HIGH PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY (HPLAC) AND ITS APPLICATION TO THE SEPARATION OF ENZYMES AND ANTIGENS

Sten OHLSON*, Lennart HANSSON**, Per-Olof LARSSON* and Klaus MOSBACH*

Divisions of Biochemistry and Technical Analytical Chemistry**, Chemical Center, University of Lund, P.O. Box 740, S-220 07 Lund 7, Sweden*

Received 14 June 1978

1. Introduction

High performance liquid chromatography (HPLC) has seen a spectacular development during the last few years due to its rapidity and its high resolving power [1–7]. Recently, HPLC has also been applied to include biological macromolecules such as polynucleotides, polysaccharides and proteins [3–7].

This paper describes the first use of bio-affinity supports (in contrast to normally used 'non-biological' stationary phases) to the resolution of biological macromolecules in combination with the technique of HPLC, and for this novel technique we suggest the term: high performance liquid affinity chromatography, HPLAC.

Examples are given of enzyme and isozyme separations using an immobilised general ligand, AMP, and of albumins employing an immobilised immunosorbent, anti-serum albumin.

2. Materials and methods

2.1. *Chemicals*

Crystalline LADH (1.85 U/mg), β-NADH (grade III), β-NAD (grade III), BSA (crystalline) and AMP were purchased from Sigma (St. Louis, MO.). LDH from pig heart (400 U/mg) and rabbit muscle (550 U/mg) were supplied by Boehringer (Mannheim, GFR). γ-Glycidoxypropyltrimethoxysilane (Silane Z-6040) was bought from Dow Corning (Midland, MA), rabbit anti-HSA from Dako (Denmark), HSA from Kabi (Stockholm, Sweden) and silica gel (Li-Chrosorb SI 60, 10 μm) from E. Merck (Darmstadt, GFR.). N^6-(6-aminohexyl)-AMP was synthesized according to a method given in the literature [8].

Abbreviations: LDH, Lactate dehydrogenase; LADH, Horse liver alcohol dehydrogenase; BSA, Bovine serum albumin; HSA, Human serum albumin; a.u.f.s., Absorption units of full scale

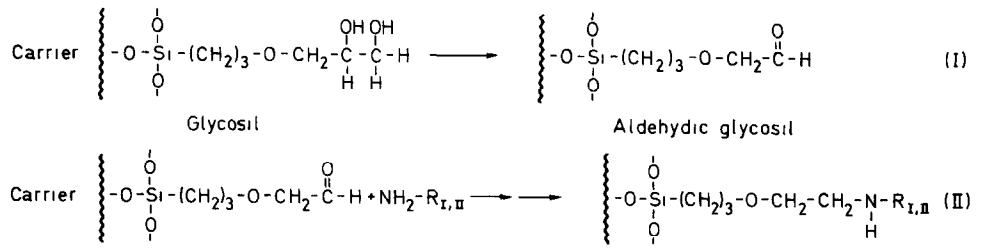
2.2. *Preparation of the affinity gels*

2.2.1. Preparation of glycerolpropylsilane bonded silica (Glycosil)

The bonding procedure used was a modification of a previously published method [7]. Silica (5 g) was suspended in 200 ml of an aqueous solution of γ-glycidoxypropyltrimethoxysilane (1%, pH 5.5). The slurry was treated under vacuum in an ultrasonic bath and was then heated to and maintained at 90°C for 2 h with occasional shaking. The pH of the mixture was adjusted to 3.0 followed by heating again for 1 h to convert the oxirane groups of the gel to glycol groups. The amount of glycerolpropylsilane coupled to the silica was determined by periodate oxidation and was found to be 530 μmol/g of silica (dried in vacuo) [9].

2.2.2. Preparation of N^6-(6-aminohexyl)-AMP bonded silica

Glycosil was first converted to its aldehydic form by periodate oxidation [9] (see scheme, I). Then N^6-(6-aminohexyl)-AMP (400 mg) was added to 2 g of aldehydic Glycosil in 5.0 ml of 0.1 M $NaHCO_3$ (pH 10) and was allowed to react for 24 h at 20°C. Following this $NaBH_4$ (80 mg) in 1.0 ml of H_2O was added at intervals of 1 h to reduce the imine formed



Scheme I and II

(see scheme, II). The amount of N^6-(6-aminohexyl)-AMP coupled to the silica was measured by UV-absorption at 268 nm of the gel slurry suspended in glycerol (87%) and 20 μmol of N^6-(6-aminohexyl)-AMP/g of silica (dried in vacuo) was coupled.

2.2.3. Preparation of anti-HSA bonded silica

1 ml of anti-HSA solution (20 mg/ml) was added to a slurry made up of 1.25 g of aldehydic Glycosil and 2.5 ml of 0.1 M $NaHCO_3$ (pH 7.9). After reaction for 50 h at 4°C, $NaBH_4$ (80 mg) in 0.4 ml of H_2O was added at intervals during a 17-h period (see scheme, II). The amount of anti-HSA coupled to the gel was 4 mg/g of silica as determined by UV-measurement at 280 nm.

2.3. *Enzyme assays*

The enzymic activities of LADH and LDH were measured by following the change in absorbance at 340 nm at room temperature (20–22°C). The assay mixtures were; LADH: 200 μl of enzyme solution (effluent from column), acetaldehyde (25 μmol) and NADH (0.3 μmol) in a total volume of 1.0 ml of 0.1 M sodium phosphate buffer, pH 7.5; LDH: 50–100 μl of enzyme solution (effluent from column), pyruvate (1 μmol) and NADH (0.3 μmol) in a total volume of 1.0 ml of 0.1 M sodium phosphate, pH 7.5.

2.4. *Chromatography*

The pumping system consisted of a Milton Roy minipump equipped with a pulse dampener (Laboratory Data Control, Riviera Beach, FL). UV detection was performed with a variable wavelength detector, 190–700 nm (LC-55, Perkin Elmer, Norwalk, CI) and the sample injections were made with a valve injector (Valco, Houston, TX).

The gels (1.2 g silica) were packed in polished '316' stainless steel columns (100 mm × 5 mm I.D.) with the upward-slurry packing technique [10]. The columns were packed in water (anti-HSA–silica) or water–methanol, 10:90, (AMP–silica) at 2000 psi. The performance of the AMP–silica columns were estimated with toluene and nitrobenzene as test samples in a mobile phase of hexane containing 1% acetonitrile. A typical HETP value (Height Equivalent to a Theoretical Plate) (nitrobenzene) for the AMP–silica column was 0.028 mm. When not in use, the anti-HSA–silica column was stored at +4°C.

All chromatographic procedures were performed at room temperature (20–22°C). The mobile phase consisted of 0.1 M sodium phosphate, pH 7.5. Pulse elution was conveniently handled by using the valve injector equipped with a 1.6 ml loop.

3. Results and discussion

3.1. *Choice of support*

HPLC requires supports that can withstand high pressures and from this point of view inorganic supports such as glass and silica are ideal. However, a major problem with the use of inorganic supports for separation of protein is nonspecific adsorption and/or denaturation of proteins on these supports. Fortunately, it has been reported [7] that a glycerylpropylsilyl layer covalently bonded to silica minimises such adverse effects. Therefore we prepared such supports for our affinity chromatography studies, namely glycerylpropylsilica particles (Glycosil). These Glycosil columns were superior to other silica supports with respect to recovery of the proteins studied (80–100% protein was recovered with Glycosil).

The AMP-analogue and anti-HSA were coupled through covalent linkages to Glycosil forming a stable ligand–silica bond (see scheme). The silica used consisted of 10 μm particles with an average pore diameter of 60 Å, which implies that the proteins are likely to interact preferentially with those affinity ligands which are located on the outer surface of the support.

3.2. *HPLAC of LDH and LADH on an AMP–silica column*

LDH and LADH were considered to be suitable model substances for evaluating HPLAC, because their behaviour in conventional affinity chromatography is well established [11]. Figures 1 and 2 show high-speed separations of LDH and LADH employing two different elution conditions namely biospecific elution, utilizing ternary complex formation (fig.1) [11] and salt elution (fig.2) [12].

In both cases the separation of the enzymes completed in less than 10 min. Neither LDH nor LADH were retarded on the reference column made up of

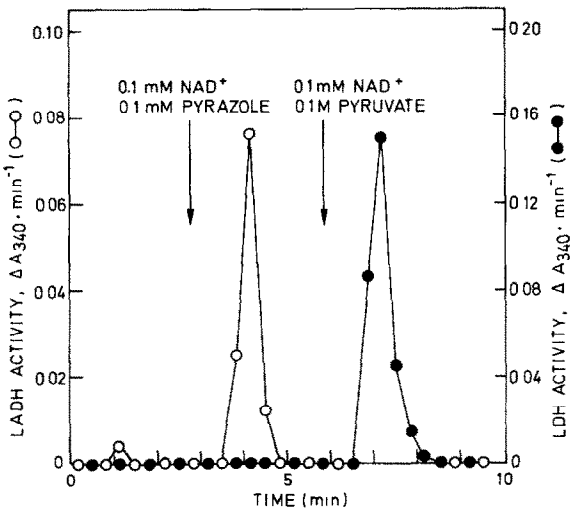


Fig.1. Separation of LADH and LDH (pig heart) with ternary complex formation on an AMP–silica column. A mixture (10 μl) of LDH (10 μg) and LADH (20 μg) dissolved in 0.1 M sodium phosphate buffer, pH 7.5 was injected onto the column and eluted, as indicated by the arrows, with pulses of 1.6 ml. Fractions of 0.5 ml were collected at a flowrate of 1.5 ml/min and subsequently assayed. Pressure, 400 psi.

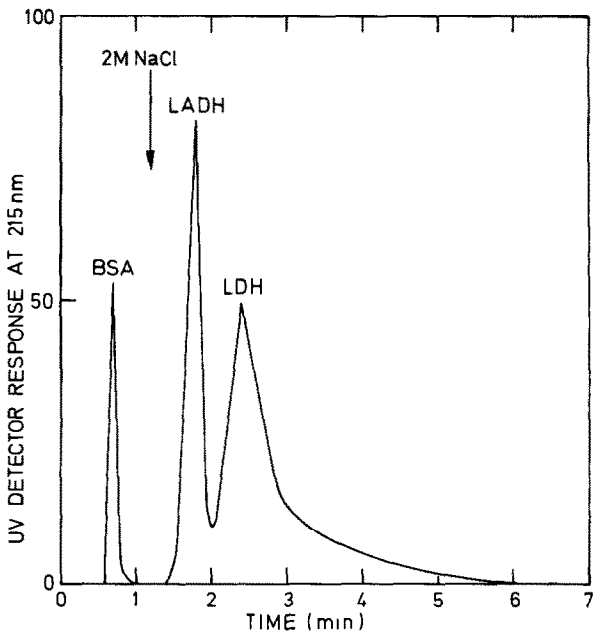


Fig.2. Separation of BSA, LADH, and LDH (pig heart) with salt elution on an AMP–silica column. A mixture (10 μl) of BSA, LADH, and LDH (10 μg of each) dissolved in 0.1 M sodium phosphate buffer, pH 7.5 was injected onto the column and eluted with 2 M NaCl in the same buffer as indicated by the arrow. The effluent was monitored at 215 nm (BSA; 1.7 a.u.f.s., LADH, LDH; 0.17 a.u.f.s.). Flowrate, 1.5 ml/min; pressure, 400 psi.

Glycosil. Both LDH and LADH were efficiently eluted as ternary complexes: LDH in combination with NAD^+ + pyruvate and LADH with NAD^+ + pyrazole (fig.1). The order of elution could be reversed, but since 2–3% LADH leaked out in the NAD^+ + pyruvate pulse, the order of elution shown in fig.1 was preferred; full recovery of the enzymes was obtained with one pulse of reagents. Since formation of the ternary complex between LDH, NAD^+ and pyruvate is a comparatively slow process, it was suspected that the residence time of the pulse on the column might be too short to give quantitative elution of the enzyme, but under the chromatographic conditions used here we did not observe such an effect. It should be added, that 2–4% of the LADH did not bind to the AMP–silica column and appeared in the effluent unretarded.

As shown in fig.2 LDH and LADH (96–98%) remained bound to the AMP–silica column, whereas

BSA was unretarded. On applying buffer containing 2 M NaCl, LADH and LDH were almost quantitatively eluted (about 90%) but tailing of the LDH was observed.

Separation of the isozymes of LDH by affinity [13] or ion exchange chromatography [14] has been thoroughly investigated. However, the main drawback of these techniques is the demand for long separation time. With the introduction of HPLC on ion exchange supports (e.g. DEAE-Glycophase, controlled pore glass) resolution and speed of separation has improved dramatically [6]. In our approach the AMP-analogue, N^6-(6-aminohexyl)-AMP, was coupled to silica, thereby making it suitable for high pressure techniques. From the results depicted in fig.3, it seems that the isozymes of LDH, i.e. H_4 and M_4 can be separated at high speed. The two isozymes used, M_4 and H_4, are from rabbit and pig respectively. An even better separation of these isozymes may be possible if they were obtained from the same species, e.g. pig H_4 and M_4, since their NADH dissociation constants are more favourable to separation than those of the system studied here [15]. The isozymes were eluted with a recovery of about 95% and no LDH was detected in the BSA-front (BSA recovery was about 99%). On changing the type of NADH-gradient profile, separation of all five isozymes of LDH may be feasible. HPLAC of isozyme mixtures (e.g. LDH) could have an advantage over conventional HPLC since the need for prepurification of the sample is lessened.

The AMP–silica column utilized in this investigation was used repeatedly for several months with no loss of efficiency. Sometimes cavities appeared at the top of the column and this presented problems, but these were easily overcome by adding new material to the top of the column and this did not require repacking.

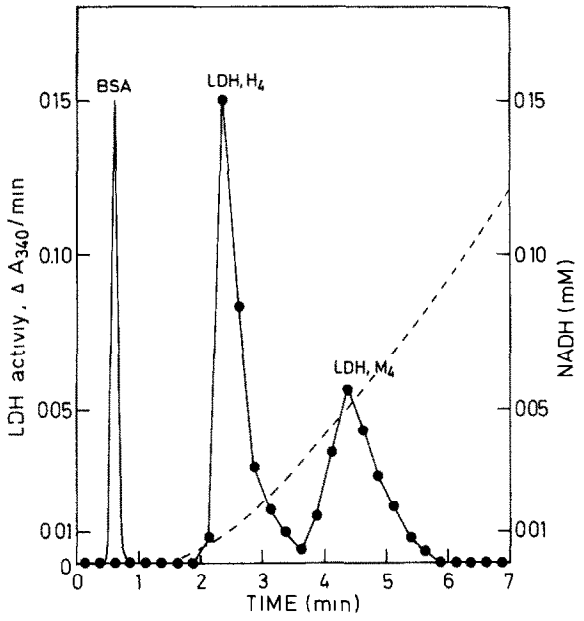


Fig.3. Separation of BSA, LDH (H_4, pig heart), and LDH (M_4, rabbit muscle) using a gradient of NADH on an AMP–silica column. A mixture of BSA, LDH, H_4, and LDH, M_4 (10 μg of each) in 10 μl of 0.1 M sodium phosphate buffer, pH 7.5, was injected onto the column. After the appearance of BSA (recorded at 215 nm) the isozymes of LDH were eluted with a gradient of NADH dissolved in the above buffer. Fractions (0.4 ml) obtained were subsequently assayed for LDH activity. The NADH concentration was measured at 290 nm. Flowrate, 1.6 ml/min; pressure, 400 psi.

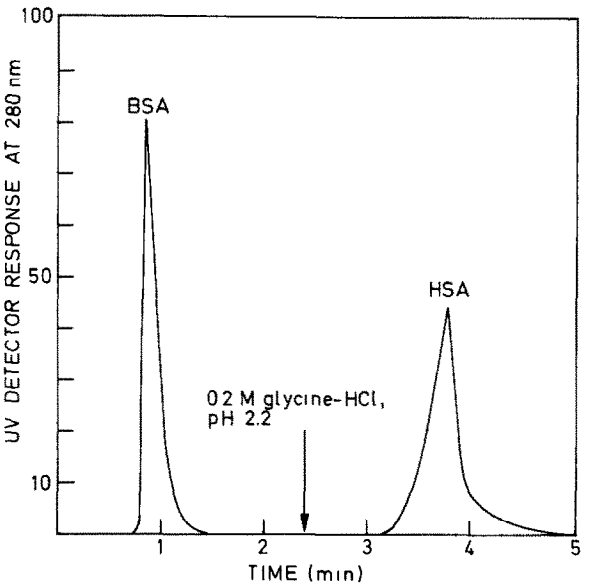


Fig.4. Separation of BSA and HSA on a column of anti-HSA–silica. The proteins were dissolved in the mobile phase buffer, 0.1 M sodium phosphate, pH 7.5, and after the appearance of BSA (50 μg), HSA (50 μg) was eluted with a pulse of 0.8 ml of 0.2 M glycine–HCl, pH 2.2. The effluent was monitored at 280 nm (0.17 a.u.f.s.). The elution profile of HSA obtained was corrected for the influence of the glycine–HCl buffer on the UV detector response at 280 nm. Flowrate, 1.5 ml/min; injection volume, 5 μl.

3.3. *HPLAC of HSA on an anti-HSA–silica column*

The technique of HPLAC can also be applied to immunosorbents as demonstrated in fig.4, that shows a high-speed separation of a mixture of BSA and HSA on an anti-HSA–silica column. At least 98% of the BSA applied to the column passed through the column unretarded. About 96% of the HSA sample added was bound to the column and on subsequent lowering of the pH to 2.2, dissociation of the anti-HSA–HSA complex was achieved and HSA was eluted with almost quantitative recovery (about 95%) [16]. A small part of the HSA sample (4%) passed unretarded through the column and probably represented contaminating proteins. As expected a reference column prepared in an analogous fashion but omitting anti-HSA, did not adsorb either HSA or BSA. The anti-HSA–silica column was used more than 20 times without loss of its antigen binding capacity.

4. Conclusion

We feel that the technique of high performance liquid affinity chromatography (HPLAC) described here will find wide application in various areas, including the clinical, where there is a need for rapid separations of biological macromolecules. Compared to stationary phases used in conventional high performance liquid chromatography the affinity ligand introduces a far higher specific component in the separation step which therefore could reduce the need for prepurification of samples. Compared with affinity chromatography this technique is much faster not only for routine separation and enrichment of macromolecules but also in their analysis in biological samples. It is conceivable that even a better resolving power with this technique will be observed compared with affinity chromatography in both the adsorption and elution steps.

Acknowledgements

The financial support by the Swedish Natural Science Research Council is acknowledged. The authors are indebted to Professor B. Smith, head of the department of Technical Analytical Chemistry, for his interest in this work and to Dr T. Griffin for linguistic advice.

References

[1] Bristow, P. A. (1976) Liquid Chromatography in Practice, hetp, Wilmslow.
[2] Kato, Y., Seita, T., Hashimoto, T. and Shimizu, A. (1977) J. Chromatogr. 134, 204–206.
[3] Kudirka, P. J., Busby, M. G., Carey, R. N. and Toren, jr., E. C. (1975) Clin. Chem. 21, 450–452.
[4] Kudirka, P. J., Schroeder, R. R., Hewitt, T. E. and Toren, jr., E. C. (1976) Clin. Chem. 22, 471–474.
[5] Mönch, W. and Dehnen, W. (1978) J. Chromatogr. 147, 415–418.
[6] Chang, S. H., Gooding, K. M. and Regnier, F. E. (1976) J. Chromatogr. 125, 103–114.
[7] Regnier, F. E. and Noel, R. (1976) J. Chromatog. Sci. 14, 316–320.
[8] Guilford, H., Larsson, P.-O. and Mosbach, K. (1972) Chem. Scripta 2, 165–170.
[9] Siggia, S. (1949) Quantitative Organic Analysis via Functional Groups, pp. 8–9, J. Wiley and Sons, New York.
[10] Bristow, P. A., Brittain, P. N., Riley, C. M. and Williamson, B. F. (1977) J. Chromatogr. 131, 57–64.
[11] Mosbach, K. (1978) in: Advances in Enzymology and Related Areas of Molecular Biology (Meister, A., ed) Vol. 46, pp. 205–278, J. Wiley and Sons, New York.
[12] Craven, D. B., Harvey, M. J., Lowe, C. R. and Dean, P. D. G. (1974) Eur. J. Biochem. 41, 329–333.
[13] Brodelius, P. and Mosbach, K. (1973) FEBS Lett. 35, 223–226.
[14] Winer, A. D. (1970) in: Mechanism of Action of Dehydrogenases (Schwert, G. W. and Winer, A. D., eds) p. 1, University Press of Kentucky, Lexington.
[15] Brodelius, P. and Mosbach, K. (1976) Anal. Biochem. 72, 629–636.
[16] Avrameas, S. and Ternynck, T. (1967) J. Biol. Chem. 242, 1651–1659.