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Short Communication

Dehydrogenase-silica as a stationary phase for the separation of alcohols and ketones

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ABSTRACT

Horse liver alcohol dehydrogenase was immobilized on tresyl-activated silica particles and slurry packed into a PTFE-coated stainless-steel column. The stationary phase enzyme column was used for the high-performance chromatographic separation of alcohols and ketones. Baseline resolution of the secondary alcohols terbutaline and bambuterol and their respective ketones was obtained. In addition, discrimination of closely related isomers, including the positional isomers of ethylphenethyl alcohol and the optical isomers of 1-phenyl-1-butanol, was achieved.

INTRODUCTION

Immobilized enzymes have been used in a number of applications, *e.g.*, in biosensors and for the selective synthesis of organic compounds. In 1983, the use of immobilized enzymes as selective adsorbents for the high-performance liquid chromatographic (HPLC) separation of organic compounds was reported [1]. More recently, immobilized hydrolytic enzymes, such as cellulase and a protease, chymotrypsin, were employed for the enantiomeric separation of various compounds [2,3]. In this paper, we show that dehydrogenase immobilized on silica can be used for the separation of alcohols and ketones and for the chiral discrimination of optically active alcohols.

EXPERIMENTAL

Silica particles (10 μm, 300-Å pores) were activated with tresyl chloride (116 mg/g silica) as described previously [4]. Sedimented silica (2.5 ml) was resuspended in a total volume of 4 ml with 5 m*M* hydrochloric acid. Horse liver alcohol dehydrogenase (HLADH, 100 mg), obtained from Boehringer, was dissolved in 4 ml of 0.5 *M* potassium phosphate (pH 7.9) and added to the activated silica particles. Coupling proceeded for 6 h at 4°C with mixing. The absorbance of the supernatant was negligible and thus the yield of immobilized HLADH was close to 100%. Subsequently, 1 ml of 1 *M* Tris–HCl (pH 8.8) was added to block remaining activated groups and incubated for an additional 12 h. The silica-bound HLADH was packed in a PTFE-coated stainless-steel column (50 × 4.6 mm

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I.D.) as described [1], connected to HPLC equipment and tested for its ability to separate a number of ketones and alcohols.

RESULTS

Active-site titration of the HLADH- silica with NADH in the presence of excess of isobutyramide gave a binding site concentration, e_0,of 0.43 mM. An initial test of the immobilized enzyme column with AMP gave a capacity factor, k', of 5.4. The capacity factor can be used to calculate the dissociation constant, K_d, of the retained compounds according to the equation

$$K_d = e_0/k'$$

if the binding site concentration, e_0, is much greater than the concentration of the substrate molecule, [S], which is to be chromatographed. Further discussion on the determination of K_d by chromatography is given in the literature [1]. The calculated K_d for AMP obtained in this study, 87 μM, agrees well with the value reported previously, 70 μM [1].

To test the ability of the immobilized enzyme column to resolve various alcohols and ketones, a mixture of β-adrenergic receptor active compounds, terbutaline and bambuterol, and their respective ketones were tested. Both the secondary alcohols and the corresponding ketones were completely resolved, as shown in Fig. 1, with k' values ranging from 0.79 for terbutaline to 4.43 for the ketone of bambuterol under the conditions described in Fig. 1.

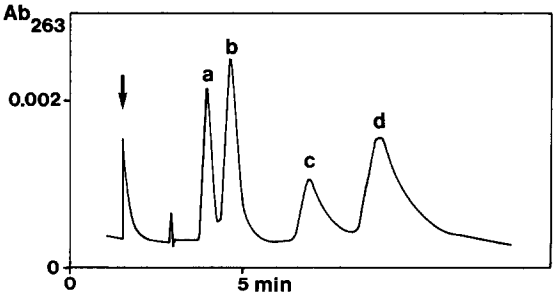

Fig. 1. Separation of β-adrenergic blocking agents, terbutaline (a) and bambuterol (b) and their respective ketones (c and d). Injection volume, 25 μl; mobile phase, 0.1 M potassium phosphate buffer (pH 7.5) containing 1 μM $ZnSO_4$; flow-rate, 0.5 ml/min. Sample concentrations of a, b, c, and d were 5, 25, 1 and 6 μg/ml, respectively.

Subsequently, the immobilized HLADH column was tested for its ability to separate various isomers of alcohols. Thus, the positional isomers of ethylphenethyl alcohol and the enantiomeric isomers of 1-phenyl-1-butanol were discriminated by the immobilized enzyme column with k' values as indicated in Table I.

HLADH contains two substrate-specific binding sites. Using eqn. 1 it can be calculated that K_d for the compounds listed in Table I are in the range 0.1–1.0 mM. This assumes that the analyte binds to the two substrate specific binding sites per immobilized enzyme molecule. It cannot be excluded, however, that interactions with other sites on the immobilized enzyme take place, although we have found that increasing the sample concentration above the calculated binding site concentration did lead to decreased retention times, which indicates, that the interaction is specific.

The immobilized enzyme column was used intermittantly during a period of 6 months. Between use, the HLADH-silica was stored at 4°C in the buffer that had been used. During this time the column retained its ability to separate the compounds above, even though the capacity fators decreased by about 50%.

DISCUSSION

Immobilized protein stationary phases, including enzymes, have previously been exploited for the separation of various organic compounds including racemic resolution of β-blockers [1–3,5]. As it has been reported previously that HLADH catalyses the stereoselective oxidation of secondary alcohols and that part of this selectivity can be attributed to different binding specificities of the enzyme for the different optically active alcohols and enantiomers

TABLE I

CAPACITY FACTORS, k', FOR VARIOUS ALCOHOLS ON HLADH-SILICA

Compound	k'
(±)-α-Ethylphenethyl alcohol	0.99
β-Ethylphenethyl alcohol	1.11
(*R*)-1-Phenyl-1-butanol	1.38
(*S*)-1-Phenyl-1-butanol	1.32

[6], we chose to investigate the ability of silica-bound HLADH to separate various β-blockers and their ketones and other primary and secondary alcohols. We found that HLADH-silica was able to separate completely a mixture of various secondary alcohols that are used as β-blockers in addition to their ketone intermediates. It is probable that the order of retention of these alcohols and ketones reflects in part the hydrophobic character of the substrate molecule, in agreement with that reported earlier [6]. In addition, we were able to separate closely related positional isomers of ethylphenethyl alcohol and to obtain racemic discrimination of the secondary alcohol 1-phenyl-1-butanol. It should also be possible to separate other alcohols, and also their oxidized or reduced intermediates, not tested here, including racemic mixtures of other optical isomers.

We are now examining other conditions in order to optimize and to extend this concept to alcohol dehydrogenases from other sources, including thermostable and secondary alcohol-specific dehydrogenases and other oxidoreductases. As there is a broad range of highly stable, especially thermostable, dehydrogenases available, in particular from microbial sources, it should be possible to prepare columns with higher stability and which can be used over a wide range of temperatures, solvents and solvent concentrations in comparison with previous chiral columns prepared from mammalian transport proteins [7,8].

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