Ann. Ist. Super. Sanità
Vol. 25, N. 3 (1989), pp. 481-486

RECENT DEVELOPMENTS IN THE ASSAY OF FERRITIN CONTAINING DRUGS

F. LA TORRE *(a)*, N. VIOLANTE *(b)*, O. SENOFONTE *(b)*, C. D'ARPINO *(a)* and S. CAROLI *(b)*

(a) Laboratorio di Chimica del Farmaco; (b) Laboratorio di Tossicologia Applicata, Istituto Superiore di Sanità, Rome, Italy

Summary. - *The advent of new stationary phases for gel permeation liquid chromatography (GPLC) capable of withstanding the high pressures of high performance liquid chromatography (HPLC) has opened new avenues for the analysis of large molecules such as ferritin. The exploitation of inductively-coupled plasma atomic emission spectrometry (ICP-AES) as a technique for detection in HPLC has evolved into a significant approach to element analysis and speciation. In general, while the on-line coupling still requires careful optimization, it expediently allows the identification of proteins and the quantification of Fe in ferritin products to be achieved.*

Riassunto (Sviluppi recenti nell'analisi di farmaci a base di ferritina). - L'avvento *di nuove fasi stazionarie per la cromatografia liquida a permeazione di gel (GPLC) capaci di resistere alle alte pressioni tipiche della cromatografia liquida ad alta risoluzione (HPLC) ha aperto nuove strade per l'analisi di macromolecole quali la ferritina. Da questo punto di vista la spettrometria di emissione atomica con plasma induttivo (ICP-AES) si è affermata come una tecnica di rivelazione per HPLC per l'analisi degli elementi e la loro speciazione. Mentre in generale l'accoppiamento in linea richiede ulteriore ottimizzazione, nel caso specifico il metodo descritto permette un'agevole identificazione della parte proteica e la determinazione quantitativa di Fe nei prodotti commerciali a base di ferritina.*

Introduction

Ferritin is a widely distributed protein in eukariotes and plants. Mammalian liver, spleen and bone marrow are especially rich in ferritin. This protein is also found in smaller quantities in serum and circulating blood cells [1]. Phytoferritins and phytosiderins have been identified in several species of plants and fungi [2]. This substance plays an important role in Fe metabolism; it is able to store the element in a soluble, non-toxic form and to recycle it for the synthesis of heme and related proteins. The need for a storage of this metal in higher animals arises from many factors such as formation of hemoglobin, sudden loss by bleeding, low dietary intake and source of Fe for the foetus during pregnancy. On the other hand, an excessive intake, as for all essential elements, may eventually lead to toxic effects.

Proteins such as ferritin, transferrin and hemosiderin can complex the metal, avoiding its precipitation and adverse influence of free Fe. Ferritin consists of a roughly spherical protein shell with an external diameter of about 13 nm capable of storing up to 4500 Fe atoms into a central cavity of about 8 nm diameter as ferrihydroxyphosphate [3, 4]. The spherical shell is a combination of 24 subunits of different types, namely the L type (light or liver) with a molecular weight of 19,000, which is more abundant in Fe-loaded tissues, the H type (heavy or heart type) with a molecular weight of 21,000, mostly found in Fe-poor tissues and malignant cells [3], and the glycosylated G type isolated from serum, with a molecular weight of 24,000 [5]. Glycosylation of serum ferritins mainly accounts for the different patterns in isoelectric focusing experiments [5]. The molecular weight of ferritin ranges from 440,000 to 500,000 depending on the relative amounts of the different subunits.

Evidence from X-ray diffraction, SDS gel electrophoresis and sedimentation are in favour of a spherical shell, with a 432 symmetry. This shell possesses several channels, allowing Fe and small molecules to enter and leave the protein cavity [6]. Iron may be removed from ferritin by chemical reduction of Fe^{3+} or by means of low molecular weight chelating agents, and his gives rise to the formation of apoferritin. Chemical reducing agents are, however, more efficient and faster than complexing molecules. Iron(II) in turn may be removed after reduction by dialysis or by chromatography on Sephadex G 25 [7].

Ferritin and apoferritin are undistinguishable on gel filtration chromatography since the outer dimensions of both are identical. Ferritin aggregates can be separated by

monomers using the aforementioned columns; unfortunately the elution time of ferritins is not affected by the different subunit composition. Monomeric ferritins are eluted behind *Escherichia coli*-galactosidase using Sephadex G 200, 4% agarose gel or TSK G 4000 SW as the stationary phase and slightly ahead of rabbit liver phosphorylase α on 4% or 6% agarose [8].

The Fe content of ferritin preparations ranges from zero (apoferritin) to 4500 Fe atoms per molecule as an average. Ferritins obtained from spleen and liver contain 2500 atoms of the element, while for those obtained from heart the Fe content is lower. The low level of ferritin circulating in plasma contains a surprisingly low amount of this metal [9, 10]. Ferritin was first isolated by Laufberger [11]. Conventional methods for the purification of horse spleen ferritins require that tissue homogenates be heated to 70-75 °C. The protein is precipitated by ammonium sulphate (35% w/v) and then purified by precipitation with 0.3 M $CdSO_4$. The homogeneity of the preparation may be checked by SDS polyacrylamide electrophoresis [12, 13].

In spite of its large size, the ferritin molecule shows a considerable stability under conditions which would normally cause denaturation of other proteins. Ferritin withstands heating at 80 °C for 10 min, and incubation in 10 mM urea or 10 g/l SDS at room temperature and can be stored for months in solution at 40 °C in the presence of sodium azide 0.02% [4]. The exceptional stability of this molecule at elevated temperatures or in the presence of denaturants is therefore exploited during the purification process. According to optical rotatory dispersion (ORD) and circular dichroism (CD) studies ferritin molecules show a relatively high helicity in aqueous solutions corresponding to about 50% of α-helix [10, 13-15].

Dissociation of native ferritin into subunits may occur in 6 M guanidinium chloride or at pH values either below 2.5 or above 11.0. This process is accompanied by unfolding of the secondary structure. Dissociation-reassociation equilibria of ferritin subunits have reportedly been followed by UV and NMR spectroscopy, sedimentation velocity and CD experiments as a function of pH value and the nature of the buffer to obtain information about the assembly pathway [16, 17]. Ferritin preparations may exist as monomers, dimers, trimers or even larger aggregates, while structures of higher level such as hexamers and octamers have also been observed after the subunit assembling process. Electrophoresis in sieving media such as starch gel or acrylamide allows the separation of monomers and oligomers in multiple bands. Ferritin monomers are readily separated from aggregates by gel permeation liquid chromatography (GPLC). The origin and physiological significance of these oligomers have not yet been clarified, although they may be thought of as precursors of hemosiderin [18-20].

Taking into account the above complex scenario and given the paucity of adequate investigative approaches, a simple and reliable analytical method has been developed to determine the amount of Fe-bound protein in pharmaceutical products containing ferritin. In particular, this procedure should prove of value for detecting possible adulterations of commercial products containing mixtures of Fe-proteins other that ferritin [21, 22].

Experimental

Apparatus

The instrumental techniques employed are GPLC and inductively-coupled plasma atomic emission spectrometry (ICP-AES). In the first phase of this research these methods were used separately exploiting their own particular features. In fact, aliquots of solutions containing samples of ferritin were routinely submitted to chromatographic separation, collected after UV detection and analyzed with ICP-AES to determine their Fe content. These operations permitted the determination of total Fe content but not its speciation.

Thereafter it became apparent that a qualitative step forward was mandatory. Thus, in order to circumvent the disadvantages inherent in a separation completely independent from the detection step, and taking into account that the output fluxes of the GPLC system were entirely compatible with the input ones of the ICP-AES excitation unit, an attempt was made to make a direct on-line connection of the two instruments. A block diagram of the instrumental assembly is shown in Fig. 1. Although with minor variations, this overall scheme is valid both for the entire program carried out thus far as well as for the investigation in progress.

In fact, this approach offers the advantages of a powerful fractionation technique with a high degree of selectivity together with the simultaneous multielement detection ability typical of ICP-AES, its relative freedom from matrix interferences, element specificity and wide dyna-

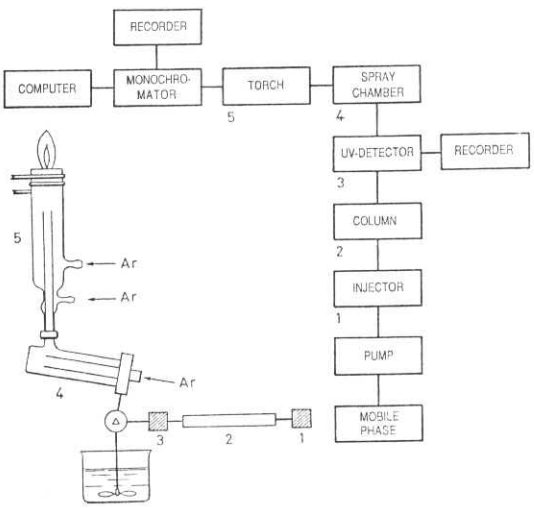

Fig. 1. - Overall view of the combined HPLC/ICP-AES system.

mic range [23-28]. In addition, it is worth emphasizing that the direct inlet of the GPLC eluates into the ICP torch in an on-line, real time fashion minimizes the risk of contamination, saves time and greatly increases the throughout of samples in order to provide reliable quantitative information.

In this connection, it should be recalled that the applications of HPLC to proteins have in general undergone a dramatic development in recent years. This was possible only when microparticulate column packings with a narrow size distribution became available on the one hand, and new stationary phases were produced capable of withstanding the requested high flow rates and pressures typical of HPLC on the other. It is also in virtue of this technical advance that an efficient coupling with ICP-AES could be achieved.

In this study two different types of size exclusion columns were used, namely the TSK G 4000 SW with a molecular weight range of 5000-1,000,000 and the Superose 6 HR 10/30 with an exclusion limit of 5×10^6 (both from Pharmacia, Uppsala, Sweden). It is well known that in the size exclusion chromatography technique molecules are separated according to their molecular size. Those which are too large to penetrate the pores of the stationary phase will be eluted at the void volume, i.e. the volume of eluent between the stationary phase particles. Molecules able to penetrate all the pores will undergo fractionation with the molecules of the eluent at the total permeation volume of the column according to their molecular size as long as the exclusion mechanism prevails over other possible processes, such as hydrophobic interactions with bonded organic groups [29].

A PVC tube was employed to join the outlet of the HPLC UV detector with the inlet of the nebulizer connected to the ICP torch, taking care to reduce dead volumes between the two parts. The salt content of the buffer eluent was very high, so that the plasma argon had to be presaturated with water to avoid clogging of the nebulizer tip.

Procedure

In testing the coupling of ICP-AES with GPLC, attention was focused on the analysis of ferritins, available to the public at large, and characterized by patterns of protein distribution different from each other as revealed by UV detector. A solid standard of ferritin was used as a reference. Its Fe content was determined by neutron activation analysis allowing calibration solutions to be carefully prepared in water as well as with the buffer eluent. The response, shown in Fig. 2, testified to the fact that calibration using the eluent provides an optimal performance of the analysis. The signals arising from the HPLC UV detector as well as from the ICP-AES photomultiplier were recorded on-line almost simultaneously by two LC Perkin-Elmer integrators. In this connection it is worth mentioning an annoying technical problem which arose as a consequence of the fact that the spectrometer monochromator had to be constantly positioned on one of the analytical lines of Fe (namely the one at 259.94 nm). In fact, thermal and mechanical variations induced random misalignment of the line peak away from the optical pathway, thus jeopardizing the validity of the entire measurement approach. An elegant solution to this otherwise serious aspect was found by periodically checking the exact position of the peak at the center of the slit just before the arrival of the Fe-containing fraction of the sample into the torch by means of Fe hollow cathode lamp for atomic absorption spectrometry placed on the optical pathway in front of the entrance slit of the spectrometer.

Quantification of the fractions eluted by the chromatographic system was performed on the basis of peak areas as measured by the first of the two on-line integrators. The same holds true for the Fe-containing fractions entering the ICP torch, for which a direct reading of the Fe amount took place simultaneously with the peak area measurement carried out by the second integrator. Detection power, reproducibility and recovery tests were also planned. The first quantity was estimated by resorting to the criterion of the 3-σ of background fluctuations injecting decreasing amounts of standard ferritin solutions and calculating the corresponding peak areas down to values still unequivocally discernible from the baseline. In turn, overall reproducibility was judged by running the same sample 10 times.

An evaluation of recovery from the various columns was possible by comparing the amounts of Fe measured on sets of four aliquots of a given sample of ferritin by direct injection into the ICP-AES system with those detected in other series. These last differed from the former for the fact that they were subjected to preliminary chromatographic separations, and that the fractions were collected and quantified as described above [30]. Figs 3 and 4 show examples of signals obtained with the two detection systems in the case of different columns as well as the behaviour of six commercial ferritins using the column with the best performance.

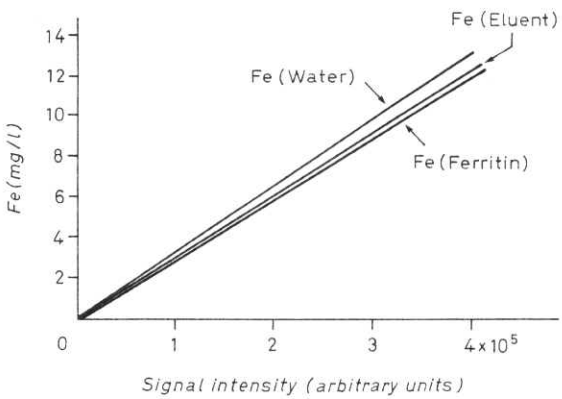

Fig. 2. - Dependence of Fe signal on ferritin concentration in different media.

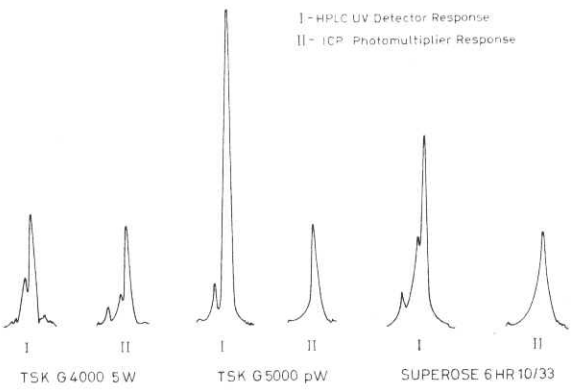

Fig. 3. - Comparison of peak profiles obtained with the HPLC and ICP-AES detection systems on different columns.

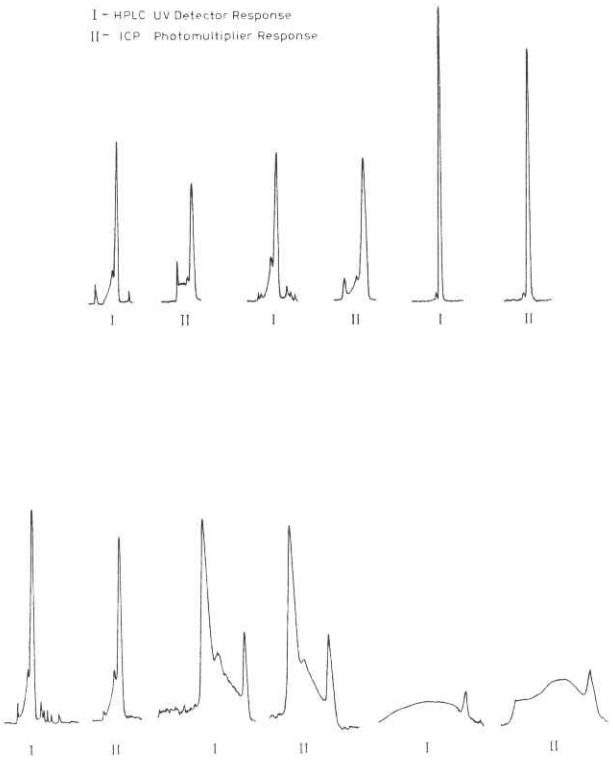

Fig. 4. - Peak profiles of six different ferritin samples obtained with the HPLC and ICP-AES detection systems after separation on the TSK G 4000 SW column.

Results and conclusions

Preliminary experiments confirm that the matrix effect in this kind of study is negligible.

The coupling of HPLC with ICP-AES gives the operator a powerful tool for both qualitative and quantitative investigation. It offers insight into new and innovative ways of analyzing Fe-bounded proteins other than ferritin. The ease of use and the possibility of analyzing compounds containing element-bound proteins facilitates the successful application of this and similar combined techniques in the field of element speciation. Efforts should consequently be addressed to the monitoring of existing pharmaceuticals containing ferritin as well as to other Fe-bound proteins by further developing the analytical approach and the combined instrumentation described in this study.

The pharmacological and clinical analytical problems related to Fe present in human body can be traced back to the identification and quantization of Fe-containing proteins, allowing in this way a deeper knowledge of the metabolism of this element. From this standpoint it is particularly worth elucidating which factors can lead to significant alterations of the normal transfer of Fe(II) to β_1-globulin to form siderophyllin in plasma and from this protein to targets like liver, muscles, bone marrow and other tissues. Moreover, how the administration of ferritin-containing drugs can be optimized to restore the physiological balance of obvious importance and requires the systematic determination of all proteic species involved. On the other hand, it certainly cannot be overlooked that presently available analytical techniques still lack the necessary specificity and detection power to afford this type of investigation. Further developments should therefore be promoted to exploit combination of separative methods with atomic emission and mass spectrometric facilities in view of their highly promising flexibility and direct applicability from a regulatory point of view.

Review submitted on invitation by the Editorial Board of the *Annali*.
Accepted for publication: 26 April 1989.

REFERENCES

1. AISEN, P. 1980. Iron transport and storage proteins. *Ann. Rev. Biochem.* **49**: 357-393.

2. CRICHTON, R.R., MILLER, J.A., CUMMING, R.L.C. & BRYCE, C.F.A. 1973. The organ-specificity of ferritin in human and horse liver and spleen. *Biochem. J.* **131**: 51-59.

3. AROSIO, P., ADELMAN, T.G. & DRYSDALE, J.W. 1978. On ferritin heterogenicity: further evidence for heteropolymers. *J. Biol. Chem.* 253: 4451-4458.

4. JOSHI, J.G. & ZIMMERMAN, A. 1988. Ferritin: an expanded role in metabolism regulation. *Toxicology* **48**: 21-29.

5. CRAGG, S.J., WAGSTAFF, M. & WORWOOD, M. 1981. Detection of glycosylated subunit in human serum ferritin. *Biochem. J.* **199**: 565-571.

6. RICE, D.W., FORD, G.C., WHITE, J.L., SMITH, J.M.A. & HARRISON, P.M. 1983. The spatial structure of horse spleen apoferritin. In: *Advances in inorganic biochemistry*. Elsevier Biomedical, New York. Vol. 5. pp. 39-50.

7. CHIANCONE, E., STEFANINI, S. & ANTONINI, E. 1982. Ferritin: structure and function. In: *Advances in red cell biology*. O.J. Wheaterall *et al.* (Eds). Raven Press, New York. pp. 19-25.

8. DE HAEN, C. 1987. Molecular weight standards for calibration of gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis: ferritin and apoferritin. *Anal. Biochem.* **166**: 235-245.

9. LINDER, M.C. & MUNRO, H.N. 1972. Assay of tissue ferritin. *Anal. Biochem.* **48**: 266-278.

10. WORWOOD, M., DAWKINS, S., WAGSTAFF, M. & JACOBS, A. 1976. The purification and properties of ferritin from human serum. *J. Biochem.* **157**: 97-103.

11. LAUFBERGER, V. 1937. Sur la cristallization de la ferritine. *Bull. Soc. Chim. Biol.* **19**: 1575-1582.

12. HEUTERSPREUTE, M. & CRICHTON, R.R. 1981. Amino acids sequence of horse spleen apoferritin. *FEBS Lett.* **129**: 322-327.

13. LISTOWSKY, I., BETHEIL, J.J. & ENGLARD, S. 1967. Conformation of ferritin and apoferritin in solution. Optical rotatory dispersion properties. *Biochemistry* 6: 1341-1348.

14. WOOD, G.C. & CRICHTON, R.R. 1971. Optical rotatory dispersion and circular dichroism studies on ferritin and apoferritin. *Biochim. Biophys. Acta*. **229**: 83-87.

15. LISTOWSKY, I., BLAVER, G., ENGLARD, S. & BATHEIL, J.J. 1972. Denaturation of horse spleen ferritin in aqueous guanidinium chloride solutions. *Biochemistry* **11**: 2176-2181.

16. STEFANINI, S., VECCHINI, P. & CHIANCONE, E. 1987. On the mechanism of horse spleen apoferritin assembly: a sedimentation velocity and circular dichroism study. *Biochemistry* **26**: 1831-1837.

17. WORWOOD, M. 1987. The diagnostic value of serum ferritin determination for assessing iron study. *Haematologia* **20**: 229-235.

18. JAENICKE, R. & BARTMANN, P. 1972. Dissociation-association properties of apoferritin in the milligram and microgram range. *Biochem. Biophys. Res. Commun.* **49**: 884-890.

19. LEE, S.S.C. & RICHTER, G.W. 1976. The monomers and oligomers of ferritin and apoferritin: association and dissociation. *Biochemistry* **15**: 65-70.

20. HOY, T.G. & JACOBS, A. 1981. Ferritin polymers and the function of haemosiderin. *Br. J. Haematol.* **49**: 593-602.

21. CAROLI, S., LA TORRE, F., SENOFONTE, O., VIOLANTE, N. & FALASCA, O. 1985. Determination of ferritin in pharmaceutical products by means of HPLC and ICP. In: *Atti del 21. Seminario spettrochimico AIM - 5. Riunione Italo-Francese di spettrometria atomica*. Frascati, June 4-7, 1985. ENEA, Serie Simposi. pp. D49-D54.

22. VIOLANTE, N., SENOFONTE, O., LA TORRE, F. & CAROLI, S. 1986. Entwicklung eines chromatographisch-spektrometrischen kombinierten Verfahrens zur Bestimmung des Ferritins. In: *Proceedings of Analityktreffen. 9. CANAS Conference on analytical atomic spectrometry*. Neubrandenburg, DDR, September 1986. p. 203.

23. EBDON, L., HILL, S. & WARD, R.W. 1987. Directly coupled chromatography - atomic spectroscopy. Part 2. Directly coupled liquid chromatography-atomic spectroscopy. *Analyst* **112**: 1-16.

24. MORITA, K., UEHIRO, T. & FUWA, K. 1980. Speciation and elemental analysis of mixtures by high performance liquid chromatography with inductively-coupled argon plasma emission spectrometric detection. *Anal. Chem.* **52**: 349-351.

25. KRULL, I.S. 1984. Plasma emission spectroscopic detectors in HPLC for trace metal analysis and speciation. *Trends Anal. Chem.* **3**: 76-80.

26. HEUSLER, D.W. & TAYLOR, L. 1981. Non-aqueous on-line simultaneous determination of metals by size exclusion chromatography with inductively-coupled plasma atomic emission spectrometric detection. *Anal. Chem.* **53**: 1223-1227.

27. DEAN, J.R., MUNRO, S., EBDON, L., CREWS, M.H. & MASSEY, C.R. 1987. Studies of metalloprotein species by directly coupled high-performance liquid chromatography inductively-coupled plasma mass spectrometry. *J. Anal. At. Spectrom.* **2**: 607-610.

28. IRGOLIC, K.J., STOCKTON, R.A., CHAKRABORTI, D. & BEYER, W. 1983. Simultaneous inductively-coupled argon plasma emission spectrometer as a multi-element-specific detector for high pressure liquid chromatography: the determination of arsenic, selenium, and phosphorus compounds. *Spectrochim. Acta* **38B**: 437-445.

29. 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.

30. LA TORRE, F., VIOLANTE, N., SENOFONTE, O., D'ARPINO, C. & CAROLI, S. 1989. A novel approach to the analysis of ferritin by inductively-coupled plasma-atomic emission spectrometry combined with high performance liquid chromatography. *Spectroscopy* **4**: 48-51.