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PREPARATIVE CAPILLARY ISOTACHOPHORESIS: A MICRO METHOD FOR THE PURIFICATION OF ERYTHROPOIETIN

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SUMMARY

The micropreparative separation of an *in vivo* active glycoprotein, erythropoietin, using isotachophoresis on an LKB Tachophor equipped with a Tachofrac, a micropreparative fraction collector is described. The preparative technique as described by earlier workers was varied by application of a new time–distance delay determination which produces highly accurate component separations. Using this technique a purification factor of 228 could be obtained for erythropoietin with a recovery of 59%.

INTRODUCTION

Hitherto the main problem in the purification of erythropoietin was the realization of a rapid, efficient fractionation procedure to obtain a product of high yield and purity. Up to now the best method is that due to Myake *et al.*[1], leading to a preparation of 70,400 IU/mg in a six-step procedure. The activity yield was 21%, demonstrating a significant loss of material during fractionation, mainly caused by loss of N-acetylneuraminic acid from erythropoietin. As the LKB Tachophor and Tachofrac had been shown to be powerful tools in protein analysis[2,3] and preparation[4,5], we tested the efficiency of preparative capillary isotachophoresis in overcoming the difficulties in the purification of erythropoietin (Epo).

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EXPERIMENTAL

*Materials**

HPMC was obtained from Dow Chem. (Midland, MI, U.S.A.), Tris from Serva (Heidelberg, G.F.R.), 1 *N* hydrochloric acid and barium oxide from Merck (Darmstadt, G.F.R.), glutamic acid, cystine and glycine from Sigma (St. Louis, MO, U.S.A.), Ampholine pH 3.5–9.5 from LKB (Stockholm, Sweden), Epo-HAI from JCL Clinical Research Corp. (Knoxville, TN, U.S.A.), indigo tetrasulphonate from LKB and ammediol from Serva.

All reagents except HPMC were of analytical-reagent grade. HPMC was purified according to the method of Delmotte[2].

Instrumentation and conditions

All investigations were carried out with the LKB Tachophor equipped with a Tachofrac, a micropreparative fraction collector. The microtitration equipment of Cooke Laboratory Products (Alexandria, VA, U.S.A.) was used for the Epo-HAI.

The Tachophor was operated with a detection wavelength of 280 nm and UV gain 175; the capillary length was 43 cm and the capillary temperature 285°K. The strip velocity of the Tachofrac was 0.43 mm/sec. The elution current was 67 μA, chart speed 0.5 mm/sec and counter flow 0.03 μl/sec.

Buffer system

The leading electrolyte was 10 m*M* hydrochloric acid in 0.3 % HPMC adjusted to pH 8.00 with Tris. The terminator was 15.38 m*M* Gly adjusted to pH 10.00 with freshly prepared barium hydroxide. Buffer preparation was carried out under nitrogen to avoid carbon dioxide adsorption[2]. Before use the buffers were degassed for 10 min.

Buffer runs

The first four runs of every sequence were carried out for buffer control and system cleaning. A constant current of 150 μA was applied, causing a voltage rise from 2.6 to 23.7 kV. The last buffer run was used to control Ampholine (Fig. 1).

Determination of time–distance delay (f value)

As there is a distance between the UV window and the T-tube outlet of the capillary, every eluted component has a delay against its UV signal. This delay, called the *f* value, is specific for every buffer system and has to be measured by a dyestuff run under preparative conditions with indigo tetrasulphonate when anionic systems are used.

A 1-μl volume of the dye (0.5 mg/ml) was applied to the system, which was kept open at the terminator side. When the dye had covered half the distance between the injection port and the UV window, a counterflow was started. The current was reduced until the zone migration stopped. As the counterflow decreased the zone sharp-

* Abbreviations: HPMC = hydroxypropyimethylcellulose; Tris = trishydroxymethylaminomethane; Glu = glutamic acid; $(Cys)_2$ = cystine; Gly = glycine; Epo-HAI = erythropoietin haemagglutination inhibition assay; ammediol = 2-amino-2-methylpropane-1,3-diol; IU = International Unit.

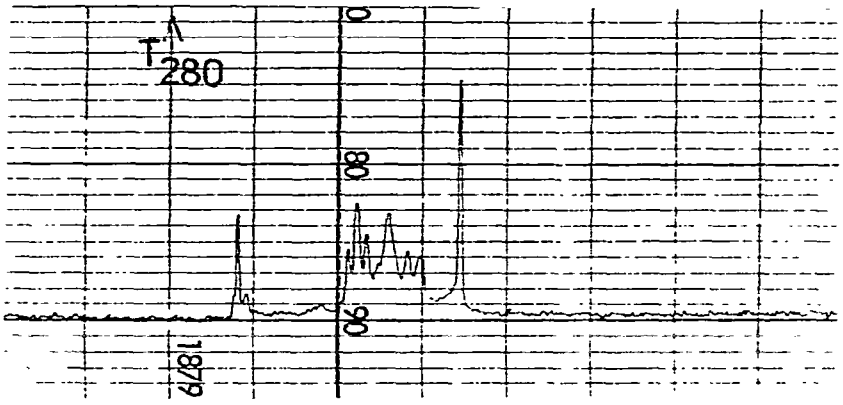

Fig. 1. Buffer run using 1.5 μl of 1% Ampholine, pH 3.5–9.5. No effect on detection is observed.

ness, the dye zone was observed for several minutes to be sure that no further migration occurred. The corresponding current reduced by 5% was the elution current. After the counterflow was stopped the system was closed with the terminator valve. After reconstitution of the dye zones, the counterflow was started again. No reduction in resolution could be observed when the system was absolutely closed.

At 0.5 kV before dye detection, the current was decreased to the elution value and the Tachofrac was switched on, followed by the counterflow pump. Each 5-cm distance was marked as a punch hole on the acetate strip, corresponding to a spike on the UV diagram. The following distances had to be estimated:

(*a*) distance of two successive spikes on the UV trace;
(*b*) distance of the first spike to the beginning of the UV signal;
(*c*) punch–punch distance on the strip (= 5 cm = constant);
(*d*) (*b*/*a*) *c*;
(*e*) punch–peak distance on the acetate strip;
(*f*) *e* − *d*.

f must be added to *d* in order to find the eluted component on the strip. The scheme for determination of the *f* value is shown in Fig. 2.

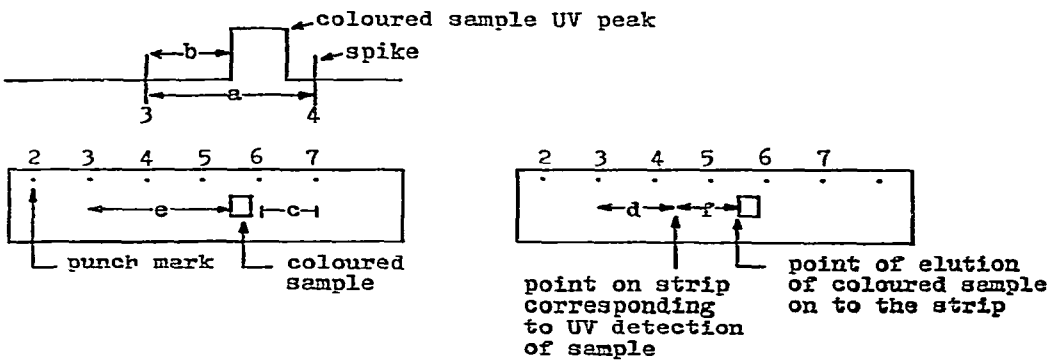

Fig. 2. Scheme for determination of *f* value.

Normally, fractionation of achromatic compounds such as proteins and peptides was carried out according to the parameters estimated in a pre-run with indigo tetrasulphonate. This produces errors when alkaline buffer systems are used because carbon dioxide adsorption changes the f value from run to run; hence the exact positions of components cannot be estimated. The f value remains constant when the buffers in the capillary and in the reservoirs are changed for every run. The f value increases when only the capillary buffers are changed. This problem may be solved by injecting 0.2 μl of dye together with the sample. The f value may then be calculated *in situ* and high accuracy is achieved for component determinations in every run. The dye does not affect the resolution (Fig. 3).

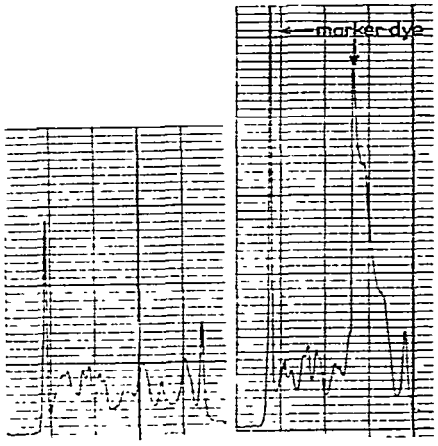

Fig. 3. Comparison of the same diagram region of two runs carried out with and without dye. Although the dye produces mixed zones with sample components, the other components are not effected.

Erythropoietin sample

A urinary protein mixture isolated from the urine of a chronic myeloid leukaemia patient by benzoic acid–acetone precipitation[6] and additional gel filtration on Sephadex G-50 (Pharmacia, Uppsala, Sweden) with an activity of 2 IU/mg standardized against the International Reference Standard (IRS) was used. Biological activity was measured by the exhypoxic polycythaemic mouse assay[7].

Determination of erythropoietin activity

Erythropoietin activity was measured with the Epo-HAI method according to Lange *et al.*[8]. The sample with a bioassayed activity of 2 IU/mg had an HAI activity of 1800 miu (milli-immunological units) calculated with the HAI quantitative chart. First, the erythropoietin activity of the regions formed by Glu and $(Cys)_2$ as discrete spacers[9] were assayed in a screening test. The acetate strip was cut into pieces corresponding to protein regions. The protruding edges of the strips were cut off so that only the small track with the components on it remained. These pieces were prewetted with phosphate-buffered saline (pH 7.4) and then incubated with 50 μl of PBS at 313°K for 20 min to resolubilize the components. The strip was then removed and the solution was assayed for HAI. For peak identification the strip region with erythropoietin activity was cut into pieces according to the calculated zone lengths.

Protein determination

Assuming Myake *et al.*'s erythropoietin preparation[1] to be absolutely pure, we could calculate the amount of erythropoietin present in our sample to be 25.5 ng/mg protein. As a 5-μl sample volume was found to be the optimum for the separation capacity, only 1.9 ng of erythropoietin could be assumed to be present in every preparative run. We tried to calculate erythropoietin concentrations using the Bouguer–Lambert–Beer law and the absorbance coefficient ($A^{1\%}_{1\,cm}$) for erythropoietin, which is 9.26 according to Espada *et al.*[10]. The other parameters necessary for calculation were obtained from the capillary geometry and the UV spectrum: I_0 = 17.5 cm (total absorbance); I = 15.85 cm (total absorbance − signal height); d = 0.05 cm (inner diameter of the capillary); ε = 0.926 (special extinction coefficient = $A^{1\%}_{1\,cm}/10$).

From these parameters, the erythropoietin concentration was calculated to be 929 ng/μl, representing the maximal concentration of the erythropoietin zone. For calculation of the amount of erythropoietin present in this zone, the zone length also had to be determined. The physical constants needed for a calculation depending on the Kohlrausch equation[11] are not known. The factor between the real zone length and UV signal length was therefore estimated by running indigo tetrasulphonate under preparative conditions. The real zone length was measured with a millimetre strip pasted parallel to the capillary. The UV signal length was measured on the UV spectrum. A linear relationship was found between the real zone length and the UV signal length and the gradient factor was 0.3. Using this value, the erythropoietin zone volume was calculated to be 0.2 μl, leading to a total amount of erythropoietin of 193 ng.

Erythropoietin fractionation

In every run, 5 μl of sample (135 miu of erythropoietin) were applied together with 1.5 μl of 1 % Ampholine, 0.3 μl of Glu (1 mg/ml), 0.3 μl of $(Cys)_2$ (1 mg/ml) and 0.2 μl of dye. During a run without fractionation the voltage increased from 2.0 to 17.6 kV at a constant current of 100 μA. During fractionation a final voltage of 14.3 kV was reached. Fig. 4 shows a typical UV spectrum of a fractionation run.

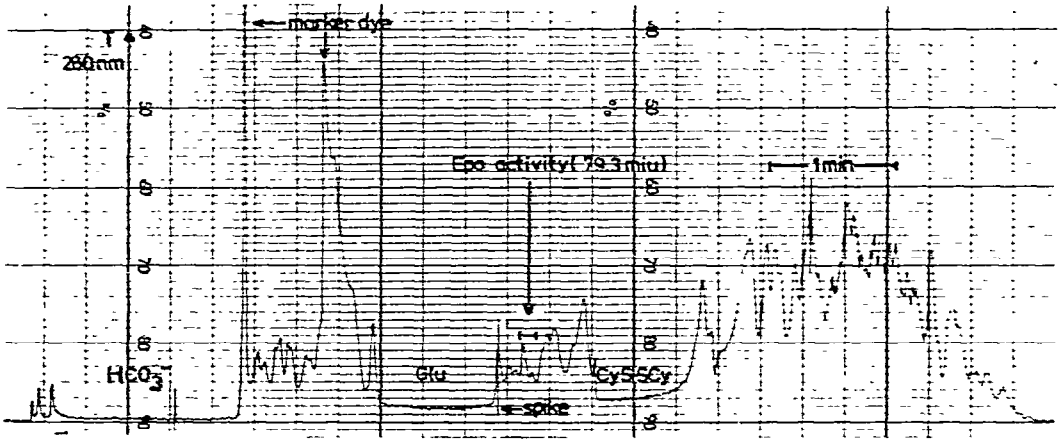

Fig. 4. UV diagram of a fractionation run. Glu and $(Cys)_2$ were used as discrete spacers. Erythropoietin activity could be detected only in the peak region marked.

RESULTS AND DISCUSSION

Preparative capillary isotachophoresis was used to purify erythropoietin with both a high yield and a high purification factor. A suitable buffer system for erythropoietin preparation was found to be 10 m*M* chloride–Tris (pH 8.00)–15.38 m*M* Gly–$Ba(OH)_2$. Chloride–ammediol (pH 9.00)–Gly–$Ba(OH)_2$, which gave a better resolution, was not useful as no erythropoietin activity could be recovered.

A modification of the method for the determination of the *f* value was tested, which gave a highly accurate isolation of components. The importance of this modification was demonstrated by measuring the influence of carbon dioxide on the *f*-value, which leads to continous changes in time–distance delay from run to run.

The mean activity yield of four determinations was 59 $\pm$ 3%. A purification factor of 228 $\pm$ 11 was calculated for the isotachophoretic fractionation. When the purification factor of benzoic acid–acetone precipitation and Sephadex chromatography is taken into account (= 25), a total factor of 5700 $\pm$ 275 for a two-step procedure was calculated from a mean activity recovery of 79.3 miu.

Compared with serum protein, uroprotein mixtures are more complex, containing large numbers of denatured proteins, peptides and fragments of these compounds. This was demonstrated by analytical isotachophoresis of chromatographic uroprotein fractions investigated with Cl^-–Tris (pH 5.8) and Gly–Tris (pH 7.2)[3] according to the method applied for macropreparation with preparative isotachophoresis on the LKB Uniphor[12]. Although the system was not optimized, up to 150 different components could be discriminated. As erythropoietin was only a minor component in our sample, a number of developments were necessary for erythropoietin fractionation. First a buffer system had to be found where the activity recovery was optimal. This proved not to be the system that gave the best resolution of components. The more alkaline pH of Cl^-–ammediol compared to Cl^-–Tris may have caused loss of sialic acid from erythropoietin, thus influencing the p*I* value and the net mobility and leading to activity spread over a wide range of mobilities with concentrations below the detection limit. This suggestion would be in agreement with Lukowsky and Painter[13], whose results of electrofocusing studies showed a significant change of erythropoietin p*I* corresponding to loss of sialic acid.

The second problem was the quantification of the protein present in the zone with erythropoietin activity. Some methods exist for component determination[14–16]. For minor components with zones thinner than the diameter of the UV slit an approximation was made by measuring the peak height in millimetres. This method is useful only when a calibration line obtained from pure standards can be calculated. This was not the case for erythropoietin; hence we were forced to estimate the real zone length of the erythropoietin zone. As calculations depending on the Kohlrausch equation were not possible because of lack of data for erythropoietin, the method described under *Protein determination* was used as an approximation. The estimation of the erythropoietin zone volume could not be carried out with high accuracy in this manner. As the zone length of any component at isotachophoretic equilibrium is directly proportional to the amount of current-transporting charges on the ions in the leading electrolyte, the Kohlrausch equation under constant conditions is reduced to

$$C_L = \text{constant} \cdot C_x$$

where C_L is the concentration of the leading ion and C_x the concentration of a dissociated component[17]. Knowing the relationship between the charge concentrations of erythropoietin and indigo tetrasulphonate, this method could be applied more accurately when physical data for a calculation are not available.

Although the method applied to the protein determination is coarse and more exact methods may lead to a lower yield and purification factor, preparative capillary isotachophoresis seems to be a very successful method. The application of a protein mixture with a higher original erythropoietin activity may open up new possibilities in the field of erythropoietin characterization, purification and quantitation.

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