ELSEVIER

Journal of Chromatography B, 656 (1994) 436–440

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short Communication

Determination of non-protein-bound iron in human synovial fluid by high-performance liquid chromatography with electrochemical detection

Nana Kawasaki*[,a], Tsuyoshi Tanimoto[a], Akira Tanaka[a], Takao Hayakawa[a], Nobuyuki Miyasaka[b]

[a]*Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158, Japan*

[b]*Division of Immunological Diseases, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113, Japan*

(First received September 23rd, 1993; revised manuscript received February 22nd, 1994)

Abstract

Non-protein-bound iron in human synovial fluid was determined using high-performance liquid chromatography with electrochemical detection. The procedure was based on the separation of the iron–diethylenetriaminepentaacetic acid (DTPA) complex formed directly on a chromatographic column containing an anion-exchange resin followed by electrochemical detection. The method enabled more than 0.1 μM Fe(III) to be determined with an injection volume of 10 μl. A mixture of synovial fluid, 20 μM DTPA and acetate buffer was incubated in the presence and absence of superoxide (O_2^-) generated by a xanthine–xanthine oxidase system and was ultrafiltered through a 30 000 molecular mass cut-off filter. No iron was detected in the ultrafiltrate at physiological pH. However, the presence of iron was observed in the ultrafiltrate at low pH, and O_2^- facilitated the release of iron into the synovial fluid. This result suggested that in an inflamed joint with generated O_2^- and decreased pH, iron may be released into the synovial fluid.

1. Introduction

Iron is an effective catalyst for the formation of the aggressive oxidant hydroxyl radical ($OH^\cdot$) from H_2O_2. In a biological system, it is proposed that $OH^\cdot$ may be responsible for oxidative damage to biological macromolecules, including lipids, proteins and DNA [1]. Most of the iron in mammals is bound to haem proteins and non-haem proteins [2–4], and is therefore not directly available to catalyse the reaction. The most likely catalyst for mediating oxidative processes is thought to be low-molecular-mass iron chelates with biological chelators such as ADP [5] and citrate [6,7].

In rheumatoid arthritis, it is suggested that $OH^\cdot$ formation may cause the degradation of synovial hyaluronic acid [8], lipid peroxidation [9] and inactivation of proteinase inhibitor [10]. Blake *et al.* [11] have argued that iron plays an important role in oxygen radical reactions in the pathology of rheumatic diseases. However, the

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00135-R

determination of a low-molecular-mass iron species in biological samples is very difficult as it constitutes only a small fraction of the total iron present.

In a previous paper [12], we described a high-performance liquid chromatographic procedure coupled with electrochemical detection (HPLC–ED) for the determination of Fe(III) and Fe(II). This method is based on the separation and detection of iron–diethylenetriaminepentaacetic acid (DTPA) complexes formed directly on the chromatographic column. It was demonstrated that the ability of this method to detect nanogram levels of iron is suitable for the determination of low-molecular-mass iron species in biological samples. In this paper, we describe a sensitive method for the determination of non-protein-bound iron in synovial fluid. This method may be useful for studying iron mobilization in synovial fluid.

2. Experimental

2.1. Materials

Transferrin (human), ferritin (horse spleen), xanthine oxidase (milk grade, IV), xanthine and superoxide dismutase (SOD; bovine erythrocytes) were purchased from Sigma (St. Louis, MO, USA). DTPA and $FeNH_4(SO_4)_2 \cdot 12H_2O$ were obtained from Wako (Osaka, Japan). Solutions were made up with demineralized water in which no iron contamination was detected by HPLC–ED.

Twelve patients with inflammatory arthritides, *i.e.*, ten patients with rheumatoid arthritis and two with osteoarthritis, were included in this study. Patients who were taking more than 5 mg/day of prednisolone and/or 100 mg/day of D-penicillamine were excluded from the study. Synovial fluid was withdrawn by puncture from the knee joint into tubes containing heparin (25 U/ml) and centrifuged at 1500 *g* for 15 min to remove the cellular components. Supernatants were collected and stored at −78°C.

2.2. Chromatographic conditions

Chromatography was carried out using a metal-free system equipped by a Tosoh (Tokyo, Japan) CCPD pump and a Rheodyne Model 7125 injector (Tosoh). The separation column containing the anion-exchange resin was a TSK gel IC-Anion-PW column (5.0 cm × 4.6 mm I.D., 10 μm particle size) (Tosoh). A Tosoh Model EC-8010 electrochemical detector with a glassy carbon working electrode was used. The detector potential was set at −0.3 V versus an Ag/AgCl reference electrode. The detector output was recorded on a Hitachi (Tokyo, Japan) Model D-2500 chromato-integrator.

The eluent was 2 m*M* DTPA solution adjusted to pH 2.0 with hydrochloric acid. The flow-rate was 1.0 ml/min. Samples were injected into a 10-μl PTFE sample loop with a plastic syringe.

A standard solution was prepared by dissolving $FeNH_4(SO_4)_2 \cdot 12H_2O$ in 50 m*M* hydrochloric acid just prior to use.

2.3. Determination of non-protein-bound iron in synovial fluids

A 100-μl volume of synovial fluid was adjusted to pH 5.1–7.4 with 700 μl of acetate buffer, and DTPA at a final concentration of 20 μ*M* was added to the mixture in order to stabilize iron as the Fe(III)–DTPA complex. After incubation at 37°C for 20 min, the mixture was subjected to ultrafiltration through a 30 000 molecular mass cut-off filter, and the filtrate was injected into the HPLC system.

2.4. Release of iron from ferritin and transferrin

The reaction mixture contained xanthine oxidase (0.02 U/ml), xanthine (0.3 m*M*) and ferritin (40 μg/ml) or transferrin (20 μg/ml) in 0.1 *M* acetate buffer containing 20 μ*M* DTPA was incubated at 37°C for 20 min. After incubation the mixture was kept at 4°C and ultrafiltered through a 30 000 molecular mass cut-off filter.

3. Results

Fig. 1A shows a typical chromatogram of the standard solution of Fe(III) at a concentration of 1 μM. A metal-free system can enhance the sensitivity. The calibration graph for Fe(III) was linear in the concentration range 0.1–10 μM with a correlation coefficient of 0.9998. The recovery of 5 μM of Fe(III) added to the ultrafiltrate of synovial fluid with dilute acetate buffer containing 20 μM DTPA was 104.9%. The within-day precision and accuracy of the assay were determined by preparing a control sample at a concentration of 5 μM in diluted synovial fluid. The control sample ($n = 6$) was assayed and the calculated concentration was 5.39 ± 0.178 μM (mean ± S.D.). The within-day precision as measured by the relative standard deviation ($n = 6$) was 3.3% and the accuracy was 107.8%.

The chromatogram of the ultrafiltrate of synovial fluid showed no significant peak at the retention time of iron. This result suggests that most of the iron in the fluid was bound to proteins such as transferrin and ferritin. Many studies have demonstrated that protonation and reduction can promote iron release from transferrin and ferritin [13–16]. In inflammatory arthritides, large numbers of phagocytic cells enter the joints, resulting in a decrease in pH at the site of inflammation [17]. It is well known that activated polymorphonuclear leukocytes produce O_2^- and release it into the surrounding medium [18,19]. Therefore, the effect of O_2^- and pH on the release of iron into the synovial fluid was studied. The xanthine–xanthine oxidase system was chosen as the source of O_2^- as it has been well studied. The chromatogram of the ultrafiltrate of the synovial fluid incubated with acetate buffer at pH 5.1 revealed a peak with a retention time of 2.7 min, suggesting the existence of iron at acidic pH (Fig. 1B). Incubation of synovial fluid with xanthine and xanthine oxidase at pH 5.1 resulted in an increase in the peak of iron (Fig. 1C). This result suggested that O_2^- facilitated the release of iron into synovial fluid. The effects of pH on the release of iron in the presence and absence of O_2^- are shown in Fig. 2. Decreasing the pH resulted in an increase in iron release. Release of iron into synovial fluid was enhanced in the presence of O_2^-.

The amounts of non-protein-bound iron in

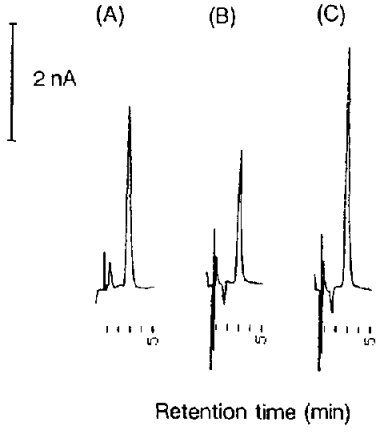

Fig. 1. Chromatograms of (A) the standard solution of Fe(III) (1 μM), (B) ultrafiltrate of synovial fluid at pH 5.1 and (C) ultrafiltrate of reaction mixture containing synovial fluid, xanthine oxidase (0.02 U/ml) and xanthine (0.3 mM) in 0.1 M acetate buffer (pH 5.1).

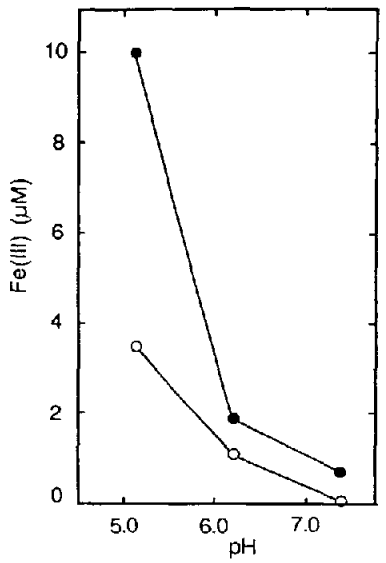

Fig. 2. Effect of decreasing pH and O_2^- on iron release from synovial fluid. (●) In the presence of xanthine oxidase (0.02 U/ml) and xanthine (0.3 mM); (○) no addition.

Table 1
Non-protein-bound iron in synovial fluid

Sample No.	Fe(III) (μM)[a]		
	A	B	C
1	6.9	8.4	6.7
2	2.5	3.6	3.0
3	3.1	3.6	
4	4.4	8.9	6.6
5	16.1	18.5	14.3
6	5.2	8.3	8.3
7	8.2	16.6	13.6
8	16.5	14.1	
9	8.7	8.1	8.4
10	3.5	10.0	7.9
11	3.1	5.1	3.7

[a] A, After incubation for 20 min with acetate buffer (pH 5.1); B, plus xanthine and xanthine oxidase; C, plus xanthine, xanthine oxidase and SOD (100 U/ml).

synovial fluid of eleven patients with inflammatory arthritides in the presence and absence of O_2^- are shown in Table 1. Non-protein-bound iron was detected in synovial fluid from all of the patients. Synovial fluid from all patients except one showed an increase in iron release in the presence of O_2^-. Addition of SOD to the incubation mixture of synovial fluid from some patients led to some decrease in the release of iron.

In order to clarify the origin of the non-protein-bound iron in synovial fluid, the effects of O_2^- and pH on the release of iron from ferritin (Fig. 3) and transferrin (Fig. 4) were studied by the proposed method. Superoxide facilitated iron release from ferritin at physiological pH. Decreasing the pH resulted in an increase in iron release in the presence of O_2^-. However, in the absence of O_2^-, only a small amount of iron was released from ferritin with decrease in pH. In contrast, the amount of iron released from transferrin at acidic pH was markedly increased in the absence of the xanthine–xanthine oxidase system. Superoxide failed to facilitate iron release from transferrin.

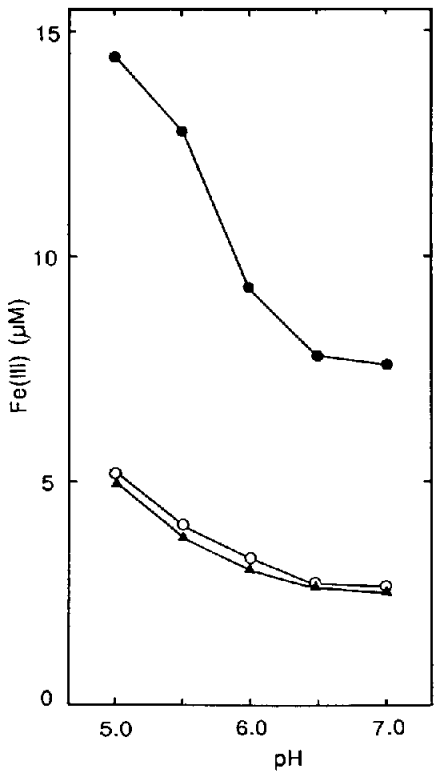

Fig. 3. Release of iron from ferritin (40 μg/ml). (●) In the presence of xanthine oxidase and xanthine; (▲) plus SOD (100 U/ml); (○) no addition.

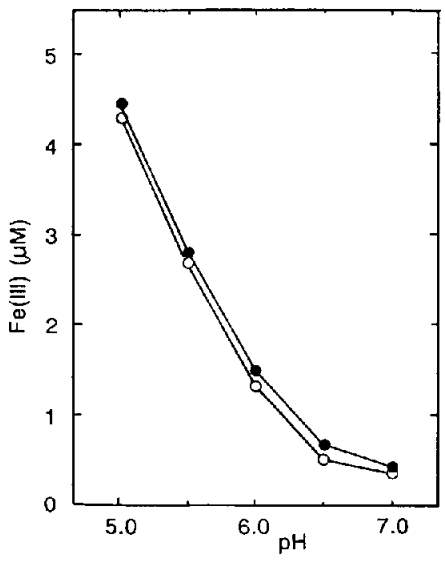

Fig. 4. Release of iron from transferrin (20 μg/ml). (●) In the presence of xanthine oxidase and xanthine; (○) no addition.

4. Discussion

It was clearly established that the formation of $OH^{\cdot}$ in systems generating O_2^- and H_2O_2 requires the presence of iron [1]. However, there are only a few reports on measurements of the catalytic iron in biological sample [5–7,20–23]. In synovial fluid from patients with inflammatory arthritides, non-protein-bound iron has been detected by the bleomycin assay method [21]. This assay seems to detect iron in a form capable of accelerating free radical reactions. Another procedure is spectrophotometry using bipyridyl [23]. Using these methods, non-protein-bound iron was detected at acidic pH in synovial fluid. This was also true in our study. In this work we also demonstrated that generating O_2^- facilitated iron release into the fluid. The results of the present study suggest that in an inflamed joint with increased O_2^- and decreased pH, iron may be released into the synovial fluid and play an important role in oxidative damage in fluid and tissue.

It was demonstrated that O_2^- facilitated the release of iron from ferritin. This is in agreement with other studies using spectrophotometric measurement with bathophenanthroline disulphonate as an iron(II) chelator [16,24–26]. However, our result that O_2^- did not facilitate iron release from transferrin even at acidic pH does not coincide with the results of Brieland and Fantone [27], who reported that the amount of iron released from transferrin by O_2^- from stimulated human neutrophils was enhanced by a decrease in pH. Our results suggest that iron was released from ferritin in synovial fluid in the presence of O_2^- and the source of iron detected at acidic pH may be mainly transferrin.

References

[1] B. Halliwell and J.M.C. Gutteridge, *Biochem. J.*, 219 (1984) 1.
[2] P. Aisen and I. Listowsky, *Annu. Rev. Biochem.*, 49 (1980) 357.
[3] R.R. Crichton and M. Charloteaux-Wauters, *Eur. J. Biochem.*, 164 (1987) 485.
[4] R.R. Crichton and R.J. Ward, *Biochemistry*, 31 (1992) 11255.
[5] D.L. Bakkeren, C.H.M. Jeu-Jaspars, C. van der Heul and H.G. van Eijk, *Int. J. Biochem.*, 17 (1985) 925.
[6] M. Grootveld, J.D. Bell, B. Halliwell, O.A. Aruoma, A. Bomford and P.J. Sadler, *J. Biol. Chem.*, 264 (1989) 4417.
[7] H.G. Parkes, R.E. Allen, A. Frust, R.D. Blake and M.C. Grootveld, *J. Pharm. Biomed. Anal.*, 9 (1991) 29.
[8] J.M. McCord, *Science*, 185 (1974) 529.
[9] D. Rowley, J.M.C. Gutteridge, D. Blake, M. Farr and B. Halliwell, *Clin. Sci.*, 66 (1984) 691.
[10] O.A. Arouma, B. Halliwell, J. Butler and B.M. Hoey, *Biochem. Pharmacol.*, 38 (1989) 4353.
[11] D.R. Blake, N.D. Hall, P.A. Dieppe, B. Halliwell and J.M.C. Gutteridge, *Lancet*, ii (1981) 1142.
[12] N. Kawasaki, T. Tanimoto and A. Tanaka, *Anal. Biochem.*, 192 (1991) 104.
[13] E.H. Morgan, *Biochim. Biophys. Acta*, 580 (1979) 312.
[14] N. Kojima and G.W. Bates, *J. Biol. Chem.*, 254 (1979) 8847.
[15] F. Funk, J.P. Lenders, R.R. Crichton and W. Schneider, *Eur. J. Biochem.*, 152 (1985) 167.
[16] P. Biomond, A.J.G. Swaak, C.M. Beindorff and J.F. Koster, *Biochem. J.*, 239 (1986) 169.
[17] D. Etherington, D. Pugh and I. Silver, *Acta Biol. Med. Ger.*, 40 (1981) 1652.
[18] B.M. Babior, *N. Engl. J. Med.*, 298 (1978) 659.
[19] B.M. Babior, *N. Engl. J. Med.*, 298 (1978) 721.
[20] C.C. Winterbourn, *Biochem. J.*, 198 (1981) 125.
[21] J.M.C. Gutteridge, D.A. Rowley and B. Halliwell, *Biochem. J.*, 199 (1981) 263.
[22] J.D. Gower, G. Healing and C.J. Green, *Anal. Biochem.*, 180 (1989) 126.
[23] J.M.C. Gutteridge, *Biochem. J.*, 245 (1987) 415.
[24] P. Biemond, H.G. van Eijk, A.J. Swaake and J.F. Koster, *J. Clin. Invest.*, 73 (1984) 1576.
[25] C.E. Thomas, L.A. Morehouse and S.D. Aust, *J. Biol. Chem.*, 260 (1985) 3275.
[26] B.J. Bolann and R.J. Ulvik, *Biochem. J.*, 243 (1987) 55.
[27] J.K. Brieland and J.C. Fantone, *Arch. Biochem. Biophys.*, 284 (1991) 78.