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Note

High-performance liquid chromatographic separation of serum erythrotropin and erythropoietin

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The development of bioassays of erythropoietic activity using the incorporation of [^{3}H]thymidine into erythroid cells has been praised as one of the most sensitive and simple methods for the bioassay of erythropoietin [1—3]. Unfortunately, this method may detect the erythrotropins, a new family of erythroid cell-stimulating factors which were originally isolated from fetal calf intestine [4]. The erythrotropins stimulate globin chain synthesis and thymidine incorporation into acid-insoluble materials in fetal bovine liver cells and act synergistically with erythropoietin in cultures of rat liver cells [4, 5]. Fetal bovine serum contains large amounts of an erythrotropin which is similar to the intestinal erythrotropin I [6]. It is then essential to develop a method for the separation of serum erythrotropin and erythropoietin in order to quantitate both peptides using the thymidine incorporation assays. In this paper we describe the reversed-phase high-performance liquid chromatographic (HPLC) separation of serum erythrotropin and sheep plasma erythropoietin and show how the fetal bovine assay is particularly sensitive for the detection of serum erythrotropin in samples of human blood.

MATERIALS AND METHODS

Sheep plasma erythropoietin was obtained from Connaught Labs. (Toronto, Canada). Serum erythrotropin was isolated from fetal bovine serum (Flow Labs., Rockville, MD, U.S.A.) using a method to be described in detail elsewhere [6] but which consists essentially of the reversed-phase extraction of serum and the purification of erythrotropin by reversed-phase and gel permeation HPLC as described for the isolation of the intestinal peptides [4]. In some

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experiments samples of human cord blood or human fetal plasma were analyzed directly by HPLC without reversed-phase extraction. A small volume of serum or plasma was acidified with 8 vols. of ice-cold 0.1% (v/v) trifluoroacetic acid (TFA), centrifuged at 10,000 *g* for 10 min and the supernatant was applied to the columns. Usually 80—100 μl of serum were used but in some instances larger amounts of serum, up to a total protein content of 10 mg as measured by the method of Bradford [7] were applied onto the columns.

The separation of erythropoietin and erythrotropin was carried out with two μBondapak C_{18} columns (Waters) and a C_{18} silica precolumn (Whatman No. 6561-403) connected in series as described in detail for globin chain separation [8]. The column was washed at room temperature with a mixture of acetonitrile—water—TFA (280:720:1) for 15 min at a flow-rate of 1.5 ml/min. The sample to be analyzed was pumped onto the columns. In some cases 10 μg coproporphyrin I (Sigma) was added as a marker to better localize the fractions containing erythrotropin. The fractions were eluted from the column using a linear gradient for 40 min starting with a mixture of acetonitrile—water—TFA (280:720:1) and finishing with 600:400:1. Fractions of 1.5 ml were collected. Each fraction was mixed with 0.5 ml acetonitrile containing 2-mercaptoethanol and TFA in order to obtain a final concentration of 3 m*M* mercaptoethanol and 0.1% TFA. Aliquots (or the complete fractions) were evaporated using a Speedvac evaporator (Savant) and suspended in 0.75 ml of F-12 medium [4]. Aliquots of 0.25 ml were used for the thymidine incorporation bioassay as described previously [4].

RESULTS AND DISCUSSION

Although the erythrotropins have been isolated using extraction and separation procedures in the presence of strong acids, it is known that erythropoietin has sialic acid residues which may be destroyed under acidic conditions [9]. However, the in vitro activity of asialoerythropoietin should remain unchanged. We found that sheep plasma erythropoietin fractionated on reversed-phase HPLC columns was able to stimulate globin chain synthesis and uridine uptake in cell cultures of fetal bovine liver [10]. We also found that thymidine incorporation into acid-insoluble materials in cell cultures of fetal bovine liver is a very sensitive bioassay for both erythropoietin and erythrotropins [4, 5]. Furthermore, these factors have different retention times on reversed-phase HPLC and therefore it should be possible to separate them using the appropriate gradients of acetonitrile in the presence of TFA. Fig. 1 shows the chromatogram of a mixture of bovine serum erythrotropin and sheep plasma erythropoietin separated with two C_{18} μBondapak columns as indicated in Materials and methods. A 7.5-mg amount of the reversed-phase extract from fetal bovine serum was purified by reversed-phase and gel permeation HPLC (steps I and II of ref. 4). The purified erythrotropin was mixed with 10 μg coproporphyrin I and 5 mg step III sheep plasma erythropoietin (3 U/mg) in 0.3 ml of 0.2% TFA and pumped directly onto the columns. Aliquots of 30 μl (1/67 of each fraction) were evaporated and used for the thymidine bioassay as previously described [4, 5]. Fig. 1 shows that there is an excellent separation of serum erythrotropin (ET) and erythropoietin (EP) in less than 40 min.

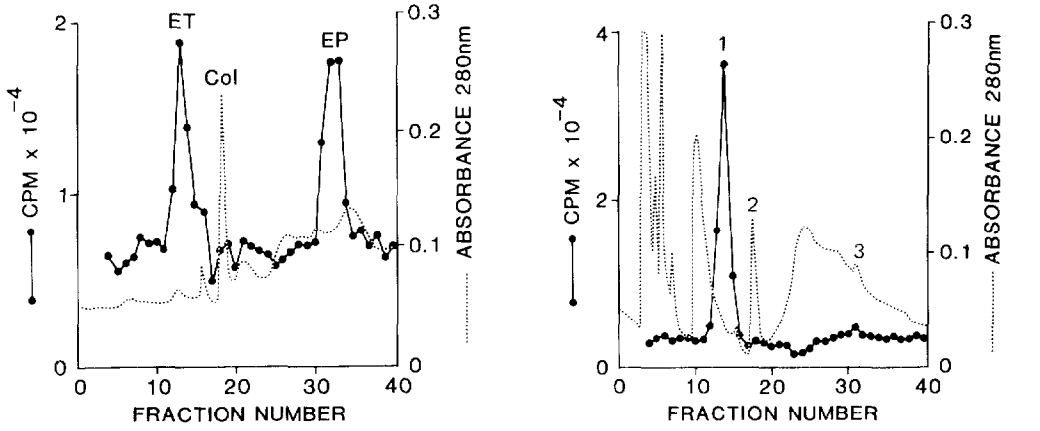

Fig. 1. Separation of erythrotropin (ET) prepared from fetal bovine serum and step III sheep plasma erythropoietin (EP) using two reversed-phase columns as indicated in the text. Coproporphyrin I (CoI) was added as an internal marker for the localization of ET and can be used to separate the fractions containing ET from those containing EP. The bioactivity is expressed as cpm of [^{3}H]thymidine incorporated into acid-insoluble materials in cell cultures of fetal bovine liver.

Fig. 2. Human cord blood serum was acidified and pumped directly onto two reversed-phase columns as indicated in the text. The bioactivity was measured as indicated in the legend of Fig. 1. 1 = Bioactivity corresponding to ET; 2 = coproporphyrin I; 3 = erythropoietin.

Furthermore, the addition of coproporphyrin I (CoI) can be very useful because its pink color can be used as a marker to localize erythrotropin and erythropoietin and as an internal standard to test the correctness of the chromatographic conditions.

The very large amounts of erythrotropin present in fetal bovine serum and human cord blood [6, 11] suggest that it should be possible to carry out a direct HPLC analysis of very small serum samples without a previous reversed-phase extraction. Fig. 2 shows the separation of 10 mg human cord blood serum which was acidified with 0.1% TFA as indicated in Materials and methods and applied to the column. Aliquots of 30 μl were used for the assay. The absorbance peak eluting after the fraction with thymidine incorporation activity corresponds to coproporphyrin I. The fraction which stimulated thymidine incorporation had the same retention time as bovine erythrotropin. Note that there is practically no bioactivity eluting in the positon of sheep erythropoietin. This has been also observed in other samples of cord blood. It is unlikely that human plasma erythropoietin (which is not commercially available) would elute at a complete different position from that of sheep plasma erythropoietin. The apparent absence of detectable amounts of erythropoietin could be explained by high lability of the human hormone but is most likely due to the very low amounts of erythropoietin in newborns [12], which is usually 30—40 mU/ml. Samples of 100 μl serum or less should be at the limits of the sensitivity of the bioassay. The amounts of erythropoietin indicated above were measured using cell culture assays with mice liver erythroid cells [12]. These determinations probably reflect the amounts of erythropoietin rather than erythrotropin for two reasons. First, the assays are carried out

in media supplemented with serum, this means in the presence of erythrotropin. Secondly, it is possible that erythroid cells from mice and probably other rodents are less sensitive to erythrotropin than fetal bovine cells, as has been shown in the case of rat liver cells [5]. Direct analysis of blood samples by HPLC will be particularly valuable in those cases where the amounts of blood available are very small. This is the case of a fetal blood plasma sample obtained from a fetus at the time of prenatal diagnosis of thalassemia, as illutrated in Fig. 3. A 100-μl aliquot of plasma was acidified with TFA and pumped directly onto the columns. Aliquots of 1 ml (one half of the fractions) were used for the bioassay. The thymidine incorporation stimulating activity is again associated with the elution position of erythrotropin and practically no activity is associated with the elution position of erythropoietin. It is interesting to see the presence of an inhibitor of thymidine incorporation eluting after erythrotropin. It remains to be seen if this inhibitor is typical of blood samples at midterm or a single case observed in this plasma sample.

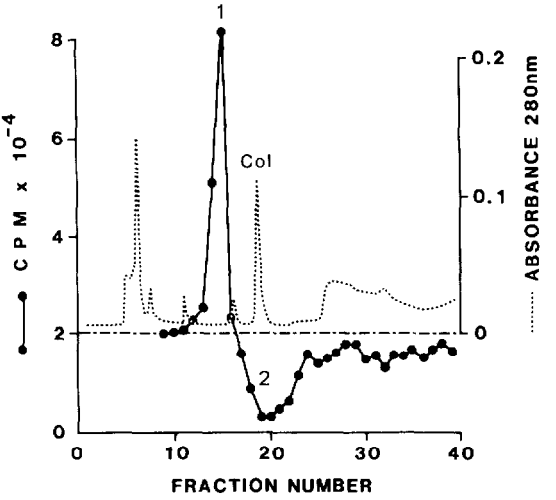

Fig. 3. Human fetal plasma taken from a fetus at midterm for prenatal diagnosis of thalassemia was acidified and pumped directly onto two reversed-phase columns as indicated in the legend of Fig. 2. The dotted line parallel to the abscissa indicate the control value which was observed in cell cultures without serum. CoI = coproporphyrin I; 1 = bioactivity corresponding to ET; 2 = inhibitor.

The chromatographic separation of serum erythrotropin and erythropoietin described above will be useful for the simultaneous determination of both erythroid cell stimulating factors with the very sensitive in vitro bioassays of thymidine incorporation. Furthermore, direct HPLC analysis of serum or plasma will be useful for the detection of erythrotropin. Further experiments indicate that this method may be applied for samples of adult patients. However, it is very important to eliminate proteins precipitated with TFA by centrifugation and to use a precolumn as indicated for globin chains [8] in order to prevent damage of the expensive reversed-phase columns.

NOTE ADDED IN PROOF

Human urinary erythropoietin has a similar retention time to sheep plasma erythropoietin in the HPLC system described above.

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