Ann. Ist. Super. Sanità
Vol. 25, N. 3 (1989), pp. 471-480

ANALYTICAL AND REGULATORY CONSIDERATIONS FOR FERRITIN-CONTAINING PHARMACEUTICAL PRODUCTS

G. CAVINA, B. GALLINELLA, F. LA TORRE and R. PORRA'

Laboratorio di Chimica del Farmaco, Istituto Superiore di Sanità, Rome, Italy

Summary. - *Ferritin, an iron-containing protein widely diffused in nature, has the important biochemical function of being the principal reserve and regulator for Fe^{3+} levels in blood and tissue. Due to its natural, effectively non-toxic iron content, ferritin has been the object of strong interest in the development of pharmaceutical products for use in iron deficiency treatment. Therefore, the need has arisen for the analytical characterization of this industrial product, be it in the hydroglyceric solution or dry powder form. The main considerations for the characterization of industrial ferritin are the following: a) iron content in both the unmodified product and the precipitated protein; b) protein content and protein/iron ratio; c) protein identification by means of polyacrylamide gel electrophoresis (PAGE); d) confirmation of protein identity and evaluation of molecular weight by means of size exclusion chromatography; e) identification and evaluation of other components of bulk ferritin preparations such as preservatives, excipients for lyophilization, water and concomitant proteins. Eight different samples were examined using the above considerations. Among the qualitative and quantitative results reported, of particular interest are those obtained by means of size-exclusion high performance liquid chromatography (SEC-HPLC). With a UV "diode array" detector it is possible to discern the peaks for ferritin and its molecular aggregates from those of concomitant proteins and preservatives; furthermore, it is possible to evaluate their molecular dimensions. Using this method, the ferritin monomer and other protein fractions can be quantitatively analyzed either by calculating area percent distribution of the chromatographic peaks or by comparing the sample with a highly pure external standard of ferritin. The parallel determination of iron in the eluted protein fractions allows for a determination of the protein/iron ratio directly from the monomer peak. The results indicate that the proposed tests and assays are each necessary and inseparable elements of a context which must remain integral for a correct, primary evaluation of the qualitative and quantitative characteristics of the samples.*

Riassunto (Considerazioni analitiche e di regolamentazione per farmaci contenenti ferritina). - *La ferritina è una proteina contenente ferro, largamente diffusa in natura ed ha la funzione biochimica molto importante di agire da riserva per il ferro ionico ematico e tessutale, regolandone i livelli a valori non tossici. Per le sue caratteristiche di composto naturale di ferro, praticamente non tossico, ha destato interesse per impieghi farmaceutici negli stati di carenza da ferro. Ne è derivata la necessità di caratterizzare dal punto di vista analitico-farmaceutico questa materia prima, generalmente prodotta o allo stato di soluzione in veicolo idroglicerico o allo stato di polvere secca. I parametri considerati fondamentali per caratterizzare il prodotto sono risultati i seguenti: a) contenuto in ferro, totale e legato alle proteine; b) contenuto in proteine e conseguente rapporto proteine/ferro; c) identificazione delle proteine mediante elettroforesi su gel di poliacrilammide; d) conferma dell'identità delle proteine e stima dei loro pesi molecolari mediante cromatografia ad esclusione dimensionale; e) identificazione di altri componenti della preparazione quali proteine concomitanti, conservanti, eccipienti di liofilizzazione. Sono stati esaminati otto differenti campioni relativamente ai parametri sopra considerati. Fra i risultati qualitativi e quantitativi ottenuti sono particolarmente interessanti quelli forniti dalla cromatografia di esclusione ad alte prestazioni (SEC-HPLC), in particolare impiegando un rivelatore UV del tipo "diode array" che consente di identificare il picco della ferritina e suoi aggregati molecolari in presenza di quelli di proteine concomitanti e di conservanti, valutandone anche le dimensioni molecolari. Con tale metodo le determinazioni quantitative delle varie frazioni proteiche, in particolare della ferritina monomero, possono essere ottenute in area per cento o mediante standard esterno, usando come riferimento un campione di ferritina di elevata purezza: la parallela determinazione del ferro sugli eluati consente di determinare il rapporto proteine/ferro operando direttamente sul picco del monomero. Dai risultati ottenuti appare chiaro che tutti i saggi propo-*

sti sono da considerare come un insieme inscindibile per una corretta valutazione delle caratteristiche qualitative e quantitative dei campioni.

Introduction

Ferritin, a widely diffused iron-containing protein, is found in many different types of tissue in both animals and plants.

By virtue of its biochemical function of sequestering and fixing trivalent ionic iron within its internal cavity, it represents the body's principal form of iron storage acting as a blood level regulator for this element, yet maintaining non-toxic levels.

The protein moiety of ferritin, apoferritin, can fix up to 4500 iron atoms for each molecular unity (460,000 Da), which is based on a structure of 24 subunits of two types: H (heavy), with molecular weight of about 21,000 Da, and L (light), with molecular weight of approximately 19,000 Da. The proportion of the two subunits varies with species and organ [1-4].

Ferritin is particularly concentrated in spleen, liver and bone marrow. Since it is a natural iron reserve, it has stimulated much interest as a possible non-toxic iron compound for oral treatment of iron deficient anemias.

Consequently, pharmaceutical ferritin-based preparations for oral use were developed and the production of industrial preparations by extraction from bovine and equine spleens - the meat of which is already used for human consumption - was initiated.

Due to its complex and extractive nature, this material renders the establishment of a monograph for a complete analytical characterization difficult. In an effort to develop a protocol by which to ascertain the quality of ferritin in preparations for human pharmaceutical use we were interested in coordinating a collaborative project together with several pharmaceutical manufacturers.

Herein we report the results of the preliminary experimental phase. Some methods used for the biochemical and clinical analysis of ferritin in biological fluids and after its extraction from tissues are reported in the literature [5-9]. However, these procedures are not easily applicable for pharmaceutical standardization purposes. The purification procedures are necessarily different when the preparations are for human pharmaceutical use; for example, the valuable use of the derivative formation of cadmium salts must be avoided in this case.

Bulk ferritin is produced either as a water-glycerol solution with some preservatives, or as a dry powder which is obtained by spray-drying or lyophilization.

We found the following to be the most important data in the characterization of industrial preparations of ferritin: a) iron content, in both the unmodified product and the precipitated protein; b) protein content and the protein/iron ratio; c) protein identification by means of polyacrylamide gel electrophoresis (PAGE); d) confirmation of protein identity and evaluation of molecular weight by means of size exclusion chromatography; e) identification and evaluation of other components of bulk ferritin preparations such as preservatives, excipients for lyophilization, water and concomitant proteins.

Materials and methods

The analyzed samples where as follows: samples A, E, F, G, I and P were preparations of ferritin from bovine spleen in hydroglyceric solution; samples B, H and L were dry ferritin preparations from the same type of tissue. Samples D and O were dry ferritin preparations from horse spleen and samples C and M were preparations from the same tissue in hydroglyceric solution. Ferritin, thyreoglobulin, catalase and amilase standard products for column calibration in size exclusion chromatography (SEC-HPLC) were obtained from Pharmacia (Uppsala, Sweden); bovine serum albumine (BSA) used as a reference for protein determinations was purchased from Sigma (no. A-8022, St. Louis, USA).

The colorimetric reagent for protein assay (BCA method, bicinchoninic acid reagent) was obtained from Pierce (no. cat. 23225, Rockford, USA).

The Folin-Ciocalteau reagent for protein determinations according to Lowry *et al.* [10] was obtained from Merck (Darmstadt, Germany). Acrylamide, bis-acrylamide and Temed (NNN'N'-tetramethylethylenediamine) for gel electrophoresis were purchased from LKB (Bromma, Sweden).

Coomassie Brilliant Blue R 250 was obtained from Fluka (Switzerland). Two chromatographic columns for SEC-HPLC were used: a TSK G-4000 SW 600 column, 600 x 7.5 mm (LKB, Sweden) and a Superose 6 HR 300 x 10 mm (Pharmacia, Sweden).

All other reagents used were analytical grade and obtained from Carlo Erba (Italy).

Iron content determination

In the unmodified sample. - The Abbey method of colorimetric analysis was performed [11]: a portion of the sample corresponding to 100-200 µl Fe^{3+} was transferred to a 200 ml beaker and diluted to a volume of 30 ml with water and 1 ml of 15% hydrogen peroxide and 1 ml of concentrated hydrochloric acid were added; the mixture was heated, boiled for 10 min and, after cooling, treated with 10 ml of 1 N ammonium thiocyanate (standardized and frequently controlled).

Five min after color development, absorbance was measured against a reagent blank at λ = 480 nm. The calibration curve was performed with ferrous ammonium sulphate at scalar amounts from 50 to 200 µg Fe^{3+}.

In the precipated protein fraction. - A portion of the sample containing about 2 mg Fe^{3+} was diluted to 20 ml with water and 20 ml of a saturated ammonium sulphate

solution (620 g/l) were added. The precipitate was separated by centrifugation and resuspended in 20 ml of 50% saturated ammonium sulphate solution.

After another centrifugation, the final precipitate was dissolved in 100 ml water and an appropriate aliquot of the resulting solution was analyzed as previously described for total iron content.

Protein content determination

The BCA colorimetric technique (using bicinchoninic acid as reagent) was perfomed as described in [12].

Samples containing from 5 to 10 µg protein in a 100 µl volume were mixed with 2 ml of composed BCA reagent (50 ml A + 1 ml B) and maintained at 60 °C for 30 min.

After cooling, and within 10 min, absorbance was read against a reagent blank at λ = 562 nm. The colorimetric assay described by Lowry *et al.* [10] was used as a comparative method utilizing phenol reagent according to Folin-Ciocaltaeau for color development (30 min at room temperature under subdued light). Color was stable for at least 2 h.

Readings were carried out (against a reagent blank) at λ = 660 nm. For both BCA and Lowry procedures, a calibration curve was performed using bovine serum albumine at scalar concentrations from 5 to 20 µg, and from 25 to 50 µg, respectively. Absorbances were measured with a mod. 554 Perkin-Elmer spectrometer.

Polyacrylamide gel electrophoresis

A 5% polyacrylamide gel was used. Samples of 10-20 µl containing 10-20 µg Fe^{3+} were applied into small wells preformed in the gel with an appropriate comb (26 x 12.5 cm slab).

Electrophoresis was carried out in a 2117 Multiphor LKB Instrument at +4 °C, using 0.05 M Tris-Glycine at pH 8.3 as the electrode buffer. A potential of 400 V was applied for 3 h.

Proteins were visualized after staining with a solution of Coomassie Brilliant Blue R 250 (0.2 g in 100 ml of methanol:water:acetic acid (45:45:10 v/v/v) for 60 min; destaining was performed in the same solvent mixture for 3 h with at least three solvent mixture changes.

Iron was visualized by staining a second gel slab in a freshly prepared solution of 1% $K_4Fe(CN)_6$ in 1% HCl for 30 min and destaining in a single washing with 1% HCl for 30 min.

Densitometric readings of wet slabs were performed by a Laser Densitometer LKB mod. 2202 Ultrascan.

Size exclusion high performance liquid chromatography (SEC-HPLC)

A Hewlett-Packard 1084 liquid chromatograph, equipped with integrator-recorder 7985A LC terminal and automatic injector was used; the UV detector was an HP 79875A model UV-visible variable wavelength detector.

As a second detection system, a Diode Array Detector HP mod. 1040 A was used in connection with an Analytical Workstation, an HP Thinkjet Printer and an HP Color-Pro Graphics Plotter.

Two types of columns were used: a TSK G-4000 SW 600 x 7.5 mm (LKB, Sweden) and a Superose 6 HR 300 x 10 mm (Pharmacia, Sweden).

Samples were dissolved in the elution buffer to a final concentration of 1 mg/ml of total product and filtered through a Millipore HAV P01300 filter.

The injection volume was kept at 50 µl. The mobile phase consisted of a M/15 solution of Na_2HPO_4 at pH 6.8 with 0.05% sodium azide; flow rate was 0.4-0.5 ml/min; detection was performed at λ = 280 nm.

For the normal gel filtration, a glass column (26 cm diameter, 46 cm length) (Pharmacia, Sweden) packed with Sephacryl 300 S Superfine was used. Eluent was 0.1 M NaCl in M/15 phosphate buffer, pH 6.8 and 0.05% sodium azide at a flow rate of 0.2-0.4 ml/min; monitoring was performed at λ = 254 nm using a fixed wavelength detector (Du Pont 840).

Results

Table 1 shows results obtained in the iron analysis of random lots (in powder or solution form) of current ferritin production. For each sample, results are given from the analysis of both the product in its natural form and after ferritin precipitation by treatment with ammonium sulphate at 50% saturation (620 g/l), a currently used procedure [13, 14] to separate ferritin. This treatment permits the discrimination between ferritin iron and iron salts of low molecular weight anions, which are not precipitable under these conditions.

Iron determination was performed with the ammonium thiocyanate colorimetric method described by Abbey [11] a simple, well considered and convenient method; other techniques can be used, provided they offer the necessary sensitivity.

Table 2 shows results obtained in the protein analysis of samples using a colorimetric method, based on the well-known reaction between protein and copper II salts. In this procedure based on the use of bicinchoninic acid ferritin is used as a colorimetric reagent revealing reduced Cu(I), as described in the BCA protein reagent procedure [12]. We compared this technique with the Lowry method [10], and obtained comparable data. Furthermore, we analyzed the protein nitrogen content according to the Kjeldahal method which allows determinations of ammoniacal nitrogen. This is advantageous since ammonium salts may be present in ferritin preparations as process impurities.

Table 2 also reports the values for the protein/iron content ratio. The use of this ratio is very convenient for a quick characterization of these industrial preparations of ferritin (Cetinkaya *et al.* [14]) and is preferred to the use of the iron by weight percent, as used by Harrison *et al.* [15].

Table 1. - *Results obtained from the analysis of the iron content of some commercial ferritin samples*

Sample	Label content (mg/g)	Value in unmodified sample (mg/g) *(a)*	Value in precipitated protein fraction (mg/g) *(a)*	Ratio of unmodified sample to precipitated sample
A *(b)*	15.0	16.5	14.9	110.7
B *(c)*	140.0	139.8	140.5	99.5
C *(b, d)*	15.0	16.9	15.9	106.3
D *(c, d)*	140.0	148.3	142.3	104.2
E *(b)*	20.2	20.1	19.5	103.1
F *(b)*	10.5	10.8	10.6	101.9
G *(b)*	12.1	12.3	11.9	103.4
H *(c)*	111.0	115.1	108.2	106.4
I *(b)*	15.0	15.1	13.7	110.2
L *(c)*	104.0	97.8	89.7	109.0
M *(b, d)*	20.0	18.9	19.8	95.4
N *(c)*	107.0	112.5	107.6	104.5
O *(c, d)*	150.0	150.4	142.2	105.8

(a) mean of two replicated analyses; (b) hydroglyceric solution sample; *(c)* dry powder sample; *(d)* obtained from horse spleen

It is worth noting that the industrial preparations of ferritin for pharmaceutical use are not generally purified to the same level of purity as the preparations obtained for biochemical studies. In addition, in these large-scale industrial preparations the iron content for each apoferritin unit may vary between 1500 and 2500 Fe atoms, depending on the origin and nutritional condition of the animals. This can be the cause of differences in the protein/iron ratios among various lots of production. Another possible source of ratio variation would be the presence of extraneous proteins, which may be co-isolated with ferritin. In our samples we found limits of 2.9-5.1 for protein/iron ratios. This ratio permits a rapid calculation of the percent content of the basic constituents of the preparations, i.e. iron and protein.

The identification of ferritin was first performed by means of polyacrylamide gel electrophoresis (PAGE) following the Zamiri and Mason [16] procedure, i.e. 5% gel concentrations having modified the buffer and pH (0.05 M, 8.3).

Table 2. - *Results obtained from the analysis of the total protein content of commercial ferritin samples and protein/iron content ratios*

Sample	BCA method protein content (mg/g) *(a)*	Lowry method protein content (mg/g) *(a)*	Ratio of protein and iron content *(b)*
A	43.7	48.3	2.6
B	460.8	459.2	3.3
C	48.8	48.3	2.9
D	423.3	448.8	2.8
E	85.5	86.9	4.2
F	43.7	44.6	4.0
G	61.2	53.1	5.0
H	416.2	451.7	3.6

(a) mean of two replicated analyses; *(b)* values from BCA method and from Table 1 (iron in the precipitated protein fraction) used for the ratio

Observing the anodic-moving bands, starting from the cathodic origin, we find different molecular aggregates: near the origin, one or two bands representing heavy polymers (first aggregate); a "dimer" band (second aggregate); a monomer band; and further along the gel, if present, traces of proteins with molecular weights lower than that of ferritin. A similar distibution of aggregates has been described elsewhere [3, 15].

With this technique it is possible to distinguish equine from bovine ferritin, the latter giving a less mobile monomer band [16].

It is also possible to submit the PAGE slabs, stained with Coomassie Brilliant Blue R-250 (as a protein reagent) [17], and with potassium ferrocyanide (as an iron III reagent) [9, 13, 14], to a densitometric reading, thereby obtaining traces which correspond to the different molecular aggregate bands, as revealed by the two reagents. Fig. 1 shows the comparison of the migration of different samples on the same slab and Figs 2 and 3 show examples

Fig. 1. - PAGE of samples A, B, C, E, F, G, H, D; for each sample two consecutive lanes were spotted at 20 and 10 μg, respectively. Staining with Coomassie Brilliant Blue reagent. 1) first aggregate a) (polymer); 1') first aggregate b) (polymer); 2) second aggregate (dimer); 3) monomer; 4) low molecular weight protein band. Samples C and D are representative of equine spleen ferritin.

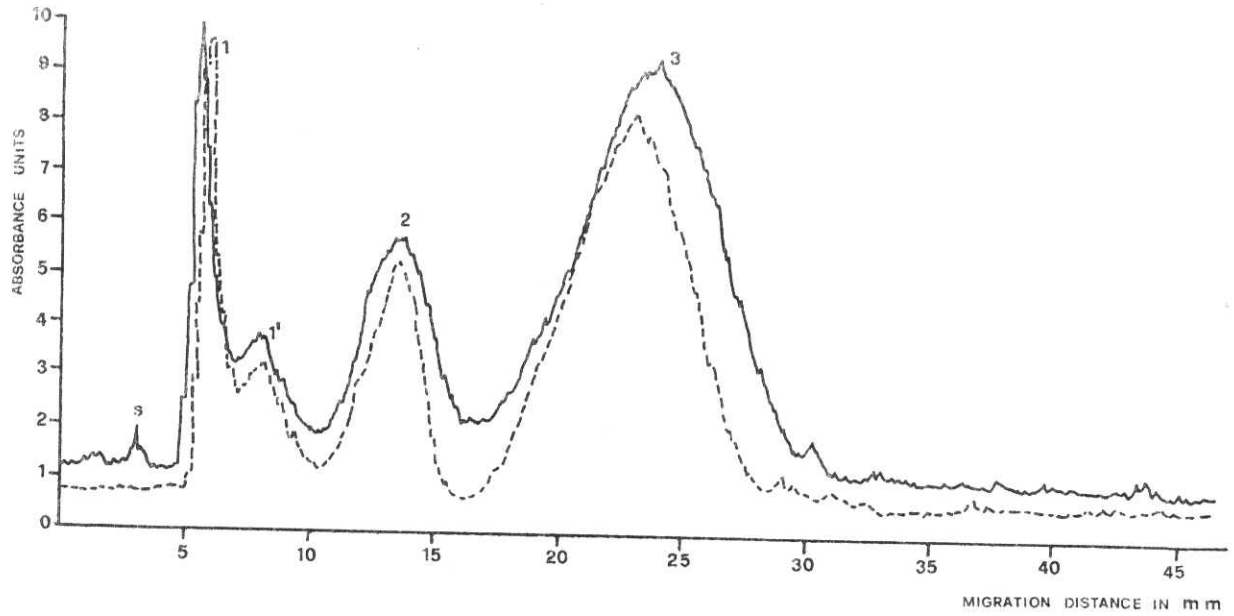

Fig. 2. - Densitometric reading of sample E (extract of bovine spleen, solution), spotted amount 20 µg. Staining with Coomassie Brilliant Blue reagent —— and iron reagent - - - - - S) start; 1) first aggregate a) (polymer); 1') first aggregate b) (polymer); 2) second aggregate (dimer); 3) monomer; 4) low molecular weight protein band.

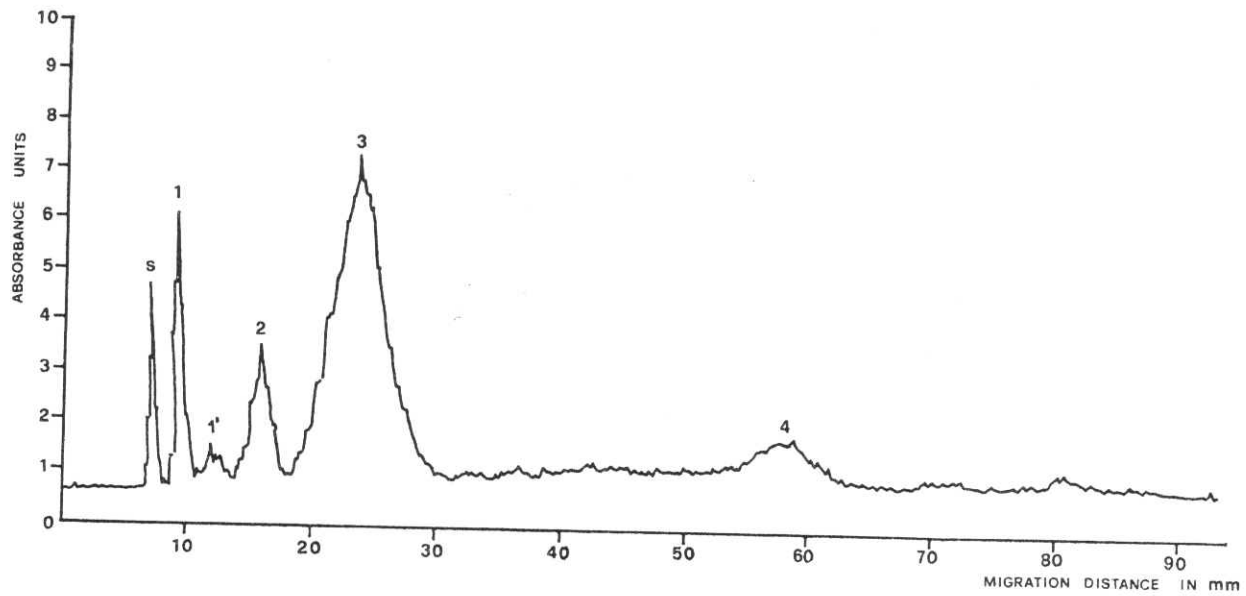

Fig. 3. - Densitometric reading of sample A (extract of bovine spleen, solution), spotted amount 20 µg; staining with Coomassie Brilliant Blue reagent.

of densitometric traces. In Table 3 values are given for the area percent distribution corresponding to the different peaks obtained with both stainings. Some samples show small, scarcely detectable, highly mobile peaks corresponding to low molecular weight extraneous proteins. Furthermore, these and other samples show the differences in percent content of the high molecular weight peak (polymer) revealed with the iron reagent compared to the percent content of the same peak obtained with protein staining. This behaviour could be evidence of a different iron content in the various molecular aggregates or of the presence of iron salts other than those of the ferritin complex. It is interesting to make a comparison between the profile of the reference ferritin showing that the less mobile band (polymer) is present at a very low percent content and the little difference between the values for two stainings; therefore, the second hypothesis is more probable. The presence of different molecular aggregates for ferritin is confirmed by submitting the samples to size exclusion high performance liquid chromatography (SEC-HPLC) which in addition permits a clear detection of the presence of extraneous components. This technique was

Table 3. - *Percent area distribution of protein and iron on the bands obtained after polyacrylamide gel electrophoresis, specific staining and densitometric readings*

Sample	Stain *(a)*	Firs aggregate band	Second aggregate band	Monomer band	More mobile band
A	1	17.0	15.6	67.4	-
	2	9.9	11.4	78.7	evident traces
B	1	15.6	17.5	66.9	-
	2	11.4	15.1	73.5	evident traces
C	1	15.0	10.1	74.9	-
	2	6.9	14.6	78.5	evident traces
D	1	14.4	10.0	75.6	-
	2	11.2	15.1	73.7	traces
E	1	10.0	18.9	71.1	-
	2	8.8	17.1	74.0	-
F	1	9.6	15.3	75.1	-
	2	11.1	10.0	78.8	-
G	1	10.4	14.4	75.1	-
	2	7.2	17.5	75.3	-
H	1	10.5	13.0	76.5	-
	2	9.9	15.3	74.7	-
Standard	1	4.3	5.8	90.5	-
	2	3.9	7.8	88.2	-

(a) stain 1 = potassium ferrocyanide for iron detection; stain 2 = Coomassie Blue for protein detection

performed by using and comparing both a TSK G-4000 SW (7.5 x 600 mm) and a Superose 6H-R (10 x 300 mm) column, with a preliminary calibration using a standard mixture of proteins for high molecular weight (Pharmacia). This procedure allows for an evaluation of the molecular weight ranges (MW) of the samples by the K_{av} shown by the different peaks.

As an index of the resolution power (Rs) of the columns we considered the value obtained between peaks of the reference compounds thyreoglobulin (669,000 Da) and ferritin (440,000 Da). The Rs was 1.65 for the TSK G-4000 SW column and 1.01 for the Superose 6H-R column. The range between these selected reference compounds corresponds to the central range of molecular weights for ferritin and its aggregates.

The relation between the log MW and K_{av} is linear between the MW of blue dextran (about 2,000,000 Da), thyreoglobulin and ferritin (440,000 Da) and deviates from linearity for reference compounds with a MW less than 300,000 Da (reference catalase 232,000 Da and aldolase 158,000 Da). For a precise evaluation at the level of these lower molecular weights a succession of two or more columns with different exclusion characteristics (as described by Barden [18]) is necessary. This type of chromatographic separation can also be obtained by low-pressure gel permation chromatography using a Pharmacia column, 26 mm in diameter, 46 cm high, filled with Sephacryl S 300 and using NaCl 0.1 M in phosphate buffer M/15 at pH 6.8 as eluent. The effluent is monitored at 280 nm. The general separation is of the same type as with SEC-HPLC (De Ligny [19] and Pagé *et al.* [20]) while the resolution value obtained is lower and the total analysis time is about 7-8 h rather than 50 min.

Table 4 shows the area percent distribution corresponding to the peaks obtained in the analysis of our samples with the described SEC-HPLC technique: the same table includes an evaluation of the MW of the principal fractions obtained from the chromatograms. These fractions are: near V_0, the polymer (a) peak (first aggregate), MW 2,000,000 Da; a closely successive aggregate (polymer B) with MW of about 1,000,000 Da present, however, in very low amounts and in only a few samples; the peak of the so-called "dimer" (second aggregate) with a MW of about 600,000 Da; and the peak of the ferritin monomer with MW of about 420,000-450,000 Da. These (not highly purified) commercial samples of ferritin produce chromatograms with peaks that are difficult to correctly integrate to obtain a reproducible percent distribution of the various corresponding areas. In our analysis, the best compromise between reproducibility and accuracy in the evaluation of peak areas, was obtained by using a valley-to-valley integration, with peak separations perpendicular to the baseline. Differences in percent distribution were found in the results from the two types of columns: the lesser resolution between the reference compounds thyreoglobulin and ferritin observed on the Superose 6-HR column produced chromatograms with an erroneous increase of the monomer peak area at the expense of the preceding "dimer" peak area. For this reason, we consider the percent distribution evaluation obtained in the SEC-HPLC using the Superose 6-HR column insufficiently accurate, at least with the dimensions used by us (10 x 300 mm).

As clearly shown in the profiles of some samples (Figs 4 and 5, Table 4) the main ferritin monomer peak (r.t. approximately 31.2 min) is followed by several small peaks with longer retention times. Through the use of a diode array detector it is possible to obtain an on-line recording of the UV spectrum of the different peaks; this can be very useful for their identification [21].

Table 4. - *Percent area distribution of the peaks* (a) *obtained by size exclusion chromatography with 280 nm UV detection. Molecular weight determinations obtained with the same procedure are indicated*

Sample	First aggregate		Second aggregate				Monomer			
	(b)	*(c)*	*(b)*	MW	*(c)*	MW	*(b)*	MW	*(c)*	MW
A	4.3	3.8	38.4	580,000	23.8	669,000	57.2	420,000	72.4	440,000
B	2.3	8.3	47.3	550,000	19.3	669,000	50.2	400,000	72.3	440,000
C	8.7	8.1	33.7	630,000	5.0	669,000	57.6	455,000	86.8	440,000
D	2.4	9.5	32.7	630,000	3.8	669,000	64.9	455,000	86.7	440,000
E	1.3	0.2	24.6	600,000	35.1	669,000	74.1	445,000	64.7	430,000
F	3.9	0.1	25.1	630,000	22.4	669,000	71.0	445,000	77.4	430,000
G	2.2	0.3	35.1	600,000	23.6	669,000	62.3	420,000	77.1	430,000
H	1.1	0.4	30.8	580,000	22.4	659,000	68.0	420,000	77.1	430,000
Standard	1.7	-	6.7	620,000	-	-	91.4	450,000	-	-

(a) only ferritin and aggregates considered; *(b)* TSK - G 4000 SW 7.5 x 600 mm column (LKB, Sweden); *(c)* Superose 6 HR/10 300 mm column (Pharmacia, Sweden)

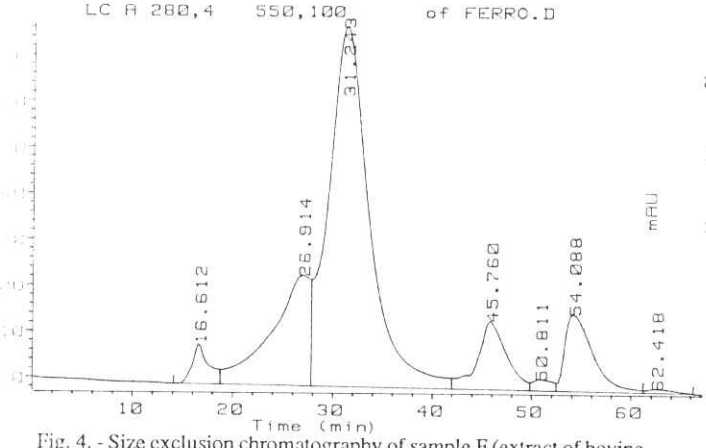

Fig. 4. - Size exclusion chromatography of sample F (extract of bovine spleen, solution): injected amount 8.1 µg of protein bound iron (total protein about 32 µg). Column TSK G-4000 SW 7.5 x 600 mm. Operating conditions: see "Materials and methods". Detector HP mod. 1040A.

Peaks	r.t.	16.6	min	first aggregate (polymer)
	r.t.	26.9	min	second aggregate (dimer)
	r.t.	31.2	min	monomer
	r.t.	45.8	min	extraneous protein
	r.t.	54.1	min	preservative (*p*-hydroxy-benzoic acid alkyl ester)

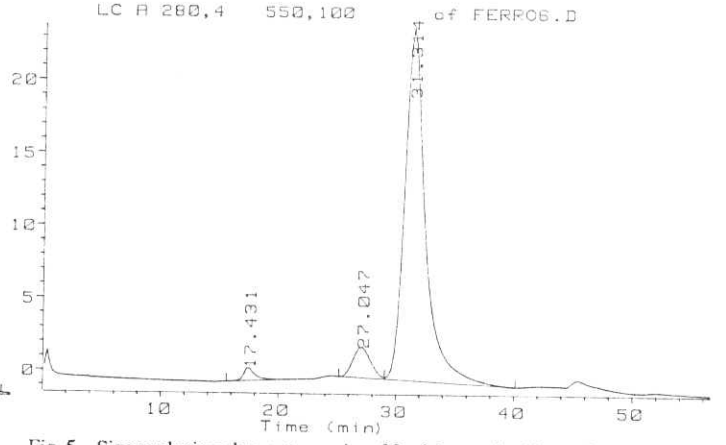

Fig. 5. - Size exclusion chromatography of ferritin standard for molecular weight: injected amount 5 µg (protein). Column, operating conditions and peaks as described in Fig. 4.

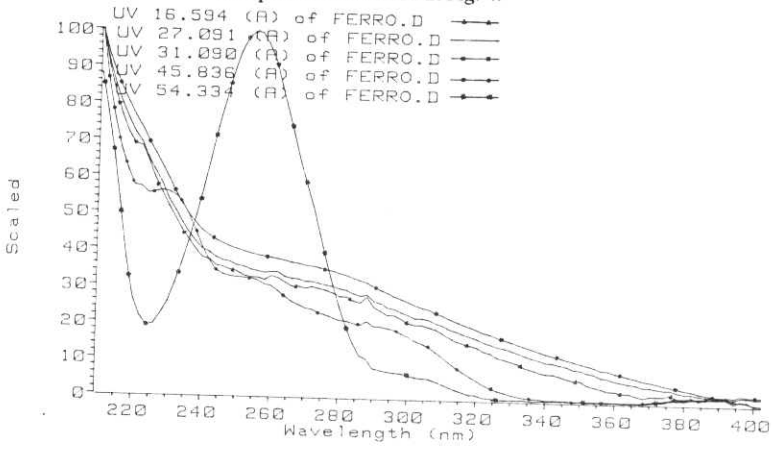

Fig. 6. - Spectrophotometric investigations of the peaks in Fig. 4, performed by means of Diode Array Detector; abscissa: wavelengths; ordinate: normalized absorbance.

Ferritin and its aggregates demonstrate a rather atypical spectrum (Fig. 6) with a single inflection at 280 nm and a long band of low-level absorbance extending to about 400 nm.

Instead, other low molecular weight proteins, observed in a few cases, show spectra with a clear maximum at 280 nm and no absorbance after 320 nm. A similar behaviour was observed in reference proteins such as albumin and catalase (Fig. 7).

All the samples in hydroglyceric solution and some in dry powder form show 3-5 small peaks with retention times between approximately 49 and 67 min. These peaks present difficult to assign UV spectra, since they do not correspond to those of the examined reference proteins (Fig. 7). Therefore these peaks were excluded from the computation of the percent area of ferritin and protein compounds, detectable at 280 nm.

Some preservatives, such as *p*-hydroxybenzoates, show an intense absorption band at 255 nm with spectrum and retention times corresponding to the results of the reference substances (Figs 4 and 6, peak with r.t. 54.3 min).

The use of this type of size-exclusion chromatography allows not only to calculate the percent distribution of the dimensionally separated protein fractions evaluated by the absorbance at 280 nm, but it also permits the evaluation of the percent distribution of iron in the individuated fractions. This iron evaluation can be performed using two

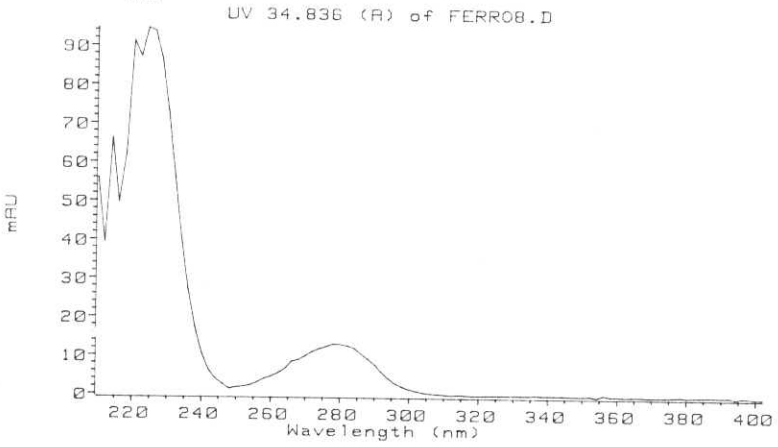

Fig. 7. - Spectophotometric investigation of the peak of the reference protein Aldolase (r.t. 34.8 min) MW 158,000 Da, performed by means of diode array detector; coordinates as in Fig. 6. A clear maximum at 280 nm is shown.

Table 5. - *Iron analysis of the eluates from SEC-HPLC, monitored at 280 nm*

Sample	Zone	Peak distribution of total recovered value (%)	Recovery of the injected amount (%)
F	lst aggregate	8.7	98.2
	2nd aggregate	11.9	
	Monomer	79.4	
	Final zone	-	
C	lst aggregate	4.0	91.7
	2nd aggregate	22.5	
	Monomer	70.4	
	Final zone	3.1	
I	lst aggregate	23.3	90.3
	2nd aggregate	22.2	
	Monomer	52.5	
	Final zone	1.8	
M	lst aggregate	-	92.9
	2nd aggregate	52.4 *(a)*	
	Monomer	47.6	
	Final zone	-	
N	lst aggregate	-	93.9
	2nd aggregate	33.5 *(a)*	
	Monomer	64.3	
	Final zone	2.3	
O	lst aggregate	-	88.3
	2nd aggregate	29.1 *(a)*	
	Monomer	70.7	
	Final zone	-	
P	lst aggregate	0.5	97.0
	2nd aggregate	19.6	
	Monomer	78.7	
	Final zone	1.2	
St	lst aggregate	8.7	98.2
	2nd aggregate	11.9	
	Monomer	79.4	
	Final zone	-	

(a) value of polymer and dimer unresolved zone

procedures. The first technique requires the use of an inductively-coupled plasma atomic emission spectrometry (ICP-AES) detector, serially connected to a UV detector and regulated for operation at the iron emission band wavelength. The result is an on-line evaluation of the iron content of the ferritin band. It is thereby possible to calculate the iron content from the resulting peak, expressing it as a percent of the total iron introduced into the column [22].

The second procedure simply requires the collection of the eluates corresponding to the various SEC-HPLC-separated protein peaks, after injection into the column of a quantity of sample equivalent to approximately 70 μg of iron. As identified by UV detection at 280 nm, the collected fractions are analyzed for their iron content with the cited colorimetric method [11].

Table 5 shows the values obtained for some of our samples with this analytical procedure. The total recovery of iron as well as the percent iron distribution in the separated fractions are reported.

Satisfactory recoveries were obtained, generally above 90% and even more for samples of higher quality. The percent iron distribution correlates with the protein distribution as monitored at $\lambda = 280$ nm, negligible differences are due to the different analytical procedures.

With the proposed procedures it is possible to calculate the effective iron content of the ferritin monomer provided that a clear chromatographic separation of the respective peak is possible.

Using the second, simpler procedure, it was also possible to verify the protein/iron ratio directly from the ferritin monomer peak.

In addition to the iron content it is necessary to determine the protein content corresponding to this peak; this can be done directly by HPLC having previously performed a calibration with a high quality ferritin sample (monomer percent > 85%) (Fig. 5). The exact value of the ferritin monomer content is determined by a preliminary SEC-HPLC analysis monitored at $\lambda = 280$ nm. Successively, using this reference ferritin, the linear relationship between peak areas and scalar amounts is verified. In our experiment, the linear regression between integrator counts (Y) and scalar amounts of true ferritin monomer (X) between 11.17 and 44.68 μg, was expressed by the equation $Y = -713.5 + 926.9X$, with the correlation coefficient $r = 0.9980$. By applying this equation it is possible to calculate the ferritin monomer content of each sample. For some samples, as reported in Table 5, the protein/iron ratios for the monomer peak were calculated and the mean values of three replicated experiments centered around 5; they were 5.34 for sample F, 5.23 for sample C and 4.92 for the reference compound.

Conclusion

We believe that the data presented herein clearly support the thesis that combining some simple procedures of chemical and physico-chemical analysis renders an eva-

luation of commercial samples of ferritin for pharmaceutical use possible, defining protein content, protein-bound iron and percent distribution of both, concerning the respective chromatographic and electrophoretic peaks.

We give special attention to the SEC-HPLC procedure because it allows the evaluation of the MW of different fractions of samples and the direct determination of the ferritin monomer content by $\lambda = 280$ nm UV analysis. It is important to consider that the proposed tests and assays are each necessary and inseparable elements of a whole. This context must remain integral for a correct, primary evaluation of the qualitative and quantitative characteristics of the samples.

Review submitted on invitation by the Editorial Board of the *Annali*. Accepted for publication: 20 June 1989.

REFERENCES

1. GRANICK, S. 1946. Ferritin: its properties and significance for iron metabolism. *Chem. Rev.* **38**: 379-403.
2. CRICHTON, R.R. 1973. Structure and function of ferritin. *Angew. Chem. Int. Ed. Engl.* **12**(1): 57-65.
3. AISEN, P. & LISTOWSKY, I. 1980. Iron transport and storage proteins. *Ann. Rev. Biochem.* **49**: 357-393.
4. JOSHI, J.G. & ZIMMERMAN, A. 1988. Ferritin: an expanded role in metabolic regulation. *Toxicology* **48**: 21-29.
5. PERROT, Y., De CERTAINES, J., RUELLAND, A. & CLOAREC, L. 1982. Serum ferritin assay using immunoenzimology and radioimmunology. Comparative study on two commercial batches. *Ann. Biol. Clin. (Paris)* **40**(3): 195-198.
6. CAZZOLA, M., AROSIO, P., BAROSI, G., BERGAMASCHI, G., DEZZA, L. & ASCARI, E. 1983. Ferritin in the red cells of normal subjects and patients with iron deficiency and iron overload. *Brit. J. Haematol.* **53**(4): 659-665.
7. AROSIO, P., ADELMAN, Th.G. & DRYSDALE, J.W. 1978. On ferritin heterogeneity. Further evidence for heteropolymers. *J. Biol. Chem.* **253**(12): 4451-4458.
8. MIYATA, Y., FURUGOURI, K. & SHIJIMAYA, K. 1984. Developmental changes in serum ferritin concentration of dairy calves. *J. Dairy Sci.* **67**(6): 1256-1263.
9. FRENKEL, E.J., VAN DEN BELD, B., KÖNIG, B.W. & MARX, J.J.M. 1983. Preparative isolation of distinct molecular forms of rabbit liver ferritin using high performance liquid chromatography. *Anal. Biochem.* **135**: 489-494.
10. LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
11. ABBEY, S. 1948. Determination of trace impurities. *Anal. Chem.* **20**: 630-634.
12. SMITH, P.K., KROHN, R.I., HERMANSON, S.T., MALLIA, A.K., GARTNER, F.M., PROVENZANO, M.D., FUJIMOTO, E.K., GOEKE, N.M., OLSON, B.J. & KLENK, D.C. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**: 76-85.
13. ADDISON, J.M., LEWIS, W.G. & HARRISON, P.M. 1984. The preparative separation of horse and human ferritin by chromatofocusing. *J. Chromatogr.* **292**: 454-457.
14. CETINKAYA, N., LENGEMANN, F.W. & KOGAN, P. 1985. Isolation, purification and characterization of bovine spleen ferritin. *Comp. Biochem. Physiol.* **80B**(4): 773-778.
15. HARRISON, P.M., HOARE, R.J., HOY, T.G. & MACARA, I.G. 1974. Ferritin and hemosiderin: structure and function. In: *Iron in biochemistry and medicine*. A. Jacobs & M. Worwood (Eds). Academic Press, London, New York. pp. 73-114.
16. ZAMIRI, I & MASON, J. 1968. Electrophorisis of ferritins. *Nature* **217**: 258-259.
17. RICHTER, J.W. 1965. Comparison of ferritins from neoplastic and non-neoplastic cells. *Nature* **207**: 616-618.
18. BARDEN, J.A. 1983. Separation of both fibrous and globular proteins on the basis of molecular weight using high performance size exclusion chromatography. *Anal. Biochem.* **135**: 52-57.
19. DE LIGNY, C.L. 1985. Gel permeation chromatography in biochemistry. *Chromatogr. Int.* **10**: 5-8.
20. PAGÈ, M., LAGUEUX, J. & GAUTHIER, C. 1980. A three step purification procedure for human liver ferritin. *Can. J. Biochem.* **58**(6): 494-498.
21. CLARK, B.J. & FELL, A.F. 1987. Multichannel spectroscopy in liquid chromatography. *Chem. Br.* 1069-1071.
22. LA TORRE, F., VIOLANTE, N., SENOFONTE, O., D'ARPINO, C. & CAROLI, S. 1988. A novel approach to the analysis of ferritin by inductively coupled plasma-atomic emission spectrometry combined with high performance liquid chromatography. *Spectroscopy* **4**(1): 48-50.