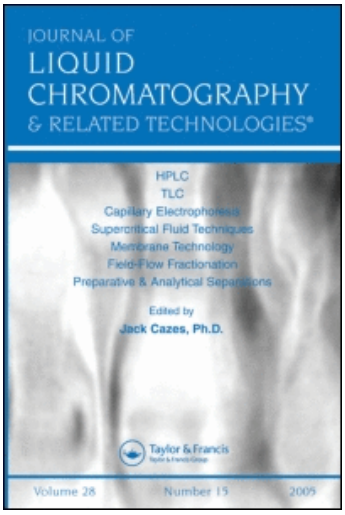

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Simultaneous Determination of Zinc(II) and Iron(III) in Human Serum by Liquid Chromatography Using Post-Column Derivatization with 4-(2-Pyridylazo)-Resorcinol

Y. Nagaosa[a]; M. Tanizaki[a]
[a] Department of Applied Chemistry and Biotechnology, Faculty of Engineering Fukui University, Bunkyo, Fukui, Japan

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SIMULTANEOUS DETERMINATION OF ZINC(II) AND IRON(III) IN HUMAN SERUM BY LIQUID CHROMATOGRAPHY USING POST-COLUMN DERIVATIZATION WITH 4-(2-PYRIDYLAZO)-RESORCINOL

Yukio Nagaosa,* Mifumi Tanizaki

Department of Applied Chemistry and Biotechnology
Faculty of Engineering
Fukui University
Bunkyo, Fukui 910, Japan

ABSTRACT

A liquid chromatographic method for the determination of sub-μg/mL levels of zinc(II) and iron(III) in human serum has been studied by use of a post-column derivatization with 4-(2-pyridylazo)-resorcinol (PAR) as a chelating agent. Human serum samples are pretreated with hydrochloric acid and trichloroacetic acid for deproteination, and then sodium acetate solution is added for adjusting the pH of the aqueous sample to 3.5. The sample is injected onto a 10 μm bonded phase, strong-acid ion exchange column, and eluted with 0.340 mol/L tartrate buffer (pH 3.75) at a flow rate of 1.0 mL/min. The eluate is mixed with 10^{-4} mol/L PAR solution as the post-column reactant at the same flow-rate of the elution of the metal ions at 30°C, and the absorbance of the mixed solution is monitored at 515 nm (Zn) and 715 nm (Fe). The human serum samples can be analyzed with good precision by the proposed method.

INTRODUCTION

Many analytical methods for the determination of metals in biological samples have been reported, using spectrophotometry,[1,2] electrochemistry,[3,4] and chromatography.[5] In clinical analysis of human serum, iron and zinc as well as copper are important elements to be determined for the evaluation of human bloods and bodies.[6] Normal concentration levels of zinc and iron in serum are in the range of 1.0-2.0 μg/mL.[7] Deficits of both metals in blood are known to cause diseases of oxygen transport and other biological process. Liquid chromatography (LC) has an advantage of enabling multi-element determinations and automatic applications. Until now, very few papers have been reported on the LC determination of zinc(II) in human serum for routine analysis because of lack of suitable chelating agents.[8,9] The purpose of this study is to develop a simple and rapid LC method for the quantitation of zinc and iron in serum samples. The proposed method is based on the separation of the two metal ions on a strong cation-exchange column with tartrate buffer, followed by post-column derivatization of the metal species with 4-(2-pyridylazo)resorcinol (PAR) as a chelating agent. The absorbance of the resultant solution is monitored spectrophotometrically at 515 nm (zinc) and 715 nm (iron). The present paper describes the post-column LC method for the routine analysis of human serum samples.

EXPERIMENTAL

Apparatus and Reagents

A Tosoh (Tokyo, Japan) CCPD liquid chromatograph equipped with a Rheodyne 7125 sample injection valve (100-μL) and a Shimadzu (Kyoto, Japan) SPD-AS UV-VIS spectrophometer fitted with a 8-μL flow cell was used. The derivatization of the eluate with PAR was carried out using a Shimadzu LC-9A delivery pump and a 1/16 x 0.4 x 1000 mm reaction coil at 30°C. A TSKgel IEC (Na^+ form) (Tosoh, Tokyo, Japan) column (150 x 4.6 mm i.d.; particle size 10 μm) was used. The flow rate was 1.0 mL/min. Data was processed with a Shimadzu model CR-5A integrator/recorder. A Toa Denpa (Saitama, Japan) HM-30s pH meter was used for pH measurements.

PAR was of analytical reagent grade from Wako Junyaku Chemicals (Tokyo, Japan). Metal ion standards of a desired concentration were prepared by dilution of stock 1000 μg/mL standard solutions for atomic absorption spectrometry. Serum samples (Wako control sera I, II) were purchased from

Wako Junyaku Chemicals. Deionized water used was prepared from a Millipore Milli-Q water purification system. The mobile phase (0.340 mol/L tartrate buffer) was prepared by dissolving 9.98 g of tartaric acid and 23.7 g of sodium tartrate in 500 mL of deionized water. The derivatization solution (10^{-4} mol/L PAR) was prepared by dissolving 5.3 mg of PAR, 7.35 mL of 17 mol/L acetic acid and 25 mL of 15 mol/L ammonia in 250 mL of deionized water. The other reagent grade chemicals were purchased from Wako Junyaku Chemicals.

LC Separation and Determination of Metal Ions

A slightly acidic aqueous solution (ca. pH 4) containing metal ions of interest, was injected onto a strong-acid ion exchange (10 μm, particle size) column and eluted with the mobile phase at 1.0 mL/min flow rate. The eluate, after LC separation, was mixed with the PAR solution and flowed to the reaction coil at 1.0 mL/min flow rate for derivatization. The absorbance of the resultant solution was monitored spectrophotometrically at 515 nm for all metal inos, except for the detection of iron(II) at 715 nm.

The concentration of the metal ion was determined by measuring the peak height on the chromatogram. In the serum analysis, the aqueous sample was injected onto the analytical column after the pretreatment procedure was carried out as described below.

Sample Preparation for Serum Analysis

Sample solution (500 μL) was placed in a 50-mL stoppered Teflon centrifuge tube and 1.0 mol/L hydrochloric acid (250 μL) added. The solution was heated in a steam bath at about 80°C for 2 min. After cooling to room temperature, the solidified mixture was stirred with 20 (w/v) % trichloroacetic acid (250 μL) and centrifuged at 2000 g for 5 min. The supernate solution was filtered with a 0.45-μm Millipore filter, and the serum concentration was decreased to 1/2 compared to that of the original serum.

The filtered solution (200 μL) was treated with 1.2 mol/L sodium acetate (200 μL) in a 5-mL Teflon test tube. Just prior to injections, hydroxylamine hydrochloride (about 5 mg) was added to the resultant solution (400 μL) for the reduction of iron(III) to iron(II).

Preparation of Calibration Curves for Serum Analysis

The calibration curves were constructed by adding known amounts of metal standards (10 μg/mL) to the serum sample I solution (500 μL) that was treated with the procedues described above. By plotting the peak heights against the metal concentrations, the slopes of the calibration curves for the determination of zinc(II) and iron(III) in serum samples were obtained. The intercepts of the curves were obtained from the reagent blank test.

RESULTS AND DISCUSSION

Post-Column LC Using PAR for Several Metal Ions

The post-column LC methods have been studied for the determination of traces of some heavy metal ions by use of citrate buffer as the mobile phase and PAR as the derivatization agent.[10] We have attempted to develop a convenient and useful post-column LC method for serum analysis. Figure1 shows a typical chromatogram obtained for direct injection of a mixture containing 1.0 mg/mL of each of the metal ions according to the procedure described above. The elution peaks appeared at retention times of 1.80 min for copper(II), 3.75 min for zinc(II), 4.45 min for nickel(II), 7.75 min for cobalt(II), 14.00 min for iron(II, III), and 30.20 min for manganese(II).

No difference in retention time was observed between iron(II) and iron(III) under the experimental conditions. The zinc(II) peak partially coeluted with the nickel(II) one when they were separated with 0.340 mol/L tartrate buffer of any pH values lower than 3.45 (1:1 molar ratio of tartaric acid to tartrate).

For the purpose of this study, however, there is no problem of peak separation between zinc(II) and nickel(II) because of a negligible presence of nickel(II) in human serum. The effect of pH of mobile phase on retention times of the six metal ions was investigated over the range of pH 3.45-4.53 under the constant concentration (0.340 mol/L) of tartrate buffers used.

Data shown in Fig. 2 indicate that the retention times gradually decreased with increasing pH of mobile phase. Indistinguishable peaks were observed among the five metal ions except manganese(II) when a 0.340 mol/L sodium tartrate buffer (pH 6.9) was used as the mobile phase.

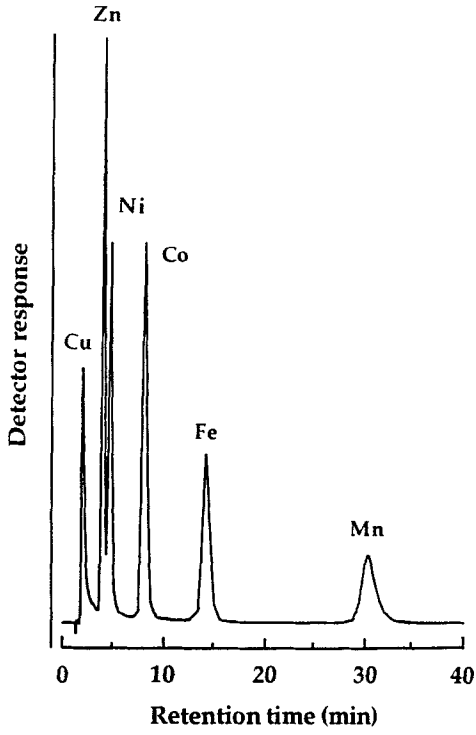

Figure 1. A typical chromatogram of some metal ions by post-column derivatization after LC separation. Concentration of metal ions:1.0 μg/mL each; mobile phase:0.340 mol/l tartrate buffer (pH3.75); derivatization solution:10^{-4} mol/l PAR solution; wavelength: 515 nm; flow rate: 1.0 mL/min; other LC conditions as in text.

The effect of concentration of tartrate buffer on the retention times was also investigated over the concentration of 0.280-0.370 mol/l at a constant pH of 3.75. It can be seen from Figure3 that the retention times gradually decreased with increasing concentration of tartrate.

The above results are understood in terms of the complex formation of metal tartrates onto the cation exchange column. We have selected a 0.340 mol/L tartrate buffer (pH 3.75) as being appropriate for the mobile phase.

The other experimental variables such as the concentrations of PAR and ammonia buffer, flow rates and column temperature were investigated, and the usual conditions were selected as described in the experimental section because

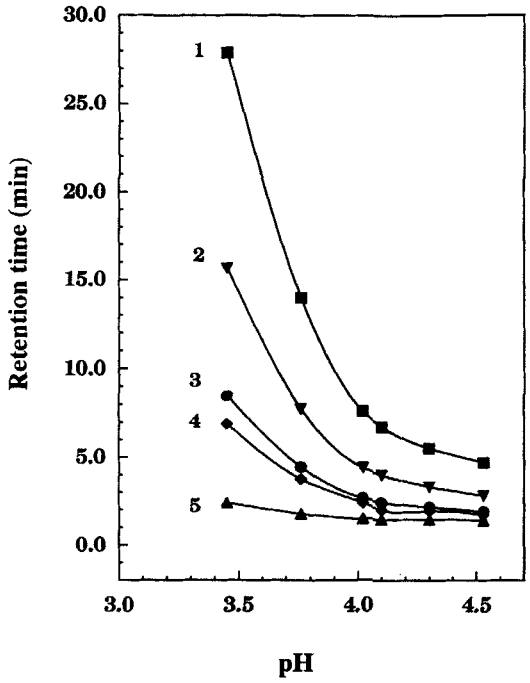

Figure 2. Effect of pH (mobile phase) on retention times. Total concentration of tartrate: 0.340 mol/L 1: Fe;2: Co; 3: Ni:; 4: Zn; 5:Cu; other conditions as in Fig.1.

they gave no serious effect on the peak heights of zinc(II) and iron(III). The iron(III) peak was relatively small and less producible in height compared with the others, and therefore, we have decided to get higher responses due to the iron(II) complex by reducing the iron(III) to iron(II) with the addition of hydroxylamine hydrochloride to samples just before injections.

Calibration Curves and Detection Limits

Calibration curves of peak height vs. metal concentration were constructed according to the procedure of the experimental section. Except for copper(II), peak heights were linearly proportional to metal concentration up to 2.0 μg/mL at correlation coefficients of above 0.998. The calibration curve for copper(II) was evaluated using the quadratic equation against the metal concentration ranging 0.1-1.0 mg/mL. It appears that the copper(II) species had an adsorption nature on the analytical column under the present conditions.

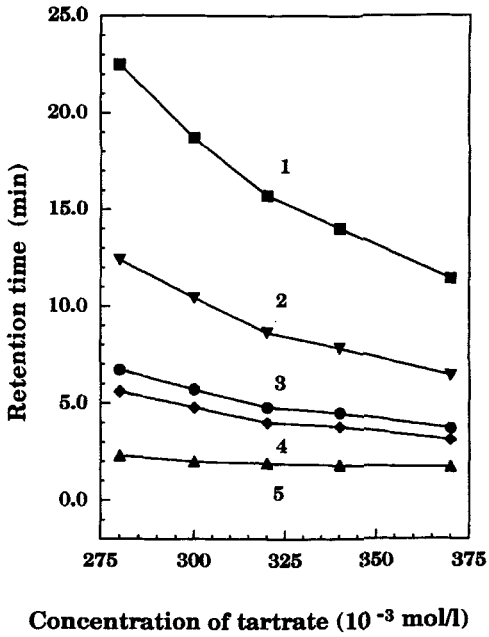

Figure 3. Effect of tartrate concentration on retention times. The pH of mobile phase: 3.75; other conditions as in Figs.1 and 2.

Accordingly, the determination of copper in serum by the proposed LC method was routinely unusable. The iron(II) complex at 715 nm had an absorbance of seven times higher than the corresponding iron(III) one at 515 nm. The detection limit for iron(II) at 715 nm attained to 0.01 μg/mL. The others at 515 nm were 0.01 μg/mL for zinc(II), 0.04 μg/mL for nickel(II) and cobalt(II), and 0.10 μg/mL for manganese(II).

Application to Serum Analysis

Based on the above results, the proposed LC method enables the five metal ions except copper(II) to be determined at least in the sub-μg/mL concentration levels. Control human sera (Wako I, II) were selected as real samples to evaluate the present post-column LC method. Taking into account the matrix effect of the serum samples, we have constructed the calibration curves for the determination of zinc(II) and iron(III) by applying the standard

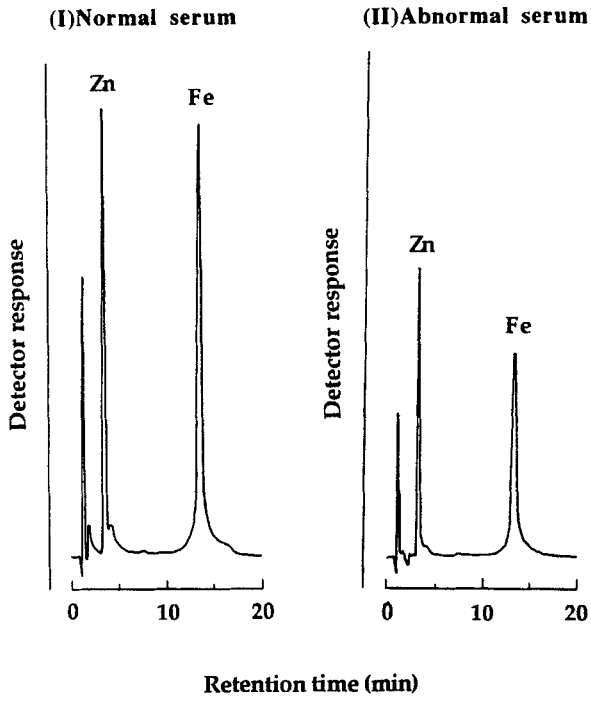

Figure 4. Determination of zinc and iron in control sera by the proposed LC method. (I) control serum I; (II) control serum II; the detections were carried out at 515 nm for zinc(II) and at 715 nm for iron(III).

addition method of analysis: known amounts of the two metal standards were added to serum sample solutions (Control serum I). The following equations were obtained by using the slopes obtained from the above experiments and the intercepts of the blank test: Y (peak height, arbitrary unit) = 53.2X (metal concentration, μg/mL) + 1 for zinc(II) and Y = 34.8X + 4 for iron(II), respectively. Fig. 4 shows the chromatograms obtained for Control sera I and II. The analytical results calculated from the peak heights, summarized in Table 1, were in good agreement with the recommended values obtained by spectrophotometry using bathophenanthroline (iron) and sodium 2-(5-bromo-2-pyridylazo)-5-(N-propyl-N-sulfopropylamino)-phenol (zinc). Therefore, the post-column LC method can successfully be applied to the simultaneous determination of zinc and iron in human serum.

Table 1

Analytical Results for Analyses of Control Sera (Wako)[a]

Sample	Metal	Concentration Found (μg/mL)	Certified Value (μg/mL)
Serum I	Zn	1.42 ± 0.02	1.34 ± 0.17
(Normal)	Fe	1.76 ± 0.07	1.80 ± 0.18
Serum II	Zn	0.84 ± 0.02	0.82 ± 0.14
(Abnormal)	Fe	0.67 ± 0.03	0.62 ± 0.07

[a] Five replicate determinations were made.

CONCLUSION

The present report presented a very useful LC method for the simultaneous determination of zinc and iron in serum. Since the method is rapid and accurate, it is recommended that this technique be used for routine analysis of biological samples as well as environmental ones.

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