Direct Determination of Zinc in Human Serum by Flow-Injection Spectrophotometric Analysis

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Flow-injection spectrophotometric analysis has been established for the determination of zinc at 1 µg ml⁻¹ level in serum using 5-Br-PAPS (2-(5-bromo-2-pyridylazo)-5-[N-n-propyl-N-(3-sulfopropyl)amino]phenol, disodium salt) as a chromogenic reagent. A 100 µl portion of serum is directly injected into the carrier stream containing 5%(v/v) TMAH (tetramethylammonium hydroxide) and 0.5 M trisodium citrate. After mixing with 0.01%(w/v) 5-Br-PAPS-0.5 M ammonium acetate-7.2X10⁻⁵ M salicylaldehyde oxime-1%(v/v) Triton X-100-0.5 M hydrochloric acid solution, the absorbance of Zn-5-Br-PAPS complex is measured at 560 nm. The proposed flow-injection system is fully controlled with a personal computer and permits 60 samples to be measured per hour. The relative standard deviation (n=50) for 1.2 µgZn ml⁻¹ solution is 3.3%.

Keywords Flow-injection, spectrophotometry, zinc, human serum

The zinc concentration in serum is important in the diagnosis of zinc deficiency. Generally, the zinc concentration in serum is routinely measured by flame atomic absorption spectrometry (AAS). Acid decomposition or protein-matrix separation technique is necessary to determine zinc in serum more accurately. Although the AAS technique is simple, there are several disadvantages: matrix effect due to the coexisting protein and contamination during the preparation of the sample solution. One solution to the problems mentioned above is the use of the flow-injection spectrophotometric method (FIA-SP). FIA is a rapid and precise technique which has found several applications to the human fluids, and spectrophotometry is still more useful than AAS in many parts of the world, because of the cost-effectiveness.

2-(5-Bromo-2-pyridylazo)5-[N-n-propyl-N-(3-sulfopropyl)amino]phenol (5-Br-PAPS), which is water-soluble pyridylazo dye, is sensitive and selective for determination of serum zinc (Zn-5-Br-PAPS, ε=1.4X10⁴ mol⁻¹ cm⁻¹, at 554 nm). However iron and copper, which are also present in serum, interfere with the determination of zinc with 5-Br-PAPS. These interferences can be eliminated by precipitation of protein matrix with trichloroacetic acid and being masked with potassium fluoride and ammonium peroxosulfate. The separation of protein matrix by precipitation is one of the essential steps to carry out accurate spectrophotometric measurement of zinc concentration in serum. In general, decomposition of the protein with a mixture of hydrochloric and trichloroacetic acids was utilized to separate matrix protein by precipitation. In the acidic solution, because of the formation of the precipitate of matrix protein, it is not appropriate for the direct determination of zinc in human serum with flow-injection-spectrophotometry. In another study, guanidine hydrochlorate was used for the determination of copper in serum by the flow-injection-spectrophotometric method. In this system, a high concentration solution of guanidine hydrochlorate containing potassium periodide and potassium iodide is utilized for deproteinization treatment. It is not appropriate for flow-injection system to pump high-salt concentration solution, because of potential of clogging of the flow line.

In this paper, we have developed an FIA system for the determination of zinc in human serum. In order to overcome the above mentioned problems, a new method was devised for on-line decomposition of protein, which enable the sample solutions to be injected directly to the FIA system. Tetramethylammonium hydroxide (TMAH) was employed to decompose zinc-protein complex so that protein matrix was retained in water-soluble state in the system. Interference from iron and copper was avoided by masking with trisodium citrate and salicylaldehyde oxime. This method was successfully applied to the determination of zinc in human serum samples.

Experimental

Apparatus
A Hitachi U-1000 ratio-beam spectrophotometer equipped with a flow cell (10-mm path length, 60 µl) was used as a detector for the flow-injection system.
Sanuki-kogyo DMX-2400T double-plunger reciprocating pump was used to deliver a carrier and a chromogenic reagent solution and an SS-3000 auto sampler was used for sample introduction. PTFE tubing (1.0 mm i.d. for analytical flow lines and 0.5 mm for the back-pressure coil) was used. All instruments were fully controlled with an NEC PC-9801LV personal computer; the operation program was written in our laboratory.

**Chemicals and solutions**

All reagents were of analytical-reagent grade and all solutions were prepared with deionized distilled water.

Standard zinc solution (1 g l⁻¹). One gram of zinc (99.99% purity) was dissolved in nitric acid and diluted to 1000 ml with water. A working standard solution was prepared by diluting the stock standard solution appropriately.

Carrier solution (5.0% tetramethylammonium hydroxide-0.1 M trisodium citrate solutions). Two hundred milliliters of 25%(v/v) tetramethylammonium hydroxide (TMAH, TAMAPURE AA-1000 grade, Tamakagaku Kogyo) and 35 g of trisodium citrate (Kanto Chemicals Co.) were dissolved in an appropriate amount of water, followed by dilution to 1000 ml with water.

Chromogenic reagent containing masking agents. About 38 g of ammonium acetate (Kanto Chemicals Co.), 1.0 g of salicylaldehyde oxime, 10 ml of polyoxyethylene-p-ocetylphenylether (Triton X-100, Kishida Kagaku), 0.5 g of 2-(5-bromo-2-pyridylazo)-5-(N-propyl-N-sulfopropylamino)phenol disodium salt (DOTTITE 5-Br-PAPS, Dojindo Lab.) and 42 ml of hydrochloric acid. When the carrier and chromogenic reagent solutions are mixed in the flow system at the volume ratio of 1:1, the final concentration for each reagent should be as follows: 3.6X10⁻³ M salicylaldehyde oxime-0.25 M ammonium acetate-1%(v/v) Triton X-100-1.0X10⁻⁴ M 5-Br-PAPS in 0.25 M hydrochloric acid solution.

**Procedure**

Figure 1 shows a schematic diagram of the manifold used. Carrier solution C (TMAH-trisodium citrate) is delivered into the analytical flow line at the flow rate of 1.2 ml min⁻¹ by using the pump P. Without deproteinization treatment the sample solution (100 µl) is introduced into the carrier stream with an auto sampler equipped with a six-way valve. After the sample solution is merged with the chromogenic reagent solution R containing masking agents at the flow rate of 1.2 ml min⁻¹, the absorbance of the zinc-5-Br-PAPS complex is monitored at the flow-through cell D at 560 nm. The peak height absorbance was utilized to determine zinc in the sample solution.

**Results and Discussion**

For the accurate determination of total zinc normally existing in the active site of the protein in body fluids, it is necessary to liberate the metals from the protein prior to analysis. We studied the decomposition method of zinc-protein complex in an alkaline medium. In trace analysis, one of the major problems is the high reagent blank involved in alkali such as sodium hydroxide or ammonia water. We employed tetramethylammonium hydroxide as an alkaline source to decompose zinc-protein complex, because of the high purity of this reagent and its low contents of impurities (less than 1 ng Zn ml⁻¹).

**Optimization of flow system**

**Effect of carrier concentration.** Because of the possible dissolution of the quartz cell equipped in the spectrophotometer, concentrated solutions of TMAH cannot be used. On the other hand, dilute solutions of TMAH can not liberate zinc ions from protein matrix. By variation of the TMAH concentration, we have found the optimal TMAH concentration to be 5%(v/v) where sensitivities for zinc in the standard and sample solutions were equal and maximal (Fig. 2). TMAH at this concentration does not seem to dissolve a quartz cell. For masking of iron, more than 0.05 M trisodium citrate is recommended. A mixture containing 5.0%(v/v) TMAH and 0.1 M trisodium citrate was used in all subsequent studies, since it ensures complete liberation of zinc from protein and elimination of matrix interferences.

**Effect of pH and buffer concentration.** The maximum absorbance was observed when the pH of the mixture of the buffer and the carrier stream was between 8.5 to 9.5. To achieve this pH range, a buffer concentration less than 0.5 M can not be employed because of the high alkalinity of the carrier solution. Therefore the buffer concentration and pH of the chromogenic reagent solution were adjusted to 0.5 M and 8.8±0.1, respectively. At the merging point of the carrier and chromogenic reagent solutions, the possibility of the appearance of the precipitation ascribed to decomposition of matrix protein is present at the interface of the carrier and chromogenic reagent solutions, because matrix protein...
decomposes and precipitates at pH of less than 6.5. In the absence of surface-active agent, in fact, the reproducibility was poor and the flow lines were clogged with the precipitate. We therefore decided to add a surface-active agent, 1%(v/v) Triton X-100 to avoid these precipitates. Accordingly the chromogenic reagent solution was 0.5 M ammonium acetate-1%(v/v) Triton X-100-hydrochloric acid.

Concentration of masking agent. Iron, copper, and nickel react with 5-Br-PAPS under the conditions employed for the zinc determination. Interference from nickel at a few ng ml\(^{-1}\) level in the real sample, is negligible. It is, however, necessary to mask iron and copper, because the serum sample solutions usually contain a few µg ml\(^{-1}\) level of iron and copper. The influence of the salicylaldehyde oxime added to the chromogenic reagent solution for masking of iron and copper was tested. It was found that the concentration of more than 7.2×10\(^{-3}\) M of salicylaldehyde oxime was necessary for proper masking of iron and copper. However, we have observed that salicylaldehyde oxime alone is not adequate to mask iron in the serum sample solution. The combination of salicylaldehyde oxime added to the chromogenic reagent solution with trisodium citrate which is dissolved in the carrier solution is suitable for masking. More than 0.1 M of trisodium citrate in the carrier solution was necessary for total masking of iron. It should be noted that this masking system can only work for iron less than 5 µg ml\(^{-1}\) in serum samples.

Chromogenic reagent concentration. The special advantages of 5-Br-PAPS used as a chromogenic reagent are its good water solubility, long-term stability and low zinc content. This reagent can be used without any further purification. Preconcentration and/or separation procedure are not required to determine zinc in human serum at the level of µg ml\(^{-1}\). The effect of the 5-Br-PAPS concentration was studied for a 2.0 µgZn ml\(^{-1}\) standard solution. The peak height for zinc increased with the increasing 5-Br-PAPS concentration and remained constant at concentrations more than 9.3×10\(^{-5}\) M. At a concentration above 9.3×10\(^{-4}\) M, unstable baselines and poor reproducibility for determination of zinc in human serum at the level of µg ml\(^{-1}\) were observed. Consequently 1.9×10\(^{-4}\) M 5-Br-PAPS was adopted to lessen the consumption of the reagent and to assure reproducibility.

Coil lengths. The length of C1 was varied from 0 to 1 m keeping the length of C2 constant at 0.5 m. In this coil the zinc-protein complex in serum sample is decomposed by TMAH in the carrier solution to form a reactable free ion of zinc. The optimum length of C1 was 0.5 m. The C1 less than 0.5 m long gave poor reproducibility for determination of 0.1 µg ml\(^{-1}\) level of zinc, whereas the same response was obtained for zinc in both the standard and the serum sample solution with the C1 0.5 m or longer. The effect of the C2 coil length was studied over the range 0 – 1 m keeping the length of C1 constant at 0.5 m. The peak height increased steadily with the increasing length of C2, reached a maximum at 0.5 m and then gradually decreased with further increase of the coil length. The optimum length of C2 was also 0.5 m.

Flow rate. The effect of the total flow rate of the pump, P, on the peak height was studied in the range of 0.6 – 3.4 ml min\(^{-1}\) by injecting 100 µl of zinc standard solution and real sample solution (Fig. 3). When the standard solution (1.06 µgZn ml\(^{-1}\)) was injected, the peak height increased steadily with the increasing flow rate from 0.6 to 1.1 ml min\(^{-1}\) and reached a maximum at 1.1 ml min\(^{-1}\), then it slowly decreased with further increase in the flow rate. For the real sample solution (1.10 µgZn ml\(^{-1}\)), the peak height increased steadily with the increasing flow rate from 0.6 to 2.4 ml min\(^{-1}\), reached a maximum at 2.4 ml min\(^{-1}\), and then slowly decreased. The equal sensitivities were obtained for both the standard and the real sample solutions in the range from 2.4 to 3.4 ml min\(^{-1}\). The optimum flow rate of P was thus 2.4 ml min\(^{-1}\), at which the flow rates of the carrier and the buffer solution are each 1.2 ml min\(^{-1}\).

Sample injection volume. The injection volume affected both the sensitivity and reproducibility. The peak height increases steadily with increasing sample injection volume, however, spread the sample zone leading to the peak split when the injection volume becomes more than 110 µl. Then 100 µl was taken as a compromise between the sensitivity and reproducibility. The signal profiles obtained using the proposed system are shown in Fig. 4.

Calibration curve
A calibration curve was prepared with the recom-
The calibration curve was linear over the range 0.1 - 10 µg ml⁻¹ zinc. Generally for determination of zinc in serum, a controlled-serum standard sample is used to prepare calibration curves. The calibration curve drawn using a standard addition method for serum sample (\( Y = a + bX, \ a = 0.0011 \pm 0.0019, \ b = 0.0415 \pm 0.0020, \ n = 3 \)) was quite in good agreement with that constructed by using the standard zinc solution (\( Y = a + bX, \ a = 0.0011 \pm 0.0019, \ b = 0.0415 \pm 0.0020, \ n = 3 \)). This indicates that zinc in serum can be determined by using calibration method. In this study, we constructed a calibration curve by using zinc standard solution.

**Interferences**
The effects of coexisting substances on the deter-
mination of zinc in human serum are listed in Table 1. Iron and copper are potential interferents to the proposed method, and other metals do not interfere at the average levels in normal serum. It should be noted that iron and copper are released in serum by hemolysis at the sampling and sample preparation steps. All organic substances examined except EDTA also do not affect the determination.

**Determination of zinc in human serum**

The recovery test using a standard addition technique gave satisfactory results (Table 2). The proposed method has been applied for the determination of zinc in serum on six healthy subjects. The results given in Table 3 are in very good agreement with those obtained by the flame-AAS method. The reproducibility is satisfactory in terms of the relative standard deviation of less than 3.3% (1.2 µg Zn ml⁻¹ level, n=50). The detection limit (signal-to-noise ratio=3) was 0.12 µg ml⁻¹ Zn. The throughput is 60 samples per hour. These results indicate that the proposed FIA method is suitable for the clinical determination of zinc in serum.

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**References**


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