A New Preparative Method for Isolation of Human Erythropoietin With Hydrophobic Interaction Chromatography

By Sylvia Lee-Huang

A new preparative method for isolation of human urinary erythropoietin (Ep) has been developed using hydrophobic interaction chromatography on Phenyl-Sepharose CL4B. Crude urine and urine concentrates from anemic patients were used directly without prior manipulation. In addition to facilitating hydrophobic interactions, Phenyl-Sepharose provided π-π interactions between its phenyl group on the gel matrix and the aromatic amino acid residues of Ep, and thus contributed to specific resolution. Over 90% of the urinary contaminants were excluded from the column, and Ep was selectively bound. Its activity was eluted with 20% ethylene glycol in 10 mM NaOH containing 4 M guanidine hydrochloride. This single step offered a mean purification factor of 110 with a recovery of 85%.

RECENTLY, we have studied the application of double-binary complex affinity chromatography in the purification of human urinary Ep, using Con-A-Sepharose 4B, wheat germ (WG) lectin-Sepharose 6MB, and phytohemagglutinin (E) (PHA(E))-Sepharose 4B. We have learned that although these affinity absorbents are effective in removing certain contaminants, they also bind irreversibly to urochrome pigments, urobilin, hematoporphyrin, and many other urinary impurities. Their binding capacities cannot be fully regenerated even with 1 M borate, 4 M sodium chloride, and 0.1% sodium deoxycholate. Thus, their resolving powers are impaired upon constant use on crude starting material. Furthermore, lectins in general are sensitive to proteolytic degradation, and urine samples are rich in these enzymes. Such operational drawbacks restrict the effective application of these lectins in the direct processing of crude urine samples. In search of practical alternatives, we investigated the use of hydrophobic interaction chromatography on Phenyl-Sepharose CL4B. This crosslinked neutral gel is biologically stable and its capacity can be fully regenerated. Most significantly, it provides a unique separation mechanism, i.e., hydrophobicity. This technique is effective in the fractionation of Ep from its urinary contaminants with similar size, charge, and monosaccharide content. Various adsorption and desorption parameters have been investigated, and optimal conditions under which Ep can be quantitatively adsorbed and effectively eluted have been established. The results of these studies are reported in this article.

MATERIALS AND METHODS

Materials

Chromatographic materials and chemicals were purchased from the following sources: Phenyl-Sepharose CL4B from Pharmacia, Inc. (Piscataway, N.J.); ethylene glycol, sodium chloride, and sodium phosphate from Fisher (Fair Lawn, N.J.); ultrafiltration membranes and hollow fibers from Amicon Corp. (Lexington, Mass.); 59FeCl3 (specific activity 22–30 mCi/mg) from New England Nuclear, Boston, Mass.; guanidine hydrochloride (ultrapure) from Schwarz/Mann (Orangeburg, N.Y.).

Erythropoietin

Two sources of starting material were used for our purification work. (1) Crude urine concentrates were distributed by NHLBI with the authorization of Dr. Anne P. Ball. These samples were designated as AL10-26, and their potencies ranged from 0.6 to 1.2 U/mg of protein. (2) Urine samples were also kindly supplied by Dr. Robert Silber and Dr. Jamshid Javid of New York University Medical Center. These samples were collected from severely anemic patients, suffering from leukemia and other chronic forms of anemia. The Ep titer of these materials ranged from 1 to 5 U/ml. Protein concentration was determined by the Lowry procedure1 and the Bradford microassay.2

Bioassay

The biologic activity of Ep was assayed in vivo using the exyphoxic polycythemic mouse method, according to the general procedure of Camiscoli and Gordon3 with some modifications as specified in the following. Briefly, CF-1 virgin female mice, 22–25 g in body weight, purchased from Charles River, were used throughout these experiments. Polycythemia was induced by hypobaric hypoxia. The animals were exposed to 0.4 atmospheres for a total of 219 hr at 19 hr/day in a decompression chamber. Ep samples for assay were made up in a solution of 0.5% albumin in 0.15 M NaCl. Testing samples were injected intraperitoneally (i.p.) in a single dose on the third day (72 hr) post hypoxia, followed by 0.5 μCi59FeCl3 intravenously (i.v.) on the fifth day. Ep activity was measured by its stimulation of 59Fe incorporation into the circulating red cells 48 hr later by way of cardiac puncture. The percent incorporation of 59Fe was determined on 0.5 ml of blood sample in a Beckman Gamma 4000 counter. The second International Reference Preparation (2nd IRP) of human urinary Ep obtained from the World Health Organization (WHO) was used as a standard. The potency was expressed in units per milligram of protein. One unit of Ep is defined as the activity contained in 0.5 mg of the 2nd IRP from WHO or 1/10 of the contents of an ampule of this preparation. Hematocrits were determined in duplicates by the microhematocrit method.

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method, using an Autocrit II centrifuge from Clay Adams. Results from animals with a hematocrit below 0.52 were discarded. Dose level means were based on quadruplicates. Dose responses were calculated on the basis of a linear regression of percent $^{57}$Fe incorporation on the logarithm of the dose of Ep. Statistical analyses were carried out by a special program on a TI 59 programmable calculator (Texas Instruments). The following statistical data were obtained: $b$ (slope of dose) 25-30, $S_b$ (standard deviation of slope) 1.1-3.3, and $S_m$ (standard deviation from regression) 2.3-3.8.

**Proteases and Sialidases Activities**

Urinary protease was assayed by its ability to solubilize vitamin-free casein. One unit is defined as the amount of enzyme that liberates acid-soluble fragments equivalent to 0.001 A$_{280}$/min at 37°C. Sialidase activity was measured by its ability to promote the release of sialic acid from bovine sialyl lactose according to the general procedure of Cassidy et al. Both of the colorimetric methods (thiobarbituric and alkali-Erlich) of Aminoff were used for the determination of free sialic acid. One unit is defined as the amount of enzyme that causes the release of 1 umole sialic acid/min at 37°C under the assay conditions.

**RESULTS**

Both Octyl and Phenyl-Sepharose CL4B were examined for their efficiency in facilitating hydrophobic interaction chromatography of Ep. Both Sepharose derivatives bound Ep quantitatively. However, the binding between Ep and Octyl-Sepharose CL4B is too firm to be eluted with good yield. On the other hand, Phenyl-Sepharose CL4B is effective. Although its phenyl group generally displays a hydrophobic intermediate between the straight-chain alkyl n-butyl and n-pentyl groups, it offers adequate binding for both good resolution and high yield. It also provides specific $\pi-\pi$ interactions with the aromatic amino acid residues of Ep and contributes to specific resolution.

**Purification of Crude Urine Concentrates**

Crude urine concentrate from NHLBI, containing 5000 mg protein, was dissolved in 10 mM sodium phosphate, pH 6.8, containing 4 M NaCl (buffer A) to a concentration of 10 mg/ml by gentle stirring for 20 min. Any insoluble material was removed by centrifugation at 10,000 g for 30 min. The clear supernatant solution contained 3300 U Ep with a potency of 0.66 U/mg protein. The sample was applied to a column (2.5 x 81.5 cm, bed volume 400 ml) of Phenyl-Sepharose CL4B, previously equilibrated with buffer A. Complete equilibration of the column is essential for its selective binding of Ep via hydrophobic interaction. Index of refraction ($n$) was used for fast and sensitive monitoring of the equilibration. For buffer A, $n$ is 1.3696; this value was matched exactly with that of the column effluent before the loading of the sample. Fractions of 25 ml were collected at a flow rate of 1.5 ml/min. Unbound impurities were washed with buffer A until the absorbance at 280 nm of the effluent was zero. The elution profile is shown in Fig. 1. Over 90% of the urinary contaminants were excluded, and no Ep activity was found in this region (peak I of Fig. 1). The column was then eluted with 10 mM sodium phosphate, pH 7.1, containing 0.5 M NaCl (buffer B). Two peaks were eluted in this region. The major peak (peak IIA), fractions 62-74, contained 1.6% of input protein with little detectable Ep activity. The minor peak (Peak IIB), fractions 75-88, represented 0.2% of the protein input and 9% of Ep units. The size and activity of peaks IIA and B varied, depending on the source of the starting material. Ep activity was eluted with 10 mM NaOH containing 20% ethylene glycol and 4 M guanidine hydrochloride (buffer C). Fractions 103-115 (peak III), containing the bulk of Ep activity, were pooled. The resulting solution, 35.42 mg protein (0.7% of input) in 325 ml, was concentrated by ultrafiltration using an Amicon YM10 membrane. The concentrated sample was then buffer changed thoroughly against PBS (5 mM sodium phosphate containing 0.15 M NaCl) to remove residual guanidine hydrochloride for further purification and for bioassay. Incomplete removal of this dissociation agent affects the testing animal and the assay results.

This step resulted in a 120-fold purification with 85% yield and a potency of 79.2 U/mg protein for this particular preparation. In general, a purification factor of 100-120 was obtained with 75-85% yield. The potency ranged from 50 to 210 U/mg protein, depending on the initial potency of the starting material.

![Fig. 1. Chromatography of human urinary Ep on Phenyl-Sepharose CL4B. This is the elution profile of crude urine concentrate on the column. The arrows indicate buffer changes as specified in the text. The Roman numerals represent pools made for in vivo bioassay as described in Results.](image-url)
**Direct Processing of Unconcentrated Urine**

In addition to urine concentrates, unconcentrated urine samples can also be applied directly to the Phenyl-Sepharose column without any prior treatment. Some of the samples collected at our New York University Medical Center were routinely processed in this manner. Urine samples were obtained by 24-hr collections from anemic patients. Any insoluble material was removed by centrifugation or filtration. The sample was either loaded directly onto the column or processed by an Amicon DC-2 concentrator/dialyzer, using a HIP10 cartridge (10,000 mol wt exclusion), and then applied to the column. The fractionation results of these samples are given in Table 1, and no significant difference was observed. Concentration and dialysis of the crude urine with the hollow fiber HIP10 resulted in the removal of low molecular weight contaminants, and thus in an increase of starting potency. However, it did not lead to a better yield and/or purification on the subsequent Phenyl-Sepharose column. This is due to the lack of hydrophobic interaction between the low molecular weight urinary impurities and the Phenyl-Sepharose gel. Any material eliminated by the hollow fiber would also be excluded from the column along with other contaminating proteins. Similarly, our results indicate that although heating, phenol extraction, ethanol fractionation, benzoic acid treatment, and deionization on Sephadex G25 also removed considerable amounts of urinary contaminants, their use prior to the Phenyl-Sepharose step did not result in any further degree of purification than that obtained by the Phenyl-Sepharose column alone, since the impurities they removed were also excluded by the column. Operationally, direct loading of the unconcentrated urine saved initial manipulation and enabled immediate processing, but it also took relatively longer to run the column due to its larger starting volume. Concentration/dialysis, on the other hand, required additional time and handling; however, the fractionation of the column was speeded up significantly due to the reduction of the sample volume. For routine purification, the urine samples were processed directly on the hydrophobic column without any prior manipulation. When the supply of urine was abundant, the samples were concentrated, dialyzed, and stored at -20°C in small lots for later purification.

**Remarks and Conclusions**

The high salt concentration in the starting buffer is unique in processing crude urine samples, since it also stabilizes the Ep activity by inhibiting the action of urinary proteases and sialidases. In buffer A, at 4 M NaCl, no protease and sialidase activity could be detected in the crude urine, whereas at 0.1 M NaCl, 1.6 U of protease and 0.1 mU of sialidase were found per milliliter of the same urine sample.

The driving force of hydrophobic interaction depends mainly on the gain in entropy obtained because of decreases in contacts of the hydrophobic groups with water. Structure-forming agents such as NaCl and certain neutral salts at high concentration tend to stabilize the structure of water and thus enhance the association of hydrophobic groups. Conversely, structure breaking agents (chaotropic

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**Table 1. Hydrophobic Interaction Chromatography of Erythropoietin on Phenyl-Sepharose CL48**

<table>
<thead>
<tr>
<th>Experiment/Fraction</th>
<th>Absorbance Unit (A)</th>
<th>Potency Unit (U)</th>
<th>Potency Percent Recovery of Activity</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Unconcentrated urine</td>
<td>3,269</td>
<td>3,596</td>
<td>1.1</td>
<td>100</td>
</tr>
<tr>
<td>Input</td>
<td>3,055</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>Buffer A (excluded)</td>
<td>110</td>
<td>605</td>
<td>5.5</td>
<td>17</td>
</tr>
<tr>
<td>Buffer C eluate</td>
<td>26</td>
<td>3,016</td>
<td>116</td>
<td>84</td>
</tr>
<tr>
<td>Buffer B eluate</td>
<td>104</td>
<td>530</td>
<td>5.1</td>
<td>17</td>
</tr>
<tr>
<td>Buffer C eluate</td>
<td>22</td>
<td>2,596</td>
<td>118</td>
<td>82</td>
</tr>
<tr>
<td>II. Concentrated/dialyzed urine (DC-2, HIP 10)</td>
<td>1,980</td>
<td>3,168</td>
<td>1.6</td>
<td>100</td>
</tr>
<tr>
<td>Input</td>
<td>1,782</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>Buffer A (excluded)</td>
<td>104</td>
<td>530</td>
<td>5.1</td>
<td>17</td>
</tr>
<tr>
<td>Buffer C eluate</td>
<td>22</td>
<td>2,596</td>
<td>118</td>
<td>82</td>
</tr>
</tbody>
</table>

*Two liters of crude urine were divided into 1-liter portions. Portion I was directly loaded onto the column, whereas portion II was concentrated/dialyzed by an Amicon DC-2 apparatus using a HIP10 cartridge and then applied to the column.
†Protein determination is not reliable in crude urine and in the excluded fraction (buffer A effluent); for this reason, absorbance unit at 278 nm was used.
§Potency is expressed as U/A, units of in vivo activity per absorbance unit, measured at 278 nm in 1 cm cuvette. In vivo activity was determined by the exhypoxic polycythemic mouse method.
and/or polarity reducing agents) such as ethylene glycol or glycerol tend to enhance the dissolution of hydrophobic groups in water, weaken hydrophobic interactions, and thus cause sample elution.

During the design and development of the purification procedure, many pilot runs were made with variations on the final method. The following results summarize our experience. (A) For effective desorption of Ep from Phenyl-Sepharose CL4B column, elution in the presence of ethylene glycol is essential. This polarity-reducing agent is very efficient in decreasing the strength of hydrophobic interactions and in eluting Ep activity. Various combinations of elution schedules with 0%–50% ethylene glycol have been studied. The optimum concentration for maximum purification as well as recovery is 20%. (B) pH is also an important factor in Ep elution. In 20% ethylene glycol, the elution characteristics were also examined under various conditions of pH and ionic strength. The yield was 35%–45% in H2O; 40%–50% in 0.5 mM sodium phosphate, pH 7.1; 50%–60% in 3 mM Na2HPO4, pH 9.6; and 75%–85% in 10 mM NaOH, pH 12, containing 4 M guanidine hydrochloride. The inclusion of the dissociation agent in the eluant enabled effective release of Ep activity from the hydrophobic gel. Ethylene glycol, guanidine hydrochloride, and alkalinity showed no detrimental effect on the activity of Ep. (C) Ammonium sulfate is a strong structure-forming agent; it has an excellent salting out effect for many proteins. However, in the case of Ep, it is unexpectedly inefficient. It failed to promote any appreciable hydrophobic interaction at a concentration of 1 M, while at 3 M, it partially precipitated the glycoprotein.

The binding capacity of the column depends on experimental conditions and on the nature of the contaminants in the starting urine. In buffer A, we found that the optimum ratio of mg protein added to bed volume (ml) was 9–13. The scale of preparations is flexible; in addition to 20–50 mg small size runs, large scale preparations have been carried out with starting materials ranging from 200 to 5000 mg protein. Removal of tightly bound material and regeneration of the gel are required before reuse of the column. We have found that this can be achieved by washing the column successively with 2–3 bed volumes of 50% ethanol and then H2O. Multiple peaks with high absorbance at 260 nm were eluted in this region, and usually no Ep activity was detected. However, if large columns were used (the ratio of mg protein to bed volume [ml] was below 1) and extensive wash-in was performed (20 bed volumes), some Ep activity (10%–15%) was also found in this region with certain lots of starting material. This may be due to the fact that more strong binding sites of the gel are made available under these conditions.

**DISCUSSION**

Aside from the problems due to the extremely low initial content of Ep in urine, purification of the hormone is quite difficult to achieve because it is contaminated with many urinary impurities with similar physiochemical properties. Many of the existing purification procedures are based on either conventional charge and size separations, sugar-specific affinity for lectin derivatives, or preparative isoelectric focusing. We thought a simple prior step separation on the basis of a different and independent property, hydrophobicity, might be important for the elimination of contaminating impurities from Ep with similar size and charge as well as similar monosaccharide content. We decided, therefore, to study the use of hydrophobic interaction chromatography in Ep purification. Our experimental results showed excellent potential and general applicability of the procedure. It is especially well suited for initial processing of crude starting material. Urine concentrates and unconcentrated urine samples can be applied directly to the column without any prior treatment. In bypassing such steps, unnecessary handling of the sample was eliminated, and the yield was increased accordingly. The studies we reported here are by no means exhaustive in defining the optimal conditions of this technique for Ep purification, but they represent a systematic investigation of some of the important parameters for high resolution and good recovery.

Affinity chromatography on Con-A-Sepharose 4B followed by WG-Sepharose 6MB or PHA(E)-Sepharose 4B served equally well as initial steps in processing crude urine concentrates. However, as already mentioned, the urinary pigments and other impurities bind irreversibly to these lectins and full regeneration of their capacities has not been possible. The cost of these columns and their maintenance is exceptionally high. In addition, their eluants, N-acetylglucosamine or N, N-diacetylglucosamine, are much more expensive than NaOH and ethylene glycol. The operational expenses are too great to justify the routine use of these lectins for preparative processing of crude urine samples. However, the incorporation of these techniques into the purification procedure at a later stage, after the initial hydrophobic interaction chromatography, proved to be valuable. The majority of urinary pigments were removed by Phenyl-Sepharose CL4B, and full regeneration of these lectins can thus be
 achieved. Furthermore, the required size of these columns was also effectively reduced. Considerable savings in time and cost were thereby achieved.

REFERENCES


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