Kinetic Fluorometric Determination of Aluminum in Serum

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We describe a simple fluorometric method for determining aluminum in serum samples by monitoring the rate of reaction of 2-hydroxy-1-naphthaldehyde-p-methoxybenzoylhydrazone with aluminum ions. The emission of the resulting fluorescent metal-chelate formed is measured at 475 nm. Aluminum was measured in the supernate of serum after proteins were removed by precipitation with concentrated nitric acid, and calculations were based on the technique of standard additions. Within-run precision (CV) was 7.8% and 4.8% at mean aluminum concentrations of 7.7 and 60.7 μg/L, respectively (n = 10); between-run precision (CV) was 8.9% and 5.7% at mean aluminum concentrations of 23.3 and 46.8 μg/L, respectively (n = 10). The standard curve for the method is linear over the range of 0–250 μg of aluminum per liter. Samples from 49 patients were analyzed for aluminum by the proposed method (y) and by electrothermal atomic absorption spectroscopy (x). Linear regression analysis of the results yielded the equation y = 0.96x + 2.3 (r = 0.989, Sx̄ = 6.7). The proposed method is comparable in sensitivity to the well-accepted atomic absorption spectrometric method but is simpler and less expensive.

Additional Keyphrases: trace metals • fluorescent chelate • atomic absorption spectrometry compared

There is increased interest in monitoring the concentrations of aluminum in serum of patients who are undergoing hemodialysis. Aluminum intoxication is suspected of being responsible for dialysis encephalopathy, renal osteodystrophy, and Alzheimer's disease, and it may be an etiological factor in renal-disease-induced osteomalacia (1, 2).

Because of its high sensitivity and freedom from serious interferences, electrothermal atomic absorption spectroscopy (AAS) is the method of choice for aluminum determination (3–5). However, the instrumentation required is fairly expensive and not generally available in clinical laboratories. Fluorometry is comparable in sensitivity with AAS and depends on less-expensive apparatus that is more commonly available in clinical laboratories.

Recently, we reported a simple, sensitive, and specific kinetic fluorometric method for determining aluminum in aqueous acetone solutions, based on the reaction of 2-hydroxy-1-naphthaldehyde-p-methoxybenzoylhydrazone (HNAMBH) with aluminum (6). Because serum components interfere seriously with direct determination of aluminum, removal of proteins—e.g., by treatment with concentrated nitric acid and heating—is required (4).

Use of a calibration curve prepared from aqueous aluminum standards gave inadequate results. Therefore we used the standard-additions method.

To evaluate the proposed method, we performed analytical recovery experiments and compared results with those by AAS.

Materials and Methods

Instrumentation

We used a Model 512 double-beam fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) with a 150-W xenon lamp, equipped with a magnetic stirrer under the sample-cell holder. The instrument settings were as follows: ratio mode; dynode voltage 750 V; excitation wavelength 420 nm, with a bandwidth of 20 nm; emission wavelength 475 nm, with a bandwidth of 20 nm; sensitivity 3. A constant temperature of 25.0 °C in the 1.000-cm sample cell was maintained with a thermostated water bath. Fluorescence intensity was recorded by a Sargent–Welch XKR recorder. For comparison studies we used an atomic absorption spectrophotometer (Model 403) with a heated graphite atomizer (Model 400) and a recorder (Model 56, all from Perkin-Elmer). An aluminum hollow-cathode lamp was used at 309.3 nm, with a deuterium arc lamp for background correction. Samples were assayed in pyrolytically coated graphite tubes (RW-0686, Perkin-Elmer).

Reagents

All solutions were prepared in de-ionized, doubly distilled water from reagent-grade materials, unless otherwise stated.

HNAMBH solution, 1.00 mmol/L, in acetone. The reagent was prepared by condensing 1 mol of 2-hydroxy-1-naphthaldehyde and 1 mol of p-methoxybenzoylhydrazide (anisic acid hydrazide) in ethanolic solution. The yellow crystalline product that separated out was purified by recrystallization from ethanolic solution (m.p. 240 °C). The HNAMBH solution in acetone is stable for several months at room temperature.

Aluminum standard solutions. Working standards were prepared daily from a stock solution of 1.000 ± 0.002 g of Al (AlCl3, Titrisol; Merck, Darmstadt, F.R.G.) diluted in 1 L of 0.1 mol/L HCl by appropriate dilution.

Succinate buffer (0.1 mol/L, pH 5.40). This stock solution was prepared by dissolving 11.81 g of succinic acid in 700 mL of water, adjusting to pH 5.40 with saturated sodium hydroxide, and diluting with water to 1 L. Stored at 4 °C, the stock solution was stable for several weeks. A working buffer solution was prepared by mixing two volumes of the stock succinate buffer solution with one volume of acetone. The apparent pH of this solution is 6.20.

Concentrated HNO3. (Merck "Suprapur") was used to rinse the plasticware and precipitate proteins from serum.

Sodium hydroxide, 1 mol/L.

Pooled sera. Pooled sera with low or high aluminum content were prepared from serum of patients with normal renal function and uremic patients, respectively. The pools were stored in 18 × 118 mm polypropylene tubes (Kartell, P.O. Box 18, Binasco, MI) at 4 °C.

Methods

Precautions are required to avoid contamination with aluminum. We tested materials coming in contact with the
samples as potential sources of aluminum. All the plasticware to be used for storing reagents and for serum collection was washed in 10-fold diluted nitric acid, then thoroughly washed with de-ionized, doubly distilled water. No glassware was used.

Sample preparation. Protein was removed by a slight modification of a procedure previously described (4). We mixed 1.00 mL of serum with 50 μL of concentrated HNO₃ in a polypropylene test tube, stirred, and heated the mixture for 5 min in a bath of boiling water. This more effectively removed proteins than did heating at 70 °C as in ref. 4. We then centrifuged the mixture for 10 min at 1500 × g.

Measurement. Transfer 300 mL of the clear supernatant to a thermostated (25.0 ± 0.1 °C) cuvette (we used a Fpinpette microcyringe) and start the stirrer. Add 150 μL of NaOH solution to neutralize the nitric acid, then add 1.50 mL of working buffer solution. Start the reaction by adding 100 μL of the reagent solution; record the fluorescence intensity for 1.0 to 1.5 min. Inject 50 μL of a working aluminum standard solution, 1000 μg/L, through a plastic septum in the cover of the cell compartment (we used a Hamilton microsyringe) and monitor the reaction for another 1.0 to 1.5 min. The initial rates of the reaction curves are estimated graphically and the concentration of the unknown is calculated by the method of standard additions as follows:

\[
v_1 = k \cdot C_x
\]

\[
v_2 = k(C_x + C_d)
\]

where \( v_1 \), \( v_2 \) are the initial reaction rates (mm · min⁻¹) before and after the addition, respectively; \( C_x \), \( C_d \) are the unknown and the added amounts of aluminum; and \( k \) is the pseudo-first-order rate constant.

Combining equations 1 and 2 we get:

\[
C_x = \frac{C_d \cdot v_2}{v_2 - v_1}
\]

and finally Al, μg/L = \( C_x \cdot 1000/300 \).

If the aluminum concentration is <10 μg/L, repeat the measurement at a greater instrument sensitivity (10), injecting 20 μL of the aluminum standard.

Analytical-recovery experiments. We pipetted 3 mL of pooled serum into each of four polypropylene tubes, then added a fixed amount of aqueous aluminum standards and mixed thoroughly. After protein precipitation we measured aluminum in serum in triplicate for each sample.

Standard-additions calibration curve. We pipetted 450 μL of neutralized low-pool serum supernate into the cuvette, added 50 μL of aqueous aluminum standards (400 to 10,000 μg/L) to give final concentrations of added aluminum from 10 to 250 μg/L, performed aluminum measurements, and constructed a kinetic calibration curve by plotting initial reaction rate (slope method) vs aluminum concentration.

Results and Discussion

The kinetic study, the optimum experimental conditions of the HNAMBH-Al chelate formation in aqueous acetone solutions (optimum pH, concentration of the organic solvent, etc.) and the interferences have been described previously (6). The proposed scheme of the reaction is as follows:

\[
\text{AlOH}^{2+} + H_2R \rightleftharpoons \text{AIHR}^{2+} + H_2O,
\]

where \( H_2R \) is the HNAMBH.

The optimum pH range, 4.7–5.1, corresponds to the maximum content of AlOH²⁺ form. To determine the optimum conditions for serum determination, we examined the effect of pH on the initial reaction rate in serum supernate. As Figure 1 shows, the optimum pH range for the assay of serum supernates was 6.0 to 6.2. The difference between this value and that for aqueous solutions can be attributed to the presence of several anions in serum (phosphate, carbonate, etc.) that affect the concentration of the active form of aluminum (AlOH²⁺).

We found satisfactory a pH 5.40 succinate buffer that, after the acetic addition, increases to pH 6.10–6.20. The acetate addition (250 mL/L) is necessary because of the low aqueous solubility of HNAMBH. The final concentration of the reagent chosen, 0.05 mmol/L, ensures an excess of reagent over the aluminum concentration and thus pseudo-first-order conditions.

In order to choose the better technique for the measurement (calibration curve or standard additions) we calculated the mean slope for aqueous standard curves (initial rate vs aluminum concentration), which we found to be 0.0368 ΔF · s⁻¹(μg/L)⁻¹ (SD = 0.0022, n = 6), and the mean slope of the standard-additions curves with serum supernate after protein precipitation, which we found to be 0.0138 ΔF · s⁻¹(μg/L)⁻¹ (SD = 0.0057, n = 14). The decreased slope of standard-additions curves with an increased variability (CV = 41.3%) obtained for various serum samples may be caused by various factors, such as ionic strength, formation of non-fluorescent complexes by various metal ions, especially Fe³⁺ and Cu²⁺ (6), etc., which cause the quenching phenomenon.

To avoid the problems of interferences, we used the standard-additions method. Linearity of the standard-additions method extended from 0 to 250 μg/L (r = 0.9994). Blank measurements gave an aluminum concentration of 1–2 μg/L, which was subtracted from the sample measurements.

The detection limit for the method, defined as three times the standard deviation of the mean blank, is 0.13 μg/L.

To determine within-run and between-run precision, we measured serum pools containing different aluminum concentrations. For within-run precision assessment, two samples, after protein precipitation, were measured 10 times each. For between-run precision we performed protein pre-
precipitation 10 times for each of the samples and measured the aluminum concentration in triplicate for each. Table 1 shows our results for serum samples of low and high aluminum concentration. The CV values obtained are sufficient for trace analysis and less than those obtained by the AAS technique (4).

The proposed method was also evaluated by performing analytical-recovery experiments on pooled serum samples (Table 2). As shown, recovery ranged from 93.3 to 108.3% (mean 100.0%) for low aluminum concentrations and 86.0 to 100.0% (mean 91.7%) for high aluminum concentrations.

Comparison data for 49 patients' samples, as assayed by the proposed and AAS methods, yielded the following linear regression equation: $y = a + bx$ ($a = 2.3$, $SD = 1.9$; $b = 0.98$, $SD = 0.02$); the standard error of estimate $= 6.7$; $r = 0.989$ ($x$-axis: AAS method; $y$-axis: kinetic fluorometric method).

The application of the direct fluorometry in analysis of real samples such as biological fluids has some inherent problems because of the several factors that interfere with fluorescence measurements. However, by applying a kinetic fluorometric method and using the standard-additions technique these problems can be avoided, and precise and accurate results can be obtained.

The proposed method is comparable in sensitivity with the well-accepted AAS method, has a wider analytical range (so sample dilution is avoided) and better precision, and the apparatus required is more common in clinical laboratories and is less expensive.

We thank T. Christopoulos for his help, the biochemistry laboratories of St. Pantaleimon's and Evangelismos Hospital (Athens, Greece) for providing the patients' samples, and Dr. M. Koupparis for helpful discussion.

### References

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**Table 1. Precision Data**

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$n=10$, throughout.

**Table 2. Analytical Recovery Data**

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