Journal of Chromatography, 566 (1991) 333–339
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5827

Direct injection high-performance liquid chromatographic method with electrochemical detection for the determination of ethanol and methanol in plasma using an alcohol oxidase reactor

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ABSTRACT

A highly sensitive reversed-phase high-performance liquid chromatographic assay for ethanol and methanol in plasma, using a post-column enzymic reactor with electrochemical detection, has been developed. The alcohols, separated on the column, were converted by immobilized alcohol oxidase into their respective aldehydes with formation of stoichiometric amounts of hydrogen peroxide, detected via oxidation at a platinum electrode. As the chromatographic column, two glass cartridges (150 mm × 3 mm I.D.) in series, packed with 10-μm HEMA-S 1000® packing, were used. Alcohol oxidase from *Candida boidinii* was immobilized onto HEMA-BIO 1000 VS-L (10 μm), packed in a 30 mm × 3 mm I.D. glass cartridge. The reaction product, hydrogen peroxide, was detected with an amperometric detector with a platinum electrode, operated at +500 mV *vs.* an Ag/AgCl reference electrode. A 20-μl volume of ten-fold diluted plasma was injected without any pre-treatment. Under the described conditions, methanol and ethanol were well resolved from each other and from the "front" of the chromatogram. The limit of detection was *ca.* 2.5 nmol for ethanol and 0.6 nmol for methanol in plasma, at a signal-to-noise ratio of 3. Excellent linearity was observed for ethanol, in the range 0.125–4 μg injected ($r = 0.9999$). In contrast, the response for methanol was markedly non-linear above 500 μg injected, presumably owing to progressive saturation of the reactor. The precision and accuracy of the assay were satisfactory, as was the reactor life (one month).

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INTRODUCTION

Alcohol determination in blood is of primary importance in clinical and forensic toxicology. The main interest is, obviously, focused on ethanol (EtOH), but, because of its higher toxicity and possible presence (as a normal minor component or, at higher levels, as an adulterant) in wines and other alcoholic beverages, methanol (MeOH) is worth testing for as well. The toxic levels in blood are above 1 mg/ml for EtOH and 0.2 mg/ml for MeOH; lethal concentrations are higher than 3.5 and 0.9 mg/ml, respectively [1].

The use of the several enzymic assays available on the market for alcohol determination in biological fluids is limited by problems of specificity [2]. On the other hand, the more specific gas chromatographic assays require "head-space" sampling devices [3] or, at least, sample deproteinization and the use of precolumns to prevent rapid column fouling [4,5].

Reversed-phase high-performance liquid chromatography (HPLC) proved to be able to separate short-chain aliphatic alcohols, such as EtOH and MeOH [6], but the sensitivity and selectivity of the commonly used refractometric detection were insufficient for the determination of alcohol levels in blood.

In recent years, post-column reactors using immobilized enzymes were used by several authors for specifically converting previously undetectable analytes into highly detectable compounds, with related improvements in sensitivity and selectivity (see refs. 7 and 8 for reviews).

According to this this approach, a micro-assay suitable for the analysis of alcohol in capillary blood was developed in our laboratory. Alcohols, separated on a reversed-phase column, were converted by an alcohol oxidase (AO), immobilized in a post-column reactor, into their respective aldehydes with formation of stoichiometric amounts of hydrogen peroxide, which was detected *via* electrochemical oxidation at a platinum electrode.

EXPERIMENTAL

Reagents, standards and samples

HPLC-grade MeOH and EtOH and analytical-grade chemicals from Carlo Erba (Milan, Italy) were used. Distilled, sterile water for intravenous injection (SIFRA, Verona, Italy) was used for the preparation of the chromatographic eluent and for the dilution of standards and samples. Nafion 117™, as a 5% alcoholic solution, was provided by Aldrich (Steinheim, Germany).

Apparatus and chromatographic conditions

The isocratic HPLC apparatus was composed of a high-pressure pump (Model 2350, Isco, Lincoln, NE, U.S.A.), a six-port injection valve (Model 7125, Rheodyne, Cotati, CA, U.S.A.) with a 20-μl loop and an amperometric detector (LC4B/17A, BAS, West Lafayette, IN, U.S.A.) with a thin-layer cell and a plati-

num working electrode, operated at +500 mV *vs.* an Ag/AgCl reference electrode.

An in-line 0.5-μm stainless-steel filter frit (Rheodyne) was inserted downstream from the injector to protect the column from clogging.

As the chromatographic column, two glass cartridges (150 mm × 3 mm I.D.) in series, packed with a 10-μm copolymer of 2-hydroxyethyl methacrylate and ethylene dimethacrylate (HEMA-S 1000®, Tessek, Prague, Czechoslovakia), were used.

The mobile phase was 0.01 *M* potassium phosphate buffer (pH 7.5) (adjusted with HCl), previously filtered through a 0.45-μm Nylon 66 membrane (Alltech, Eke, Belgium). The flow-rate was 250 μl/min. Under these conditions, at room temperature, the corresponding operating pressure was always lower than 90 bar.

Plasma samples were directly injected after ten-fold dilution with the mobile phase.

Enzymic reactor

The enzymic reactor was made of 10-μm HEMA-BIO 1000 VS-L (Tessek), a methacrylate polymer ready for protein immobilization via the reactive vinylsulphone groups (reported density 5–10 μm/g), packed into a 30 mm × 3 mm I.D. glass cartridge.

The enzyme used was AO from *Candida boidinii* (Boehringer, Mannheim, Germany) with an approximate activity of 6–7 U/mg (unit definition: one unit will oxidize 1.0 μmol of EtOH per min at pH 7.5 at 25°C). More details on AO were reported previously [9].

AO was bound to the support as follows: after conditioning the cartridge with 5 ml of 0.2 *M* phosphate buffer at pH 8.5, 50 U of AO, in 400 μl of the same buffer, were slowly (roughly 50 μl/min) injected with a plastic syringe. The cartridge was then incubated at 4°C for 48 h. It was extensively rinsed with the working eluent and then inserted between the column and the electrochemical cell.

Membrane electrode protection

In order to protect the electrode from fouling by sample constituents, and to increase the selectivity of the assay, we tested the shielding effect of Nafion 117, a perfluorinated cation-exchange material. A 10-μl volume of Nafion 117 solution, diluted 1:20 (v/v) in ethanol, was deposited on the electrode, covering the entire surface, and left to dry at room temperature.

Hydrodynamic voltammograms of hydrogen peroxide at bare and Nafion-protected platinum electrodes were obtained under flow injection conditions, by connecting the injection valve to the electrochemical cell with a 30 cm × 0.25 mm I.D. tube; the other chromatographic conditions were unchanged. Constant amounts of 20 nmol of hydrogen peroxide were injected. In order to enhance the possible influence of the membrane on the electron exchange at the electrode, it

was shielded with 10 μl of a four-fold more concentrated Nafion solution (*i.e.* 1%).

RESULTS AND DISCUSSION

Separation

HEMA-S 1000, according to our previous experience [10], enabled the separation of MeOH and EtOH. However, in order to achieve an almost complete resolution with a discrimination factor of 0.86 [11], it was necessary to use two 15-cm-long cartridges coupled in series. On the other hand, because of the hydrophilic character of the HEMA matrix, plasma proteins were almost unretained and eluted in a narrow front peak, well separated from EtOH and MeOH, leaving a clean baseline. Even though no problems of irreversible protein adsorption were encountered, it should be pointed out that, owing to the wide pH range of stability of HEMA [2–12], washings with basic and/or acidic buffers can be adopted in order to remove proteins and restore column performance.

Enzymic reactor

Since MeOH and EtOH eluted very close to each other, post-column band spreading became crucial. Despite a considerable internal volume (212 μl), the enzymic reactor proved able to maintain the chromatographic efficiency, owing to the "HPLC size" of the packing (see also ref. 9).

By using the described coupling procedure, *ca.* 1.5 mg of AO from *C. boidinii* was bound to the cartridge, as evaluated by measuring the absorbance of the effluent at 280 nm [7]. Assuming that the activity of the enzyme was completely maintained, this means that *ca.* 10 U were immobilized.

The reactor life was was *ca.* one month. The only precaution adopted was storing the reactor at 4°C, during the night and at weekends.

Previous experience with a more active AO from *Pichia pastoris* [9] revealed a limited stability of the enzymic reactor during storage. This was ascribed to non-specific production of hydrogen peroxide, which, not removed by the flowing eluent, would concentrate inside the reactor, thus damaging the enzyme. Consistent with this hypothesis, the *C. boidinii* enzyme, which is retro-inhibited by hydrogen peroxide, proved much more resistant during storage and was therefore preferred.

Analytical data

Because AO is more active towards MeOH than EtOH, the response to the former was *ca.* four times greater. Therefore the absolute limit of detection, in plasma, was found to be *ca.* 2.5 nmol for EtOH and 0.6 nmol for MeOH, at a signal-to-noise ratio of 3. Under the described conditions, a fairly stable background current of 10–20 nA was observed.

The dose–response curve of the assay is shown in Fig. 1. Excellent linearity

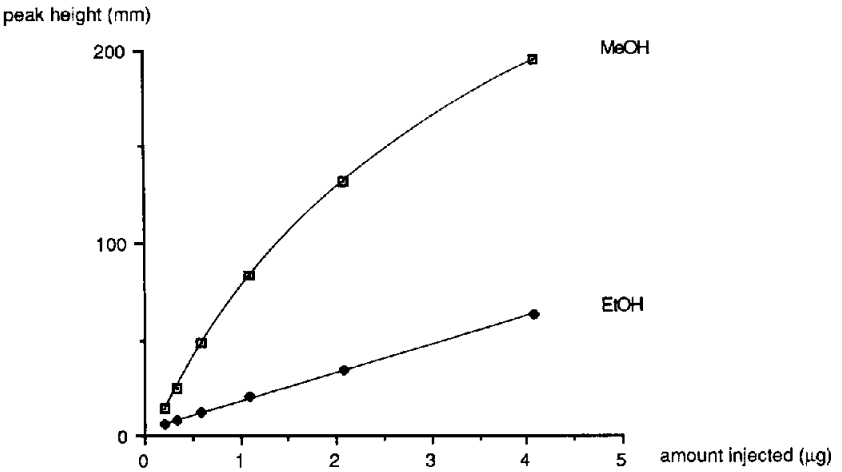

Fig. 1. Dose–response curve for MeOH and EtOH. For analytical conditions, see text.

was observed for EtOH, in the range 0.125–4 μg injected, as described by the equation $y = 14.586x - 0.792$, $r = 0.9999$ [where x is the injected amount (in μg) and y the corresponding peak height (in mm)]. In contrast, the response for MeOH was markedly non-linear, above 500 ng injected, indicating progressive

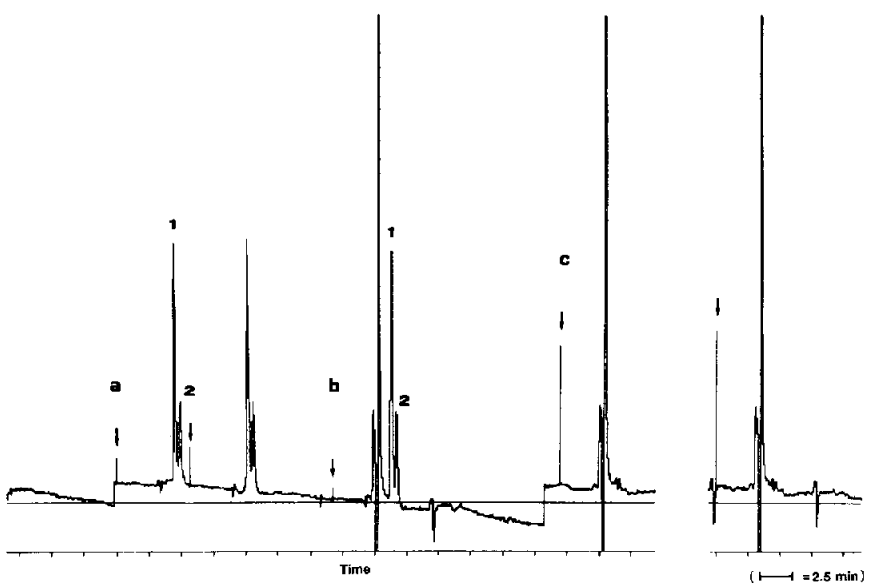

Fig. 2. Chromatograms of (from left to right): (a) standard solution of MeOH (0.05 mg/ml) and EtOH (0.1 mg/ml) (two injections); (b) plasma spiked with MeOH and EtOH at 0.5 and 1 mg/ml, respectively, diluted ten-fold with the mobile phase and injected; (c) blank plasma diluted as before and injected (two injections). Injection volume: 20 μl. Peaks: 1 = MeOH; 2 = EtOH. The arrows indicate injections. Full scale deflection, 5 nA. Other analytical conditions are described in the text.

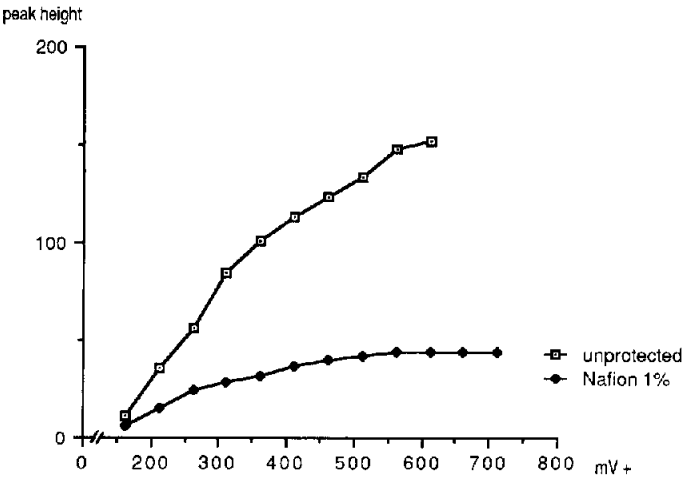

Fig. 3. Hydrodynamic voltammograms of hydrogen peroxide oxidized at a bare platinum electrode and at the same electrode previously protected with 10 μl of 1% Nafion solution. Constant amounts of 20 nmol of hydrogen peroxide were injected under flow-injection conditions.

saturation of the reactor (under this limit the linearity was good with $r = 0.9997$). This is in agreement with the very different Michaelis constants of AO for EtOH and MeOH (9.0 *vs.* 0.7 m*M*, respectively, at pH 7.5; see ref. 12). As is well known, the linear dynamic range of every enzymic measurement of a substrate is limited up to the Michaelis constant. However, since the toxic concentration of MeOH are much lower than that of EtOH, the narrower range of linearity, in our opinion, does not impair the usefulness of the method. In any case, it could be extended by increasing the amount of enzyme immobilized in the reactor and/or by increasing the residence time of the substrates [7].

The precision and accuracy of the assay were satisfactory, as shown in Table I.

Fig. 2 shows typical chromatograms obtained by injecting standard solutions of MeOH and EtOH, spiked plasma and blank plasma.

TABLE I

PRECISION AND ACCURACY DATA FOR METHANOL AND ETHANOL IN PLASMA

Concentration (mg/ml)	*n*	Coefficient of variation (%)		Accuracy (%)
		Intra-assay	Inter-assay	
Methanol				
0.5	5	1.8	5.3	+1.3
1.0	5	1.5	5.0	+1.2
Ethanol				
1.0	5	1.5	5.3	+3.4
2.0	5	1.3	6.2	+2.5

Electrode protection

Even if the column alone was able to separate MeOH and EtOH from matrix interferences, the use of a very thin Nafion membrane protecting the electrode gave additional selectivity, through a sieving action. Its thinness excluded any appreciable ion-exchange retention of charged compounds, such as proteins. The membrane stability was good, owing to the fully aqueous eluent. When necessary, the membrane was easily removed with ethanol and redeposited using fresh Nafion solution. The hydrodynamic voltammograms of hydrogen peroxide oxidized at shielded or bare electrodes did not change significantly, except the absolute current, as shown in Fig. 3. It should be pointed out that the Nafion solution used in this case was four times more concentrated than for the actual use.

In conclusion, the present method provides an interesting alternative to gas chromatography for rapid, simple and reliable determination of EtOH and MeOH in micro-volumes of plasma. In addition, it demonstrates a further application of bioreactors, an expanding biotechnological approach in liquid chromatography.

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