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DIRECT ANALYTICAL AND PREPARATIVE RESOLUTION OF ENANTIOMERS USING ALBUMIN ADSORBED TO SILICA AS A STATIONARY PHASE

PER ERLANDSSON*
Department of Technical Analytical Chemistry, Chemical Center, University of Lund, P.O. Box 124, S-221 00 Lund (Sweden)
LENNART HANSSON
Pierce Sweden AB, Sölvegatan 41, S-223 70 Lund (Sweden)
and
ROLAND ISAKSSON
Division of Organic Chemistry 3, Chemical Center, University of Lund, P.O. Box 124, S-221 00 Lund (Sweden)
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SUMMARY

A rapid and simple method for the preparation of high-performance liquid chromatography columns for chiral separations is described. The stationary phase is prepared by adsorbing bovine serum albumin on silica. Both analytical and preparative applications are described. A polarimeter was used as a detector to determine the enantiomer elution orders.

INTRODUCTION

During the last decade, interest in the determination of enantiomeric composition has increased dramatically, especially for pharmaceutical applications. A number of stationary phases for direct chiral separation by liquid chromatography, *i.e.*, resolution of enantiomers without prior derivatization, have been developed[1]. These have been based on, *e.g.*, naturally occurring chiral polymeric materials such as cellulose[2], starch, α_1-acid glycoprotein[3] and albumin[4].

In 1973, Stewart and Doherty[5] immobilized bovine serum albumin (BSA) on succinoylaminoethyl-Sepharose and completely resolved DL-tryptophan. Later, Allenmark *et al.*[4] covalently immobilized BSA on 10-μm silica in order to increase the column efficiency. This column has successfully been used to resolve various polar aromatic compounds, such as N-aroylamino acids and aromatic sulphoxides[1]. To the best of our knowledge, the immobilization technique used by Allenmark *et al.*[4] has not been described in the literature. BSA has also been used as a chiral additive in the mobile phase by, *e.g.*, Pettersson *et al.*[6].

The purpose of this work was to prepare analytical and preparative columns

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by irreversible adsorption of BSA to silica and secondly to compare the efficiency and selectivity of these columns with covalently bonded BSA columns.

EXPERIMENTAL

Materials

Bovine serum albumin (BSA) (Art. A-7030) was obtained from Sigma (St. Louis, MO, U.S.A.), D-tryptophan from United States Biochemical Corp. (Cleveland, OH, U.S.A.), DL-tryptophan and L-tryptophan from Merck (Darmstadt, F.R.G.) and DL-benzoin from BDH Chemicals, (Poole, U.K.). DL-Warfarin was a gift from AB Ferrosan (Malmö, Sweden), as were DL-Oxazepam from KabiVitrum (Stockholm, Sweden) and DL-Omeprazol from AB Hässle (Mölndal, Sweden).

Chromatography

Analytical columns were prepared by packing Vydac silica (5 μm, 300 Å, 100 m^2/g) (The Separations Group, Hesperion, CA, U.S.A.) or Nucleosil (5 μm, 300 Å, 120 m^2/g) (Macherey-Nagel, Düren, F.R.G.) into stainless-steel 200 mm × 4.6 mm Li-Chroma tubing (Skandinaviska GeneTec AB, Kungsbacka, Sweden) with an ascending slurry-packing technique[7]. A 3-g amount of silica was suspended in chloroform–methanol (2:1) and poured into a 75-ml packing bomb. The slurry was packed into the column at 400 bar using methanol as displacing medium.

For the preparative column, Matrex silica (20–45 μm, 500 Å) (Amicon-Grace, Helsingborg, Sweden) was packed into a 500 mm × 22 mm Valco stainless-steel column (Skandinaviska GeneTec AB) by a descending sedimentation technique. A 1120 mm × 22 mm glass tube was connected to the top of the open steel column with a plastic tube and the assembly was filled with a slurry of BSA–silica in 0.05 *M* phosphate buffer at pH 5.0. A water aspirator was connected to the bottom of the steel column and operated at the maximum available reduced pressure. During the sedimentation of the silica, more phosphate buffer was added until the bed had completely settled.

Potassium phosphate buffer mobile phases were prepared in MilliQ grade water obtained by purifying demineralized water in a MilliQ filtration system (Millipore, Bedford, MA, U.S.A.). In some cases, 1-propanol was used as the organic modifier. All mobile phases were degassed and filtered through a 0.45-μm Millipore filter prior to use. A standard chromatographic set-up was used comprising an LDC Constametric III pump or an Altex 110A pump with a preparative head (maximum flow-rate 28 ml/min). UV detection was performed with an LDC Spectromonitor III variable wavelength UV detector connected to a W+W 1200 dual-channel potentiometric recorder. The samples were injected with a Rheodyne 7120 injector with a 10-μl loop, or for preparative experiments with a 2-ml loop.

Specific rotation was monitored with a Perkin-Elmer 241 MC polarimeter using a 1 ml/l dm flow-cell to establish the elution order between the enantiomers[6].

Determination of BSA adsorption

To optimize the conditions for adsorption of BSA to silica, the influences of the phosphate concentration and pH were investigated. To 10 mg of 5-μm, 300-Å silica in a 10-ml glass test-tube were added 5 ml of a BSA–phosphate solution. The

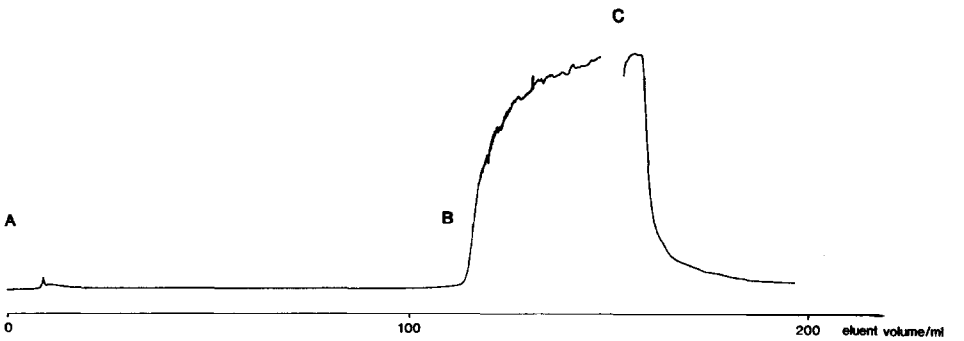

Fig. 1. Breakthrough of the eluting liquid from a 100 mm × 4.6 mm silica column during *in situ* adsorption of BSA on silica. UV detection at 280 nm. At A, a solution of 1 mg BSA/ml in 0.05 *M* phosphate, pH 5.0, is pumped into the column and at B, the breakthrough point is reached. At C, the column is washed with 0.05 *M* phosphate, pH 5.0.

tube was placed in an ultrasonic bath for 1 min and then on a vortex mixer for 10 s. The pH was measured and the contents of the tube were then centrifuged in a bench-top centrifuge to sediment the silica. The change in absorbance at 280 nm of the supernatant was monitored with an Hitachi Perkin-Elmer 124 spectrophotometer and the amount of adsorbed BSA per mg silica was calculated.

In situ immobilization of BSA by adsorption

The prepacked silica column was washed with approximately 50 column volumes of water and equilibrated with the same volume of 0.05 *M* phosphate buffer, pH 5.0. A solution of 1 mg BSA/ml in 0.05 *M* phosphate buffer pH 5.0 was pumped through the column until breakthrough of BSA was detected at 280 nm (see Fig. 1). From the breakthrough point the amount of BSA immobilized on the column was calculated. The column was then equilibrated with the same buffer but without BSA until a stable UV baseline was obtained. The leakage of BSA into the eluent was checked with Pierce BCA-protein assay reagent (Pierce Chemical Co., Rockford, IL, U.S.A.). The void volumes used for calculating k' and α were determined by injection of sodium nitrite, water or by observing the first baseline disturbance. Plate numbers, N, were calculated from the band widths at half-height[8].

RESULTS AND DISCUSSION

The amount of BSA adsorbed to silica is strongly dependent on the pH of the phosphate buffer (see Fig. 2), but the ionic strength has only a minor effect (see Fig. 3). The isoelectric point of BSA is located at pH 4.9[9], which is also the point of maximum adsorption. The maximum amount of BSA adsorbed is approximately 0.1 g per 100 m^2 silica (1 g). The surface area values employed were those stated by the supplier.

BSA has been described as a 140 Å × 40 Å prolate ellipsoid[10]. Assuming that, with a molecular weight of 66 500, it occupies an area of 140 × 40 $Å^2$ on silica, this will give a surface coverage of 60%. The surface area of silica that is accessible to the large BSA molecule is lower than the specific surface, 100 m^2/g, as measured by

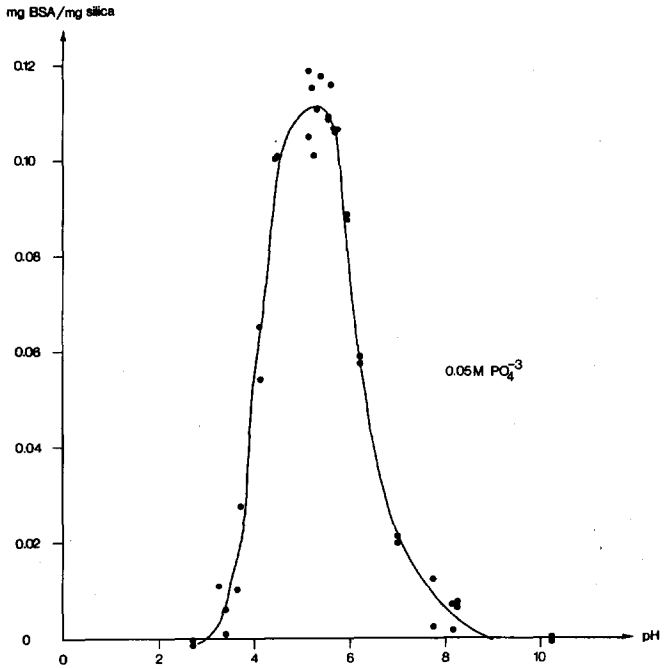

Fig. 2. The effect of pH on the adsorption of BSA to silica in 0.05 *M* phosphate buffer.

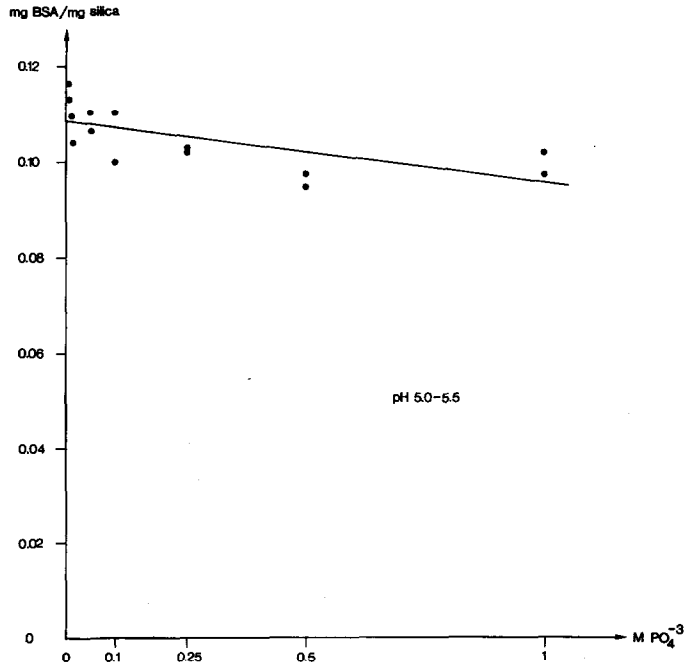

Fig. 3. The effect of the phosphate concentration on the adsorption of BSA to silica in phosphate buffer.

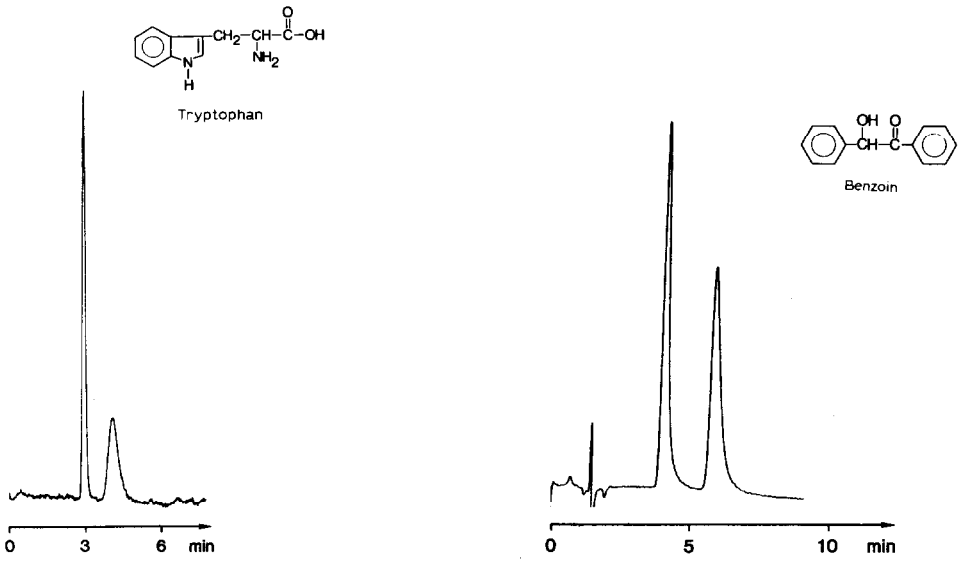

Fig. 4. Resolution of DL-tryptophan (10 μl, 0.37 m*M* injected) on a 200 mm × 4.6 mm column packed with 5-μm BSA–silica; mobile phase 0.05 *M* phosphate, pH 7.0; flow-rate 1.0 ml/min; UV detection at 278 nm. $k'_1 = 0.26$, $k'_2 = 0.78$, $\alpha = 3.0$, $N_1 = 2500$ and $N_2 = 460$.

Fig. 5. Resolution of DL-benzoin (10 μl, 0.01 mg/ml injected). Details as in Fig. 4, except 2% 1-propanol as modifier and UV detection at 250 nm. $k'_1 = 1.7$, $k'_2 = 3.0$, $\alpha = 1.7$, $N_1 = 2000$ and $N_2 = 1600$.

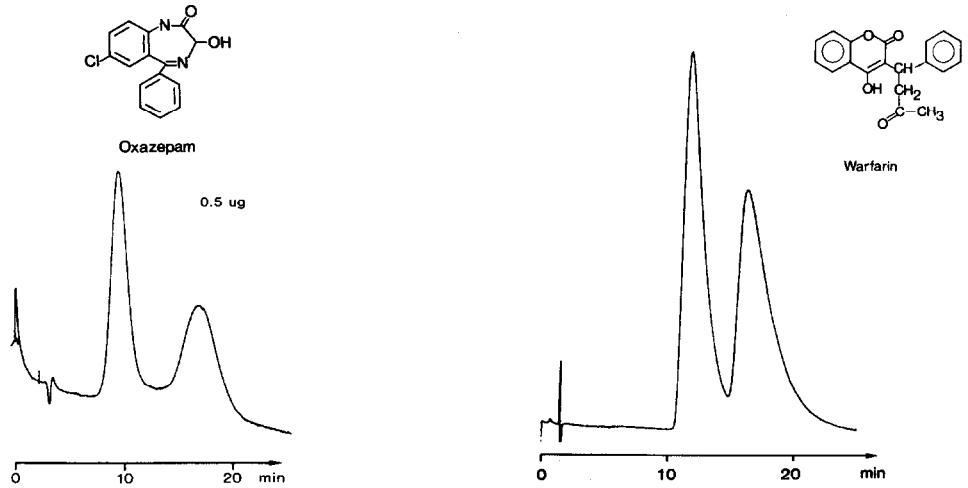

Fig. 6. Resolution of DL-Oxazepam (10 μl, 0.05 mg/ml injected) on a 200 mm × 4.6 mm column packed with 20–45 μm BSA–silica; mobile phase 0.1 *M* phosphate, pH 7.0; 2% 1-propanol as modifier; flow-rate 1.0 ml/min; UV detection at 230 nm. $k'_1 = 2.1$, $k'_2 = 4.5$, $\alpha = 2.1$, $N_1 = 170$ and $N_2 = 120$.

Fig. 7. Resolution of DL-Warfarin (10 μl saturated solution in water, (less than 0.1 mg/ml injected) on a 200 mm × 4.6 mm column packed with 5-μm BSA–silica; mobile phase 0.05 *M* phosphate, pH 7.0; 2% 1-propanol as modifier; flow-rate 1.0 ml/min; UV detection at 308 nm. $k'_1 = 7.0$, $k'_2 = 10.3$, $\alpha = 1.5$, $N_1 = 250$ and $N_2 = 200$.

TABLE I

SUMMARY OF CHROMATOGRAPHIC DATA

0.05 *M* phosphate buffer, pH 7.0 was used as the mobile phase and the column size was 200 mm × 4.6 mm.

Solute	*Silica (μm)*	*1-Propanol (%)*	k'_1	k'_2	α	N_1	N_2	*Fig.*
DL-Tryptophan	5	–	0.26	0.78	3.0	2500	460	3
DL-Benzoin	5	2	1.73	3.0	1.73	2000	1600	4
DL-Oxazepam	20–45	2	2.13	4.53	2.13	170	120	5
DL-Warfarin	5	2	7.0	10.3	1.48	250	200	6

BET nitrogen adsorption[11] and it seems reasonable that a monolayer of BSA is obtained.

Our results are in good agreement with those obtained for porous glass (particle size 10 μm, 240 Å, surface area 97 m^2/g) by Mizutani[12] where 136 mg albumin per g glass were adsorbed under optimum conditions, *i.e.*, in phosphate buffer pH 5. Mizutani deduced that albumin was adsorbed as a monolayer and that adsorption was caused by two factors; one is the amine–silanol ionic bonding and the other is a cooperative aggregative force between silica and proteins. It was also stated that both hydrogen bonding and hydrophobic interaction are of minor importance for protein adsorption.

Once the BSA is immobilized at pH 5 only a small fraction of BSA is lost from the column by changing the pH in the range 4–7.5.

The separations obtained for DL-tryptophan, DL-benzoin, DL-Oxazepam and DL-Warfarin (see Table I and Figs. 4–7) are in good agreement with results published by Allenmark *et al.*[13–16] from columns with covalently bonded BSA. Preliminary results show however differences in chiral selection between adsorbed and covalently bonded BSA. Chiral sulphoxides such as DL-Omeprazole and DL-2-methylsulphinylbenzoic acid could not be resolved on the adsorbed BSA–silica column. The reason for this behaviour is not yet known.

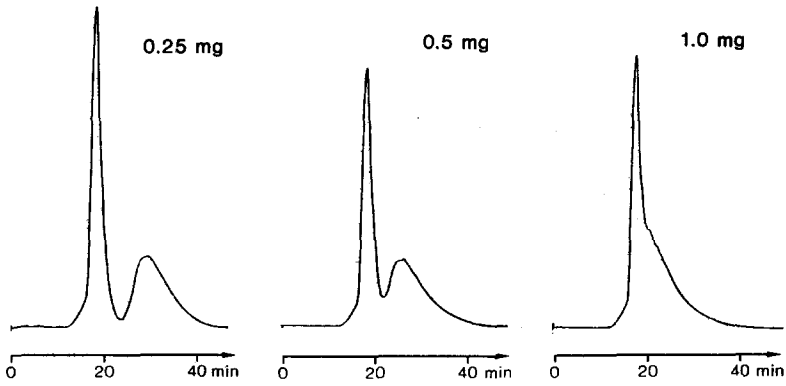

Fig. 8. Chromatograms showing the loadability of DL-tryptophan (0.25, 0.5 and 1.0 mg) on a 500 mm × 22 mm column packed with 20–45 μm BSA–silica; mobile phase 0.05 *M* phosphate, pH 7.5; flow-rate 11 ml/min; UV detection at 278 nm.

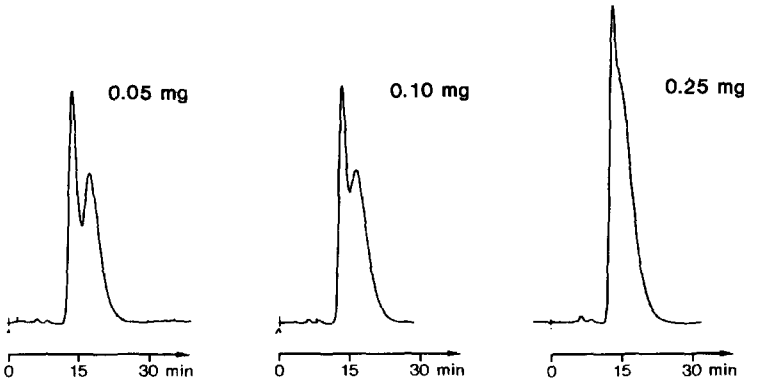

Fig. 9. Chromatograms showing the loadability of DL-benzoin (0.05, 0.10 and 0.25 mg). Details as in Fig. 8, except flow-rate 28 ml/min and UV detection at 250 nm.

The preparative use of columns with chiral discriminators of high molecular weight is limited because of the intrinsically low loadabilities of such columns[17]. In spite of this, the use of these columns in preparative work is of interest in studies, of, *e.g.*, the biological activity of potent drugs where milligram quantities are sufficient. In such cases, the stationary phase must be accessible in large quantities or easily made in the laboratory from commercially available materials.

To evaluate the adsorbed BSA–silica stationary phase for preparative purposes, a preparative column was prepared and the loadability of DL-tryptophan (see Fig. 8) and DL-benzoin (see Fig. 9) was investigated. The amount of adsorbed BSA on

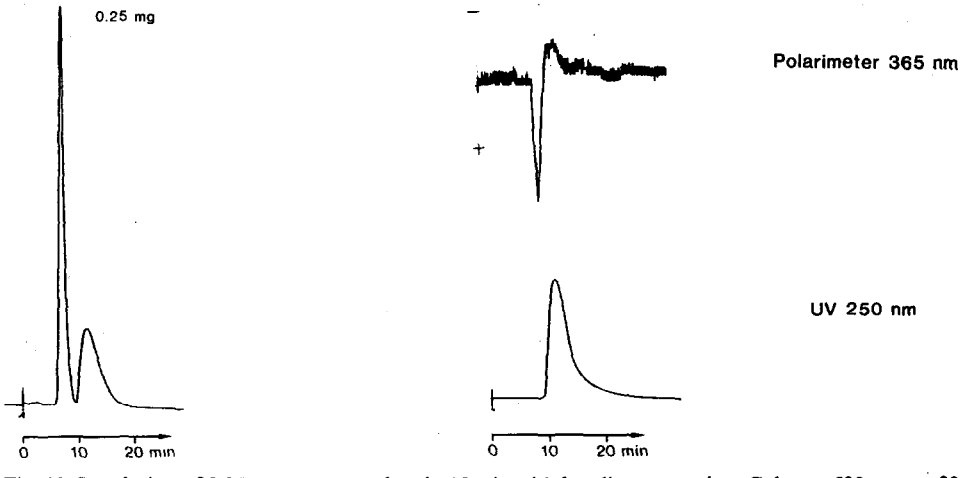

Fig. 10. Resolution of 0.25 mg DL-tryptophan in 15 min with baseline separation. Column: 500 mm × 22 mm packed with 20–45 μm BSA–silica. Mobile phase: 0.05 *M* phosphate, pH 7.5; flow-rate 28 ml/min. UV detection at 278 nm.

Fig. 11. Polarimetric detection at 365 nm and simultaneous UV detection at 250 nm of 5 mg DL-benzoin eluted from a 500 mm × 22 mm column packed with 20–45 μm BSA–silica. Mobile phase: 0.05 *M* phosphate, pH 7.5; flow-rate 28 ml/min.

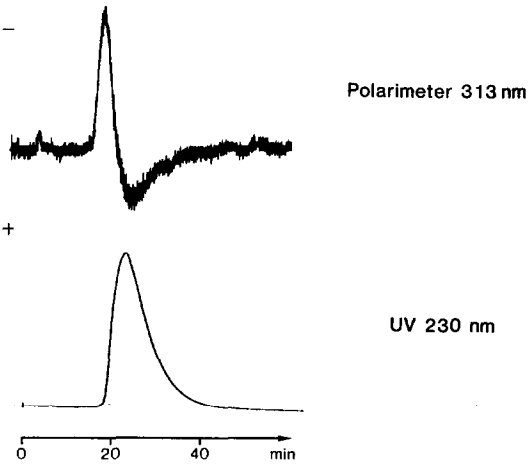

Fig. 12. Polarimetric detection at 313 nm and simultaneous UV detection at 230 nm of 2.5 mg DL-Oxazepam. Details as in Fig. 11.

the 500-Å silica was approximately 0.1 g per 50 m^2 (1 g), which gives a surface density of BSA of twice that for 300-Å silica (surface area values according to the supplier). The reason for this may be that a larger portion of the pores of the 500-Å silica is accessible to the BSA. The total amount of BSA in the preparative column was approximately 8 g. In favourable cases, a throughput of approximately 1 mg/h of racemic compounds could be obtained with baseline separation (see Fig. 10). It was also possible to use a polarimeter as a detector to determine the elution order of enantiomers (see Figs. 11 and 12) which facilitates, *e.g.*, recycling techniques. Because of the low sensitivity of the polarimeter and the low specific rotations of benzoin and Oxazepam, overloading of the column was necessary for these compounds.

Leaching of BSA from the equilibrated column was found to be below the detection limit of the assay, 0.01 mg/ml. Changes in the pH of the mobile phase and injection of solutes in organic solvents such as methanol, ethanol and 1-propanol displaces small amounts of BSA from the column. The displaced BSA is unretained and causes no interference with the retained solutes. Of the above mentioned alcohols, methanol causes the smallest amount of BSA to be displaced.

The lifetime of the columns is more than 6 months if they are stored properly, *i.e.*, at +4°C, pH 5–7 when not in use. The pH stability under alkaline conditions is primarily limited by the silica. At pH 8, some BSA is lost and exposes the silica surface onto which BSA can be recoated. After two to four recoatings the columns do not show acceptable performance and must be discarded.

REFERENCES

1 S. Allenmark, *J. Biochem. Biophys. Methods*, 9 (1984) 1–25.
2 G. Hesse and R. Hagel, *Liebigs Ann. Chem.*, (1976) 996–1008.
3 J. Hermansson, *J. Chromatogr.*, 269 (1983) 71–80.
4 S. Allenmark, B. Bomgren and H. Borén, *J. Chromatogr.*, 264 (1983) 63–68.
5 K. K. Stewart and R. F. Doherty, *Proc. Natl. Acad. Sci. U.S.A.*, 70 (1973) 2850–2852.

6 C. Pettersson, T. Arvidsson, A.-L. Karlsson and I. Marle, in C. Pettersson, *Dissertation*, University of Uppsala, 1984.
7 P. A. Bristow, P. N. Brittain, C. M. Riley and B. F. Williamson, *J. Chromatogr.*, 131 (1977) 57–64.
8 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979, p. 31.
9 A. L. Lehninger, *Biochemistry*, Worth, New York, 2nd ed., 1978, p. 162.
10 P. G. Squire, P. Moser and C. T. O'Konski, *Biochemistry*, 7 (1968) 4261–4272.
11 B. H. Kaye, in F. J. Welcher (Editor), *Standard Methods of Chemical Analysis*, Vol. 3, Van Nostrand, New York, 6th ed., 1966, pp. 829–831.
12 T. Mizutani, *J. Liq. Chromatogr.*, 8 (1985) 925–983.
13 S. Allenmark, B. Bomgren and H. Borén, *J. Chromatogr.*, 237 (1982) 473–477.
14 S. Allenmark, B. Bomgren and H. Borén, *J. Chromatogr.*, 316 (1984) 617–624.
15 S. Allenmark, *LC, Liq. Chromatogr. HPLC Mag.*, 3 (1985) 348–353.
16 S. Allenmark, *J. Liq. Chromatogr.*, 9 (1986) 425–442.
17 W. Lindner and C. Pettersson, in I. W. Wainer (Editor), *Liquid Chromatography in Pharmaceutical Development*, Aster Publishing Corp., Springfield, OR, 1985, p. 99.