Journal of Chromatography A, 658 (1994) 399–406
Elsevier Science B.V., Amsterdam

CHROM. 25 499

Calculation of peak profiles in preparative chromatography of biomolecules

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ABSTRACT

Preparative chromatography under overloaded conditions is used for the recovery of biomolecules in different disciplines. The process consists of loading, washing out the unbound material and elution using a step gradient or a linear gradient. The advantage of this adsorption–desorption process is the concentration of the biomolecule and the high productivity. Nevertheless, optimization of the process is still performed with small-scale experiments, varying the loading and elution conditions. This study involved the evaluation of the feasibility of the external mass balance theory for the prediction of protein profiles and salt profiles in preparative ion-exchange chromatography. Prior to the calculation of the peak profile, the separation parameters are characterized by a few runs. The salt gradient is characterized by a pulse experiment and the separation efficiency by a few isocratic runs. As a basis for calculation, the approach of the continuous-flow plate model was used. The calculation simulates closely the real separation conditions, because loading and washing are also taken into consideration. The calculated results were compared to experimental data. As a model, the purification of human superoxide dismutase by anion-exchange chromatography was used.

INTRODUCTION

Various models have been described for the prediction of peak profiles [1]. According to the rate theory (also called the mass balance theory), one can be distinguish between equilibrium and non-equilibrium models [2]. In most instances, preparative chromatography of proteins is performed under equilibrium conditions to achieve high resolution of highly concentrated biomolecules in the peak fractions. Equilibrium conditions are easier to reproduce because of standardized parameters. Many biotechnological processes use preparative chromatography as the predominant purification step [3]. Safety regulations and good manufacturing practice (GMP) regulations require rigid, simple and reproducible processes. To meet all these strict requirements, it is preferable to perform preparative chromatography in an adsorption–desorption mode [4]. Gel filtration is an exception.

The protein solution is loaded on to the ion-exchange column after equilibration, unbound material is washed out and elution (also called desorption) is accomplished by changing the composition of the mobile phase. This process also can be described as a displacing process [5] in ion-exchange chromatography. Salt ions effecting elution are also called the displacing salt. Desorption of proteins from the ion-exchange resin can be performed by continuous alteration of the mobile phase (linear gradient elution) or by an instantaneous change of the mobile phase (step-gradient elution). The different mobilities of the proteins and of the contaminating sub-

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SSDI 0021-9673(93)E0918-K

stances under elution conditions lead to the selective desorption of the protein from the stationary phase of the column, resulting in concentration of each protein by the adsorption–desorption process. Nearly all the stationary phase can be used to remove the protein from the mobile phase.

In most instances, an excess of the protein solution (several times the total column volume) is pumped through the gel. The adsorption process itself contributes to the concentration of the feed. Additionally, the desorption process can increase the concentration of the feed protein, if substantial zone spreading does not occur. During elution, the protein is desorbed from the ion-exchange resin and migrates as a very sharp zone. Concentration factors of up to 200 are observed [6] depending on the dilution of the starting material and the efficiency of the adsorption–desorption step.

Ion-exchange chromatography using DEAE-Sepharose fast flow applying a stepwise elution of biomolecules was used as a model to evaluate our method for predicting peak profiles. Human copper–zinc superoxide dismutase (SOD) produced by haemolysis of outdated human erythrocyte concentrate was used as a model protein. Significant non-specific adsorption phenomena were not observed in our model system using this model protein. SOD is highly soluble in aqueous buffers.

The experimental peak profiles obtained were compared with values calculated using a modelling approach described by Yamamoto *et al.* [7,8]. Excellent prediction of peak profiles was observed, suggesting that mass balance theory can be applied to the prediction of peak profiles by applying crude SOD to the ion-exchange column.

THEORY

The theory used in this study is based on the mathematical model of the plate theory under equilibrium conditions first proposed by Martin and Synge [9]. At equilibrium the protein concentration bound to the stationary phase, C_s, is a function of the protein concentration and ionic strength in the mobile phase, C and I:

$$C_s = K[C, I] \tag{1}$$

where K is the distribution coefficient of the protein, which usually decreases with increase in C and I.

In the equilibrium theory, the rate of movement of the protein zone in the column can be described by the equation

$$\frac{d_{zp}}{dt} = u(1 + HK[C, I]) \tag{2}$$

where d_{zp} is the peak position of the protein zone, $H = (V_t - V_0)/V_0$ and V_t and V_0 are the total volume and the void volume, respectively. In the model used by Yamamoto *et al.* [7,8], the zone spreading effects are represented by the number of plates (N_p is the number of plates for proteins and N'_p is that for salt). The compression effects are calculated by the change in the ionic strength in each plate. The change in ionic strength is also predicted by the plate theory.

The following assumptions are made in the model using the continuous-flow plate theory:

(1) the column consists of a certain number of equivalent virtual (theoretical) plates, and the ratio of the volume of the mobile phase to that of the stationary phase remains constant over all plates;

(2) the flow is continuous without mixing between the plates;

(3) equilibrium of solutes between the two phases is attained instantaneously,

(4) the distribution coefficient of a salt, K', is not dependent on either concentration or ionic strength and is not influenced by the proteins in the plate; and

(5) the number of plates for a protein, N_p, and that for a salt, N'_p, are constant.

Under these assumptions and the general mass balance equation for proteins [10],

$$u \cdot \frac{\delta C}{\delta z} + \frac{\delta C}{\delta t} + H \cdot \frac{\delta \bar{C}_s}{\delta t} = \frac{1}{2N} \cdot \frac{\delta^2 C}{\delta z^2} \tag{3}$$

can be expressed as

$$v[C_{(n-1)} - C_{(n)}] = \frac{V_0}{N_p} \cdot \frac{dC_{(n)}}{dt} + \frac{V_t - V_0}{N_p} \cdot \frac{dC_{s(n)}}{dt} \tag{4}$$

according to Yamamoto and co-workers [7,8,11]. The axial diffusion term is neglected because this band broadening process is covered by N_p. $C_{(n)}$ and $C_{s(n)}$ are the protein concentrations in the mobile phase and stationary phase, respectively, at plane n, v is the flow-rate, V_0 is the void volume V_t is the total column volume and t is time.

Eqn. 4 is rearranged in the reduced form as

$$C_{(n-1)} - C_{(n)} = \frac{1}{N_p}\left[\frac{dC_{(n)}}{d\theta} + H \cdot \frac{dC_{s(n)}}{d\theta}\right] \quad (5)$$

where H is the dimensionless fraction of the stationary phase and θ is the dimensionless time, tv/V_0.

Differentiation of eqn. 1 with respect to θ gives

$$\frac{dC_{s(n)}}{d\theta} = \frac{dC_{s(n)}}{dI} \cdot \frac{dI}{d\theta} + \frac{dC_{s(n)}}{dC_{(n)}} \cdot \frac{dC_{(n)}}{d\theta} \quad (6)$$

Assumption (3) allows the insertion of eqn. 1 into eqn. 6. The first term of the right-hand side of eqn. 6 then becomes

$$\frac{dC_{s(n)}}{dI} \cdot \frac{dI}{d\theta} = \frac{d\langle K[C_{(n)}]C_{(n)}\rangle}{dI} \cdot \frac{dI}{d\theta} = \frac{dI}{d\theta} \cdot \frac{dK[C_{(n)}, I]}{dI} \cdot C_{(n)} \quad (7)$$

and the second term is represented by

$$\frac{dC_{s(n)}}{dC_{(n)}} = \frac{dC_{(n)}}{d\theta} \cdot \frac{d\langle K[C_{(n)}, I]C_{(n)}\rangle}{dC_{(n)}} = K[C_{(n)}]\frac{dC_{(n)}}{d\theta} \cdot \frac{dK[C_{(n)}, I]}{dC_{(n)}} \cdot C_{(n)} \quad (8)$$

Substitution of eqns. 6–8 into eqn. 5 gives

$$\frac{dC_{(n)}}{d\theta} = \frac{N_p[C_{(n-1)} - C_{(n)}] - C_{(n)}H\{dK[C_{(n)}, I]/dI\}(dI/d\theta)}{1 + H(K[C_{(n)}, I] + C_{(n)}\langle dK[C_{(n)}, I]/dC_{(n)}\rangle} \quad (9)$$

In a similar manner, the basic equation for the distribution of ionic strength $I_{(n')}$ in the mobile phase at plate n' is derived under assumptions (1)–(5).

Eqn. 10 is a linear differential equation:

$$\frac{1 + HK'}{N'_p} \cdot \frac{dI_{(n')}}{d\theta} + I_{(n')} = I_{(n'-1)} \quad (10)$$

Hence K' and N'_p remain constant, and the differential equation can be solved analytically with known initial and boundary conditions:

$$dI_{(n')}/d\theta = (I_{elu} - I_0)g'(\theta, n')R' \quad (11)$$

The derivation of eqn. 11 was described by Yamamoto *et al.* [7]. R' is given by

$$R' = N'_p/(1 + HK') \quad (12)$$

and g' is defined by

$$g'(\theta, n') = \frac{\exp(-\theta R')}{(n' - 1)!}(\theta R')^{n'-1} \quad (13)$$

Eqn. 9 is numerically calculated by the Runge–Kutta method. The value of N'_p is greater than that of N_p. Therefore, the salt plate corresponds to that protein plate where the majority of the volume overlaps with the protein plate. As the step width (h) for the Runge–Kutta method, $1/N_p$ was chosen.

The boundary conditions of the differential equations are determined by the loading and washing procedure. These are the starting conditions for elution and the distribution of the protein among the plates closely simulates reality. Therefore, an assumption such as an infinitesimally small sample volume is not necessary.

EXPERIMENTAL

Starting material

SOD, an enzyme from human erythrocytes, was extracted by osmolysis and the main impurities were removed by heat precipitation [12]. The suspension was clarified by centrifugation in a laboratory centrifuge at *ca.* 5000 *g* and the supernatant was subjected to further investigations.

Enzyme activity

SOD activity was quantified by an enzyme activity assay using xanthin–xanthine oxidase as radical generator and nitro blue tetrazolium as indicating scavenger [13].

Chromatography

For anion-exchange chromatography, DEAE-Sepharose fast flow (Pharmacia, Uppsala, Sweden) was used. The column was equilibrated with 10 m*M* potassium phosphate buffer (pH 6.4). The clarified starting solution was loaded on to the column and unbound material was washed out with equilibration buffer. Elution was carried out with 10 m*M* potassium phosphate buffer supplemented with 100 mmol/l NaCl. The gel was regenerated with 1 *M* NaCl in equilibration buffer. The linear velocity was 25 cm/h. Packing of the gel was performed by pouring the slurry into the column and after the adapter had been fitted on the column the pump was turned on. The packing velocity was 400 cm/h. The packing was carried out in 20% ethanol.

Determination of K by batch experiments

A 300-μg amount of swollen and equilibrated DEAE-Sepharose Fast Flow gel was transferred into 1.5-ml screw microtubes (Sarstedt, Nürnberg, Germany). Before the protein was added to the gel the protein was transferred into the appropriate buffer by desalting on small gel filtration columns. The mixture was shaken for 3 h, then the gel was sedimented by low-speed centrifugation and the enzyme concentration was determined.

K was then calculated with the equation

$$K = \overline{m_p}/m_p \tag{14}$$

at a defined ionic strength, where $\overline{m_p}$ is the molarity of the protein bound to the ion exchanger and m_p is the molarity of the protein in solution. The concentration of the protein in the gel is easily calculated with the equation

$$m_p = \frac{w_s}{w_g}(m_p^i - m_p^e) \tag{15}$$

where m_p^i and m_p^e are the molarities of the protein in the outer solution at the initial and equilibrium states, respectively, w_s is the amount of protein solution and w_g is the amount of gel.

Pulse response experiment for determination of K, N_p' and N_p

DEAE-Sepharose fast flow was packed into a column, saturated with 1 *M* NaCl and equilibrated with the appropriate buffer. A 500-μl volume of 1 *M* NaCl solution was injected and the conductivity of the column effluent was continuously monitored. The monitor signal was changed to a digital signal and the data were acquired by a data acquisition system (Nelson Analytical, Cupertino, CA, USA; Version 5.0). The first normalized statistical moment, μ_1', was calculated according to Foley and Dorsey [14] and the K value was obtained with the equation

$$\mu_1' = \frac{Z}{u}(1 + HK) \tag{16}$$

where Z is the column length and u is the linear mobile phase velocity. The K values was plotted against the salt concentration (I) and the best fit was calculated. Eqn. 16 can also be expressed as

$$K = \frac{V_e - V_0}{V_t - V_0} \tag{17}$$

Eqn. 17 is easier to handle than eqn. 16. N_p' can be obtained from

$$N_p' = (\mu_1')^2/\mu_2' \tag{18}$$

where μ_1' is the first and μ_2' the second normalized moment.

RESULTS

The starting materials for the experimental verification of the predicted peak profiles were produced from outdated human erythrocyte concentrate. All experiments for determining K and N_p were performed with purified material. Only a balance of the purification step is shown (Table I).

Determination of K', K, N_p' and N_p

K' and N_p' were determined by a salt pulse experiment (Fig. 1). The value for K' was calculated according to eqn. 8 as $K' = (86\text{ ml} - 98\text{ ml}/3)/(98\text{ ml} - 98\text{ ml}/3) = 0.82$. The plate number for the salt (N_p') was calculated according to eqn. 9 as $N_p' = (43.34 + 60)^2/18\,003 = 370$. The plate number for protein (N_p) was also determined in a pulse experiment using the same equation; as $N_p = (58.26 \cdot 60)^2/76\,661 = 160$. The plate number was assumed to be constant for all simulated cases.

TABLE I

SUMMARY OF THE PURIFICATION OF SOD FROM OUTDATED HUMAN ERYTHROCYTE CONCENTRATE

The purified material was concentrated by ultrafiltration to the appropriate concentration required for different experiments.

Purification procedure	Volume (ml)	Protein (μg/ml)	SOD (μg/ml)	Yield (%)
Haemolysate	5051	3300	6.3	100
DEAE-Sepharose FF	475	674	320	94.6
Phenyl-Sepharose FF	719	80	32.7	73.9
Cu-chelate-Sepharose FF	123	332	190.2	73.6
Ultrafiltration	54.3	671	400	72.6
Desalting[a]	150	210	150	70.8

[a] Desalting was performed by gel permeation using Sephadex G-25.

The change in dK/dI was determined by batch experiments and by isocratic runs (Fig. 2). In the range 0–5 mg/ml SOD, linear adsorption isotherms could be obtained (Fig. 3). This means that for the respective salt concentration (0–100 mM) the term $dK[C_{(n)}, I]/dC_{(n)}$ can be neglected, because there is no influence of the protein concentration on K. The dependence of K on $[I]$, $dK[I]/dI$, could be fitted by a second-order polynomial, $dK[I]/dI = (-0.1052 + 0.002I - 1.087I^2)$. This polynomial was used for the prediction of the peak profiles. The best fit was calculated by a non-linear regression.

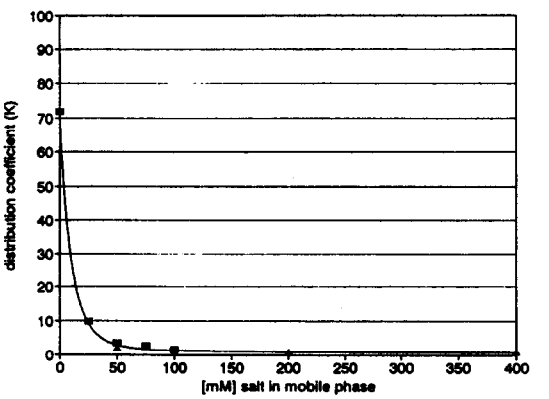

Fig. 2. Determination of dK/dI by batch experiments and by isocratic runs. In the isocratic runs a DEAE-Sepharose fast flow column (17.5 × 2.6 cm I.D.) was used at 25 cm/h. The best fit was calculated using a second-order polynomial.

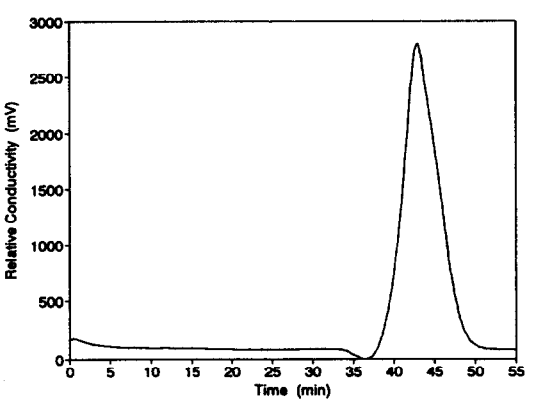

Fig. 1. Pulse response experiment for determination of K' and N'_p. A DEAE-Sepharosse fast flow column (17.5 × 2.6 cm I.D.) was operated at 25 cm/h. Conductivity was continuously monitored with an on-line conductivity meter. Data were acquired with a data aquisition system from Nelson Analytical.

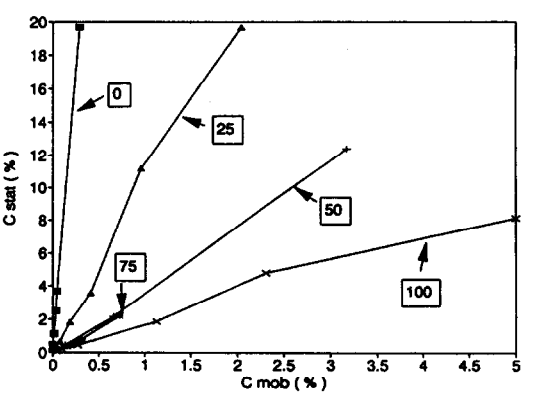

Fig. 3. Adsorption isotherm of SOD on DEAE-Sepharose fast flow. The isotherms were determined by batch experiments at different salt concentrations in the mobile phase (0, 25, 50, 75 and 100 mM NaCl) using 0.01–5.0 mg/ml SOD.

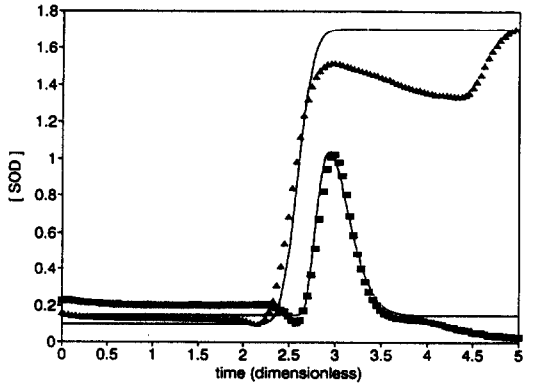

Fig. 4. Comparison of experimental by obtained data and predicted peak profiles: 6 ml of SOD solution were applied to an equilibrated DEAE-Sepharose fast flow column (17.5 × 2.6 mm I.D.) and eluted by applying 100 m*M* salt solution. The scale of the predicted values was fitted to the range of the experimental values. ■ = SOD (experimental); ▲ = salt (experimental); solid lines = predicted peak profiles of SOD and salt.

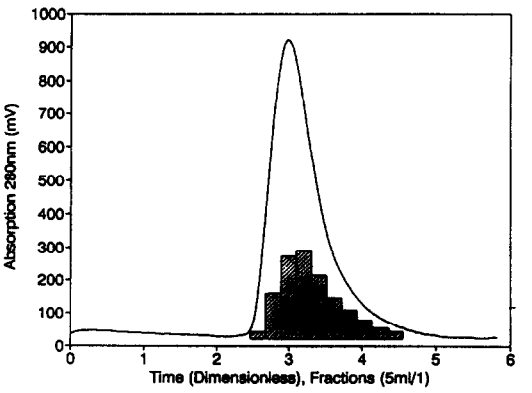

Fig. 5. Peak profiles of HSA-contaminated SOD. The column effluent was also collected and SOD activity determined.

Prediction of peak profiles with pure SOD

In Fig. 4, the predicted peak profile and the experimental values are compared. As boundary conditions for the calculation of the derivative equation we put 1 arbitrary unit of SOD in the first plate. The initial condition is $C_{(1)} = 1$, $C_{(2)} = \cdots = C_{(N_p)} = 0$ at $\theta = 0$. The following parameters for calculation using eqn. 9 were used: $h = 0.00625$, $N_p = 160$, $N'_p = 370$, $I_0 = 0$, $I_{elu} = 100$, $K' = 0.8$, $V_t = 93$ and $V_0 = 31$. That should closely simulate the real conditions and a type I elution curve is obtained. For verification of the predicted elution curve, 1 mg of SOD was applied to a 93-ml DEAE-Sepharose fast flow column and elution was carried out as described under Experimental.

Prediction of peak profiles with contaminated SOD

For confirmation that the peak prediction is also valid under real separation conditions (processing raw material), the enzyme was contaminated artificially by adding human serum albumin (HSA) in different ratios. We observed a minor shift of each peak maximum where the SOD was desorbed. Also, compounds originating from HSA co-migrated with the SOD peak. With the highest contamination (SOD:HSA = 1:100) the experimental obtained peak position did not move significantly and corresponded to the predicted position. The peak shape could be directly compared with pure SOD. In experiments with contaminated material we had to collect small fractions and we determined the enzyme activity of each. The profile of the enzyme activity in each fraction resembles to the peak profile (Fig. 5).

In an experiment in which the haemolysate was processed with DEAE-Sepharose fast flow, the eluted protein was collected in small fractions and the SOD activity was determined. Also in this instance for the profile of enzyme activity, the position of the fraction with the maximum enzyme activity is comparable to the predicted values (Fig. 6).

DISCUSSION

Prediction of peak profiles is a valuable tool for optimizing the preparative chromatography of biomolecules. The biomolecules are never present as single components nor are they accompanied by only a few substances in most circumstances. Depending on the stage of the purification process, the biomolecule is often only a minor component in the solution. Chromatographic methods are preferred to other purification procedures owing to the sensitivity to environmental conditions and the simple oper-

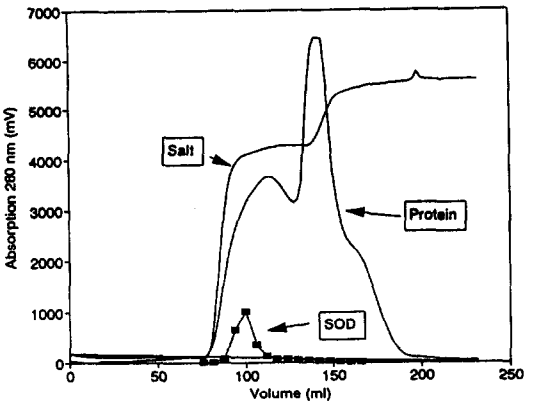

Fig. 6. Purification of SOD by DEAE-Sepharose fast flow using haemolysate from outdated human erythrocytes. A 335-ml volume of haemolysate was applied to the column (17.5 × 2.6 cm I.D.), which was washed with 0.8 l of equilibration buffer and eluted by a step gradient using 100 m*M* NaCl in equilibration buffer.

ation. The aim of this investigation was to determine the manner in which impurities may influence peak profiles and whether the experimental peak profiles are comparable to those predicted by the model. One has to consider that important parameters such as K, K', N_p and N'_p are measured with purified material. The assumption was made that the distribution coefficient for a salt (K') and the plate number for a salt (N_p) are not influenced by the mass of protein. The distribution coefficient for the protein (K) does not change with variation of the concentration in the mobile phase within the working range of the model system. The change of K with salt in the mobile phase was fitted by a second-order polynomial $dK/dI = a + bI - cI^2$. To achieve sufficient precision over the entire working range of [I], that is 0–100 m*M* NaCl, K was determined by batch experiments and by isocratic runs, where K is calculated from eqn. 8. At low ionic strength, the residual protein concentration in the mobile phase is very low in batch experiments, thus not allowing an accurate measurement. Therefore, batch and isocratic run data were combined and the best fit was calculated by non-linear regression.

Yamamoto and co-workers [8,11] published a similar relationship, $K = AI^b + K_{crt}$, where K_{crt} is the critical distribution coefficient of the protein at high ionic strength and electrostatic interaction can be ignored, and A and b are constants that have to be determined for each case. The derivative of this equation (dK/dI) resembles the second-order polynomial which we found to be the best fit.

After determining N_p, N'_p, K and K', the peak profiles were calculated according to the modified approach of Yamamoto *et al.* (see eqns. 1–13) and the predicted values were compared with the results of the ion-exchange chromatographic experiment using purified SOD. An excellent prediction was observed, especially for the retention time and the shape of the peak. In contrast to Yamamoto *et al.* [7], we used as initial conditions $C_{(1)} = 1$, $C_{(2)} = 0 \cdots = C_{(N_p)}$ at $\theta = 0$ to circumvent negative θ values which may be responsible for an unstable numerical calculation. They assumed as initial conditions $C_{(1)} =$, $C_{(2)} = \cdots = C_{(N_p)} = 0$ at $\theta = -X_0$, where X_0 is the ratio of the sample volume to V_0 and the boundary condition is C_0 for $-X_0 < \theta \leq 0$ and $C_0 = 0$ for $\theta > 0$, where C_0 is the initial concentration of the sample protein. The calculated and experimental profiles of the salt gradient were only comparable in the phase where the step gradient developed. In the flattening phase, the calculated value indicated an even transition.

In the experiments, an interaction between the salt and the ion-exchange resin may occur owing to replacement of other ion species. The conductivity increases and after about $0.5V_0$ the value decreased to 100 m*M* salt after this transition. The ions released by the chemical reaction may not contribute to the separation process of the protein.

To determine whether the peak profile is influenced by impurities present in the starting solution, chromatography was carried out with artificially contaminated SOD and with a crude SOD solution. The SOD peak profile could not be recorded directly from the in-line monitor, because contaminants co-eluted with the model protein. Therefore, the column effluent was fractionated into small volumes, the enzyme activity was measured and the profile was compared with the calculated data. No significant difference was observed between the calculated and the experimental profiles (Fig. 4). These

findings indicate that the SOD and the contaminants do not influence each other and that crossed isotherms do not appear to be involved in the adsorption–desorption process. Investigations to determine the exact chromatographic behaviour of the impurities are required.

The results of the model used for the prediction of peak profiles in ion-exchange chromatography using stepwise elution are comparable to the experimental values with respect to retention time and peak shape. The external mass balance model is suited for the prediction of protein and salt profiles in preparative ion-exchange chromatography, not only using pure material.

SYMBOLS

$C_{(0)}$	feed concentration (% or mg/ml)
$C_{(n)}$, $C_{s(n)}$	concentration of protein at plate n in the mobile phase (% or mg/ml)
C, C_s	concentration of protein in mobile phase and stationary phase (% or mg/ml)
d_{zp}	peak position of the protein zone (cm)
H	dimensionless fraction of stationary phase, $(V_t - V_0)/V_0$
h	step width for Runge–Kutta calculation (dimensionless)
I	ionic strength (mM)
I_0	ionic strength with which column is initially equilibrated (mM)
I_{elu}	ionic strength of the elution buffer in stepwise elution (mM)
K, K'	distribution coefficient (dimensionless)
m_p	concentration of protein in solution (% or M)
$\overline{m_p}$	concentration of protein bound to ion exchanger (% or M)
n	number of plates numbered from the top of the column (dimensionless)
N_p, N'_p	number of plates of protein and salt (dimensionless)
R'	$N_p/(1+HK')$
t	time(s)
u	linear velocity (cm/s)
v	flow-rate (cm^3/s)
V_0, V_t	void and total volume of the column, respectively (cm^3)
θ	dimensionless time
$\mu'_1 f$	first normalized peak moment (dimensionless)
μ'_2	second normalized peak moment (dimensionless)

ACKNOWLEDGEMENTS

The Österreichische Blutspende Zentrale of the Rotes Kreuz Wien supplied the outdated erythrocyte concentrate. We thank James L. Wittliff for critical comments and helpful discussions.

REFERENCES

1 J.C. Bellot and J.S. Condoret, *Process Biochem.*, 26 (1991) 363.
2 S. Golshan-Sirazi and G. Guiochon, *J. Chromatogr.*, 603 (1992) 1.
3 J. Bonnerja, S. Oh, M. Hoare and P. Dunnhill, *Biotechnology*, 4 (1986) 954.
4 A. Jungbauer, *J. Chromatogr.*, 639 (1993) 3.
5 W. Kopaciewicz and F. Regnier, *Anal. Biochem.*, 133 (1983) 251.
6 B. Malm, *J. Immunol. Methods*, 104 (1987) 103.
7 S. Yamamoto, K. Nakanishi, R. Matsuno and T. Kamikubo, *Biotechnol. Bioeng.*, 25 (1983) 1465.
8 S. Yamamoto, K. Nakanishi, R. Matsuno and T. Kamikubo, *Biotechnol. Bioeng.*, 25 (1983) 1373.
9 A.J.P. Martin and R.L.M. Synge, *Biochem. J.*, 35 (1941) 1358.
10 S. Yamamoto, T. Suehiza and Y. Sano, *Chem. Eng. Commun.*, 119 (1993) 221.
11 S. Yamamoto, K. Nakanishi, and R. Matsuno, *Ion-Exchange Chromatography of Proteins*, Marcel Dekker, New York, 1988.
12 A. Jungbauer, W. Schönhofer, D. Pettauer, G. Gruber, C. Uhl, E. Wenisch and F. Steindl, *Bioscience*, 8 (1989) 21.
13 K. Vorauer-Uhl, M. Skias, A. Trkola, W. Schönhofer, F. Unterluggauer, P. Schulz and A. Jungbauer, *Bioseparation*, 3 (1993) 217.
14 J.P. Foley and J.G. Dorsey, *Anal. Chem.*, 55 (1983) 730.