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Application of non-size-related separation effects to the purification of biologically active substances with a size-exclusion gel

Hartmut Schlüter* and Walter Zidek

Medizinische Poliklinik, Universität Münster, Albert-Schweitzer Strasse 33, 48149 Münster (Germany)

ABSTRACT

Most reports on the size exclusion of biomolecules describe chromatographic conditions designed to ensure a pure size-exclusion process. However, other retention mechanisms are also associated with size-exclusion chromatography, resulting in non-size-related separation effects. Applications of the size-exclusion gel Sephacryl S-100 High Resolution (HR) employing non-size-related separation effects, are described. With the help of non-size-related effects, purification and desalting of a biologically active, low-molecular-mass substance (<1000 g/mol) was achieved. To gain a better understanding of the non-size-related effects, the retention behaviour of the S-100 HR gel was examined with low-molecular-mass substances (amino acids and metabolites) and the K_{AV} values of the substances, chromatographed with high- and low-ionic-strength eluents, were compared. Coulombic interactions were most prominent at basic pH values and with low-ionic-strength eluents. Electrostatic interactions led to cation-exchange and anion-exclusion separation effects. The results demonstrate that, utilizing non-size-related separation effects, the application range of the S-100 HR gel is not limited to the fractionation of macromolecules, but may also be useful for the purification of low-molecular-mass biomolecules.

INTRODUCTION

Size-exclusion chromatography (SEC) has become a routine technique for biopolymer separation and characterization [1]. Typically, mild, non-aggressive mobile phase conditions are used, which preserve the native structure and functionality of the biopolymers [2]. Ideally solute molecules are fractionated in terms of size by equilibrium partitioning via diffusion between the mobile, fluid phase and gel pores [3]. However, the migration of the biopolymers on SEC columns is in fact influenced by electrostatic, hydrophobic, hydrogen bonding and steric effects [4]. For the analytical characterization of macromolecules it is desirable to minimize these effects. For preparative purposes non-size-related effects have been demonstrated to improve the separation of molecules of similar hydrodynamic volume [5]. Especially for the separation of peptides, non-ideal SEC has attracted increasing interest [6–9].

The utilization of non-size-related separation effects appeared to be a suitable solution for a separation problem in our laboratory. A biologically active substance with a molecular mass between 300 and 1000 g/mol [10], present in a perchloric acid extract from human platelets, had to be separated from other low-molecular-mass organic molecules and inorganic salts. To detect the biological activity a bioassay was used [11]. The bioassay only yields reproducible results if the compounds in the fractions to be tested for their vasoactive action do not exceed special physiological limits. Because of these conditions it is impossible to purify the bioactive substances chromatographically with eluents that contain

* Corresponding author.

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non-volatile components. Aqueous 1 *M* acetic acid is completely volatile and therefore fitted to the needs of the bioassay. With this eluent, the size-exclusion gel Sephacryl S-100 High Resolution (HR) shows remarkable separation results, especially if low-molecular-mass substances with high affinities to proteins have to be separated from the proteins [12]. A study of Johansson and Gustavsson [13] on the interactions of some proteins with the gel matrix suggested solving the separation problem with S-100 HR employing non-size-related separation conditions. Therefore, in this study, the elution behaviours of small organic molecules with similar molecular masses, diverse aliphatic and aromatic amino acids and metabolities, chromatographed with different eluents, were examined. The results of this investigation should be of help in finding suitable separation conditions for purifying the biologically active substance from platelets.

EXPERIMENTAL

Materials

Sephacryl S-100 HR was supplied by Pharmacia Biosystems (Freiburg, Germany), HPLC-grade water by J.T. Baker (Gross-Gerau, Germany), aspartic acid (Asp), arginine (Arg), glycine (Gly), phenylalanine (Phe), vanillylmandelic acid (VMA), norepinephrine (NE), bovine insulin and tris(hydroxymethyl)aminomethane (Tris) by Sigma (Deisenhofen, Germany) and ferritin, ovalbumin, cytochrome *c* and vitamin b_{12} by Fluka (Neu-Ulm, Germany). All other chemicals were purchased form Merck (Darmstadt, Germany).

Equipment

All chromatographic equipment was obtained from Pharmacia Biosystems unless specified otherwise. The HPLC equipment consisted of a Model 2248 HPLC pump, coupled to a Rheodyne injector (Latek, Heidelberg, Germany), a Uvicord S II spectrophotometer, a flowcell for conductivity measurement (LE-191, WTW, Germany), a two-channel compensation recorder and a RediFrac fraction collector. A column with 1000 mm × 16 mm (C16/100) and a 600 mm × 10 mm column (Superformance 600-10; Merck) were used.

Eluents

Five eluents were used (see Figs. 1 and 2): (1) 1 *M* acetic acid in HPLC-grade water; (2) 30 m*M* Tris buffer (pH 9) in HPLC-grade water; (3) 20 m*M* Tris buffer (pH 9) with 200 m*M* sodium chloride in HPLC-grade water; (4) 10 m*M* hydrochloric acid; and (5) HPLC-grade water. Prior to chromatography all eluents were filtered through a 0.2-μm filter (Anotop; Merck) and degassed.

Packing procedure

The columns were packed according to the packing instructions [14]. Briefly, the gel suspension was degassed under vacuum, fines were removed and the flow-rates for packing the C16/100 column were 1 ml/min (step 1, 2 h) and 2.5 ml/min (step 2, 1 h) and for the Superformance 600-10 column 0.4 ml min (step 1, 2 h) and 1.2 ml/min (step 2, 1 h). The efficiency of the column packing procedure was confirmed by passing 200 μl of acetone (10 mg/ml in water) through the C16/100 column at 0.5 ml/min or 80 μl of acetone (10 mg/ml in water) through the Superformance 600-10 column at 0.2 ml/min. The column was repacked if the peak shape of the eluting acetone was not symmetrical.

Chromatography

The flow-rate used for the C16/100 column was 1 ml/min and that for the Superformance 600-10 column was 0.4 ml/min. UV absorbance and conductivity were measured continuously. More details are given in the captions on the figures.

Determination of partition coefficients (K_{AV})

The void volume (V_0) was determined with ferritin in eluents 1 and 3 and the total volume of the packed bed (V_t) with acetone (5 μl/ml in water). Acetone was chosen for the determination of V_t because acetone had the same elution volume with all the eluents used. The partition coefficient (K_{AV}) was calculated with the equation $K_{AV} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the individual substances.

Preparation of platelet extract

The preparation of the platelets was described by Agha *et al.* [10]. Briefly, the isolated, washed, frozen and rethawed platelets from 200 ml of blood were deproteinized with 0.6 *M* perchloric acid, followed by neutralization using potassium hydroxide. The clear supernatant (2 ml) was applied directly to the Sephacryl S-100 HR size-exclusion column (see Fig. 2).

Bioassay

Vasoactivity was measured in an isolated perfused rat kidney [11]. The isolated perfused kidney was prepared as described by Hagel [1]. As the perfusion system, a single-pass system at a constant flow-rate of 9 ml/min with Tyrode solution kept at 37°C and equilibrated with CO_2–O_2 (5:95) was used as described previously [15]. A typical dose–response curve was given by Schlüter *et al.* [16].

RESULTS

Fig. 1 shows the calibration graph for S-100 HR. The eluent used for the calibration was 1 *M* acetic acid. Within the molecular mass range 1000–100 000 g/mol S-100 HR exhibits linear elution behaviour when the logarithm of molecular mass is plotted *versus* the partition coefficient.

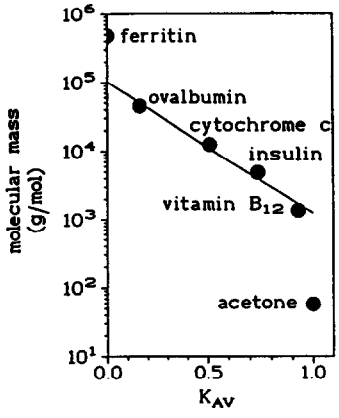

Fig. 1. Calibration graph for Sephacryl S-100 HR. Plot of molecular mass of typical calibration substances *versus* the corresponding partition coefficients (K_{AV}). Molecular masses of the substances: ferritin, 440 000; ovalbumin, 44 000; cytochrome *c*, 12 600; insulin (bovine), 4800; vitamin b_{12}, 1350; acetone 58 g/mol. Column, 1000 × 16 mm; bed size, 920 mm; eluent, 1 *M* acetic acid; flow-rate, 1 ml/min.

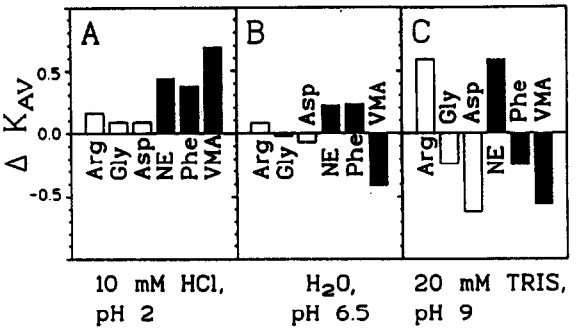

Fig. 2. Elution behaviour of standard molecules chromatographed on Sephacryl S-100 HR under non-ideal size-exclusion conditions. Plot of the deviation of the partition coefficients K_{AV} from the partition coefficients K_{AV} under ideal size-exclusion conditions. Standard molecules: open bars, aliphatic substances; filled bars, aromatic substances. (A) 10 m*M* hydrochloric acid (pH 2); (B) distilled water; (C) 20 m*M* Tris buffer (pH 9). Column, 600 × 10 mm; bed size, 550 mm; flow-rate, 0.4 ml/min; detection, UV absorbance at 280 nm or conductivity; amount of substance injected, 0.1 mg; volume injected, 100 μl. For the determination of K_{AV} single substances were injected.

In Fig. 2 the deviation from ideal size-exclusion retention behaviour of the standard molecules aspartic acid, vanillylmandelic acid, glycine, phenylalanine, arginine and norepinephrine chromatographed on S-100 HR in Tris buffer (pH 9), in distilled water and in 10 m*M* hydrochloric acid, is summarized. The retention values under ideal size-exclusion conditions were measured with 20 m*M* Tris buffer (pH 9) with electrostatic interaction suppressing 200 m*M* sodium chloride. In this buffer system all standard molecules eluted at $K_{AV} = 1$, owing to their molecular masses. In Fig. 2 negative differences from the origin reflect exclusion effects whereas positive differences reveal retention effects. In the acidic, low-ionic-strength eluent (0.01 *M* HCl, Fig. 2A) the K_{AV} values of the aliphatic amino acids glycine, arginine and aspartic acid differ only slightly from unity. The aromatic substances were retained significantly more strongly. In Fig. 2B, the deviation of the K_{AV} values of the low-molecular-mass standard substances, chromatographed in doubly distilled water, from ideal values is shown. Arginine, glycine and aspartic acid revealed only slight

differences from the ideal K_{AV} of 1. Norepinephrine and phenylalanine were clearly retained. The elution of vanillylmandelic acid was influenced by exclusion effects. In the low-ionic-strength Tris buffer (pH 9) (Fig. 2C), the retention of the standard molecules differs greatest from ideal retention behaviour. The basic molecules arginine and norepinephrine were retained whereas the neutral and acidic molecules eluted earlier than expected under ideal size-exclusion conditions.

The chromatogram in Fig. 3 demonstrates the resolving power of S-100 HR under non-ideal size-exclusion conditions. It was possible to separate vanillylmandelic acid, sodium chloride and phenylalanine from each other in water, whereas under ideal size-exclusion conditions no separation of these molecules was achieved.

The first attempt to chromatograph the platelet extract with S-100 HR was carried out in 1 *M* acetic acid (Fig. 4A). According to the calibration graph (Fig. 1), the UV absorption elution pattern indicates that most of the substances in the platelet extract have a low molecular mass. The biological activity (Fig. $4A_2$) eluted together with the main conductivity peak (Fig. $4A_1$). As this separation was not satisfactory with respect to further purification steps, the question arose of whether changes in the composition of the eluent can improve the separation.

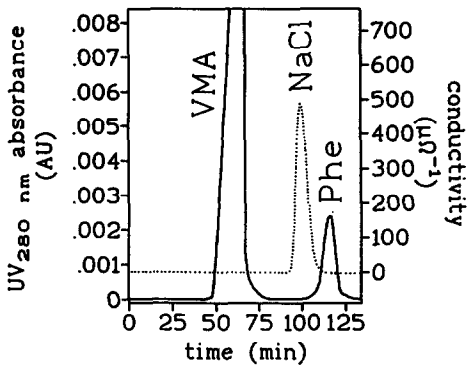

Fig. 3. Chromatogram of the separation of vanillylmandelic acid, sodium chloride and phenylalanine on Sephacryl S-100 HR. Column, 600 × 10 mm; bed size, 550 mm; flow-rate, 0.4 ml/min; left ordinate = UV absorbance at 280 nm (AU = arbitrary units) (solid line); right ordinate = conductivity (dotted line); sample, 0.25 mg of vanillylmandelic acid, 10 mg of sodium chloride and 5 mg phenylalanine, dissolved in 1 ml of water; volume injected, 100 μl.

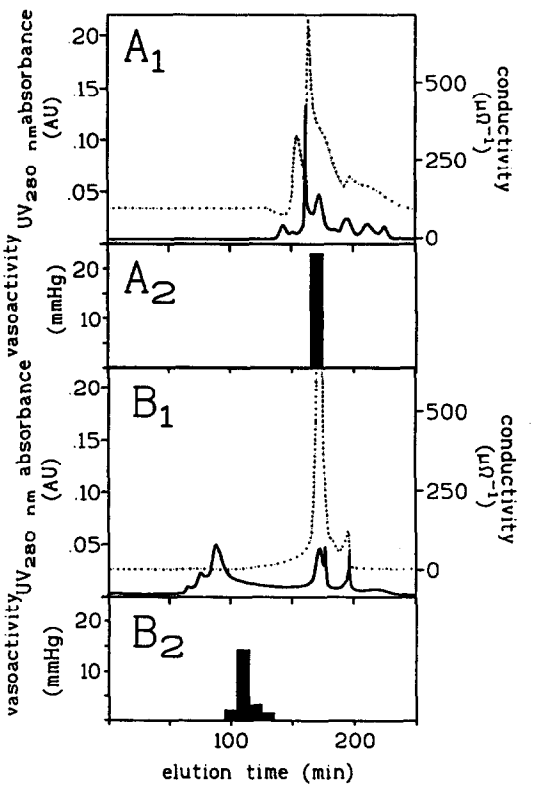

Fig. 4. (A_1, B_1) Chromatograms of typical separations of human platelet extracts in (A) 1 *M* acetic acid and (B) water and (A_2, B_2) corresponding profiles of the vasopressor actions of the fractions. Column, Sephacryl S-100 HR (1000 × 16 mm); bed size, 90 cm; flow-rate, 1 ml/min; samples, perchloric acid extract from human platelets from 200 ml of blood, dissolved in (A) 1 *M* acetic acid or (B) water; sample volume, 2 ml; A_1 and B_1, left ordinate = UV absorbance at 280 nm (solid line) (AU = arbitrary units), right ordinate = conductivity (dotted line); A_2 and B_2, ordinate = vasoactivity, measured as changes in perfusion pressure (mmHg; 1 mmHg = 133.322 Pa) after injection of the individual fractions into an isolated perfused rat kidney; abscissa, elution time.

Fig. 4B depicts the chromatography of the platelet extract with S-100 HR in water. The biologically active substance elutes between a pair of UV-absorbing peaks and the increase in conductivity (Fig. $4B_2$).

DISCUSSION

The Sephacryl S-100 HR matrix consists of allyldextran cross-linked with N,N'-methylenediacrylamide [17]. Dextrans contain small

amounts of carboxylic groups [18]. Johansson- and Gustavsson [13] demonstrated that these anionic groups are responsible for the non-size-related elution behaviour of proteins. The electrostatic interactions of the gel matrix with the proteins result in an ion-exchange and ion-exclusion mechanism. The question arose of how the electrostatic interactions affect the elution behaviour of low-molecular-mass substances and if these effects can be utilized for the purification of the biologically active platelet-derived substance. It could be considered that the biologically active substance, which binds to anion exchangers, interacts with the Sephacryl support via ion-exclusion.

Further, to evaluate the extent of interactions between molecules smaller than 1000 g/mol and the Sephacryl support, aliphatic and aromatic amino acids and metabolites were chosen as standard substances and the elution behaviours of these standard substances in different eluents were compared. Johansson and Gustavsson [13] assumed that proteins with intermediate p*I* values between 5 and 9 may not interact with the Sephacryl support when the pH of the mobile phase was 7 or 10 and the sodium concentration was higher than 0.2 *M*. According to these findings, an aqueous Tris buffer with a pH of 9 and containing 200 m*M* sodium chloride was chosen as the eluent, which guarantees minimum support–solute interactions. Because the electrostatic interactions are most prominent at basic pH values in the absence of sodium chloride, 20 m*M* Tris buffer (pH 9) was chosen as an eluent that promotes electrostatic interactions. The comparison of the retention of the low-molecular-mass substances in the two buffer systems revealed significant differences (Fig. 2C). Acidic molecules undergo ion exclusion. These molecules eluted about 0.5 K_{AV} units earlier under conditions where electrostatic interactions were present. In the case of basic molecules without suppression of the electrostatic interactions an increased retention was observed owing to ion-exchange effect.

The next question that had to be answered was whether water used as the eluent exhibits similar separation effects (Fig. 2B). Although the pH of water is lower than pH 7 it could be assumed that ion-exclusion and ion-exchange mechanisms are still present. Fig. 2B verifies this assumption. The deviation of the elution behaviour from the size-related separation in water is not as distinct as in the basic eluent with low ionic strength, but still the elution order is comparable, indicating an interaction between the standard molecules and the negative groups of the S-100 HR support. In an acidic eluent with low ionic strength (Fig. 2A) it seems that only minimal electrostatic interactions were present, but it is obvious that the aromatic molecules interact with the S-100 HR support. This interaction may be due to interactions between the aromatic systems and the ether bridges introduced by the cross-linker [19].

The calibration graph (Fig. 1) suggests that non-specific interactions of the standard proteins with the S-100 HR packing were minimal in 1 *M* acetic acid. With this eluent the purification of the platelet extract was tried first. As Fig. 4A indicates, in 1 *M* acetic acid a separation of the biologically active substance from the salts was not achieved. This result was expected because a previous study [10] showed that the biologically active substance has a molecular mass smaller 1000 g/mol. Molecules smaller 1000 g/mol, which do not interact with the S-100 HR matrix, elute near V_t, the total liquid volume of the column. With regard to the purification of the platelet extract, water as eluent seemed advantageous because it is completely volatile. Fig. 4B shows the chromatogram of the separation of the platelet extract in water. The K_{AV} value of the biologically active substance decreased from 1 in 1 *M* acetic acid (Fig. 4A) to 0.5 in water (Fig. 4B). This behaviour suggests an ion-exclusion effect. This observation corresponds to the previous finding [10] that the biologically active substance has an anionic group.

CONCLUSIONS

The utilization of non-size-related effects in the purification of biomolecules with similar size may be beneficial with size-exclusion supports, as this study demonstrates. Therefore, the knowledge of the possible interactions between support and solute is most important. With this knowledge the chromatographic conditions can be manipulated to achieve separations that are

not possible under ideal size-exclusion conditions. With the S-100 HR support the most prominent separation effects observed in this study can be obtained with low-ionic-strength buffers at basic pH values. These effects result in ion exclusion and ion exchange of the solutes. To work with low-ionic-strength eluents offers the opportunity to use even water as the eluent, which may be beneficial if complete volatility of the eluent is necessary.

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