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Journal of Chromatography B, 731 (1999) 199–205

JOURNAL OF CHROMATOGRAPHY B

Automated isolation of high-purity plasma albumin for isotope ratio measurements

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Received 6 January 1999; received in revised form 9 April 1999; accepted 11 May 1999

Abstract

Measurement of the incorporation of labeled amino acids in plasma albumin, isolated from plasma sampled at different time points after infusion start is a well-known technique to study human albumin synthesis. Unfortunately, no chromatographic method has been described yet, enabling the automated isolation of high-purity albumin from large numbers of plasma samples as is required to study the kinetics of this process. Therefore, we developed a fast protein liquid chromatographic method, capable of processing 200 μl amounts of plasma in 74 min (injection to injection). The system can run unattended as the FPLC system is connected to a sample processor equipped with a polyether ether ketone (PEEK) sample loop and a cooled sample tray. Albumin isolation was divided into three steps. First, plasma samples were injected onto a 1-ml Blue Sepharose HiTrap affinity column, equilibrated with 50 mmol/l phosphate buffer (pH 7.0). After elution of non-binding protein, switching the solvent to phosphate buffer with 1.5 mol/l sodium chloride eluted albumin. The resulting albumin fraction was desalted on-line by directing it through two consecutive HiTrap 5-ml desalting columns, whereafter it was retained in the system within a 5-ml PTFE loop, connected to a motor valve. After switching this valve, thus bypassing the sample loop, the phosphate buffers were changed automatically to Tris buffers. Final purification involved elution of the captured fraction over a 1-ml ion-exchange Resource Q column, using a sodium chloride gradient, ranging from 0 to 0.5 mol/l in Tris buffer (20 mmol/l, pH 7.5). A more than 99% purity of the final albumin fraction was confirmed by capillary electrophoresis.

Keywords: Isotope ratio; Albumin

1. Introduction

Hypoalbuminaemia is a feature associated with malnutrition and disease [1–4]. Although several efforts have been made to clarify its mechanism, the etiology of hypoalbuminaemia is still not completely understood. Using the decay-curve of injected [^{131}I]-labeled albumin [1,2,5], some authors found a decrease in albumin synthesis, while others have observed an increase in albumin breakdown. It has been assumed that this discrepancy can be explained by the fact that this technique facilitates an indirect measurement, which is not a good indicator of the actual situation [1,3].

An alternative method approach to measure albumin synthesis is through continuous infusion of

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00222-4

labeled leucine and determination of its incorporation rate into albumin [1,6–8]. Accuracy of this method depends largely on the degree of (im-) purity of the isolated albumin fraction. Even a small contamination (a few percent) with a protein possessing a high fractional synthetic rate (FSR) results in an overestimation of the albumin FSR. Traditional methods like cold ethanol purification according to Cohn [9] result in a purity of about 96% as can be seen from the product guarantee label of chemical suppliers. The remainders are mostly globulins, which may increase drastically in the acute phase response. Alternatively, single-step chromatographic methods are employed [6,10], but these also cannot ensure the desired high degree of purity [10,11]. In contrast, multi-step methods, capable of producing the desired purity, usually require a laborious and time costly re-introduction of collected fractions into the next chromatographic step [11,12]. Considering the large number of samples required to reach statistical significance it may not be a surprise that thus less complicated isolation methods [13] are favorably used to measure albumin synthesis rates, which are more sensitive to protein contamination, especially in the acute phase response. This may explain (part of the) discrepancies in albumin synthesis rates described in the literature.

The aim of this study was therefore to develop a fast, automated, low cost, multi-step chromatographic method for the isolation of high-purity plasma albumin.

This was achieved using a fast protein liquid chromatography (FPLC) method based on two consecutive chromatographic principles, affinity chromatography and ion-exchange chromatography. The method enables the purification of mg amounts of high-purity plasma albumin from a large number of samples. The fraction's purity was certified by capillary electrophoresis (CE).

2. Experimental

2.1. Equipment

Our FPLC system consisted of a Model LCC 501 controller, two Model P1 pumps, two Model MV8 motor valves, a Model MV7 motor valve, a Model UVM II UV detector, equipped with a 5 mm analytical flow cell and a Model Superfrac fraction collector, all from Pharmacia Biotech (Roosendaal, The Netherlands). Three PTFE three-way low-pressure solenoid valves (Cole Parmer, Applikon, Schiedam, The Netherlands) were included in the system to select the solvents and to divide the flow to the fraction collector. The FPLC separation program (Table 1) was written on a personal computer running FPLC-manager.

Chromatographic data were processed on a separate computer running Turbochrom data system (version 4.12; Perkin-Elmer, Gouda, The Netherlands). Samples were introduced using a Model 233 XL Gilson sample processor (Meyvis, Bergen op Zoom, The Netherlands), equipped with a 200 μl polyether ether ketone (PEEK) sample loop and cooled (4°C) sample trays.

2.2. Solvents and columns

Two solvent systems were used. The first system contained phosphate buffer and was used to trap and elute albumin from the Blue Sepharose affinity column. Buffer A was 50 mmol/l phosphate buffer, pH 7.0 and buffer C was phosphate buffer with 1.5 mol/l sodium chloride. The second solvent system contained Tris buffer and was used for further purification on the ion-exchange column. Buffer B was 20 mmol/l Tris buffer, pH 7.5 and buffer D was Tris buffer with 0.5 mol/l sodium chloride.

Three different columns were used to isolate the albumin fraction. The first column was a 1-ml HiTrap Blue column. Next, two 5-ml HiTrap desalting columns were used in series to remove the buffer salts from the first purification step. The last column was a 1-ml Resource Q ion-exchange column (all from Pharmacia Biotech).

2.3. Samples and standards

Heparinized blood samples from human volunteers, submitted for the resection of a tumor of the large bowel, were obtained (after informed consent) by venapuncture and collected on ice. Samples were collected after an 8-h period of [1-^{13}C]leucine infusion before and after surgery. Plasma was prepared immediately by 10 min centrifugation at 8500 *g* at

Table 1
FPLC program

Time (min)	Setting	Code	Time (min)	Setting	Code
0	Conc% B	0	22.1	Valve.pos	3.1
0	Port.set	0.0	28	Conc% B	35
0	Port.set	3.0	28.5	Port.set	6.1
0	Port.set	4.0	28.5	Feed tube	
0	Valve.pos	1.1	28.5	Port.set	0.1
0	Valve.pos	2.2	31	Conc% B	35
0	Valve.pos	3.1	32	Feed tube	
0.1	ml/min	1	32	Port.set	5.1
5	Conc% B	0	32	Port.set	0.0
5.1	Conc% B	100	37	Conc% B	100
5.1	Valve.pos	1.2	40	Conc% B	100
7	Valve.pos	1.1	40.1	Conc% B	0
9	Valve.pos	2.1	50	Valve.pos	1.1
16	Conc% B	100	60	Port.set	3.0
16.1	Port.set	3.1	60	Port.set	4.0
16.1	Port.set	4.1	60	Valve.pos	1.2
16.1	Conc% B	0	60.1	Wash A.B	1.1
16.2	Wash A.B	1.1	60.2	Valve.pos	2.2
16.3	Valve.pos	3.3	60.3	Valve.pos	1.1
17	Valve.pos	1.3	72	Port.set	0.1
22	Conc% B	0	74	ml/min	0

4°C and stored at −80°C until analysis. Before analysis samples were centrifuged again for 5 min.

Albumin standards were prepared weekly by dissolving weighed amounts (Sigma, Zwijndrecht, The Netherlands) into 50 mmol/l phosphate buffer (pH 7) and stored at 4°C until analysis.

2.4. Procedure

2.4.1. Isolation

First, 200 μl plasma was injected into the FPLC system, equilibrated with phosphate buffer (A). Non-binding protein was eluted to waste by switching a Model MV8 motor valve. Next, the buffer was switched to phosphate buffer with 1.5 mol/l sodium chloride (C). Solvent lines were flushed with the new buffer, whereafter the motor valve was switched and albumin was eluted from the Blue Sepharose column. The passing albumin fraction was directed through the desalting columns and stored temporarily into a 5-ml PTFE loop, mounted to a Model MV7 motor injection valve. Solvents were changed to Tris buffers (B, D) and a motor valve Model MV8 mounted prior to the Blue Sepharose column was switched to bypass this column and the desalting columns. Next, the desalted fraction was eluted in back-flush from the sample loop onto the ion-exchange column, which was equilibrated with 20 mmol/l Tris buffer, pH 7.5. Albumin was then eluted using a gradient between buffer (B) and (D). The purified albumin fractions were collected in 6-ml glass scintillation vials.

2.4.2. Determination of the purity

Purity was confirmed by CE, using a Beckman Model P/ACE 5500 system, equipped with an unfused-silica capillary of 27 cm (effective length of 20 cm)×50 μm I.D. The system was operated with 100 mmol/l borate buffer, pH 10.2 and set to a detection wavelength of 200 nm. Samples were introduced using a pressure injection (34.5 kPa), after which a 10 kV voltage was applied for 5 min.

3. Results and discussion

The determination of albumin synthesis rate under conditions of metabolic stress has been subject for a number of investigations [1–6,17,18]. The results of these studies are not always conclusive, sometimes

even contradictory. It has been suggested that these differences in the results may be explained by the design of the study (direct or indirect measurement), but also by the analytical method used to isolate albumin. It is known that albumin purified by the commonly used ethanol fractionation according to Cohn, still contains impurities [10,11]. In contrast, sedimentation of total plasma proteins, followed by dissolution of albumin in acidified ethanol has been claimed to produce pure albumin [13]. Combined with its ease of use, the latter method has become popular in isotope enrichment studies [1,7,8]. However, it cannot be excluded that this single-step approach completely prohibits contamination of proteins, which are elevated during the acute phase response. Furthermore, it has been described that conformational changes resulting from pathology and polymerization influence the ability to re-dissolve in ethanol [11]. Recovery percentages will thus be lower and it is not known what the consequences are considering the determination of turnover rates.

In contrast to this approach, a chromatographic isolation is a much better guarantee of a pure fraction. A single-step chromatographic method has already been described for this purpose [2], but this still does not exclude significant contaminations [10,11]. A multi-step chromatographic procedure is more likely to exclude a high degree of these remaining impurities, but usually these procedures are laborious and have a long cycle time (6 to 8 h), which therefore do not facilitate efficient processing of studies with large numbers of samples.

In the present method, first isolation step involved affinity chromatography using a Cibacron Blue column, thus enabling the highest clean-up in one step. It was our goal to isolate an amount of 10 mg albumin. Normal human plasma albumin concentration ranges from 40 to 60 g/l. We therefore expected that the injection and separation of a 200 μl-plasma portion best could be performed on a 1-ml Blue Sepharose HiTrap affinity column, possessing a binding capacity of about 40 mg (according to the manufacturer). The first eluting peak consists of non-binding protein eluted with the 50 mmol/l phosphate buffer, while the second peak mainly consists of albumin (Fig. 1). Although it was recommended to dilute the sample into the starting buffer (for a better recovery), we found that a direct injection was also adequate. In this way, up to 100 samples could be injected onto the column, after which its back-pressure increased and the binding-capacity decreased (notable by a shift in response of a standard from the second peak to the first peak). The albumin fraction, eluting from the column in a high-salt phosphate buffer, still contained impurities.

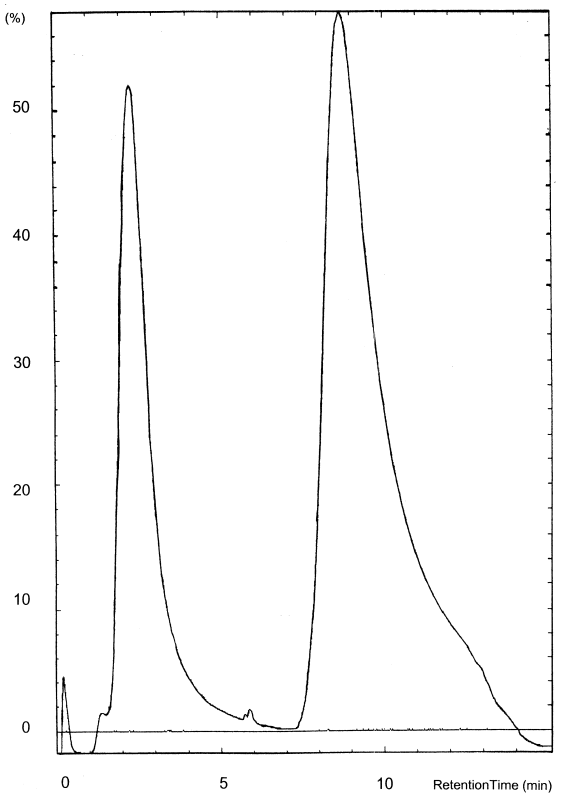

Fig. 1. Separation of plasma proteins on a Blue Sepharose HiTrap column.

To remove these impurities, gel-filtration and ion-exchange chromatography were considered. Because gel-filtration is known to require long run-times, we chose to use ion-exchange chromatography. This choice induced another problem. The albumin-containing fraction eluting from the affinity chromatography contains high salt concentrations of the collected fractions, which prohibit the efficient application of ion-exchange chromatography. Direct injection of the collected fraction onto the ion-exchange column would therefore result in the unretained elution of all proteins present. Therefore, a desalting

step of the obtained fractions is required. Furthermore, best resolution on an ion-exchange column is obtained using Tris-based buffers. A change in the buffer system is thus required also.

To automate this step, two consecutive HiTrap 5-ml desalting columns were incorporated into the FPLC system, mounted in series with a 5-ml PTFE loop, attached to a motor driven (Model MV-7) injection valve. This setup prohibited the subjection of the (disposable) desalting columns to a too high back-pressure (above 5 bar). Furthermore, it enabled the on-line storage of the albumin fraction, while the buffer system was changed to Tris buffers and the application of a sharp salt gradient to the ion-exchange column at a relative high flow-rate (1 ml/min). The desalting columns lasted for about 100 injections after which they were replaced.

To reduce costs, the final purification involved separation on a 1-ml Resource Q column, rather than using the four-times more expensive 1-ml Mono Q column. The performance of both columns was nearly identical, while the back-pressure of the Resource Q column is only a quarter of the Mono Q column (not shown). The resulting separation allowed the isolation of a high purity albumin fraction (Fig. 2), which are collected into 6-ml glass scintillation vials.

The next problem was how to establish the purity of the isolated albumin fraction. Most biochemical methods are designed to identify proteins on the bases of an activity test. Recovery percentages of each purification step are also based on these tests. Our problem was however to determine the amount of impurities in the isolated fraction, as especially contamination with quickly turning proteins could change the results of the enrichment measurement. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was found unsuitable (not shown) as a large amount of sample must be applied to visualize any contamination, which in turn goes at the cost of resolution. The resulting albumin spot could thus easily hide remaining other proteins [10,11]. Alternatively, CE was considered, a fast and sensitive method, requiring only small amounts of sample. Using this technique, we could not detect any contaminants in the isolated fractions (Fig. 3).

After the methodological setup was now completed, performing two pilot studies tested the validity of the described technique. In the first experiment, a primed continuous infusion of L-[4,5-^{3}H]leucine was given to a conscious laboratory pig, operated previously in conformity to our standard protocol as is described elsewhere [14]. At $T=0$, 1, 2, 3, 4, 5 and 6 h arterial plasma samples were collected on ice. Plasma free and albumin bound leucine specific activity was determined as is described elsewhere [15]. Mean plasma free leucine specific activity was 14 030 desintegrations per minute (dpm)/μmol. A mean amount of 11.1 mg purified albumin was isolated from 200 μl plasma portions, from which a mean amount of 3.15 μmol leucine was isolated after hydrolysis. In these samples, a linear increase ($R^2=0.99$) in activity was found (Table 2), from which the FSR was calculated as 7.7%/day.

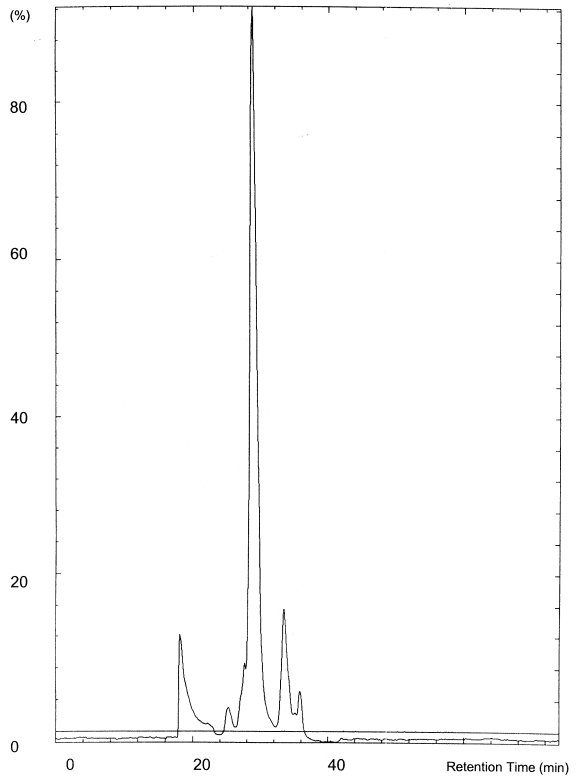

Fig. 2. Ion-exchange separation of the Blue Sepharose fraction (highest peak is albumin; separation conditions as in text).

In the second pilot experiment, a group of five postabsorptive patients, submitted for gastrointestinal diseases, received a primed continuous

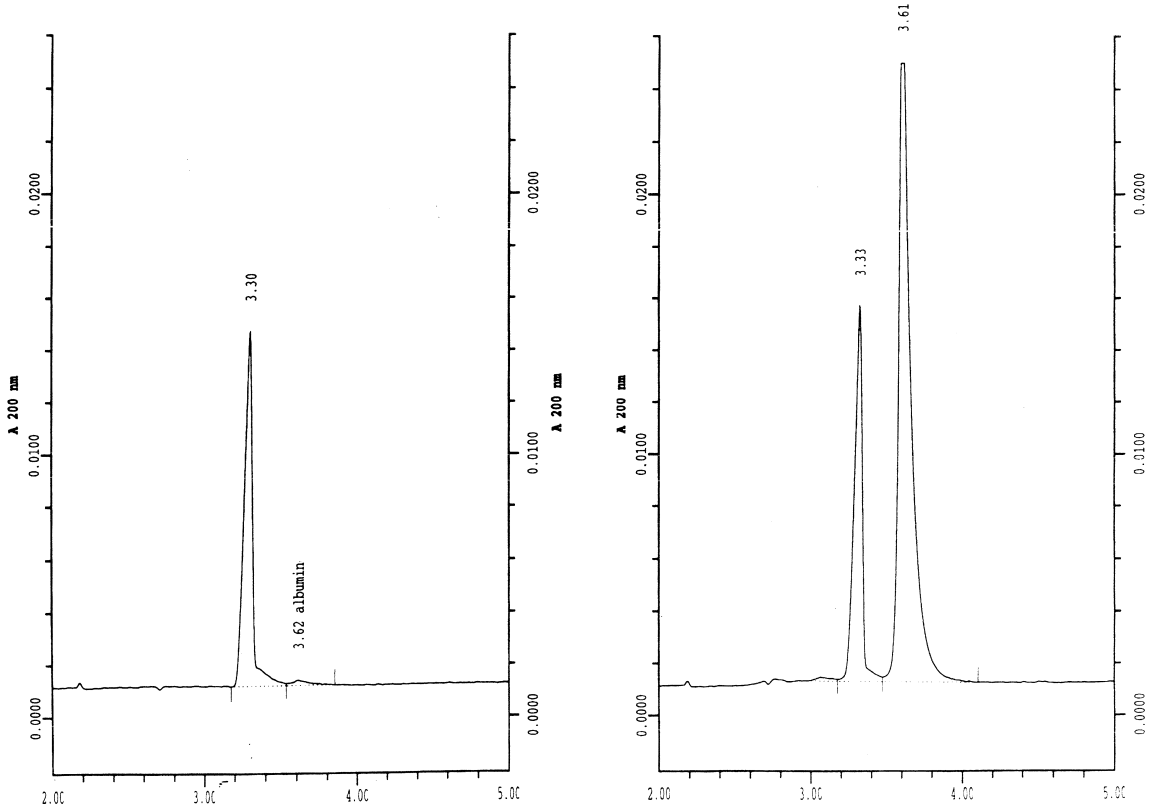

Fig. 3. Purity check of the final albumin fraction by capillary electrophoresis (upper panel: blank Tris buffer; lower panel: purified albumin fraction).

[1-^{13}C]leucine infusion for 8 h after their informed consent was obtained. Plasma samples were ice-chilled immediately and collected in time. Albumin fractions were isolated, hydrolyzed and leucine enrichment was determined on a gas chromatograph combustion isotope ratio mass spectrometer Model MAT 252 (Thermoquest, Veenendaal, The Netherlands) as their *tert*.-butyldimethylsilyl (TBDMS) derivatives as is described elsewhere [16]. Again, a linear increase in enrichment (expressed as the tracer tracee ratio: TTR) was found (R^2=0.99). Mean plasma precursorpool enrichment (KIC) was 10.2% TTR (standard error of the mean; S.E.M.=0.5% TTR), resulting in an albumin synthesis rate (FSR) of 8.8%/day±1.1% (mean±S.E.M.; Table 2), which are well within the range of recent literature [17,18].

Table 2
Determination of albumin synthesis rate

Pig pilot (n=1)		Patient pilot (n=5)	
Time (h)	Increase in activity (DPM)	Time (h)	Increase in enrichment (TTR, %)
1	81	2	0.052 (±0.009)
2	197	3	0.084 (±0.014)
3	329	4	0.120 (±0.021)
4	457	6	0.192 (±0.033)
5	594	8	0.276 (±0.046)
6	726		
FSR	7.7%/day	FSR	8.8%/day

In conclusion, in contrast to the traditional methods, the here described method enables the *automated* isolation of high-purity albumin for isotope incorporation studies. Furthermore, the multi-step character of the present method minimizes the risk of isolating an albumin fraction, contaminated with proteins with a high turnover rate, which would result in an overestimation of the FSR. In traditional

single-step methods, the risk increases when studying patient populations with an acute phase response, thus possessing elevated concentrations of potential interfering proteins.

Future studies must distinguish if, and to what extent differences in the albumin purification method may explain the described differences in albumin synthesis rate.

Acknowledgements

The authors wish to thank Mr. A.J.M. Wagenmakers for the mass spectroscopy enrichment measurements, Mr. B. Van Kreel and Mrs. Wijnen for performing the capillary electrophoresis analysis.

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