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Short Communication

Preparation of heparin-glyceryl controlled-pore glass affinity media for the separation of α- and β-lipoproteins

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ABSTRACT

The preparation of a stable affinity medium with heparin as the affinity ligand has been investigated. Glyceryl controlled-pore glass (CPG) was activated with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate and coupled with heparin. This affinity medium was used to separate some simple proteins, trypsinogen and lysozyme, in a high-performance liquid chromatographic configuration. The heparin-glyceryl-CPG was also used to separate α- and β-lipoproteins in human serum. The effectiveness of the separation is confirmed by radial immunodiffusion and the determination of the cholesterol content of each of the separated fractions.

INTRODUCTION

The determination of total serum cholesterol is an important assay which can be used to assess a patient's risk for developing coronary heart disease. There has been an increasing emphasis, however, on the measurement of cholesterol in individual lipoprotein classes as a more reliable means by which to make this assessment. The cholesterol associated with the β-lipoproteins, which correspond approximately to low-density (LDL) and very-low-density lipoprotein (VLDL), is thought to have atherogenic properties [1,2]. On the other hand, cholesterol associated with the α-lipoproteins, corresponding roughly to the high-density lipoproteins (HDL), is thought to have anti-atherogenic properties [2,3]. Presently, methods for determining these quantities lack the precision and accuracy necessary for reliable diagnoses [4]. This is due to the lack of efficient and reliable methods for separating the lipoprotein classes.

Heparin, a sulfated polysaccharide, is commonly used as a precipitating agent for the isolation of HDL (α-lipoprotein) [5,6]. Alternatively, immobilized heparin (*e.g.* heparin-Sepharose) can be used as an affinity medium for the separation of α- and β-lipoproteins [7] as well as for the separation of a variety of simpler proteins [8]. We decided to immobilize heparin onto a more stable support, controlled-pore glass (CPG), in the hope that this medium may eventually be incorporated into a flow system for the on-line determination of cholesterol in the separated lipoprotein classes.

The reaction of cross-linked polysaccharides, such as Sepharose, with cyanogen bromide (CNBr) to form active cyanate esters followed by ligand coupling has become one of the most widely used procedures for the immobilization of affinity ligands [9]. However, the overall yield of these reactions is usually extremely low at 1–2%. The basic reaction conditions used in the activation (pH 11) tend to hydrolyze CNBr and cya-

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nate esters, once they are formed. This leaves a very small percentage of active sites capable of coupling with the affinity ligand. Kohn and Wilchek [10] proposed that a more electrophilic cyanylating agent, an N-cyano derivative, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP), be used instead of CNBr. The activation step using CDAP is performed under less basic conditions and, as in the case of conventional CNBr activation, leads to the formation of active cyanate esters, but without the competing side-reactions. As a result, the activation yield should be higher.

Glyceryl-CPG is a commercially available CPG which has been chemically modified with a hydrophilic non-ionic coating in order to reduce the possibility of non-specific adsorption of proteins from aqueous buffers. Vicinal hydroxyl groups on the glyceryl-CPG can be used to immobilize ligands for affinity chromatography [11].

In this paper, a simple method for the preparation of a durable affinity medium consisting of glyceryl-CPG activated with CDAP and coupled with heparin is described. This affinity medium is utilized for the separation of some simple proteins under HPLC conditions. Preliminary results of experiments utilizing heparin-glyceryl-CPG under low-pressure conditions, for the separation of α- and β-lipoproteins, are also presented.

EXPERIMENTAL

Preparation of heparin-glyceryl-CPG

Glyceryl-CPG (1.5 g, 515 Å mean pore diameter, 200–400 mesh, Sigma, St. Louis, MO, USA) was washed several times with deionized water then with 60% acetone. The CPG was suspended in 5 ml of 60% acetone and cooled to 0°C in an ice bath. CDAP (150 mg, synthesized according to the method of Wakselman *et al.* [12]) was added and the solution stirred. This was followed by the addition of 1.2 ml of 0.02 *M* triethylamine. The solution was stirred for an additional 10 min then filtered and washed with water and borate buffer (0.05 *M*, pH 9). The beads were placed in 5 ml of borate buffer and the solution placed in an ice bath. Heparin (200 mg, sodium salt, from ovine intestinal mucosa, approximately 182 I.U./mg, Sigma) was added and the solution stirred at 0°C for 3 h followed by storage overnight at 4°C.

Chromatographic procedure

The chromatographic system for the separation of proteins consisted of two Waters Model 590 pumps, a Waters Model 660 solvent programmer (Waters Canada), and a Rheodyne Model 7125 injector (20-μl sample loop) (CSC, Montreal, Canada). The detector was a Waters Model 440 UV absorbance detector (280 nm). A stainless-steel column (200 mm × 2 mm I.D.) was packed with heparin-glyceryl-CPG using a Shandon column packer (CSC). Packing was performed downward under 67 bar pressure with water as the packing solvent.

Trypsinogen and lysozyme were obtained from Sigma. A 60-min linear gradient from 0 to 1.0 *M* NaCl in 0.02 *M* phosphate buffer, pH 7.0, running at 0.5 ml/min, was used to separate the proteins.

Separation of lipoproteins by affinity chromatography

Fresh, pooled serum was obtained from the Royal Victoria Hospital (Montreal, Canada). Heparin-glyceryl-CPG beads (2 ml) were packed into a 4.0 cm × 0.8 cm I.D. disposable polyethylene column (BioRad, Mississauga, Canada). The beads were rinsed several times with a 0.1 *M* NaCl solution. Serum (200 μl) was applied to the column and the unretained lipoproteins were washed through the column with 2 ml of 0.1 *M* NaCl and collected (fraction 1). The retained lipoproteins were then eluted off the column with 2 ml of 1.0 *M* NaCl and collected (fraction 2).

The cholesterol content of each of the fractions was determined according to an enzymatic procedure described elsewhere [13].

Radial immunodiffusion studies were performed with Behring M-Partigen (apolipoprotein A) and Nor-Partigen (apolipoprotein B) plates (Hoechst, Montreal, Canada), according to the manufacturer's instructions.

RESULTS AND DISCUSSION

The separation of some simple proteins, trypsi-

nogen and lysozyme, was attempted with a packed heparin-glyceryl-CPG column in an HPLC system in order to test the utility of this material as an affinity chromatography medium. The successful separation of the two proteins is shown in Fig. 1.

For the separation of lipoproteins, the heparin-glyceryl-CPG beads were placed into disposable polyethylene columns. The two lipoproteins fractions were collected and immunodiffusion studies performed. Apolipoprotein A-I is the major protein of the α-lipoproteins and apolipoprotein B is the major protein of the β-lipoproteins. By testing for the presence of these two proteins in each of the separated fractions, we can determine whether or not the separation was successful. As expected, we found that fraction 1 contained apolipoprotein A-I, but no apilipoprotein B. Fraction 2 contained apolipoprotein B but no apolipoprotein A-I. This qualitative analysis was repeated for four separations, and in none of the experiments was any cross-contamination seen. In a separate experiment, the cholesterol content of each fraction was determined and results are presented in Table I.

These experiments indicate that heparin-glyceryl-CPG can be used as an affinity medium for the separation of α- and β-lipoprotein. The immobilization procedure using CDAP-activated glyceryl-CPG could be used for the immobilization of a wide variety of affinity ligands. The strength of the CPG support allows for its incorporation into high-performance chromatographic systems making this a potentially useful alternative to the popular CNBr-activated polysaccharide affinity supports.

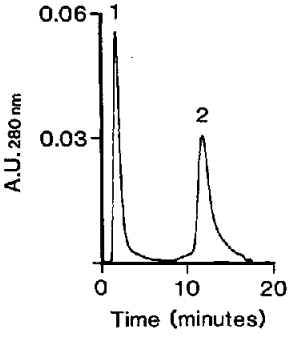

Fig. 1. Chromatogram of a mixture of (1) trypsinogen (1 mg/ml) and (2) lysozyme (0.5 mg/ml), separated on heparin-glyceryl-CPG.

TABLE I

CHOLESTEROL DETERMINATION IN α- AND β-LIPOPROTEIN FRACTIONS AFTER SEPARATION ON HEPARIN-CLYDERYL-CPG

	Cholesterol concentration			
	α-Lipoprotein		β-Lipoprotein	
	mg/dl	mmol/l	mg/dl	mmol/l
Heparin-CPG column	75.3	1.95	141.6	3.66
Hospital assay	71.2	1.84[a]	139.2	3.60[b]

[a] Reported as HDL-cholesterol.
[b] Calculated by total cholesterol − HDL cholesterol.

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