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Determination of serum aluminium using an ion-pair reversed-phase high-performance liquid chromatographic-fluorimetric system with lumogallion

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Abstract

An ion-pair reversed-phase high-performance liquid chromatographic method with fluorimetric detection, using lumogallion [4-chloro-3-(2,4-dihydroxyphenylazo)-2-hydroxybenzene-1-sulphonic acid] as a ligand, has been successfully applied to the determination of aluminium in human serum. The highly fluorescent aluminium–lumogallion complex (λ_{ex} 505 nm, λ_{em} 574 nm) was separated on a LiChrosorb RP-18 column with an eluent consisting of 30% acetonitrile, 70% 0.02 *M* potassium hydrogen phthalate and 10 μM lumogallion. The proposed system offers a simple, rapid, selective and sensitive method for the determination of aluminium in serum. The detection limit for aluminium was 0.05 μg/l in aqueous solution and the limit of determination was 2.2 μg/l in serum. The recovery of the method is generally over 90%.

1. Introduction

There is increasing evidence that aluminium is neurotoxic to humans [1,2]. It is well established that aluminium is causally implicated in the generally fatal brain disease dialysis encephalopathy [3] and there is considerable evidence that aluminium may be related to Alzheimer's disease [1,4]. The average intake of aluminium is about 20 μg/day via the gastrointestinal tract and 3–15 μg/day via inhalation, leading to a median plasma level of 7 μg/l (range 1.5–15) [5].

The importance of determining aluminium concentrations in serum of patients undergoing haemodialysis has been recognized because of the suspicion that aluminium intoxication may be responsible for osteopathy [6].

Graphite-furnace atomic absorption spectrophotometry (GF-AAS) and inductively coupled plasma atomic emission spectrometry (ICP-AES) have been the two most commonly used techniques for the determination of aluminium. However, the use of AAS is often limited due to matrix interferences and insufficient precision, especially for serum samples, since high concentrations of both organic and inorganic endogenous compounds are present, and aluminium

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concentrations in these fluids are normally close to the limits of determination of line-source graphite furnace AAS [7]. Furthermore, these instruments, especially ICP-AES, and their maintenance are rather costly.

The use of high-performance liquid chromatography (HPLC) for the determination of aluminium in the form of complexes has been gaining popularity over the recent years [8–12]. The method has been successfully applied to tap water, natural waters, solar salt, alkali pellets and biological samples such as oyster tissue and bovine liver. The most frequently used complexing agents include 8-quinolinol [8], salicylalhydebenzoylhydrazone (SAB) [9], 3-(5-chloro-2-hydroxyphenylazo)-4,5-dihydroxy-naphthalene-2,7-disulphonic acid (plasmocorinth B) [10], 2,2′-dihydroxyazobenzene (DHAB) [6,11] and 1-phenyl-3-methyl-4-benzoyl-5-pyrazolone (PMBP) [12]. The structures of these compounds are shown in Fig. 1. Unfortunately, most of these methods cannot be used to detect aluminium at μg/l levels, and some of them are tedious and suffer from poor recoveries. For example, plasmocorinth B and aluminium form a complex with a high molar absorptivity in the UV region, but because of the low detection sensitivity, an off-line enrichment procedure prior to HPLC analysis is necessary [10]. Only SAB forms a highly fluorescent complex with aluminium and therefore by using an HPLC with fluorimetric detection, higher sensitivity can be obtained. However, SAB is not commercially available, necessitating in-house synthesis and purification procedures. For analysis of aluminium in serum, DHAB has been used as a precolumn reagent and the complex was analyzed by HPLC with UV detection at 510 nm [6].

Lumogallion [4 - chloro - 3 - (2,4 - dihydroxyphenylazo) - 2 - hydroxyben - zene - 1 - sulphonic acid] is one of the DHAB derivatives and it reacts readily with all inorganic aluminium species. It is very likely that lumogallion acts as a planar tridentate ligand in these reactions, two phenolic oxygen ions (2 and 2′) and the azo group apparently being bound to the metal, forming complex rings in an aromatic linkage [13] (Fig. 1). Such a ring system is presumably responsible for the high stability of the complex (the stability constant is higher than $5.62 \cdot 10^7$) within the pH range 2.0–5.7 [14]. The aluminium ion is characterized by the presence of two aqua ligands in the *ortho* positions, while the sulfonato group, not involved in the complexation, allows the retention of the complex on the HPLC analytical column.

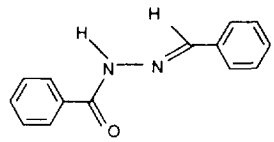
In this paper, we describe a simple and sensitive method based on ion-pair reversed-phase HPLC with fluorimetric detection for the detection of trace amounts of aluminium in serum with lumogallion as a pre-analysis reagent. This represents the first report on the use of HPLC with fluorimetric detection for the determination of aluminium via analysis of the aluminium–lumogallion complex. The proposed method was validated by using serum from 20 healthy persons who had no occupational history of exposure to aluminium.

2. Experimental

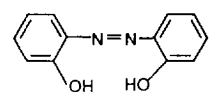
2.1. Reagents

All chemicals used were of analytical reagent grade, unless otherwise indicated. Water was deionized and distilled by glass and polypropylene apparatus.

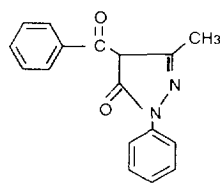
Stock standard aluminium solution containing 1 g/l Al(III) in the aluminium nitrate form ('SpectrosoL') for AAS was purchased from BDH (Poole, UK). All working aluminium standard solutions were prepared fresh daily by appropriate dilution of the stock solution with 0.03 *M* HNO_3 in standard flasks and transferred immediately to polypropylene tubes. The ligand, lumogallion, was purchased from Tokyo Kasei Kogyo (Tokyo, Japan) (purity: 94.0%) and a 3 m*M* solution was prepared by dissolving 0.52 g of the compound in 500 ml of deionized-distilled water. The 0.2 *M* potassium hydrogen phthalate ('Baker Analyzed', purity 100.02%) (J.T. Baker, Phillipsburg, NJ, USA) solution was prepared by adding 2 g of the reagent to 50 ml of deionized-distilled water. Sodium thiosulphate solution (0.2 *M*) was prepared by dissolving 12.40 g sodium thiosulphate pentahydrate (purity: 99.5%) (BDH) in 250 ml of deionized-distilled water.



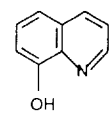
SAB



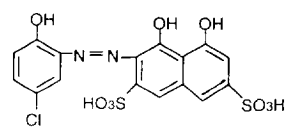
DHAB



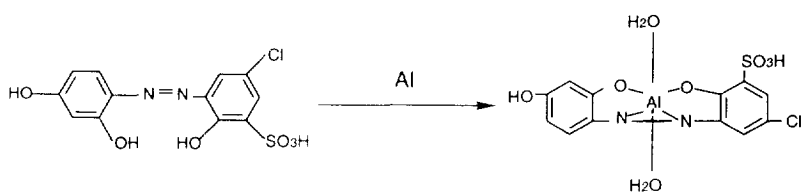
PMBP



Oxine



Plasmocorinth B



Lumogallion

Aluminium-lumogallion complex

Fig. 1. Structures of some ligands used in aluminium determinations, and possible structure of aluminium–lumogallion complex [13]. SAB = salicylalhydebenzoylhydrazone, DHAB = 2,2′-dihydroxyazobenzene, PMBP = 1-phenyl-3-methyl-4-benzoyl-5-pyrazolone, plasmocorinth B = 3-(5-chloro-2-hydroxyphenylazo)-4,5-dihydroxy-naphthalene-2,7-disulphonic acid.

Trichloroacetic acid (TCA) (purity: 99.5%; BDH) solutions (1.2 *M* and 0.3 *M*) were prepared by dissolving 49.05 and 12.26 g of the reagent into 250 ml of deionized-distilled water, respectively. The mobile phase was prepared by adding 2.04 g of potassium hydrogen phthalate and 1.7 mg lumogallion to 500 ml of deionized-distilled water. NaOH solution (1 *M*) was used to adjust the pH values of the reaction mixture as well as of the mobile phase buffer.

2.2. Apparatus

The λ_{max} for the aluminium–lumogallion complex was determined with a Shimadzu (Kyoto, Japan) spectrofluorophotometer RF-5000.

The HPLC was a Waters Powerline system (Millipore Corporation, Milford, USA), which includes a Model 600E multisolvent delivery system controller, a Model 470 scanning fluorescence detector and a Model 700 Satellite WISP autosampler. The separation column used was a LiChrosorb RP18 10 μm (25 cm × 4.6 mm I.D.) purchased from HiChrom (Reading, UK). Waters Maxima 825 HPLC software was used for quantitation.

2.3. Procedure

For deproteinization, 0.5 ml of serum was placed in a 1.5-ml Eppendorf vial with an equal amount of 1.2 *M* TCA [15]. The mixture was stirred for 1 min with a vortex-mixer and centrifuged for 10 min at 1500 *g* using a desk-top centrifuge. The supernatant was then transferred to a 10-ml calibrated polypropylene test-tube. An additional 0.5 ml of 0.3 *M* trichloroacetic acid was added to the vial containing the precipitate, and after vortex-mixing vigorously for 2 min, the mixture was centrifuged for 10 min at 1500 *g*. The supernatant obtained was combined with the earlier aliquot.

A 1-ml volume of 0.2 *M* potassium hydrogen phthalate was added to the supernatant and the pH was adjusted to 4.7 using 1 *M* NaOH. The solution was mixed with 0.25 ml of 0.2 *M* sodium thiosulphate solution and the mixture was allowed to stand for 15 min and subsequently 0.25 ml of 3 m*M* lumogallion solution was added. The volume was made up to 5 ml with deionized-distilled water and the mixture was vortex-mixed vigorously for 2 min. After being allowed to stand for 15 min at ambient temperature, the mixture was analyzed by HPLC. All samples were prepared in duplicate.

2.4. HPLC conditions

The separation was achieved isocratically with a mobile phase of 30% acetonitrile and 70% of 10 μM lumogallion in 0.02 *M* potassium hydrogen phthalate buffer. The optimum pH of the mobile phase was found to be 4.2 (see section 3). The mobile phase was degassed with helium for 10 min before use. The aluminium–lumogallion complex was detected at 505 nm (excitation wavelength), and 574 nm (emission wavelength). The flow-rate was 1 ml/min and the injection volume was 100 μl. The analysis was carried out at room temperature. Aluminium concentrations were determined by the method of standard addition.

2.5. Contamination control

Because of the widespread occurrence of aluminium, rigorous precautions were taken to avoid contamination of the samples with aluminium from other sources. All reagent solutions were stored in polycarbonate vessels, and all materials, reagents and solutions used in this study were screened for possible aluminium contamination. To obtain absolute blank values for the described procedure, sample solutions were replaced with deionized-distilled water, treated and subjected to identical operations as for the analytical determination. Despite all the precautions, serious contamination was found to arise from the sodium hydroxide solution. As high as 1 ng/g of aluminium could be found in the analytical-grade sodium hydroxide. However, since the background was stable, the signals superimposed on the blank value could be measured with sufficient precision.

2.6. Serum samples

Blood samples were randomly taken from 20 healthy blood donors at the National University Hospital, National University of Singapore. Serum was obtained by centrifugation at 1000 *g* for 10 min. The samples were stored in polypropylene test tubes at −70°C until analysis.

3. Results and discussion

3.1. Reaction conditions

A chemometric approach, orthogonal array design, has previously been applied for the optimization of conditions for HPLC, a polaro-

graphic reaction system, and microwave dissolution; this procedure has been described in greater detail elsewhere [16–20]. In the present work, conditions for aluminium–lumogallion complex formation were also optimized using this systematic approach. The assignment of initial values was based on literature references, past experience and intuition. Two sets of experiment were carried out following a three-level orthogonal array design with an OA_9 (3^4) matrix and a two-level design using an OA_8 (2^7) matrix [16–20]. The temperature, pH, concentration of sodium thiosulphate and reaction time were considered and further investigations were made to optimize the procedures. Little difference was noted for temperatures ranging from 25 to 80°C and reaction times from 5 to 50 min. In subsequent studies, a pH of 4.7 and 15 min of reaction time at room temperature were selected. The complex was stable for more than 24 h. A previous study has shown that the aluminium–lumogallion complex has a stoichiometry of 1:1 at pH below 7.5 [21].

3.2. Conditions for HPLC analysis

The conditions for HPLC analysis were also optimized following a two-level orthogonal array design with an OA_8 (2^7) matrix [16–20]. For the mobile phase, the type and concentration of the organic solvents, pH and concentrations of lumogallion and potassium hydrogen phthalate in buffer solution, were examined. Acetonitrile was found to perform better than methanol in terms of peak shapes and resolution. A buffer of pH 4.2 was found to be the most favourable for analysis of the aluminium–lumogallion complex although, as pointed out earlier, a pH of 4.7 appeared optimal for the formation of the complex.

3.3. Linear range, precision, limit of determination and recovery

In the fluorimetric detection mode, both peak area and peak height of the complex increased linearly up to 0.5 g/l of aluminium in standard solutions. The correlation coefficients (r) were over 0.99 (mean of 6 replicate analyses). The correlation coefficients were 0.97 and 1.0 for two serum samples spiked with Al(III) standard covering the range 10–50 μg/l (mean of 3 replicate analyses).

The reproducibility (relative standard deviation, R.S.D.) of the method was 2.4% for 6 duplicate analyses of 50 μg/l aluminium solution and 2.1% for the mean value of these 6 replicate analyses repeated on three consecutive days. The reproducibility of the method in serum was 2.1% for 3 replicate analyses of the same sample and 1.7% for the between-day assays on three consecutive days.

The detection limit (the concentration equivalent to three times the standard deviation of the blank) was 0.05 μg/l of aluminium for aqueous solution and the limit of determination was 2.2 μg/l of aluminium for human serum. These values are much lower than that obtained either by AAS (10 μg/l in serum) [22], or by HPLC with UV detection (6 μg/l in serum) where DHAB was used as the ligand [6].

Aluminium standards of concentrations ranging from 10 to 50 μg/l were prepared by spiking to test the recovery of the method. As shown in Table 1, recoveries higher than 92% were obtained.

3.4. Interferences

Fig. 2 shows examples of liquid chromatograms generated in this work. The peaks at 5.3 min represent the aluminium–lumogallion complex at different concentrations. Thus, in the present method, it is unnecessary to remove the excess lumogallion, reaction medium compo-

Table 1
Aluminium recovery

Al(III) added (ng)	Al(III) found (ng)	Recovery (%)	R.S.D. (%)
0	7.8 ± 0.9	–	11.5
10	16.9 ± 0.5	91.0	3.0
25	30.9 ± 0.4	92.4	1.3
50	55.7 ± 0.7	95.8	1.3

n = 4, 0.5 ml of serum.

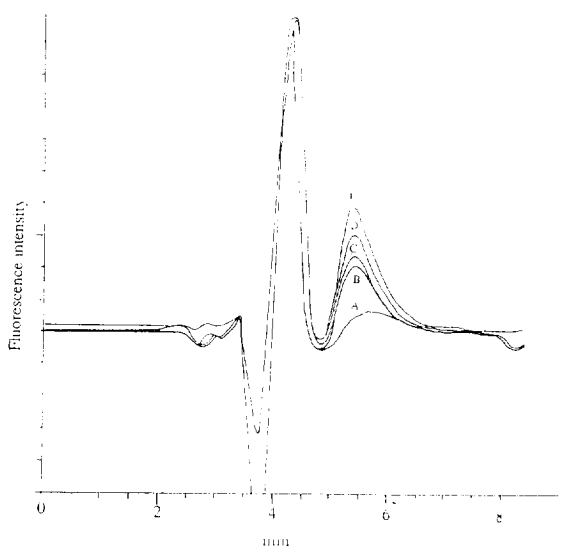


Fig. 2. Typical chromatograms obtained by fluorimetric detection of aluminium in serum and serum spiked with aluminium standards. (A) Blank (no serum); (B) serum sample; (C) serum sample spiked with 2.5 μg/l Al(III); (D) 5 μg/l Al(III); (E) 10 μg/l Al(III). Chromatograms from different runs have been superimposed on one another.

nents and other metal–lumogallion complexes before the aluminium–lumogallion complex is determined. The complex was well separated with no spectroscopic evidence of coelution with other metal complexes in serum. Lumogallion itself shows very low emission intensity under the conditions used, and thus was not detected. It can be seen that the analysis is free from interferences from ions such as Cr(VI), Zn(II), Co(III), Ni(II,III), Ca(II), Mg(II), Na(I), K(I), Mn(II), P(V), V(V) and Sn(II), which are commonly found in human serum. This is because the majority of the transition metal–lumogallion complexes are essentially non-fluorescent except for those of Fe(III) and Cu(II), which were also not detected with the proposed method. The interference from Cu(II), if present, can be eliminated by the reaction of Cu(II) with $S_2O_3^{2-}$ in acidic solution. For the interference of Fe(III), tests were carried out to demonstrate that the analysis of 10 μg/l of Al(III) (normal level for healthy person) was not affected until the Fe(III) concentration was above 4 mg/l, which is about 4 times the normal level (1 mg/l) found in human serum [23].

Fig. 2 also shows the presence of a peak at 4.2 min. The identity of this component is unknown. It also appeared in the blank run, and could be an impurity. (Preliminary experiments conducted with a sodium acetate–acetic acid buffer instead of the potassium hydrogen phthalate did not show a similar interference, suggesting that the impurity could be present in our batch of phthalate.) Nevertheless, the presence of this component did not severely compromise the analysis.

3.5. Aluminium concentration in serum

Serum samples from 20 healthy persons were analyzed using the described method. The serum aluminium concentration ranged from 2.2 to 15.7 μg/l with a mean value of 6.6 μg/l, which is in accordance with the published data for healthy individuals [6,7,16,22,24].

4. Conclusions

Lumogallion is a commercially available reagent which reacts cleanly (i.e. without side reactions) with aluminium, forming a stable and highly fluorescent complex. These characteristics permit the use of lumogallion in the determination of aluminium at trace levels. Coupled with RP-HPLC as a separation technique and fluorimetric detection, interferences from the reaction media and matrix are eliminated. The technique offers a rapid and sensitive means for the determination of aluminium in serum samples, as demonstrated in this work. As low as 0.05 mg/l of aluminium in aqueous solution and 2.2 mg/l of the metal in serum could be analyzed with this method, the recovery being higher than 92%. Due to its operational simplicity and general convenience, the method may be adopted for the routine determination of aluminium in serum.

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