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Determination of albumin and myoglobin in dialysate and ultrafiltrate samples by high-performance size-exclusion chromatography

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Abstract

A high-performance size-exclusion chromatographic method was developed, validated and implemented for simultaneous and quantitative determination of albumin and myoglobin along with inulin, vancomycin and creatinine in dialysate and ultrafiltrate samples from in vitro hemodialysis experiments. The experimental parameters including mobile phase pH, ionic strength, detection wavelength, flow-rate, injection volume were first optimized for the determination of albumin, myoglobin, inulin, vancomycin and creatinine. The peak height ratio and detection limits of the proteins were then comparatively studied at 210, 254 and 280 nm by UV and diode array detection. The method was further validated by evaluating the linearity, precision and accuracy of the proteins. The assay was finally implemented to the simultaneous and quantitative determination of the proteins in dialysate and ultrafiltrate samples.

Keywords: Proteins; Albumin; Myoglobin

1. Introduction

Approximately 77% of all United States hemodialysis centers reuse hemodialyzers and the reuse saves hundreds of millions of dollars per year in the treatment of patients with end stage renal disease (ESRD). However, hemodialyzer reuse also has been associated with concerns such as the decreased removal of solutes and the changes in the removal of solutes with varying molecular mass (M_r) [1–4]. Some evidence may even suggest that the reuse is linked to an increased mortality. The overall goals of our in vitro hemodialysis experiments is to determine the effect of hemodialysis reuse on the removal of drugs/solutes over a large M_r range. The solutes that we use in our research are creatinine, vancomycin, inulin, myoglobin and albumin. Creatinine (M_r 112) provides clearance information regarding small solute in patients with ESRD. Vancomycin, (M_r 1448) commonly used in the ESRD patients, represents a clinically relevant middle M_r in the spectrum of study solutes. Inulin, (M_r 5200) often used pharmaceutically for the determination of glomerular filtration rate, represents a larger middle M_r. Myoglobin (M_r 17 000) provides information about the removal of small protein. Albumin (M_r

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66 000) is used as a marker for the removal of a large protein that represents a significant indicator of clinical status in patients with ESRD. The methods have been developed to individually quantify inulin, vancomycin or creatinine in our laboratory [1,5] but the method for the simultaneous and quantitative determination of albumin and myoglobin has not been previously reported.

Size-exclusion chromatography (SEC) has widely been used for the separation and purification of proteins [6–8]. SEC is an entropically controlled separation technique that depends on the relative size of a macromolecule with respect to the size and shape of the pores of the packing. High-performance SEC (HP-SEC) columns are favored because of their speed, high resolution and high sensitivity. Silica-based packing seems to be preferred for quality control and quality assurance because of its higher efficiency, shorter analysis time, and more robust nature. However, the columns exhibit some unwanted solute–packing interaction under certain conditions [9–11]. HP-SEC has been developed as a useful tool for the analysis of biomolecules of clinical importance [12]. Flapper et al. compared several commercially available HP-SEC columns for the separation of serum proteins [13]. SEC assays for the analysis of specific binding proteins present in tissue and fluids have been described [14]. SEC has also been used for the quality control of immunoglobins for the therapeutic use [15–17]. Ricker and Sandoval have demonstrated a fast, reproducible SEC of biological macromolecules [18]. Qiu et al. has described identification of myoglobin in human smooth muscle [19]. Nevertheless, the method for the simultaneous and quantitative determination of albumin and myoglobin in a biological matrix has not been reported although there have been several publications related to the qualitative separation of albumin and myoglobin in a standard aqueous solution [9–11,18].

The major aim of this present study was to develop, validate and implement a rapid, reliable, accurate and efficient method for simultaneous and quantitative determination of albumin and myoglobin in dialysate and ultrafiltrate samples from in vitro hemodialysis experiments. In addition, we explored the possibility of separating, identifying and quantifying inulin, vancomycin and creatinine along with the simultaneous determination of two proteins in a single run.

2. Experimental

2.1. Apparatus

The optimization and partial validation of the method were performed on Shimadzu high-performance liquid chromatography (HPLC) equipment (Shimadzu, Columbia, MD, USA), which comprised a liquid chromatograph (LC-10 ADVP), a system controller (SCL-10AVP), a diode array detection (DAD) system (SPD-M 10AVP) and an automatic injector (SIL-10ADVP). The subsequent validation and application of the method were performed on Beckman HPLC equipment, consisting of a 118 solvent module, a 166 UV detector and a 507e autosampler (Beckman Instrument, Fullerton, CA, USA). Data and chromatograms were collected by Class-VP software from Shimadzu system and by Gold Nouveau software from the Beckman system. Data were analyzed with Class-VP and Gold Nouveau software and Microsoft Excel 2000.

2.2. Chromatography

Analytical separation and guard columns were from Hewlett-Packard Zorbax Bio Series columns (Agilent Technologies, Wilmington, DE, USA). The separation column GF-250 (serial No. Lx12464, Lx12495, part No. 884973901) was 250 mm×9.4 mm I.D. packed with spherical silica (size 4–4.5 μm, pore diameter 150 Å, surface area 140 m^2/g) bonded with a hydrophilic molecular monolayer (diol-type). The guard column cartridge (serial No. USGS002525, part No. 820950-911) was 12.5 mm× 4.6 mm I.D. with 5 μm Zorbax packing of identical chemistry to the separation column. Between consecutive analyses, a needle for the automated injector was washed with 70% aqueous methanol. All experiments were carried out at ambient temperature at approximately 23°C.

Between the runs, the injection needle was washed with 70% methanol. To protect the column and increase its life, guard columns were used and changed after every 100 to 200 injections. The

column was flushed with 0.1 *M* phosphate at pH 6.5, containing 0.005% NaN_3 as an anti-microbial agent daily after each batch of experiments. The next day the column was equilibrated with new mobile phase for 60 min before analysis. When the column efficiency decreased, the column was washed with 50% acetonitrile containing 0.05% trifluoroacetic acid for 2 h to regenerate the column. When the column became plugged, the blockage was cleared by back flushing the column or replacing the column inlet frit.

2.3. Chemicals

All chemicals were of analytical-reagent grade: potassium chloride, sodium phosphate monobasic dihydrate ($NaH_2PO_4 \cdot 2H_2O$) from J.T. Baker (Phillipsburg, NJ, USA); sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$), sodium azide and methanol from EM Science (Gibbstown, NJ, USA); sodium chloride from VWR Scientific Products (West Chester, PA, USA); bovine albumin fraction V (purity≥98%) from ICN Biomedicals (Aurora, OH, USA); horse heart myoglobin (purity≥95%), creatinine (purity≥99%) and inulin (purity≥99%) from Sigma (St. Louis, MO, USA), and vancomycin (purity≥99%) from Abbott Labs. (Chicago, IL, USA). Water used for dilution and mobile phase solutions was purified by reverse osmosis and further filtered through a 0.45-μm membrane filter from Schleicher & Schuell (Keene, NH, USA).

2.4. Preparation of various solutions

2.4.1. Mobile phase

The stock solutions of 0.5 *M* $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 7H_2O$, 1 *M* NaCl and KCl were first prepared and stored in a refrigerator and discarded after 1 month. The buffer solutions containing 70 m*M* sodium phosphate, 50 m*M* NaCl and 40 m*M* KCl were prepared from the stock solutions and the pH adjusted to 6.0, 6.5, 7.0, 7.5 and 8.0. Moreover, the solutions containing 25, 50, 70, 100, 150, 200, 250 or 300 m*M* phosphate with 50 m*M* NaCl and 40 m*M* KCl; 50, 100, 150, 200, 250 or 300 m*M* NaCl with 70 m*M* phosphate and 40 m*M* KCl; 0, 40, 100, 200, 300, 400 or 500 m*M* KCl with 70 m*M* phosphate and 50 m*M* NaCl were prepared and adjusted each to pH 6.5 to produce solutions containing different concentrations of phosphate, NaCl and KCl, respectively. The final optimal mobile phase was composed of 70 m*M* phosphate, 50 m*M* NaCl and 40 m*M* KCl at pH 6.5. The storage solution for the columns was 0.1 *M* phosphate at pH 6.5, containing 0.005% NaN_3 as an anti-microbial agent. The above mobile phase and storage solutions were filtered through a 0.45-μm membrane filter and degassed by an ultrasonic bath for 10 min before use.

2.4.2. Simulated biological matrix

Artificial kidney machine hemodialysis concentrate solution from Minntech (Minneapolis, MN, USA) was diluted with water at 1:34 and then filtered through a 0.45-μm membrane filter and degassed by ultrasonic bath for 10 min before using. The matrix was used as simulated serum during in vitro dialysis experiments [1,4].

2.4.3. Calibration standards and quality control samples

The calibration standards and quality control samples were freshly prepared on every day of analysis. Stock solutions of 0.6 and 1.0 μg/μl of myoglobin, 3.0 and 8.0 μg/μl of albumin were prepared in the biological matrix. Six albumin (0.125–4.0 μg/μl) and six myoglobin (0.0156–0.5 μg/μl) calibrators composing the standards curve and three albumin (0.38, 0.77, 1.5 μg/μl) and three myoglobin (0.05, 0.15, 0.30 μg/μl) quality control samples were prepared by making serial dilutions from stock solutions and spiking them into the biological matrix.

2.5. Assay validation

2.5.1. Linearity of standard curve

In our experiment, peak area was linearly proportional to analyte concentration via standardization procedures. This relationship was then used to convert a sample's peak area to its apparent analyte concentration. The linear range is the interval between the lower and upper analyte concentration in dialysate and ultrafiltrate samples. The lowest and highest concentrations of albumin and myoglobin in samples from hemodialyzers were predicted based on previous in vitro hemodialysis experiments and pilot

experiments. The concentration range of them was established from 80% of the lowest predicted level to 120% of the highest predicted level.

Four standard curves were prepared on four separate days. The curves were fit by a least-squares linear regression method. For each curve, the slope, intercept, determination coefficient, standard error, *F* statistic, degrees of freedom, regression sum of squares and residual sum of squares were calculated.

2.5.2. Precision

For the intra-assay, six replicates of each low, middle and high concentration standards were analyzed. The mean, standard deviation, relative standard deviation (RSD) and accuracy for each concentration were calculated. For the inter-day assay, six replicates of each low, middle and high concentration standards were analyzed daily for five days. The mean, standard deviation, RSD and accuracy of total 30 samples for each concentration were calculated.

2.5.3. Accuracy

The accuracy of mean predicted concentration compared with nominal concentration was calculated as:

$$\text{Accuracy (\%)} = 100 \times \{1 - [(\text{predicted concentration} - \text{nominal concentration})/\text{nominal concentration}]\}$$

which should be between 90% to 110%.

$$\text{Accuracy deviation (\%)} = 100 \times [(\text{predicted concentration} - \text{nominal concentration})/\text{nominal concentration}]$$

which should be $\leq \pm 10\%$.

2.6. Analysis of albumin and myoglobin in dialysate and ultrafiltrate samples

Approximately 1000 dialysate and ultrafiltrate samples were collected in August 1999 from dialyzers used in vitro dialysis experiments after 0, 1, 5, 10, 15, and 20 reuse cycles and frozen at −70°C until assayed. Frozen samples were thawed and 200 μl of each sample was then transferred to a polypropylene HPLC sample vial. A 10-μl volume of each sample was injected into the column and the concentrations of proteins were determined based on the daily standard curve. The six concentrations of calibration standards and three quality control samples were analyzed with each analytical set of samples. Sufficient quantities of each calibration standard and quality control concentration were prepared for 50 analytical runs. These calibrator and control batches were separated into aliquots in HPLC sample vials, stored at −70°C and used in the sample analyses. Both the original and freshly prepared were analyzed concurrently to be sure that they are statistically equivalent. To ensure that the assay and chromatographic system were working properly, calibrators were placed at the beginning and end of each analytical run. Quality control samples were evenly interspersed among the clinical samples.

3. Results and discussion

3.1. Method development

3.1.1. UV spectra and identification of analytes

The UV spectra of inulin, vancomycin, creatinine and the blank biological matrix were obtained using the Shimadzu DAD system (Fig. 1A–D). Albumin

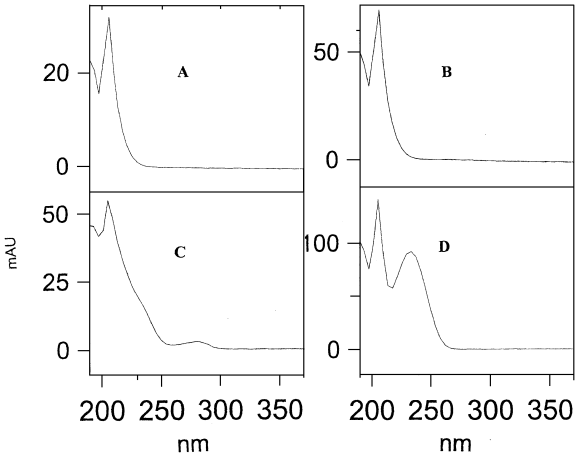

Fig. 1. Typical UV spectra obtained for the blank biological matrix and for inulin, vancomycin and creatinine in the biological matrix and in the range of 190–370 nm using Shimadzu HPLC equipment with DAD. (A) Blank biological matrix, (B) inulin, (C) vancomycin and (D) creatinine.

and myoglobin had the strong maximum at 210 nm and the weak maximum around 280 nm. The blank biological matrix had UV maximum at 210 nm but no absorbance above 230 nm (Fig. 1A). Fig. 2A shows the blank matrix peak was eluted at 6.4 min. Inulin, a polysaccharide, should not have UV absorbance at 210 nm. However, inulin dissolved in the matrix intensified the matrix peak height/area and UV absorbance (Fig. 1C and D and Fig. 2A and B) while individual albumin, myoglobin, vancomycin and creatinine in the exactly same matrix did not. Initially, we thought probably because there was some impurity in inulin and the impurity had the same UV spectrum as the matrix. However, the hypothesis was denied because we did not find the impurity. Alternatively another possible explanation is that inulin interacted with some components in the matrix. The matrix, an artificial kidney machine hemodialysis solution, contains a variety of inorganic and organic components. It seems probable that inulin formed a complex with some component in the matrix and the complex had the same resident time in SEC and UV absorption as the matrix, thus intensifying the matrix peak and UV absorption. Certainly, a lot of experiments need to be done to completely explain the interaction between inulin and the matrix.

The UV spectrum of vancomycin, a peptide, was similar to those of the proteins because they belong to the same family of UV absorption. Creatinine had UV maxima at 210 and 230 nm. At 254 nm, it still had relatively strong absorbance but no absorbance above 280 nm.

In addition to the comparison of retention time,

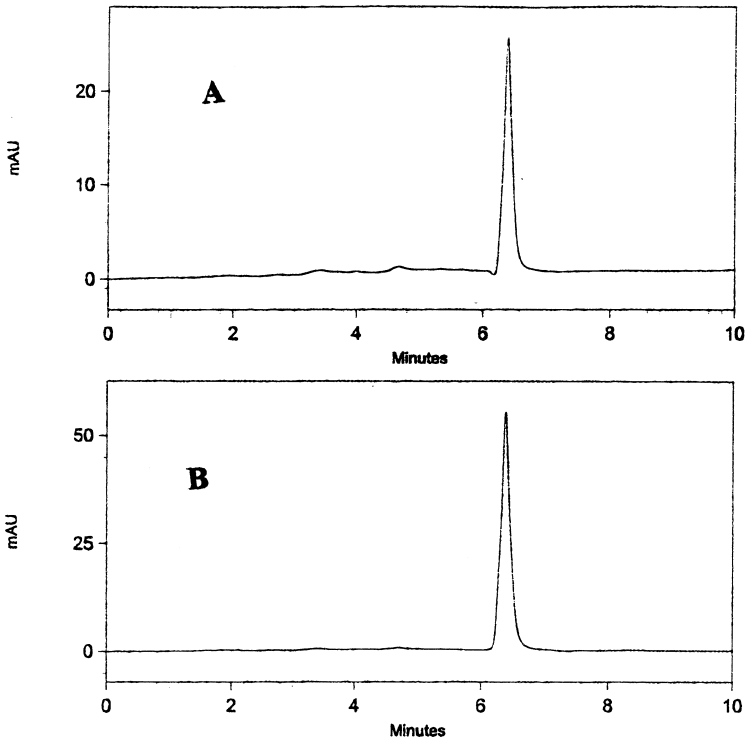

Fig. 2. Chromatograms of blank biological matrix and inulin in the matrix. Experimental conditions: the experiment was performed on Shimadzu HPLC equipment with DAD. The mobile phase was composed of 70 m*M* phosphate, 50 m*M* NaCl and 40 m*M* KCl at pH 6.5; detection wavelength at 210 nm, injection volume: 10 μl, and flow-rate 2 ml/min. The separation column was Zorbax GF-250, 250 mm×9.4 mm I.D. packed with silica (size 4–4.5 μm,) bonded with diol. The guard column cartridge was 12.5 mm×4.6 mm I.D. with 5 μm packing of identical chemistry to the separation column. Between consecutive analyses, a needle for the automated injector was washed with 70% aqueous methanol. All experiments were carried out at ambient temperature around 23°C. (A) Blank biological matrix and (B) 0.3 μg/μl of inulin in the matrix.

the UV spectra of the analytes could aid in identification of the separated peaks. As seen in Fig. 3A–C, peak 3 disappeared at 254 and 280 nm because inulin/matrix did not absorb at those wavelengths. At 254 nm peak 4 was identified as both vancomycin and creatinine (Fig. 3B) but at 280 nm only identified as vancomycin by comparison of UV spectra due to creatinine's very weak absorption at that wavelength (Fig. 3C).

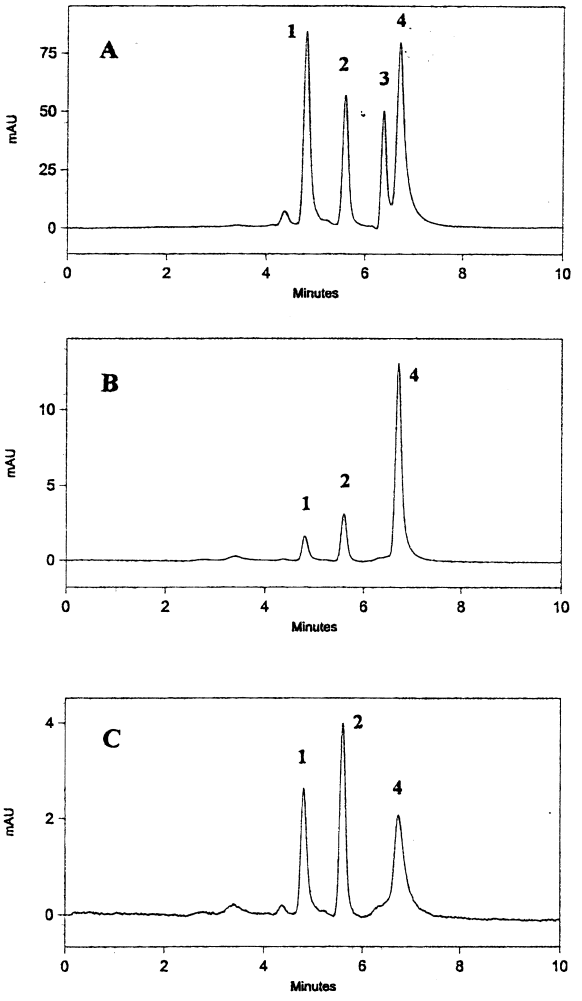

Fig. 3. Chromatograms of a mixture of five analytes in the matrix with simultaneous DAD at 210, 254 and 280 nm. Detection wavelength (A) at 210 nm, (B) at 254 nm and (C) at 280 nm. Experimental conditions as in Fig. 2. Peaks: 1=albumin (0.01 μg/μl), 2=myoglobin (0.03 μg/μl), 3=inulin (0.2 μg/μl)/matrix and 4=vancomycin/creatinine (0.01 μg/μl).

3.1.2. Effect of mobile phase pH

The column used in this study was silica-based and surfaced stabilized with zirconium to withstand operation at higher pH (up to pH 8.5). The pK_{a2} of phosphate buffer is around 7 and the most effective buffering range of a buffer is within its p$K_a \pm 1$. Thus, the effect of pH (6.0, 6.5, 7.0, 7.5 and 8.0) of the mobile phase containing 70 m*M* phosphate, 50 m*M* NaCl and 40 m*M* KCl was studied. Resolution for an adjacent pairs of analytes is defined as the distance between band peaks divided by the average bandwidth. The best resolution for the three adjacent pairs of analytes was obtained at pH 6.5 (Fig. 4). At this pH, myoglobin (isoelectric point, p*I* 7.2) bore some net positive charge and ionization of silanols to $-Si-O^-$ occurs [20] but the secondary interaction between myoglobin and $-Si-O^-$ was not observed. This is probably because the ionic strength of mobile phase was high enough to shield the charge and restore expected elution. The resolution between inulin/matrix and vancomycin or creatinine steadily decreased from pH 6.5 to 8.0, mainly because the peak width of vancomycin or creatinine increased,

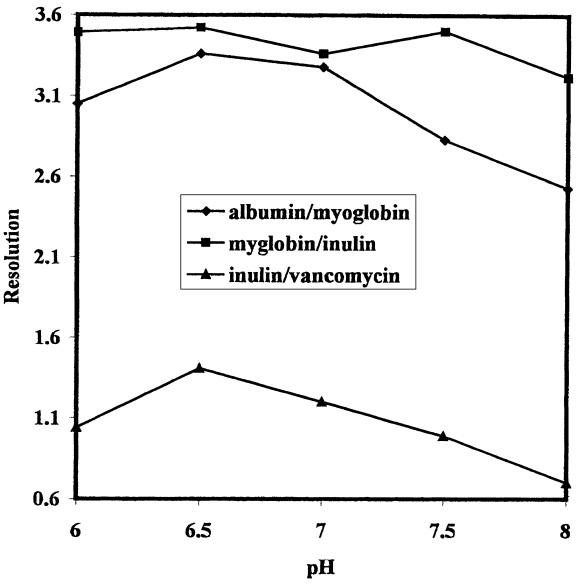

Fig. 4. Effect of pH on resolution of five analytes. Experimental conditions: pH varied from 6.0 to 8.0. Other experimental conditions as in Fig. 2. Resolution of two adjacent peaks is defined as the distance between band peaks divided by the average bandwidth.

while inulin had a relatively stable retention time and peak shape over this pH range.

3.1.3. Effect of mobile phase ionic strength

The components and ionic strength of mobile phase at pH 6.5 were further investigated, including eight sodium phosphate concentrations from 25 to 300 m*M*, six NaCl concentrations from 50 to 300 m*M*, and seven KCl concentrations from 0 to 500 m*M*. The results showed albumin and myoglobin had proper size-exclusion behavior, as indicated by a stable retention time and peak shape within the investigated range. They did not display nonideal (electrostatic or hydrophobic) interactions at either low or high ionic strength. However, the best resolution for inulin and vancomycin/creatinine was achieved by using 70 m*M* phosphate, 50 m*M* NaCl and 40 m*M* KCl (Fig. 3). Inulin and vancomycin could not be completely resolved with different pH, components and ionic strength of mobile phase, indicating that the GF-250 column is not suitable for separating the analytes between M_r 5000 and 1500. In addition, vancomycin and creatinine were always co-eluted under any conditions because the both were totally included.

3.1.4. Peak height ratio and detection limits at different wavelengths on DAD and UV detection systems

The signal intensity and detection limits of the proteins were investigated at 210, 254 and 280 nm using a Shimadzu DAD system and a Beckman UV detector (Tables 1 and 2). The peak height ratio with respect to 254 nm for the proteins on the DAD system was similar to that on the UV detector (Table 1). Consistent with their UV spectra, 210 nm produced the largest signals for the proteins on the both detectors. Albumin had smaller signal intensity at 254 nm than at 280 nm, while myoglobin had nearly the same signal intensity at 254 and 280 nm.

Large detector signals alone do not always provide high sensitivity, thus the detection limits (defined as three times the baseline noise) at the three wavelengths were further investigated. As shown in Table 2, UV detection was more sensitive than DAD for the detection of albumin and myoglobin, especially at 254 and 280 nm. With DAD, 210 nm provided the best detection limits and 254 nm was the second best. With the Beckman UV detector 210 and 254 nm provided similar detection limits for them (Table 2).

Table 1
Peak height ratio of albumin and myoglobin with respect to 254 nm at the three wavelengths of 210, 254 and 280 nm using a Shimadzu DAD system and a Beckman UV detector[a]

Protein	Peak height ratio (with respect to 254 nm)		
	Detection wavelength (nm)		
	210	254	280
Shimadzu DAD system			
Albumin	47.8	1	1.5
Myoglobin	15.0	1	1.1
Beckman UV detector			
Albumin	37.1	1	1.4
Myoglobin	14.5	1	1.1

[a] Experimental conditions: The mobile phase was composed of 70 m*M* phosphate, 50 m*M* NaCl and 40 m*M* KCl at pH 6.5 with 10 μl injection volume. The separation column was Zorbax GF-250, 250 mm×9.4 mm I.D. packed with silica (size 4–4.5 μm) bonded with diol. The guard column cartridge was 12.5 mm×4.6 mm I.D. with 5 μm packing of identical chemistry to the separation column. Between consecutive analyses, a needle for the automated injector was washed with 70% aqueous methanol. All experiments were carried out at ambient temperature approximately 23°C.

3.1.5. Selection of detection wavelength

Based on the above results, the selection of detection wavelength depended upon what equipment and detector were used. If a Shimadzu HPLC system with DAD was used and highest sensitivity

Table 2
Detection limits of albumin and myoglobin defined as three times baseline noise in ng at the three wavelengths of 210, 254 and 280 nm using a Shimadzu DAD system and a Beckman UV detector[a]

Protein	Detection limit (3×baseline noise in ng)		
	Detection wavelength (nm)		
	210	254	280
Shimadzu DAD system			
Albumin	8	155	167
Myoglobin	7	45	70
Beckman UV detector			
Albumin	5	8	15
Myoglobin	3	2	4

[a] Experimental conditions as in Table 1.

was required, 210 nm was optimal. If a Beckman HPLC system with UV detection was used, 210 or 254 nm could be chosen. The concentrations of myoglobin in many samples were from 0.03 to 0.3 μg/μl while the concentrations of albumin were up to 50 μg/μl. Although the determination of high concentrations of albumin was not our interest, its presence in samples would influence the detection of low concentrations of myoglobin. At 254 nm, the detection limit for myoglobin was four-times as sensitive as that for albumin while at 210 nm only about 1.5 times (Table 2). Therefore, 254 nm was the best detection wavelength for the determination of myoglobin in the presence of very high concentration of albumin. However, 280 nm was optimal for the detection of vancomycin because the presence of inulin, matrix and creatinine would cause less interference at the wavelength.

3.1.6. Effect of flow-rate

The effect of flow-rate at 1, 1.5 and 2 ml/min was investigated on the retention time and resolution of the analytes (Fig. 5). The resolution for albumin and myoglobin decreased with increasing the flow-rate from 1 to 2 ml/min due to their decreasing rate of diffusion into and out of pores in the packing, which conformed to a van Deemter curve. In contrast, the efficiency of vancomycin and creatinine increased at higher flow-rate, which seemed abnormal. Ricker and co-workers [11,18] got similar results when they used the same type of column to separate proteins along with sodium azide, i.e. the efficiency of sodium azide also decreased at lower flow-rate. We thought the ''abnormal effect'' was probably related to the packing, pore and particle size of GF-250 columns. On this type of column, longitudinal diffusion of small solutes became pronounced at lower flow-rate, causing their broadening peaks and lower efficiency. The contribution of longitudinal diffusion is inversely proportional to the mobile phase velocity and thus becomes more significant at lower flower rate. Relative to large solutes (proteins), vancomycin, and creatinine are small solutes and their diffusion rates are fast. Then the high diffusion rates of small solutes in the mobile phase cause the solute molecules to disperse axially while slowly migrating through the column, thus causing peak broadening and lower efficiency of the small solutes. In addition to the explanations described above, probably there are other reasons. For example, the small solutes possibly adsorbed onto the column or some sample matrix at lower flow-rate, and the adsorption was limited at higher rate.

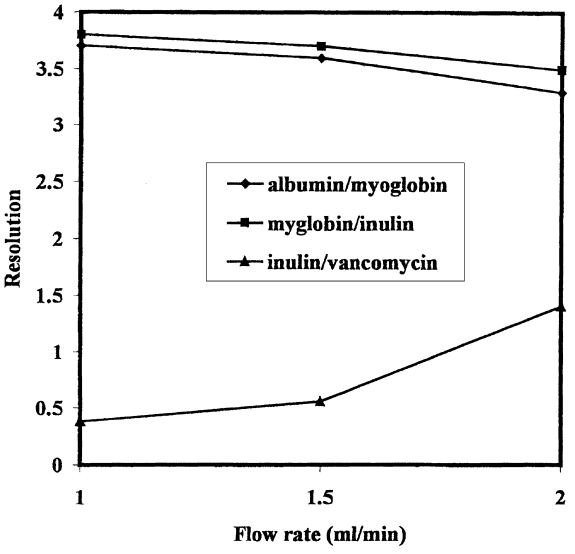

Fig. 5. Effect of flow-rate on resolution of five analytes. Experimental conditions: flow-rate changed from 1.0 to 2.0 ml/min. Other experimental conditions as in Fig. 2. Resolution of two adjacent peaks is defined as the distance between band peaks divided by the average bandwidth.

The resolution for albumin and myoglobin at 2 ml/min was sufficient to resolve them completely, so that flow-rate could be chosen to minimize run time. However, the concentrations of albumin were 100 times higher than those of myoglobin in many samples and thus keeping high resolution was needed (Fig. 6). After many sample injections, the column efficiency decreased and the peak width of albumin and myoglobin increased significantly. Under this circumstance, the flow-rate of 1.5 ml/min was chosen to keep the desired resolution for the two proteins.

3.1.7. Optimization of injection volume

To select the optimal injection volume, we prepared the standard mixture containing 80% of the lowest expected concentration of albumin and myoglobin in the samples. The protein mixture of 5, 10, 20 or 40 μl with six replicates each was injected

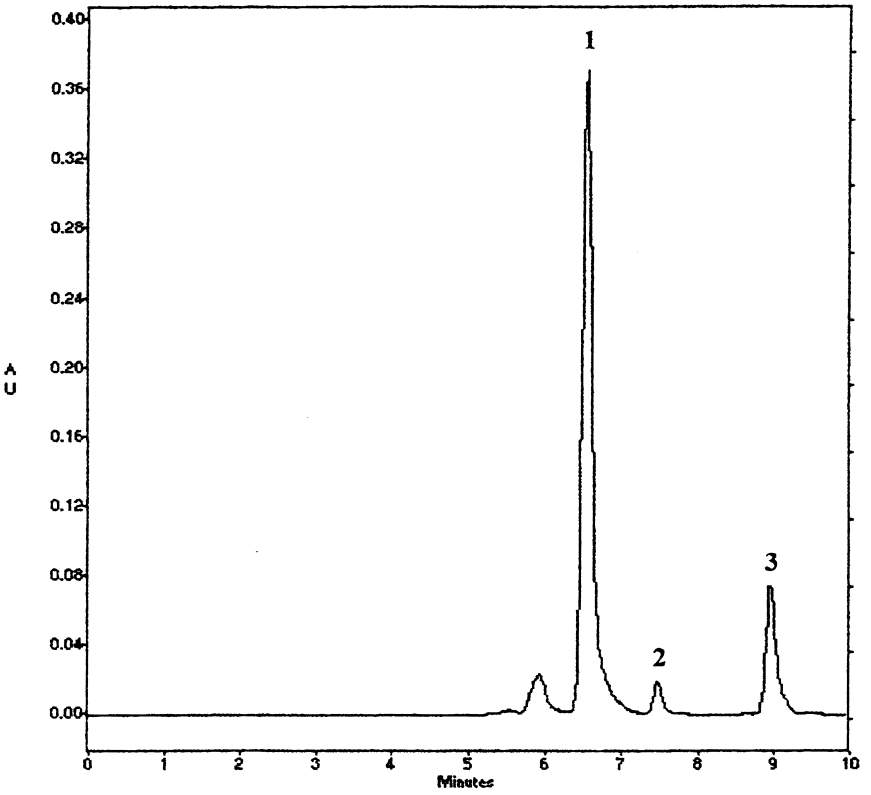

Fig. 6. A typical chromatogram of dialysate and ultrafiltrate samples. The experiment was performed on Beckman HPLC equipment with UV detection at 254 nm at a flow-rate of 1.5 ml/min with a 10-μl injection volume. Other experimental conditions as in Fig. 2. Peaks: 1=albumin (ca. 40 μg/μl); 2=myoglobin (0.317 μg/μl); 3=vancomycin/creatinine.

onto the column and the precision and accuracy were calculated (see Section 3.2). The accuracy of myoglobin was less than 90% when the injection volume was 5 μl. The injection volume of 10 μl was found to be optimal.

Based on the above results, the optimized conditions were 70 m*M* phosphate, 50 m*M* NaCl, and 40 m*M* KCl at pH 6.5 and at the detection wavelength of 210 or 254 nm with 10 μl injection volume at a flow-rate of 1.5 or 2 ml/min.

3.2. Method validation

3.2.1. Linearity

The method exhibited linearity over the 0.125–4.0 μg/μl range for albumin, and over the 0.0156–0.5 μg/μl range for myoglobin. The mean coefficient of determination R^2, *y*-intercept, and slope values of 4-day standard curves for albumin and myoglobin are shown in Table 3.

3.2.2. Precision and accuracy

Table 4 shows 4-day standard curve validation of albumin and myoglobin. Overall mean precision (RSD) ranged from 0.11 to 4.17% for albumin and from 0.00 to 4.05% for myoglobin. The accuracy of these calculations ranged from 99.60 to 103.00% for albumin and from 97.44 to 101.20% for myoglobin. Furthermore, the analysis of independent quality control samples was used to determine intra- and inter-day precision and accuracy. The intra-day RSD for albumin and myoglobin changed from 0.24 to 0.58% and the accuracy from 95.53 to 100.67% (Table 5). The inter-day RSD for albumin and myoglobin varied from 0.29 to 1.7% and the accuracy from 95.08 to 102.00% (Table 6). The above

Table 3
Mean R^2, a and b of 4-day standard curves ($y=ax+b$)[a] for albumin and myoglobin in biological matrix ($n=2$[b])[c]

Protein	Mean $R^2\pm$SD	Mean $a\pm$SD	Mean $b\pm$SD
Albumin	$0.99998\pm2.00\cdot10^{-5}$	$1.10\cdot10^{-5}\pm9.06\cdot10^{-8}$	0.00414 ± 0.00156
Myoglobin	$0.99993\pm1.24\cdot10^{-4}$	$2.75\cdot10^{-6}\pm1.10\cdot10^{-8}$	0.00219 ± 0.00415

[a] The regression equation is $y=ax+b$; R^2 is coefficient of determination; y is concentration of albumin or myoglobin (μg/μl); x is peak area $\cdot10^{-3}$; a is the slope and b is the y-intercept.
[b] n means the daily number of replicates.
[c] The experiment was performed on Beckman HPLC equipment; detection wavelength at 254 nm. Other experimental conditions as in Table 1.
SD, Standard deviation.

results indicated that the method was reliable, reproducible and accurate.

3.3. Method application

The validated method was applied to quantify albumin and myoglobin in approximately 1000 of dialysate and ultrafiltrate samples from dialyzers A, B, C, D, E, F and G. During the analysis of samples, the method validation was still continued. We insist the method validation be only a beginning, as the method was monitored continually during its applica-

Table 4
Four-day standard curve validation of albumin and myoglobin[a]

Concentration (μg/μl) ($n=2$)[b]		RSD (%)	Accuracy[d] (%)
Nominal	Mean predicted[c]$\pm$SD		
Albumin			
0.1250	0.121±0.005	4.17	103.00
0.2500	0.247±0.002	0.81	101.20
0.5000	0.501±0.003	0.59	99.80
1.0000	1.004±0.006	0.58	99.60
2.0000	2.005±0.007	0.37	99.76
4.0000	3.997±0.005	0.11	100.08
Myoglobin			
0.0156	0.016±0.000	0.00	97.44
0.0312	0.031±0.000	1.60	99.84
0.0625	0.062±0.003	4.05	101.20
0.1250	0.126±0.002	1.38	99.60
0.2500	0.251±0.002	0.88	99.70
0.5000	0.500±0.001	0.20	100.10

[a] Experimental conditions as in Table 3.
[b] n means the daily number of replicates.
[c] Mean predicted concentration of albumin or myoglobin was calculated by daily linear regression equation.
[d] Accuracy (%)=100×{1−[(predicted concentration−nominal concentration)/nominal concentration]}.

Table 5
Intra-day precision and accuracy of albumin and myoglobin[a]

Concentration (μg/μl) ($n=6$)[b]		RSD (%)	Accuracy[d] (%)
Nominal	Mean predicted[c]$\pm$SD		
Albumin			
0.386	0.393± 0.000	0.24	98.19
0.772	0.805± 0.001	0.38	95.73
1.545	1.614± 0.001	0.39	95.53
Myoglobin			
0.050	0.050± 0.000	0.28	100.00
0.150	0.149± 0.001	0.33	100.67
0.300	0.298± 0.002	0.58	100.67

[a] Experimental conditions as in Table 3.
[b] n means the number of replicates.
[c] Mean predicted concentrations of albumin or myoglobin.
[d] Accuracy (%)=100×{1−[(predicted concentration−nominal concentration)/nominal concentration]}.

Table 6
Inter-day precision and accuracy of albumin and myoglobin for 5 days[a]

Concentration (μg/μl) ($n=30$)[b]		RSD (%)	Accuracy[d] (%)
Nominal	Mean predicted[c]$\pm$SD		
Albumin			
0.386	0.394±0.001	0.29	97.93
0.772	0.810±0.005	0.68	95.08
1.545	1.617±0.007	0.46	95.34
Myoglobin			
0.050	0.049±0.001	1.70	102.00
0.150	0.148±0.001	0.56	101.33
0.300	0.296±0.002	0.84	101.33

[a] Experimental conditions as in Table 3.
[b] n means the number of replicates.
[c] Mean predicted concentrations ($n=30$) of albumin and myoglobin.
[d] Accuracy (%)=100×{1−[(predicted concentration−nominal concentration)/nominal concentration]}.

Table 7
Concentration of myoglobin in dialysate and ultrafiltrate samples from dialyzer A[a]

Sample	Concentration (μg/μl) of myoglobin					
	Reuse times of dialyzers[b]					
	R0	R1	R5	R10	R15	R20
Dialysate						
1	0.367	0.307	0.295	0.324	0.314	0.336
2	0.357	0.341	0.311	0.331	0.259	0.343
Ultrafiltrate[c]						
1	0.017	0.029	0.028	0.027	0.033	0.061
2	0.020	0.045	0.050	0.032	0.013	0.060

[a] Experimental conditions as in Table 3.
[b] The samples from dialyzer A were collected after 0, 1, 5, 10, 15, and 20 reuse cycles (R0, R1, R5, R10, R15 and R20).
[c] At each reuse cycle, the dialysate and ultrafiltrate samples were collected at different times.

tion of real samples to ensure that it performs as originally validated. The six concentrations of calibration standards and three quality control samples were analyzed with each analytical set of samples. Daily standard curves were generated to determine the sample concentrations. Acceptance of assay results was determined by monitoring the predicted calibrators and quality control results. All the accuracy deviation of the predicted calibrators and quality control results during the analysis of samples was within ±10%. Table 7 shows the representative concentrations of myoglobin in dialysate and ultrafiltrate samples from dialyzer A after 0, 1, 5, 10, 15 and 20 reuse cycles. Fig. 6 shows a representative chromatogram of the samples, where the concentration of albumin was 100-times higher than that of myoglobin.

4. Conclusions

A rapid, efficient, sensitive, reliable and accurate HP-SEC method has been developed, validated and implemented for the simultaneous and quantitative determination of albumin and myoglobin in dialysate and ultrafiltrate samples.

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