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Journal of Chromatography B, 791 (2003) 13–19
JOURNAL OF CHROMATOGRAPHY B
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Kinetic differentiation mode chromatography using 8-quinolinol and fluorimetric detection for sensitive determination of aluminum adhering to the gastric mucosa

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Received 11 July 2002; received in revised form 20 January 2003; accepted 5 February 2003

Abstract

A highly sensitive method of kinetic differentiation (KD) mode high-performance liquid chromatography (HPLC) with fluorimetric detection was established using 8-quinolinol to measure aluminum adhering to the gastric mucosa. After sucralfate was hydrolyzed by 1 mol/l hydrochloric acid, an 8-quinolinolate–aluminum complex was produced by reacting aluminum with an 8-quinolinol solution. Then contaminants in the gastric mucosa and sucralfate were removed by liquid–liquid extraction with chloroform. Next, the 8-quinolinolate–aluminum complex was separated on a reversed-phase column that was specifically designed to detect aluminum (50×4.6-mm I.D.). Separation was done at a flow-rate of 0.8 ml/min, using BES buffer containing sodium dodecyl sulfate (pH 7.0) as the mobile phase. Fluorescence was detected at 370 nm (excitation) and 504 nm (emission). The sensitivity of this method was more than 1000 times greater than that of absorptiometry using 8-quinolinol. The detection and quantitation limits were 1.68 and 5.11 ng/ml, respectively. When tested with aluminum solutions of 10, 30, and 90 ng/ml, the intra-assay and inter-assay coefficients of variation were below 7.1%, with an error of less than 8.3%. Aluminum adhering to the gastric mucosa was determined by HPLC and absorptiometry after administration of sucralfate to rats. The HPLC method showed that aluminum levels were higher at sites of ulceration than in the normal mucosa at all times after sucralfate administration. When the values above zero obtained for absorptiometry were assessed, there was a significant correlation ($r=0.993$, $P<0.0001$) between the aluminum concentrations measured by the two methods. This new HPLC method could be applied to the determination of aluminum in small samples, such as human gastric mucosal biopsy specimens.

Keywords: Kinetic differentiation mode chromatography; 8-Quinolinol; Aluminum

1. Introduction

Aluminum is an element that exists widely in nature, but its physiological functions have not been completely clarified [1]. On the other hand, it has been persuasively argued since the early 1970s that there is a relationship between aluminum and a syndrome that has been termed aluminum encephalopathy [2] or osteopathia [3], and a cause–effect relationship between aluminum and Alzheimer's disease has also been proposed [4,5]. Accordingly, it

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1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00159-4

has become more important to develop a method for highly sensitive measurement of aluminum in biological specimens.

Aluminum is currently measured using various methods. Although the most powerful methods for assaying metal ions are inductively coupled plasma atomic emission spectrometry (ICP-AES) and ICP-mass spectrometry, these methods show poor selectivity for aluminum and require expensive dedicated equipment [6]. Therefore, graphite furnace atomic absorption spectrometry (GF-AAS) is generally used for the determination of aluminum in biological specimens. However, GF-AAS is limited by interference from the matrix and insufficient precision, especially when measuring serum samples that contain high concentrations of organic and inorganic endogenous compounds, and the aluminum concentrations in body fluids are normally close to the limit of determination for this method [7].

Derivation with 8-quinolinol (oxine) followed by liquid–liquid extraction is another well-known method for the assay of metal ions [8–11]. Evaluation of sucralfate adhering to the gastric mucosa, which was used as the source of aluminum in the present study, has already been performed by a modified absorptiometry method using 8-quinolinol [12,13]. Sucralfate is an oral antiulcer drug, consisting of sucrose sulfuric ester (SSE) and aluminum hydroxide. It is insoluble in water, but partly dissociates to yield SSE and aluminum under acidic conditions, such as in the stomach. Dissociated sucralfate shows an antiulcer effect by selective adherence to gastroduodenal ulcers [14,15]. The above-mentioned absorptiometry method using 8-quinolinol is simple and can be employed to process many specimens simultaneously. However, a specimen with a size of several grams is needed for the assay, because its sensitivity is not so high [16,17]. Unfortunately, it is difficult to collect large specimens from patients. Progress in endoscopic technology has led to the collection of very small specimens as current practice. Therefore, to investigate the adhesion of sucralfate to the gastric mucosa in humans, there is a need to develop a method for the selective and highly sensitive determination of aluminum.

Recently, Sato et al. found that 8-quinolinol acts as a specific reagent for aluminum in kinetic differentiation (KD) mode HPLC with fluorimetric detection [16]. They reported that this HPLC method for the assay of aluminum was less influenced by other metallic elements coexisting in the matrix. Sato et al. also established a highly sensitive method for determination of serum aluminum, which excluded the effect of protein in the matrix by a micellar-HPLC procedure [6]. However, contaminants present in the gastric mucosa and drugs such as sucralfate could not be sufficiently removed using this method.

Therefore, the present study was performed to attempt the application of KD mode micellar-HPLC in combination with liquid–liquid extraction by chloroform for the assay of aluminum adhering to the gastric mucosa. As a result, we established a simple and highly sensitive method for the determination of aluminum.

2. Experimental

2.1. Separation of aluminum adhering to the gastric mucosa

Six-week-old (colony-bred) male Sprague–Dawley rats were obtained from Charles River Japan (Atsugi, Japan), and were housed under controlled conditions with a 12-h light/dark cycle, a temperature of 23±2 °C, and a relative humidity of 55±10%. The animals were given a pellet diet and tap water ad libitum throughout the acclimatization period. Acetic acid ulcers were created in accordance with a previously reported method [19]. Three days later, harvesting of the stomach was done. After the rats had been fasted for 24 h, a 100 mg/ml sucralfate suspension in 3% hydroxypropyl starch (HPS) was administered orally at 1 ml/kg. Sucralfate and HPS were obtained from our own factory (Fujieda, Japan) and from Freund Industrial (Tokyo, Japan), respectively. Then the animals were sacrificed at 1, 3, 6, 12 and 24 h after administration of the sucralfate suspension. The stomach was immediately excised from each rat, opened by a longitudinal incision along the greater curvature, rinsed gently in purified water, and spread out on a cork plate. The ulcerated area and a non-ulcerated region of equal size (normal mucosa) were cut out from the glandular portion of the stomach. Then the specimens were immersed in

2 ml of 1 mol/l hydrochloric acid for 24 h to promote the dissociation of sucralfate into its SSE moiety and aluminum. Hydrochloric acid (1 mol/l) was prepared by diluting 20% (v/v) hydrochloric acid (ultrapure grade, Tama Chemicals, Kawasaki, Japan) with ultrapure water (Tama Chemicals).

To obtain validation data, spiked samples of gastric wall tissue were prepared. About 30 mg of rat gastric wall tissue was immersed in 2 ml of 1 mol/l hydrochloric acid for 24 h. Then aluminum solutions were prepared by mixing 900 μl of the resulting matrix solution with 100 μl of a 10-fold concentration of aluminum solution.

2.2. Liquid–liquid extraction of the 8-quinolinolate–aluminum complex

In order to prepare the 8-quinolinolate–aluminum complex, 50 μl of R1 solution and 400 μl of R2 solution were added to 150 μl of the extract in 1 mol/l hydrochloric acid. R1 and R2 reagents were, respectively, the chelate reagent solution (8-quinolinol hydrochloric acid solution) and the chelation solution [R-2: *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer (pH 7.5)] in the aluminum detection kit (Dojindo Laboratories, Kumamoto, Japan), which was prepared in accordance with the report of Sato et al. [6]. After the mixture was reacted at 25 °C for 60 min, 0.9 ml of chloroform was added, and the mixture was agitated for 10 min. After centrifugation for 5 min at 3000 *g*, the chloroform layer was isolated and dried using a vacuum desiccator. Before injection into the HPLC apparatus, the specimen was re-dissolved in an aqueous solution of 20% (v/v) acetonitrile.

A similar procedure was used to produce 8-quinolinolate–aluminum complex from an aluminum standard solution (Wako Pure Chemical Industries, Osaka, Japan), which was diluted with 0.5 mol/l nitric acid (ultrapure grade, Tama Chemicals) to the required concentration.

2.3. Separation of the 8-quinolinolate–aluminum complex, and determination of aluminum

The HPLC system (Hitachi, Tokyo, Japan) consisted of an L-7100 pump, L-7610 Degasser, L-7480 fluorescence detector with a 12-μl cell, L-7300 column oven, L-7200 autosampler, D-7000 interface, and D-7000 system manager. The analytical column was a Capcell Pak AL (50×4.6-mm I.D. (phenyl) from Shiseido, Tokyo, Japan). Fluorescence was detected at 370 nm (excitation) and 504 nm (emission). The flow-rate of the mobile phase was set at 0.8 ml/min, and a 200-μl sample was injected. As the mobile phase, the R-3 reagent (BES (*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid) buffer solution containing sodium dodecyl sulfate (pH 7.0)) from the same kit was used.

For comparison with the new method, absorptiometry using 8-quinolinol was also performed [13]. An oxine reagent was prepared by dissolution of 50 mg of 8-quinolinol in 0.15 ml of warm glacial acetic acid, after first mixing with 30 ml of pure water and then with 50 ml of 2 mol/l sodium acetate solution. Finally, the volume was adjusted to 100 ml with water. To 1 ml of the assay sample diluted 10-fold with pure water, 2 ml of the oxine reagent was added, and immediately mixed. After addition of 5 ml of chloroform, the mixture was shaken, and then the aqueous layer was removed. Next, the absorbance of the chloroform layer was measured at 386 nm using a model UV-1600 spectrophotometer (Shimadzu, Kyoto, Japan).

2.4. Minimization of aluminum contamination

Because of the widespread occurrence of aluminum, minimization of contamination is important in order to reduce errors during microanalysis of aluminum [7]. In this study, we took several precautions to avoid contamination of the samples with aluminum from other sources. The vessels, such as the sample cups of the autosampler, stock bottles of all reagents, and storage containers for biopsy specimens, were all disposable polypropylene containers. Reagents and solutions were screened for possible aluminum contamination. Since pure water, hydrochloric acid, and nitric acid are often contaminated by aluminum, we used ultrapure-grade reagents, in which the aluminum content was not more than 20 ppt.

In the routine assay, ultrapure water and standard river water containing 61 ng/ml of aluminum (The Japan Society for Analytical Chemistry) were each

measured three times and contamination of the HPLC system was confirmed by the height of the aluminum peak. The acceptable peak intensity (and the C.V. values) of ultrapure water and standard river water were set at below 15 000 (C.V.: 10%) and 100 000 (C.V.: 5%), respectively. When absolute value or C.V. of the peak height was outside these criteria, the HPLC system was washed overnight with 10^{-4} mol kg^{-1} disodium EDTA [18]. Samples were diluted so that the aluminum concentration was below 100 ng/ml prior to application to the HPLC column in order to avoid retention of aluminum in the system that could interfere with the subsequent assays.

2.5. Statistical analysis

The difference between aluminum levels in the ulcer and the normal mucosa was calculated using the paired *t*-test.

3. Results and discussion

3.1. Specification of the 8-quinolinolate–aluminum complex peak, and separation of the SSE moiety and 8-quinolinolate–aluminum complexes by liquid–liquid extraction

Under the present analytical conditions, the peak of the 8-quinolinolate–aluminum complex had a retention time of about 5 min (Fig. 1a). This peak was also seen in the background (Fig. 1b). However, since background peak was stable, the signal superimposed on the blank value could be measured with sufficient precision.

The chromatogram of sucralfate hydrolyzed with 1 mol/l hydrochloric acid showed the effect of interfering substances (Fig. 1c). Since the SSE moiety and aluminum were produced by hydrolysis of sucralfate, separation of these peaks might be affected by the SSE moiety itself. Moreover, the SSE moiety is readily absorbed to the column, leading to

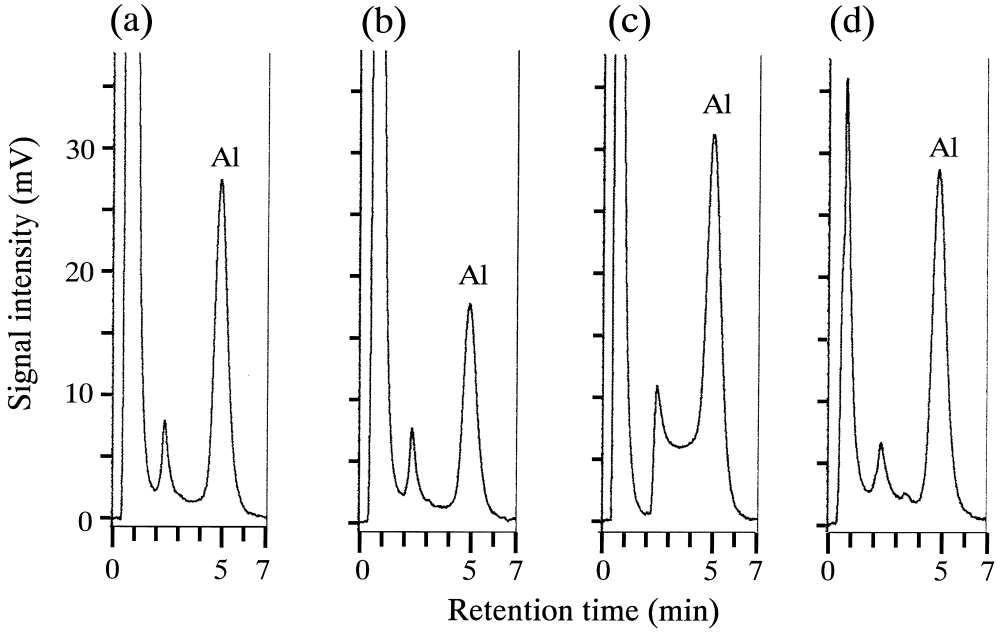

Fig. 1. Chromatograms of the 8-quinolinolato-aluminum complex. Column, Capcell Pak AL (50×4.6-mm I.D. (phenyl), Shiseido, Tokyo, Japan); mobile phase, BES buffer solution containing sodium dodecyl sulfate; pH 7.0); detection, fluorescence detection Ex=370 nm, Em=504 nm; flow-rate, 0.8 ml/min; column temperature, 25 °C; sample volume, 200 μl. (a) A gastric mucosal sample spiked with 10 ng/ml aluminum standard solution; (b) a blank gastric mucosal sample; (c) a gastric mucosal sample spiked with 50 ng/ml sucralfate without chloroform extraction; (d) a gastric mucosal sample spiked with 50 ng/ml sucralfate and subjected to chloroform extraction.

deterioration of column properties. Therefore, it was suggested that the contaminants needed to be removed from the sample, which was done liquid–liquid extraction (Fig. 1d).

3.2. Linearity of the calibration curve

When the linearity of the calibration curve was investigated using matrix samples containing standard aluminum solutions from 5 ng/ml up to 500 ng/ml, the calibration curve showed good linearity up to 100 ng/ml. The concentration of the aluminum standard solutions (x) up to 100 ng/ml and the ratio of the peak height (y) showed the following relation: $y = 16147\ (\pm SE\ 723.6) + 1010.5\ (\pm SE\ 13.9)\ x$, $r = 0.999$, $p < 0.0001$.

3.3. Detection limit and quantitation limit

To determine the limit of detection (LOD) and the limit of quantitation (LOQ), the aluminum concentration was analyzed in matrix samples spiked with standard aluminum solutions (0, 1, 2, 4, 6, 8 and 10 ng/ml). The LOD and LOQ were calculated from the slope of the regression line obtained, as: $LOD = 3.3\sigma/\text{slope}$, $LOQ = 10\sigma/\text{slope}$ (σ, standard deviation at a concentration of 0 ng/ml) [19]. In the present study, the LOD and LOQ were 1.69 and 5.11 ng/ml, respectively.

3.4. Reproducibility and accuracy, and determination of aluminum in the standard reference

Intra-day and inter-day reproducibility and accuracy were determined by performing assays on a single day or on three consecutive days, using matrix containing standard aluminum solutions of 10, 30 and 90 ng/ml (Table 1). Reproducibility was expressed as the coefficient of variation (% C.V.), and accuracy was calculated as the deviation from the nominal value (% bias) [20]. At all concentrations, the intra-assay and inter-assay variation of the HPLC method was below 7.1%, with an error of less than 8.3%.

Determination of aluminum in the standard river water offered by the Japan Society for Analytical Chemistry was performed by the HPLC method. The value obtained for this standard reference was 61.4±3.5 ng/ml ($n = 6$), which was sufficiently close to the certified value of 61 ng/ml.

3.5. Comparison of sensitivity and working range between the HPLC method and absorptiometry using 8-quinolinol

To compare the sensitivity and working range between the HPLC method and absorptiometry using 8-quinolinol, samples of the same volume (150 μl of the extract in 1 mol/l hydrochloric acid) were tested by each method. The aluminum concentration was determined in matrix samples spiked with sucralfate (Table 2). Recovery rates were calculated by comparison of the peak heights for samples with those for the corresponding standard solutions. Using the HPLC method, the recovery of aluminum from samples with concentrations of 5, 50, and 500 ng/ml was 4.71±0.14 (94.1%, $n = 3$), 48.6±1.83 (95.9%, $n = 3$), and 503.5±6.83 ng/ml (100.5%, $n = 3$), respectively. On the other hand, the working range of the absorptiometry method was 5–500 μg/ml, and

Table 1
Reproducibility and accuracy of the aluminum assay

Concentration added (ng/ml)	Intra-day ($n = 6$)			Inter-day ($n = 2 \times 3$)		
	Concentration found (ng/ml)	C.V. (%)	Bias (%)	Concentration found (ng/ml)	C.V. (%)	Bias (%)
10	10.0±0.4	3.8	−0.3	10.8±0.8	7.1	8.3
30	29.4±0.5	1.8	−2.0	29.5±0.8	2.7	−1.6
90	90.2±1.4	1.5	0.2	90.1±0.8	0.9	0.1

Reproducibility was expressed as the coefficient of variation.
Accuracy was calculated as deviation from the nominal value (% bias).

Table 2
Comparison of the sensitivity and working range between the HPLC and absorptiometry methods

Addition of aluminum (ng/ml)	HPLC method		Absorptiometry method	
	Aluminum measured (ng/ml)	Recovery (%)	Aluminum measured (ng/ml)	Recovery (%)
500 000	N.T.	–	444 350.7±13 168.1	88.9
50 000	N.T.	–	41 774.9±18 34.2	83.5
5000	N.T.	–	3333.3±396.1	66.7
500	503.5±6.8	100.5	ND	–
50	48.6±1.8	95.9	N.T.	–
5	4.7±0.1	94.1	N.T.	–

N.T., not tested; ND, not detected. For comparison, both assays were carried out using the same sample volume.

the sensitivity of this method was at least 1000 times inferior to that of the HPLC method.

3.6. Detection of aluminum adhering to gastric mucosa

Fig. 2 shows the results of determination of aluminum adhering to ulcerated and normal rat gastric mucosa by the HPLC method. Aluminum levels were higher in the ulcerated mucosa than in the normal mucosa at all times after sucralfate administration, as was previously reported in studies using absorptiometry with 8-quinolinol [14,15,17].

Although the aluminum values determined by absorptiometry were below the LOD in several samples at 6 h after administration, the HPLC method could detect aluminum in all samples up to 24 h. When the values above zero obtained using absorptiometry were assessed, there was a significant correlation ($r = 0.993$, $P < 0.0001$) between the aluminum concentrations measured by the two methods (Fig. 3).

4. Conclusions

We established a highly sensitive method for determination of aluminum adhering to the gastric mucosa, involving a combination of liquid–liquid extraction and KD mode micellar HPLC. This method can be applied to the measurement of aluminum

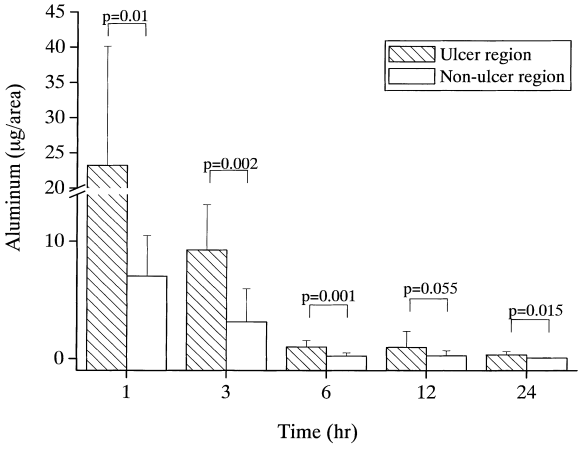

Fig. 2. Detection of aluminum adhering to the gastric mucosa using the HPLC method after oral administration of 100 mg of sucralfate to rats. Data represent the mean±SD for nine rats. Each *P* value was calculated by the paired *t*-test.

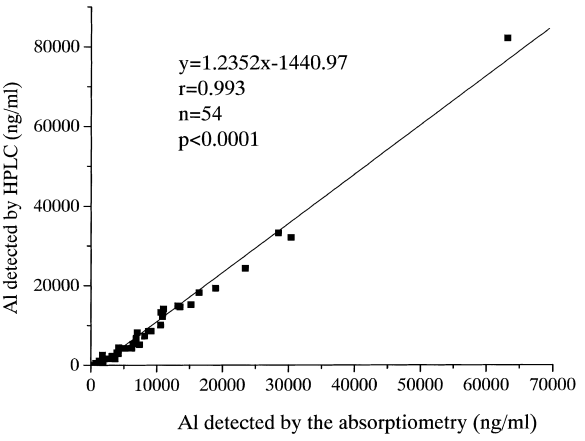

Fig. 3. Comparison of aluminum concentrations measured using the HPLC and absorptiometry methods.

in small samples, such as human gastric mucosal biopsy specimens. The application of this method to determine aluminum in other biological samples (brain, kidney, and bone) is now being investigated.

Acknowledgements

We thank M. Sato, Ph.D. (Marketing and Planning Department, Shino Test Corporation) for his useful advice.

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