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Rapid reversed-phase high-performance liquid chromatography method for quantitation, at high pH, of the recombinant apolipoprotein A-IMilano in *Escherichia coli* fermentation broth

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Abstract

An automated reversed-phase high-performance liquid chromatography method for quantitative determination of recombinant apolipoprotein A-IMilano (r-Apo A-IM) in *E. coli* fermentation broth has been developed and evaluated. The use of a unique matrix (Poros IIR/H) makes it possible to achieve rapid separation and good resolution at high pH. The r-Apo A-IM-containing fraction is well separated from other proteins allowing a reliable quantification. The automation and high sample throughput of this method makes it very useful for routine determination of r-Apo A-IM in fermentation broth and in eluates from the various purfication steps. With suitable modifications and adaptions this method is likely to be useful for similar rapid analytical determination of recombinant proteins in complex solutions. © 1998 Elsevier Science B.V.

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1. Introduction

Apolipoprotein A-I is the major protein component in the high density lipoprotein (HDL) particle. It plays an important role in the reversed cholesterol transport system which controls the balance of cholesterol in the body [1–5]. Apolipoprotein A-IMilano (Apo A-IM) is a naturally occurring mutant with an amino acid substitution at position 173, where an arginine residue has been replaced by a cysteine [6,7].

The Apo A-IM molecule has thus the ability to form disulfide-linked dimers. Therapeutic use of apolipoproteins, for treatment of coronary artery diseases, has been suggested [8].

Adequate and reliable determination of the amount of recombinant proteins in fermentation broth are often difficult due to the complexity of the sample matrix. Within the pharmaceutical industry the demands for rapid analyses, in order to shorten the time for process development, are also major issues.

Usually, the need for quantitation of biomolecules (proteins) are achieved by use of immunologically based techniques like enzyme-linked immunosorbent assays (ELISA) and/or radioimmunoassay (RIA) [9]. These techniques are complex, time-consuming, difficult to automate and are operator dependent [10,11].

Furthermore, such methods always run the risk of unwanted cross-reactions hampering the reproducibilities and thus giving unreliable determinations.

In certain cases, specific affinity properties of the target molecule has been used for chromatographic analysis [12–14].

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The useable pH range for mobile phases in RP-HPLC is limited, especially for silica-based stationary phases, to about pH 2–8 [15]. Therefore polymer-based packings are recommended for separations at higher pH intervals [16].

We here present a rapid reversed-phase HPLC method, for determination of the amount of recombinant apolipoprotein A-IMilano (r-Apo A-IM) in different solutions, that uses a polystyrene–divinylbenzene matrix (Poros IIR/H) for separation of r-Apo A-IM at pH 11.0. Integration of peak areas corresponding to the apolipoprotein A-IMilano fraction gives reliable determination of the protein concentration in different samples. Some performance characteristics of the method are also given.

Adaptation of this method for rapid and reliable determination of specific protein species in complex solutions, during process development of protein-based pharmaceuticals, seems likely to be useful.

2. Experimental

2.1. Materials

All chemicals were of analytical grade, if not otherwise stated. Trisodium phosphate, Tris–HCl and 2-mercaptoethanol were purchased from Sigma (St. Louis, MO. USA). Acetonitrile (far UV-grade) and 2-propanol was from LabScan (Dublin, Ireland). All solutions were prepared in deionized water from a Milli-Q Plus water purification system (Millipore, Bedford, MA, USA) and filtered through 0.45-μm filters before use. Calibration proteins for electrophoresis (low-molecular-mass-markers: 14 000–94 000) were from Pharmacia BioTech, Uppsala, Sweden.

2.2. Preparation of r-Apo A-IM reference

Recombinant apolipoprotein A-IMilano was expressed in an *E. coli* system and thereafter purified from procaryotic components by a series of chromatographic steps.

All these procedures were carried out by the Departments of Fermentation and Down-Stream Processing, respectively, at Technical Operation, Process R&D, Pharmacia and Upjohn, Stockholm, Sweden.

The purified reference batch was checked for purity by SDS–PAGE and HPLC before further use. The reference was stored in 50-μl portions at −70°C. On each occassion of use, a new portion was thawed.

2.3. Preparation of a monoclonal antibody directed towards r-Apo A-IM

Antibodies directed towards r-Apo A-IM was produced by immunization of mice with pure r-Apo A-IM followed by hybridization techniques to give an antibody-producing clone. Obtained monoclonals were purified by Protein-G chromatography, biotinylated and finally stored at −70°C before further use. All these procedures were done by the Department of Bioanalytical Immunology, Development, Pharmacia and Upjohn.

2.4. Reversed-phase HPLC at alkaline pH

The development and evaluation of the presented method was done on a Waters 600 multisolvent delivery system, an UV–VIS detector 486 from Waters (Milford, MA, USA) and an CMA200 autosample injector (CMA/Microdialysis, Stockholm, Sweden). Acquisition of data was done by a Millenium 2010 chromatography work station (Waters). For collection of fractionated material, a Frac-200 fraction collector (Pharmacia BioTech AB, Uppsala, Sweden) was used.

The analytical reversed-phase chromatography was done at ambient temperature on a Poros IIR/H, 10-cm×4.6-mm I.D., analytical column (Perseptive Biosystems, Cambridge, MA, USA). The polystyrene–divinylbenzene matrix has a particle size of 10 μm with a pore size of 500–1000 Å. The matrix also contains large (5000–8000 Å) 'through-pores' which enables high resolution and capacity at high flow-rates. This polymeric stationary phase is chemically stable in broad pH-range.

Mobile phases were: (A), 2.2 m*M* trisodium phosphate pH=11.0 and (B), 2.2 m*M* trisodium phosphate, pH=11.0 containing 70% (v/v) acetonitrile.

The flow-rate was 2 ml/min. The column was

equilibrated in a mixed mobile phase A/B solution of 39%B. Elution was performed in a gradient running from 39–54%B over 8 min. A sample volume of 100 μl was injected for each analysis and detection was done at 220 nm.

The total analysis time was 16 min, including column regeneration before the next sample injection.

The samples were centrifuged at 18 000×*g* for 20 min to remove cell debris and other particulate matter. The supernatant was treated in a 85 m*M* Tris–HCl pH=6.5 buffer with 5.9 *M* guanidine hydrochloride and 16.9% isopropanol. The sample was also reduced with 140 m*M* 2-mercaptoethanol at 60°C for 15 min. Thus the r-Apo A-IM was determined in its monomeric form (cf. Refs. [6,7]).

Standard curves were generated for quantitative determinations of unknown samples, by injections of known amounts of the r-Apo A-IM reference.

Separated peaks from a fermentation broth sample were collected for further analysis on purity and identity. The fractionation and collection was repeated three times in order to get sufficient material.

2.5. Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) was done on 12% gels in an electrophoresis unit from Hoefer (San Francisco, CA, USA).

The electrophoresis was performed in 20 m*M* Tris–100 m*M* glycine (pH 8.6) containing 0.1% SDS. The gels were run at a constant current of 16 mA/gel and maximum setting of 400 V (essentially according to Laemmli [17]). The gels were silver-stained according to the method of Johansson and Skoog [18].

2.6. Western blotting

SDS–PAGE separated material was transferred to Hybond-ECL nitrocellulose membranes (Amersham Life Science, Little Chalfont, UK) in an transfer-blot cell (BioRad, Richmond, VA, USA). The transfer buffer was 48 m*M* Tris–39 m*M* glycine (pH 9.1) containing 20% methanol and 2 m*M* SDS. Both the nitrocellulose membrane and the SDS–PAGE gel were equilibrated for 10 min in this solution prior to the transfer. Transfer of the proteins was done during 60 min at 12°C.

The voltage was 200 V resulting in 0.45–0.65 A. After transfer nonoccupied binding sites of the membrane were blocked in a phosphate-buffered saline (PBS) buffer (pH 7.4) containing 0.05% Tween and 5% dried 'fat free' milk as blocking agents (cf. Refs. [19,20]). After three wash steps in the PBS-Tween solution the membrane was incubated overnight at 4°C with the r-Apo A-IM specific monoclonal antibody, followed by three 20-min wash steps, in the PBS-Tween solution. The nitrocellulose membrane was then incubated with a secondary (goat–anti-mouse–IgG HRP conjugate) antibody from Bio-Rad. Finally the proteins were visulized by use of enhanced chemiluminiscence detection (Reagent-ECL kit No. RPN 2106, Amersham Life Science).

2.7. Performance characteristics

The method was evaluated with regard to accuracy, limit of detection (LOD), limit of quantification (LOQ), linearity and precision. The accuracy was determined as recovery of spiked samples, using known amounts of the r-Apo A-IM reference as spiking material. LOD and LOQ were investigated through serial dilution of a known sample. LOD was set at a signal-to-noise ratio of 10:1. The linearity was calculated from the obtained calibration curves.

3. Results and discussion

Preliminary attempts for separation of the r-Apo A-IM containing fermentation broth material at acidic pH on the Poros matrix gave poor resolutions (data not shown).

At high pH, required performances were achieved and pH=11.0 was chosen for the method development.

3.1. Qualitative evaluation of the method

Chromatographic profiles from reversed-phase separations of r-Apo A-IM-containing samples, at pH=11.0, are shown in Fig. 1a–c.

The fermentation sample shows several more or

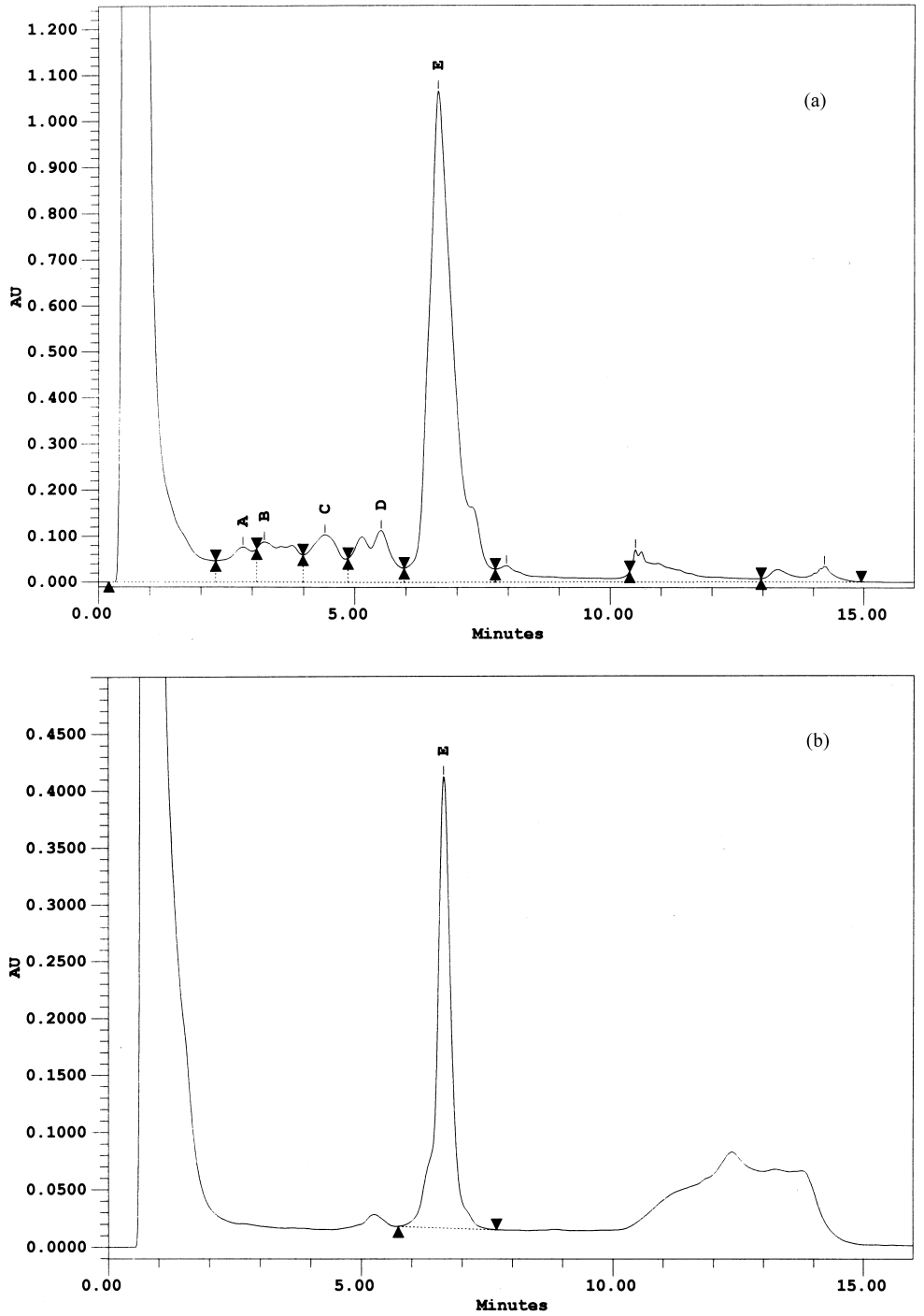

Fig. 1. Reversed-phase chromatography on Poros IIR/H of r-Apo A-IM containing samples. (a) *E. coli* fermentaion broth, (b) Semipurified material from a purification step and (c) r-Apo A-IM reference. Denotations A–E correspond to collected peak fractions (cf. Figs. 2 and 3). Chromatographic conditions are described in Section 2.

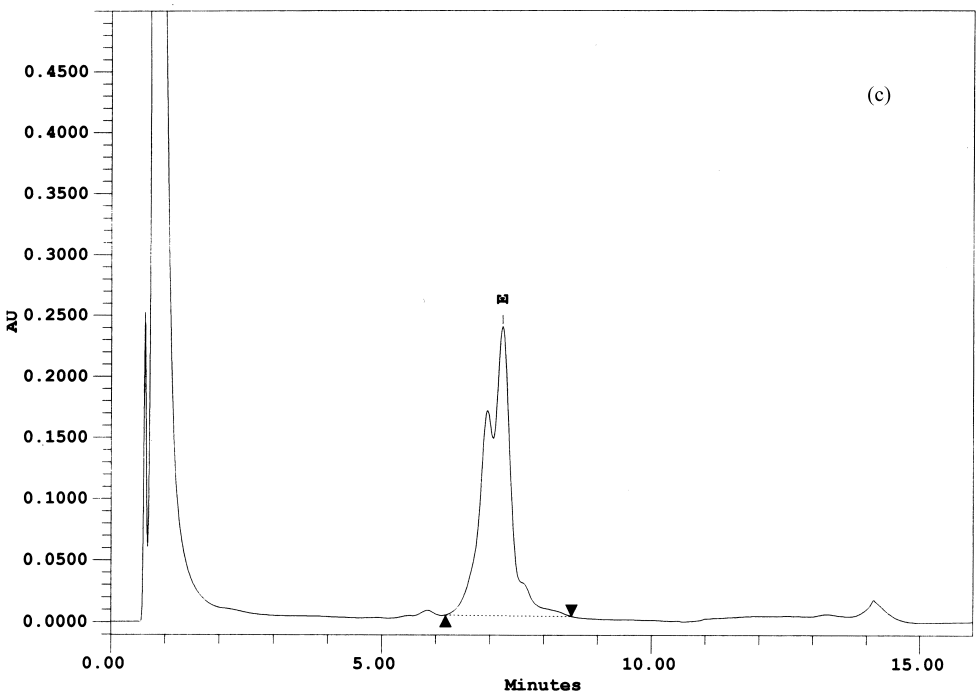

Fig. 1. (*continued*)

less resolved peaks (Fig. 1a). Analysis of material that has been partially purified reveals one major peak (Fig. 1b). A run with the r-Apo A-IM reference is shown in Fig. 1c. The retention time for peak E (Fig. 1a) and the major peak in Fig. 1b were similar to the retention time of the reference material (Fig. 1c), indicating that these peaks were r-Apo A-IM-containing fractions.

For further investigation on the identity and purity of the peak-E fraction (cf. Fig. 1a) fermentation broth material was fractionated and the collected fractions were further analyzed. SDS-PAGE of the collected fractions (Fig. 2) shows that the peak-E material contains two main bands (Fig. 2, lane E). These two bands migrate corresponding to an apparent molecular mass of roughly 30 000. The stained band patterns of other fractions (lanes A–D in Fig. 2, cf. Fig. 1a) contain bands of similar apparent molecular masses. The molecular mass of monomeric r-Apo A-IM is, according to sequence data [21], about 28 000.

To verify if all these bands corresponded to r-Apo A-IM, the SDS–PAGE pattern was subjected to western blotting using r-Apo A-IM specific monoclonal antibody (Fig. 3, cf. Fig. 1a and Fig. 2). Immunointeractions were found with material in the

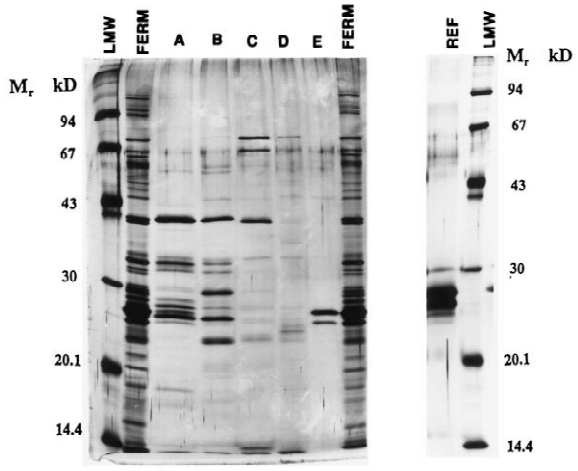

Fig. 2. SDS–PAGE of collected chromatographic peak fractions A–E (cf. Fig. 1a). Lanes A–E: correspond to peak fractions A–E in Fig. 1a. Lanes FERM: fermentation broth material. Lane REF: r-Apo A-IM reference. Lanes LMW: low-molecular-mass markers. kD=‘kilodalton’.

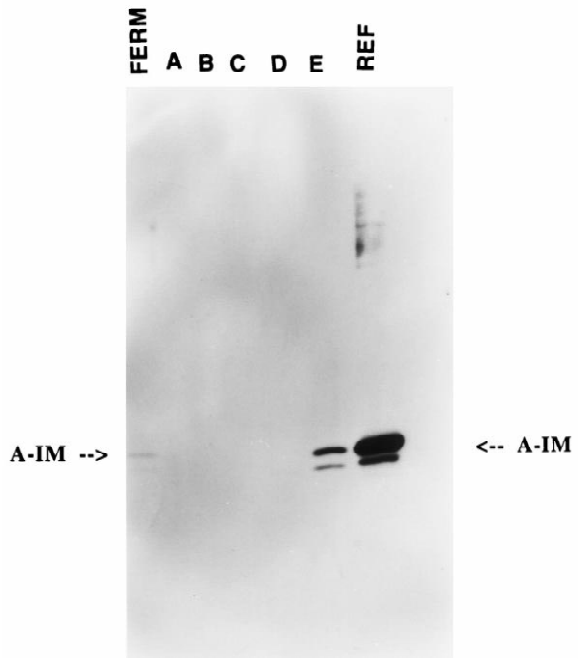

Fig. 3. Western Blot of SDS–PAGE (Fig. 2) separated chromatographic peak fractions A–E (cf. Fig. 1a) using a r-Apo A-IM-specific monoclonal antibody for detection. Lanes A–E: correspond to the lanes A–E in Fig. 2 (cf. peaks A–E in Fig. 1a). Lane FERM: fermentation broth material. Lane REF: r-Apo A-IM reference. The position of r-Apo A-IM is indicated by the arrows.

unfractionated fermentation sample (Fig. 3, lane FERM) and in the peak-E fraction material (lane E, Fig. 3). The immunostained bands in these materials appeared at similar positions as for the reference (lane REF, Fig. 3). The obtained patterns for silver-stained and immunostained bands in the peak-E fraction and the reference are very similar (compare lanes E and REF in Fig. 2 with the corresponding lanes in Fig. 3). In both analyses, trace amounts of aggregated r-Apo A-IM material are seen in the reference (lanes REF in Figs. 2 and 3, respectively). This is most likely due to incomplete reduction of the sample before electrophoresis or due to aggregation during analysis.

No interactions are seen with materials in the other chromatography fractions (lanes A–D in Figs. 2 and 3, respectively. (cf. Fig. 1a).

Thus the material in peak-E is pure r-Apo A-IM although there seems to be two variants of the r-Apo A-IM molecule. Indeed, analyses with HPLC and mass spectrometry (data not shown) indicate that the lower band corresponds to a truncated form of r-Apo A-IM whereas the main band is the full-length form.

The reason for the splitted pattern of peak-E in the reference (Fig. 1c) has not been fully investigated. However, as seen in Fig. 1a and b, peak-E in the process samples are not splitted. Peak-E material, collected from both the fermentation sample and the reference, contain full-length as well as truncated r-Apo A-IM (see lanes E and REF in Figs. 2 and 3, respectively). Thus the splitted peak pattern of the reference is probably not due to a separation between full-length and truncated r-Apo A-IM but might be caused by various chemical or conformational modifications of the protein.

The main aim of the method development was to determine the total amount of r-Apo A-IM.

3.2. Quantitative evaluation of the method

A summary of the performance characteristics of this reversed-phase method is given in Table 1.

Recovery of spiked samples showed an accuracy value of 92% ($n=8$).

The calibration curve showed a linearity of $r^2=0.99$ over a range of 2 to 70 μg r-Apo A-IM on column. This corresponds to a concentration range of

Table 1
Performance characteristics for separation of r-Apo A-IM-containing fermentation broth by reversed-phase chromatography, at pH=11.0, on the Poros IIR/H column. Conditions are described in Section 2

Accuracy (recovery of spiked sample)	LOD (ng protein)	LOQ (μg protein)	System precision (R.S.D.% of area response)	Method precision (R.S.D.% of area response)
92% ($n=8$)	40	2	1.3 ($n=10$)	3.5 ($n=8$)
Linearity:	$r^2=0.999$			
Range:	20 μg/ml–700 μg/ml corresponding to 2–70 μg on column			

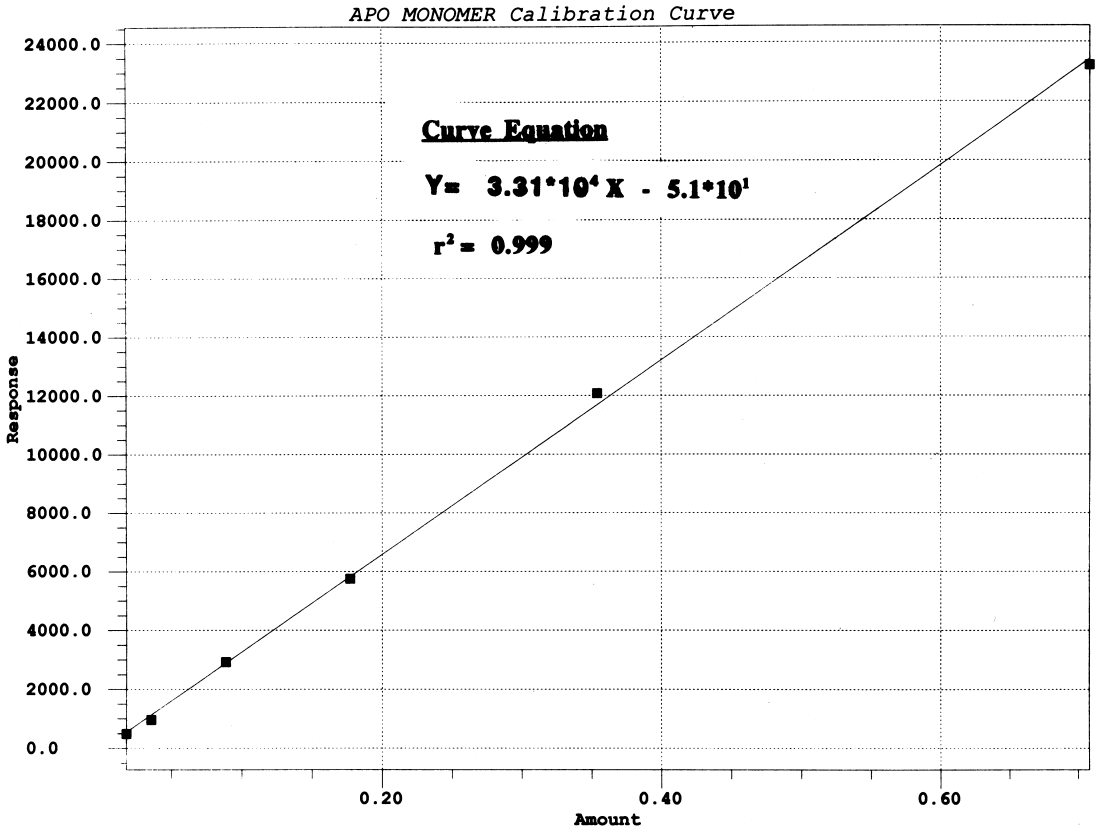

Fig. 4. Calibration curve obtained from injections of known amounts of the r-Apo A-IM reference onto the Poros IIR/H column. The curve equation is inserted in the figure.

20 to 700 μg r-Apo A-IM/ml in solution (see Fig. 4). Limit of detection and limit of quantification was found at 40 ng and 1.8 μg of r-Apo A-IM, respectively.

The system and method precision, determined by measuring the response areas for r-Apo A-IM peaks, expressed as relative standard deviation, were found to be 1.3% ($n=10$) and 3.5% ($n=8$), respectively (Table 1).

The over-all performance of this method shows that reliable determinations of the r-Apo A-IM content in complex fermentation broth are achieved.

4. Conclusions

The main advantages of the presented method, compared to other commonly used RP-HPLC methods for protein separations, are the high sample throughput and good chromatographic performance achieved by the Poros IIR/H matrix, operating at a high pH. The method is well suited for reliable determinations of the total content of r-Apo A-IM in fermentation broth as well as in r-Apo A-IM-containing samples in down-stream purification processes. The method might be useful for analytical preparation for further qualitative analyses of the r-Apo A-IM molecule. It has, however, to be considered that the high pH has a risk for deamidation. A qualitative analysis might then be inaccurate. A thorough investigation addressing this issue has to be done before interpretation of such data.

The Poros IIR/H matrix is stable over a broad pH range, so useful adaptation of the presented method for rapid and reliable determinations of specific protein species in complex solutions seems likely.

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