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Determination of iron(III) ion using ion chromatography with electrochemical detection and its application to the assay of the ferroxidase activity of ceruloplasmin

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SUMMARY

A simple, rapid and sensitive method for the determination of iron(III) ion by ion chromatography coupled with electrochemical detection was developed. The method reduced the interferences of iron(II) ions and enabled more than 5 pmol of iron(III) to be determined with an injection volume of 10 μl. The method was applied to the determination of ferroxidase activity of ceruloplasmin with good reproducibility. The production of iron(III) ion by ceruloplasmin was found to be linear with respect to reaction time and protein concentration.

INTRODUCTION

Iron is an essential element in biological systems. It exists in an aqueous solution in two oxidation states, Fe^{II} and the Fe^{III}, on which the transport and storage of iron in biological systems is based[1].

Most methods now used for determination of Fe^{II} are spectrophotometric, using, for instance, bathophenanthroline as a reagent[2], but the determination of Fe^{III} is complicated as it must first be reduced to Fe^{II} and the total iron is determined, making the trace determination of Fe^{III} in the presence of excess Fe^{II} difficult. Osaki[3] developed an assay method for ferroxidase activity of ceruloplasmin which catalyses the oxidation of Fe^{II} to Fe^{III} in the presene of oxygen[4] and apparently detects ferroxidase-generated Fe^{III} by spectrophotometric measurement following Fe^{III}–transferrin formation. However, in this process, the form in which iron is incorporated into the apotransferrin molecule is unknown, and it is often influenced by the presence of contaminating substrates.

An ion chromatographic method was introduced for use in the separation and determination of some metal cations, the cations separated with an ion-exchange column being detected with a conductivity detector[5] or a spectrophotometric detector[6–8]. Recently, there has been increasing interest in the use of electrochemical

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detectors for the trace determination of electroactive compounds[9], as this method usually enhances both selectivity and sensitivity. However, few methods for the determination of Fe^{III} with an electrochemical detector have been reported[10,11], as it must be determined via a reduction step which introduces problems associated with oxygen interference.

In this work we attempted to develop a selective and sensitive method for the determination of Fe^{III} by combining an ion-exchange column with electrochemical detection. This method may be useful for determining the ferroxidase activity of ceruloplasmin.

EXPERIMENTAL

Materials

Lactic acid, lithium hydroxide, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and $FeNH_4(SO_4)_2 \cdot 12H_2O$ were obtained from Wako (Osaka, Japan), trichloroacetic acid from Nacalai Tesque (Kyoto, Japan) and ceruloplasmin from Green Cross (Osaka, Japan).

Chromatographic conditions

The chromatographic system consisted of a Hitachi (Tokyo, Japan) Model L-6000 pump and a Rheodyne Model 7125 injector (Tosoh, Tokyo, Japan). An HPIC-CS5 separation column with an HPIC-GS5 guard column (Dionex, Sunnyvale, CA, U.S.A.) was used to separate Fe^{III}. The latter was detected with an Irica Σ-875 amperometric detector with a glassy carbon working electrode and Ag/AgCl reference electrode (Irica Kogyo, Kyoto, Japan), and the detector output was recorded on a Hitachi Model D-2000 chromato-integrator.

The eluent was 250 m*M* lactic acid adjusted to pH 3.2 with lithium hydroxide and purged with nitrogen. The flow-rate was 1.0 ml/min.

Before the beginning of operation, 0.1 *M* sodium sulphite solution was pumped through the column at 1.0 ml/min for 1 h every day to remove oxygen from the system.

Samples were injected into a 10-μl PTFE sample loop with a plastic syringe.

Determination of Fe^{III}

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

Determination of ferroxidase activity

A mixture of 530 μl of 0.2 M acetate buffer (pH 5.0) and 70 μl of 20 m*M* iron(II) ammonium sulphate solution was preincubated at 30°C for 3 min. A 100-μl volume of solution containing various amounts of ceruloplasmin (0.5–100 μg) was added to the mixture and mixed well. After incubation for 3 or 20 min at 30°C, the reaction was terminated by the addition of 300 μl of 12.5% trichloroacetic acid. The mixture was centrifuged at 6500 *g* for 10 min to remove the precipitated proteins and 10 μl of the supernatant were used for analysis. The enzyme activity was determined as the amount of Fe^{III} produced per minute.

RESULTS

Determination of Fe^{III}

The separation and detection of Fe^{III} was performed by ion chromatography with electrochemical detection as the lactic acid-chelated complex formed directly on the chromatographic column. In order to establish the optimum conditions for the rapid and sensitive determination of Fe^{III}, we investigated the effect of different factors on the chromatographic behaviour of the Fe^{III}–lactic acid complex.

The pH of the mobile phase was varied from 3.0 to 4.5. The retention times of Fe^{III} at pH 3.5 and 3.0 were 2.1 and 3.6 min, respectively. Hydrodynamic voltammograms of Fe^{III} in lactic acid at various pH are shown in Fig. 1. As the pH increased, the hydrodynamic voltammogram shifted to the negative side, and therefore it was found that the peak area increased with decreasing pH at a certain potential. However, when a more acidic mobile phase was used, the peak shape was broadened considerably, and the retention time was prolonged. Therefore, lactic acid at pH 3.2 was found to be most suitable with respect to both sensitivity and separation.

Another important factor is the applied potential of the working electrode to detect the Fe^{III} complex. The maximum peak area for Fe^{III} in lactic acid at pH 3.2 is obtained at a potential near -0.5 V, but this potential is unfavourable from the standpoint of interference by oxygen. A potential of -0.2 V was chosen to obtain an optimum balance between the sensitivity and stability of the electrode.

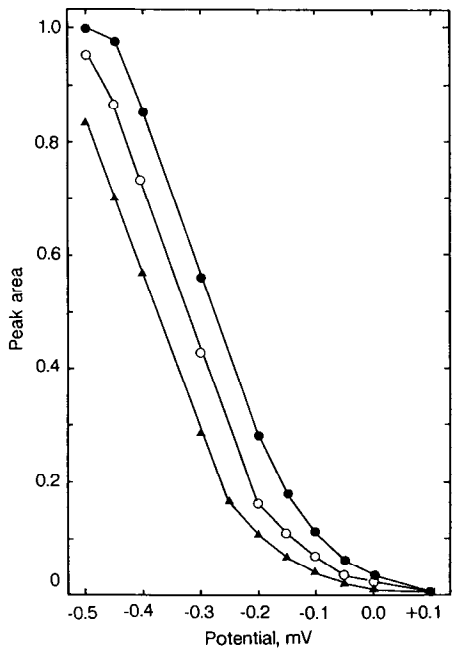


Fig. 1. Hydrodynamic voltammograms of Fe^{III} in 250 mM lactic acid at (●) pH 3.2, (○) pH 3.6 and (▲) pH 4.0. The peak area at pH 3.2 at a potential of -0.5 V was taken as 1.0.

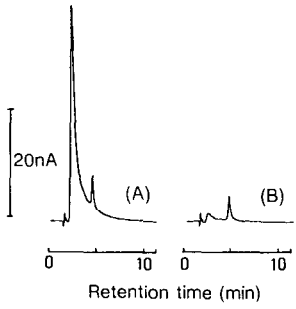
Peak-area calibration was superior to peak-height calibration. The calibration graph for Fe^{III} obtained for a 10-μl injection was linear at concentrations of more than 5 pmol, and the correlation coefficient between 1000 and 5 pmol was 0.9997.

Determination of ferroxidase activity

The proposed method for the determination of Fe^{III} was applied to the determination of ferroxidase activity. Fig. 2 shows chromatograms of the reaction mixture consisting of ceruloplasmin and substrate Fe^{II}. Under the condition described, Fe^{III} was eluted with a retention time of 2.2 min, and a minor peak associated with trichloroacetic acid was detected at 4.3 min. The presence of a large excess of the substrate Fe^{III} did not interfere with the measurement of the product.

The enzymatic reaction proceeded linearly for 20 min up to 10 μg of ceruloplasmin. In the range from 10 to 100 μg of ceruloplasmin, the maximum rate of Fe^{III} production was obtained with incubation for 3 min (Fig. 3). Increasing the time of incubation lowered the detection limit.

In order to examine the stability of Fe^{III}, Fe^{III} solutions containing Fe^{II} or ceruloplasmin were incubated as described in Table I. Fe^{III} could be recovered quantitatively even if the mixtures were incubated for 20 min (Table I). Moreover, no over-recovery of Fe^{III} was found so that the non-enzymatic oxidation of excess Fe^{II} to Fe^{III} could be neglected under these conditions.



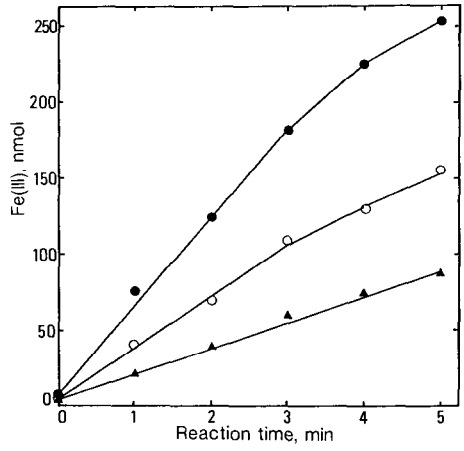


Fig. 2. Chromatograms of reaction mixtures consisting of ceruloplasmin and Fe^{II}. (A) Ceruloplasmin (50 μg) in acetate buffer (pH 5.0) containing 2 m*M* Fe^{II} was incubated for 3 min at 30°C. The reaction was stopped by addition of trichloroacetic acid. (B) Ceruloplasmin (50 μg) was added to a solution of trichloroacetic acid and acetate buffer containing 2 m*M* Fe^{II} and incubated for 3 min at 30°C. Each sample was centrifuged at 6500 *g* for 10 min, and 10 μl of sample were injected for ion chromatography.

Fig. 3. Time course of the generation of Fe^{III} with ceruloplasmin. Assay mixture containing (●) 100, (○) 50 or (▲) 25 μg of ceruloplasmin was incubated for the indicated time at 30°C, then the reaction was terminated by addition of 12.5% trichloroacetic acid.

TABLE I

RECOVERY OF IRON(III) ADDED TO THE INCUBATION MEDIUM

Iron(II) salt was added to acetate buffer (pH 5.0) containing Fe^{II} or ceruloplasmin, and the mixture was incubated for 3 or 20 min at 30°C. After incubation, trichloroacetic acid was added to the mixture. A 10-μl volume of each sample was used for analysis, and the appropriate blanks for correction were prepared. The peak areas of Fe^{III} in these samples were compared with that obtained from a 10-μl injection of 120 or 70 μM Fe^{III} standard solution.

Final concentration of Fe^{III} (μM)	*Incubation medium*		*Time (min)*	*n*	*Recovery (%)*	*Reproducibility (R.S.D.[a], %)*
	Fe^{II} (mM)	*Ceruloplasmin (μg)*				
120	2	0	3	6	100	1.1
120	0	50	3	6	98	0.67
70	2	0	20	6	99	1.4
70	0	5	20	6	97	2.4

[a] Relative standard deviation.

The method allowed the measurement of the activity of 0.5 μg of ceruloplasmin. The oxidation rate was proportional to the amount of the enzyme from 0.5 to 100 μg with a correlation coefficient of 0.9999 when the peak areas were measured at −0.2 V (Fig. 4).

The reproducibility of ferroxidase assay was determined by measuring the ferroxidase-generated Fe^{III} six times. The relative standard deviations for amounts of enzyme of 50 and 5 μg were 1.3 and 2.9%, respectively.

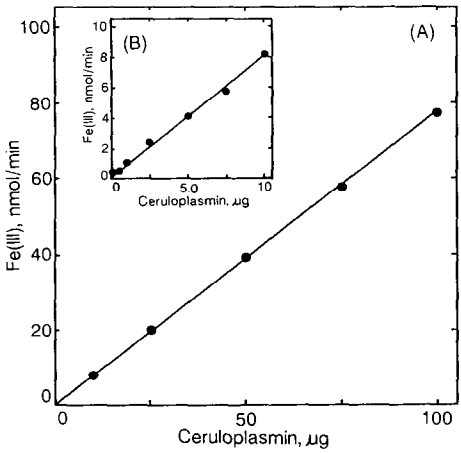


Fig. 4. Relationship between the rate of Fe^{III} generation and ceruloplasmin concentration. The reaction time was (A) 3 min or (B) 20 min.

DISCUSSION

The determination of Fe^{III} by electrochemical detection must necessarily be via the reduction step. However, the reduction process frequently introduces problems associated with oxygen interference. In addition, it is occasionally difficult to obtain the maximum response for the determination of Fe^{III} because the negative shift of the potential required for reduction is often dependent on the type of chelating agents used. Bond and Nagaosa[11] reported the determination of Fe^{III} as the Fe^{III}–8-hydroxyquinoline complex by reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection. To determine more than 1 ng of Fe^{III}, they had to select a potential of -0.5 V, which is unfavourable for routine analysis. Compared with their method, the use of lactic acid as the chelating agent enabled us to determine more than 0.3 ng of Fe^{III} even at -0.2 V, which is a practical and stable potential for the electrode.

Most HPLC methods for the determination of Fe^{III} are based on spectrophotometric detection, in which the eluted metal ions are detected after a post-column reaction with a chelating agent such as 4-(2-pyridylazo)resorcinol[12]. Compared with this detection method, electrochemical detection is selective and simple as the separated Fe^{III} can be detected directly without a post-column reaction. This method is approximately ten times more sensitive than ion chromatography with spectrophotometric detection[13].

Another HPLC method for the assay of ferroxidase activity based on the determination of ferroxidase-generated Fe^{III} was reported by Shioiri *et al.*[14], who used an ion-exchange HPLC system connected with a UV detector, using sulphuric acid containing acetonitrile as the mobile phase. This procedure was 100 times less sensitive for the determination of Fe^{III} than our method and the measurement of the activity was limited to a narrow range of ceruloplasmin concentrations.

Takayanagi and Yashiro[15] reported a ferroxidase assay procedure based on the measurement of the decrease in substrate Fe^{II} by ion chromatography coupled with spectrophotometric detection. However, this method was unsuitable for the microassay of iron species, as Fe^{II} was gradually oxidized to Fe^{III} under their conditions, and ferroxidase activity could not be measured accurately. In the method described here, this problem was overcome by using lactic acid at pH 3.2. This eluent reduced the blank value so that good reproducibility was obtained and over-recovery of Fe^{III} was not observed.

The ion chromatographic method coupled with electrochemical detection not only enhanced the sensitivity for the determination of Fe^{III} but also provided rapid, simple and sufficient reproducibility and recovery. This method was applicable to the determination of the ferroxidase activity of ceruloplasmin, and may be suitable for the investigation of other biological reactions associated with Fe^{III}.

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