling buffer at 500 μl/min, then a void dummy column (internal volume 400 μl) filled with 50 U of enzyme diluted in the same buffer (filtered through a 0.45-μm cellulose acetate membrane) was inserted upstream from the cartridge. The solution was passed through the cartridge at 30 μl/min. After *ca.* six dummy column volumes, the flow-rate was gradually increased to 250μl/min, and the eluent was replaced with the assay buffer. Extensive washing was carried out before connecting the enzymic reactor to the electrochemical cell.

In order to estimate roughly the protein binding capacity, 50 μg of enzyme were repeatedly injected through the injection valve, and the signal of the UV detector was recorded. The heights of the peaks corresponding to the leached protein increased, with a sigmoid shape, up to a plateau, which was considered the signal of cartridge saturation. The amount of protein loaded up to the flex point was arbitrarily considered the binding capacity of the cartridge.

It must be pointed out that, although the Chrompack cartridges (CHR) were employed a few weeks after receipt, the Tessek material (TES) was used after the expiry date stated by the producer (epoxy groups of the support were unstable in the shippig solvent, water). Therefore quantitative comparisons are beyond the limits of this study.

The AO activity was assayed in bulk solution with an electrochemical analyser PAR M270 (EG&G, Princeton, NJ, U.S.A.) with a rotating platinum electrode (A = 3.14 mm^2; 1600 rpm) polarized at 600 mV *vs.* a standard calomel reference. EtOH or MeOH (3 μmol) was added to 2.5 ml of suitable buffer (see further on) containing 6.6 U of AO. The formation of hydrogen peroxide was recorded for 1 min at room temperature. Equal amounts of alcohols were injected in a flow-injection configuration to test the activity of the enzyme reactors.

RESULTS AND DISCUSSION

The reversed-phase chromatography of short-chain, linear aliphatic alcohols, such as MeOH and EtOH, is problematic using derivatized silica columns, because of the effect of residual silanol groups and the instability of silica at the slightly alkaline pH (7.5–9) required to achieve the best AO activity. The 10-μm polymeric packing Separon HEMA-S 1000 proved able to give quite acceptable separation of MeOH and EtOH, especially when two 15-cm columns were put in series. Its broad pH stability and its slight hydrophobicity, making it able to deal with large amounts of proteins such as in direct injection methods [14], led to the adoption of this matrix as the stationary phase [8].

However, because MeOH and EtOH elute very close to each other ($\alpha = 1.33$), the post-column band spreading is crucial. After separation by the column, the alcohols were converted by the post-column AO reactor, into aldehyde and H_2O_2. Enzymically produced H_2O_2 can readily be detected by an electrochemical detector at a Pt elecrode at $+500$ mV (*vs.* Ag/AgCl), according to the reaction: $H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$.

First, we evaluated the influence on the post-column band spreading of the internal volume of the reactor and of the size of the packing. The main characteristics of the reactors and the relative resolution obtained for EtOH and MeOH peaks, expressed as the Schoenmaker discrimination factor (DF) [18], using the same chromatographic column, are summarized in Table I. The DF varies between 0, when the valley between two peaks is not visible, and 1 for completely separated peaks.

It is clear that coarse supports (EP45 and CHR/3) gave unacceptable overlapping of the MeOH and EtOH peaks. On the other hand, HPLC-sized packings (CHR/1 and TES/1) maintained the efficiency of the chromatographic separation, with a moderate influence of the reactor volumes, varying by a factor of 6 between the two reactors.

Another point investigated was the ability of different supports and immobilization chemistries to bind the enzyme without activity loss.

It must be pointed out that, unlike other enzymes such as glucose oxidase or choline oxidase, which proved quite robust, AO is considered to be a weak enzyme susceptible to inactivation.

Because of the difficulty of exactly determining the amount of the bound protein and its enzymic activity (see ref. 15), results are quite rough and qualitative, but seem good enough for preliminary conclusions to be drawn.

Covalent binding, via either aldehyde or epoxy groups, was tested. Some comparisons were also be made with the commercial ready-to-use reactor (EP45), employing a proprietary binding chemistry based on the reactivity of isocyanate groups at the end of long spacer arms. The nature of the support was either silica or polymers of different pore and particle size, a shown in Table I.

Reactor EP45 (Biometra)

The reactor showed a high alcohol conversion, owing to the high enzymic activity immobilized (reportedly 80 U). On the other hand, a high background current (*ca.* 500–2000 nA) and a remarkable continuous negative drift of the baseline were observed. This hindered the use of the most sensitive part of the range of the detector. Nevertheless, a high analytical sensitivity was achieved, with an absolute limit of detection (ALD) of *ca.* 0.1–0.5 nmol of MeOH. The reactor remained active for a couple of weeks, but with rapid and progressive loss of sensitivity. As previously pointed out, another serious shortcoming of this kind of reactor is the considerable post-column band spreading.

Reactor CHR/1 (Chrompack)

The reactor, consisting of HPLC-sized aldehyde-activated silica with pores of 120 Å, retained *ca.* 200–300 μg of protein with low enzymic activity. The ALD was 20–40 nmol. However, compared with the reactor EP45, the background current was much lower (25–100 nA) and more stable. Therefore the high sensitivity ranges of the detector could be used. The reactor life extended over several weeks. On the other hand, the nature of the support (silica) hardly met the optimum pH (slightly to moderately basic) for AO activity.

Reactor CHR/2 (Chrompack)

This was similar to the CHR/1, but had a much larger pore diameter (500 Å). It proved able to bind very high amounts of protein, up to 1–2 mg, but enzymic activity was almost completely lost. On the other hand, background currents as high as 10 000 nA were observed.

Reactor CHR/3 (Chrompack)

The reactor packing consisted of coarse vinyl acetate–divinylethyleneurea copolymer (VADEU) with epoxy groups for enzyme coupling. The average pore diameter was 300 Å. Protein binding was roughly comparable with CHR/1, but the activity was higher (ALD 1–5 nmol). The background current was conversely very low, in the range 5–20 nA. Reactor life exceeded one month, although with considerable loss of activity. Unfortunately, the high band broadening due to the coarseness of the packing made this reactor unsuitable for use with the HEMA-S column.

Reactor TES/1 (Tessek)

The reactor was packed with 10-μm epoxy-derivatized Separon HEMA-BIO 1000 (copolymer of 2-hydroxyethylmethacrylate and ethylenedimethacrylate). Approximately 300 μg of protein seemed to have been retained with good activity. The ALD, background current and reactor life were comparable with those of CHR/3. Despite the larger dimensions of the reactor, compared with CHR/1, the post-column loss of the efficiency was still acceptable, even maintaining the reso-

lution of closely eluted peaks. Furthermore, the polymeric nature of the support allowed the use of basic eluents.

These data suggest that polymeric supports are superior to silicas in maintaining the enzyme activity; in addition, HEMA is the only tested polymeric support with a size compatible with high-efficiency separation.

The loss of enzymic activity with silica supports may be related to the microenvironment, which is very different from that pertaining in the bulk eluent. Indeed, the negatively charged silica support will attract protons, resulting in a lower pH locally around the immobilized enzyme, which could to out of the optimum range 7.5–8.0 and, maybe, even of the working range 6.0–9.5. Other problems could arise from mass-transfer limitation.

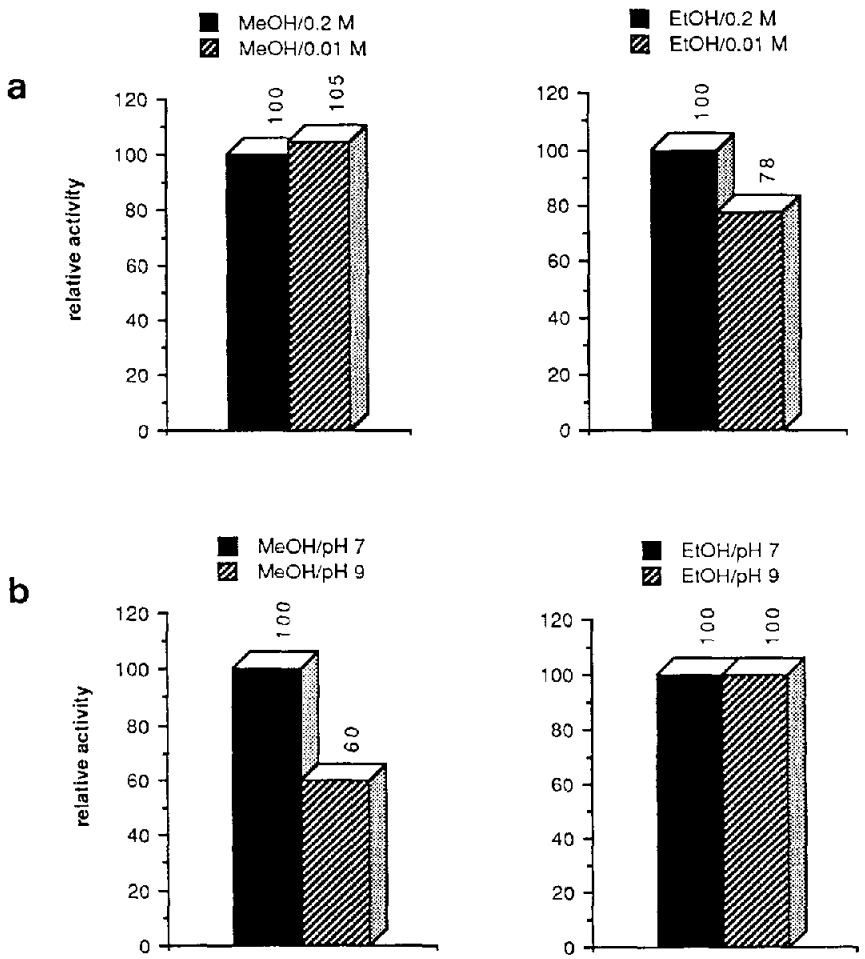

Fig. 1. (a) Relative activities of AO on MeOH and EtOH dissolved in 0.2 *M* or 10 m*M* phosphate buffer, at pH 7.5 and room temperature. (b) Relative activities of AO on MeOH and EtOH dissolved in pH 7.0 or 9.0 phosphate buffer, 10 m*M*.

A further point investigated was the substrate specificity, under different pH and ionic strength conditions. Relevant differences between the enzyme in bulk solution and after immobilization were found. The relative activities of AO on MeOH and EtOH at two different potassium phosphate concentrations and different pH values are shown in Fig. 1a and b. After immobilization the behaviour of AO changed, showing an increased activity at low ionic strength (Fig. 2a) and a change in the relative activity on MeOH and EtOH at basic pH (Fig. 2b).

Although these phenomena probably depend on changes of enzyme conformation due to binding to the support, given the practical approach of the present study, we did not investigate the reasons. Yet these data have to be taken into account even in the development of a simple HPLC assay.

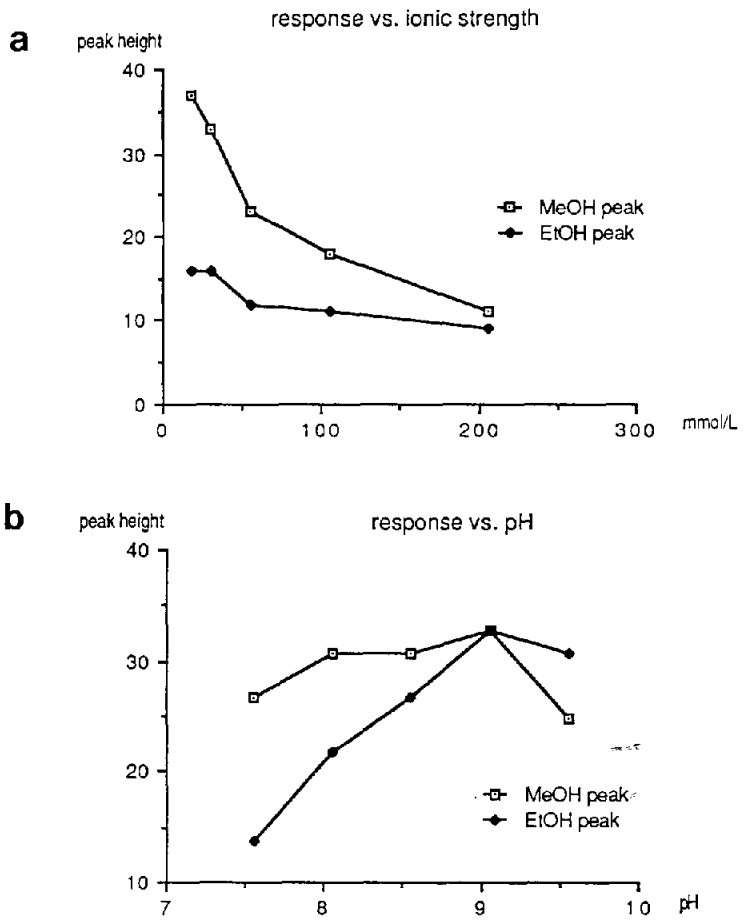

Fig. 2. (a) Responses to MeOH and EtOH of an AO reactor (in a flow-injection assay), using eluents (phosphate buffer) of different ionic strengths in the range 0.2 *M*- 10 m*M*, at pH 7.5. (b) Responses to MeOH and EtOH of an AO reactor (in a low-injection assay), using eluents (phosphate buffer) of different pH in the range 7.5–9.5, at 10 m*M*.

CONCLUSIONS

The use of enzymic reactors in flow systems has been reported as a promising approach for the development of simple and sensitive assays for alcohols in complex matrices, even omitting chromatographic separation [16,17]. However, a preliminary chromatographic step can ensure additional selectivity and specifcity to the enzymic reaction, on which continuous-flow and flow-injection methods rely.

Owing to the slightly alkaline pH required for the optimum activity of AO, polymeric columns seem preferable for chromatography. HEMA has been chosen as the stationary phase, but the close elution of MeOH and EtOH peaks poses severe problems of limiting the post-column band spreading. The importance of using HPLC-sized supports for limiting the post-column band spreading is stressed, as well as the advantages of using polymeric matrices for enzyme immobilization.

Although some problems, such as the linearity of response and the storage stability of the reactor, have not yet been dealt with, the use of an AO reactor in an HPLC assay for MeOH and EtOH seems to be feasible.

However, once again, it is stressed that the development of every HPLC method using enzymic reactors requires a careful optimization of specific separation and detection conditions, to meet several, often unforeseen and conflicting, needs and problems.

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