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Use of nitric acid in sample pretreatment for determination of trace elements in various biological samples by ETAAS

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Abstract

Trace elements in liquid biological samples may be determined by direct electrothermal atomic absorption spectrometry (ETAAS). In our previous work it was found that samples containing proteins or DNA may leak out of the graphite tube before the drying step, despite the addition of various modifiers. In order to keep the sample to the graphite tube, samples were diluted before analysis 1 + 1 with 32% v/v nitric acid, or 5 µl of 32% v/v nitric acid was added to the graphite tube before ETAAS determination. Applying the proposed procedure, the concentrations of lead in eluted fractions after gel chromatographic separation of human cerebellar nucleus dentatus supernatant and platinum in isolated DNA samples were determined. The use of nitric acid in sample pretreatment prevent sample leakage out of the graphite tube, provided for even drying and considerably reduced nonspecific absorption in lead determination. The repeatability of measurements was better than ± 6%. The accuracy of the procedure was checked by spiking samples. The recoveries for both elements lay between 93–104%. Nitric acid was found to be a better modifier than TRITON X-100. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Trace elements; ETAAS; Matrix modifier; Nitric acid; Liquid biological samples

1. Introduction

Liquid biological samples with a high content of biological macromolecules (e.g. proteins, DNA) and/or a high salt content are complex matrices. Trace elements in these samples may be determined directly by electrothermal atomic absorption spectrometric (ETAAS) analysis, but measurement parameters should be optimised for each particular element and each specific sample matrix. There are many reports on direct determination of trace elements by ETAAS in human serum [1–7], plasma [1,8–10], whole blood [11–14] and spent continuous peritoneal dialysis fluids (CAPD) [15–18]. In order to reduce matrix effects, samples were simply diluted with water [1–3], or various matrix modifiers such us magnesium

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nitrate [1,10], copper nitrate [5], copper and magnesium nitrate [7] or nitric acid [1,14,18] were added. TRITON X-100 was also frequently recommended as a matrix modifier. It has been used alone [1,3,13,17] or in combination with nitric acid [1,8,9], magnesium nitrate [4,9], nickel nitrate [6,11] and ammonium hydrogen phosphate [12]. The choice of matrix modifier depended not only on matrix composition but also on specific problems in the determination of a particular element by ETAAS. Special care was devoted to elements like selenium [5–7,11], lead [12–14] and cadmium [12], which are liable to preatomization losses. In our laboratory an analytical procedure has been reported recently for direct determination of copper, aluminium and iron in spent CAPD fluids by ETAAS. It was found experimentally that some samples were leaked out of the graphite tube before the drying step, although various modifiers were used. In order to keep the sample in the graphite tube and to obtain reproducible measurements, samples were diluted before analysis $1+1$ with 32% v/v nitric acid [18].

In addition to serum, plasma, whole blood and spent CAPD fluids, other liquid biological samples can also be analysed by ETAAS. Trace element speciation in biological fluids is usually performed by separation on chromatographic columns and ETAAS detection. If trace elements in the separated fractions are bound to biological macromolecules, the problem of sample leakage out of the graphite tube may also appear. Similar problems can be observed when trace elements are determined in isolated DNA samples. The aim of our work was to examine the applicability of nitric acid in sample pretreatment when analysis of lead in eluted fractions after gel chromatographic separation of human cerebellar nucleus dentatus, and analysis of platinum in isolated DNA samples were performed. The measurement parameters for reliable determination of both elements by ETAAS in these biological samples were also optimised. The repeatability of measurements was tested and the accuracy of the procedure was checked by spiking samples.

2. Material and methods

2.1. Instrumentation

Lead and platinum were determined by ETAAS on a Hitachi Z-8270 polarized Zeeman atomic absorption spectrophotometer (Tokyo, Japan) equipped with an autosampler.

2.2. Reagents

Merck Suprapur acids and water doubly distilled in quartz were used. Stock standard solutions of lead and platinum (1000 mg/l $\pm$ 2 mg/l in 5% HNO_3) were obtained from Merck. Triton X-100 (98–102%, for molecular biology) and palladium nitrate (10.0 g/l $\pm$ 0.2 g/l of Pd in 15% HNO_3) were purchased from Merck.

2.3. Sample preparation for determination of lead in eluted fractions after gel chromatographic separation of human cerebellar nucleus dentatus supernatant

The autopsy sample from brain nucleus dentatus was homogenised with nitrogen-saturated buffer and ultracentrifuged as described by Falnoga et al. [19]. Supernatant was applied to a calibrated Sephadex G-75 column, and eluted at 4°C with buffer [19]. The column eluate was collected in 5 ml fractions in glass tubes and the concentration of lead determined ‘off line’ by ETAAS. Before analysis samples were equilibrated to room temperature.

2.4. Sample preparation for determination of platinum in isolated DNA samples

Subcutaneous tumours were induced dorsolaterally in mice [20]. After tumours developed mice were treated with cisplatin or electrochemotherapy treatment was applied [21]. For determination of platinum bound to DNA, mice were sacrified at various times after the treatment. Tumours were excised, treated with collagenase and DNA isolated by a modified salting method [22]. The volume of the isolated DNA samples, dissolved in bidistilled water, was 100 μl. After isolation, sam-

ples were frozen at −20°C and equilibrated to room temperature just before analysis. The concentration of platinum in isolated DNA samples was determined by ETAAS.

2.5. Sample pretreatment before ETAAS analysis

Before ETAAS analysis samples were diluted 1+1 with 32% v/v nitric acid, or 5 μl of 32% v/v nitric acid was added to the graphite tube before each determination. When Triton X-100 modifier was applied, 5 μl of 0.2% Triton X-100 containing 0.5 g/l of palladium (as palladium nitrate) was added to the graphite tube before each determination.

3. Results and discussion

3.1. Optimisation of measurement parameters for determination of lead in eluted fractions after gel chromatographic separation of human cerebellar nucleus dentatus supernatant by ETAAS

Analysis of lead in biological samples after chromatographic separation is a difficult task because of severe matrix interferences in ETAAS determinations. Matrix effects due to the presence of proteins are minimised by addition of TRITON X-100 which improves the drying characteristics of the sample. In order to reduce nonspecific absorption, an ashing temperature of at least 800°C should be applied. To prevent preatomization losses of lead at this temperature, a matrix modifier, e.g. palladium nitrate, is recommended. The use of a combined TRITON X-100-palladium nitrate modifier, added to the graphite tube before each determination, could therefore have beneficial effects in reducing interferences in analysis of lead in biological samples after chromatographic separation. In order to prevent sample leakage out of the graphite tube and to reduce matrix effects of proteins, the use of nitric acid in sample pretreatment was also tested. It was found experimentally that samples stayed in the graphite tube when just before the determination they were diluted 1+1 with 32% v/v nitric acid, so that the final concentration of acid was 16%. This concentration of nitric acid resulted in a colloidal solution, but did not cause precipitation of proteins during the time of analysis (8 h). The application of 5 μl of 32% v/v nitric acid to the graphite tube before each determination was tested as well. The measurement parameters for determination of lead by ETAAS are presented in Table 1.

Pyrolytically coated graphite tubes were used throughout the measurements. Zeeman background correction was employed to compensate for nonspecific absorption. It is evident from the data of Table 1 that careful drying of the sample was applied with a slow temperature ramp to 150°C. Special attention was paid to efficient cleaning (10 s at a temperature of 2800°C) of the salt deposit from the graphite tube before the next determination.

3.2. Determination of lead in eluted fractions after gel chromatographic separation of human cerebellar nucleus dentatus supernatant by ETAAS and accuracy checks

Analysis of lead in eluted fractions after gel chromatographic separation by ETAAS was not possible without the use of a matrix modifier due to extremely high (over range) nonspecific absorption signals and coincidental sample leakage from the graphite tube. In order to reduce matrix interferences the use of nitric acid in sample pretreatment and a Triton X-100-palladium nitrate matrix modifier were critically evaluated. Samples were analysed in 5 ml fractions throughout the chromatographic run under the optimised measurement conditions (Table 1). It was found experimentally that the standard addition method should be applied in the calibration procedure. Comparative analyses were made by the use of nitric acid and Triton X-100-palladium nitrate modifier. The results are presented in Table 2.

It is evident from these data that lead after gel chromatographic separation was eluted in two chromatographic peaks which on the basis of elution volumes from 37 to 47 ml corresponded to lead bound to high molecular weight proteins (≥70 000 Da) and from 107 to 127 ml to very low molecular weight proteins (<10 000 Da) [19]. In other fractions throughout the chromato-

Table 1
Measurement parameters for determination of lead in eluted fractions after gel chromatographic separation of human cerebellar nucleus dentatus supernatant by ETAAS with Zeeman background correction[a]

Electrothermal atomization programme

Stage no.	Stage	Temperature (°C) start	Temperature (°C) end	Time (s) ramp	Time (s) hold	Gas flow (ml/min)
1	Dry	60	90	10	0	200
2	Dry	90	100	10	5	200
3	Dry	100	150	10	0	200
4	Ash	150	400[b] 800[c]	10	20	100
5	Atomization	2200	2200	0	4	0
6	Clean	2800	2800	0	10	200
7	Cool	–	–	0	5	200

[a] Wavelength, 283.3 nm; spectral bandwidth, 1.30 nm; lamp current, 7.5 mA; sample volume, 10 µl.

[b] Nitric acid pretreatment: samples were diluted 1+1 with 32% v/v nitric acid before determination, or 5 µl of 32% v/v nitric acid was added to the graphite tube before each determination.

[c] Triton X-100-palladium nitrate modifier: 5 µl of 0.2% Triton X-100 containing 0.5 g/l of palladium (as palladium nitrate) was added to the graphite tube before each determination.

Table 2
Concentrations (μg/l) of lead in eluted fractions (5 ml) after gel chromatographic separation of human cerebellar nucleus dentatus supernatant determined by ETAAS with Zeeman background correction, using the standard addition method in the calibration procedure[d]

Elution volume (ml)	Pb (μg/l)		
	A[a]	B[b]	C[c]
2–37[e]	<1.0	<1.0	<1.0
37–42	54.0 ± 1.0	51.1 ± 3.2	57.0 ± 6.9
42–47	47.0 ± 1.5	48.1 ± 2.9	65.0 ± 10.5
47–107[e]	<1.0	<1.0	<1.0
107–112	6.4 ± 0.2	7.7 ± 0.2	7.6 ± 0.3
112–117	18.6 ± 0.4	19.4 ± 0.1	23.1 ± 2.0
117–122	19.8 ± 1.0	20.0 ± 0.5	21.8 ± 0.4
122–127	5.0 ± 0.2	6.3 ± 0.4	5.3 ± 0.3
127–162[e]	<1.0	<1.0	<1.0

[a] A: samples were diluted 1+1 with 32% v/v nitric acid before determination.

[b] B: 5 μl of 32% v/v nitric acid was added to the graphite tube before each determination.

[c] C: 5 μl of 0.2% Triton X-100 containing 0.5 g/l of palladium (as palladium nitrate) was added to the graphite tube before each determination.

[d] Results are expressed as the mean of three parallel determinations ± standard deviation of measurement.

[e] Concentration of lead was <1.0 μg/l in each particular 5 ml fraction.

graphic run the concentration of lead was below 1.0 μg/l. It is further evident from the data of Table 2 that the results obtained when samples were diluted 1 + 1 with 32% v/v nitric acid, agree with those when 5 μl of 32% v/v nitric acid was added to the graphite tube before each determination. It was found experimentally that addition of nitric acid appreciably reduced nonspecific absorption at an ashing temperature not higher than 400°C. When Triton X-100-palladium nitrate modifier was used, the results in fractions eluted under the chromatographic peak from 107 to 127 ml in general agreed with those obtained when nitric acid was used in sample pretreatment, while in the chromatographic peak eluted from 37 to 47 min the results obtained by the use of Triton X-100-palladium nitrate modifier were higher. It should be pointed out that despite the use of palladium nitrate which enabled a higher ashing temperature to be applied (800°C), the signal for nonspecific absorption was over range in fractions eluted under the chromatographic peak from 37 to 47 min. Therefore, Zeeman background correction did not compensate efficiently for nonspecific absorption. On the basis of the observations from Table 2, it can be concluded that the use of nitric acid in sample pretreatment in analysis of lead in eluted fractions after chromatographic separation is more successful than Triton X-100-palladium nitrate modifier.

The accuracy of the procedure using nitric acid in sample pretreatment (dilution of samples 1 + 1 with 32% v/v nitric acid) was checked by spiking of samples with 10 or 50 μg/l of lead. Results are presented in Table 3.

It is evident from these data that recoveries for spiked samples lay between 94 and 103%. From these data and from the data of Table 2 it can be concluded that reliable analysis of lead can be performed by ETAAS determination when nitric

Table 3
Recoveries in spiked samples in determination of lead by ETAAS in eluted fractions under chromatographic peaks after gel chromatographic separation of human cerebellar nucleus dentatus supernatant[a]

Elution volume (ml)	Pb (μg/l)	Pb added (μg/l)	Pb found (μg/l)	Recovery %
37–42	54.0 ± 1.0	50.0 ± 0.5	97.3 ± 2.0	93.5
32–47	47.0 ± 1.5	50.0 ± 0.5	97.1 ± 2.0	100.0
107–112	6.4 ± 0.2	10.0 ± 0.1	16.0 ± 0.4	97.5
112–117	18.6 ± 0.4	10.0 ± 0.1	27.7 ± 0.6	97.0
117–122	19.8 ± 1.0	10.0 ± 0.1	30.7 ± 0.5	103.0
122–127	5.0 ± 0.2	10.0 ± 0.1	15.0 ± 0.3	100.0

[a] Results are expressed as the mean of three parallel determinations ± standard deviation of measurement. Samples were diluted 1+1 with 32% v/v nitric acid before determination. The standard addition method was applied in the calibration procedure.

Table 4
The repeatability of measurement of lead in eluted fractions under chromatographic peaks after gel chromatographic separation of human cerebellar nucleus dentatus supernatant[a]

Elution volume (ml)	Replicate 1 Pb (μg/l)	Replicate 2 Pb (μg/l)	Replicate 3 Pb (μg/l)	Replicate 4 Pb (μg/l)	Replicate 5 Pb (μg/l)	Replicate 6 Pb (μg/l)	Mean Pb (μg/l)	RSD (%)
37–42	47.3	55.2	53.7	52.1	50.3	47.7	51.1	±6.3
42–47	51.3	47.5	45.0	44.3	50.0	50.3	48.1	±6.1
107–112	7.4	8.0	7.8	7.5	7.8	7.9	7.7	±3.0
112–117	19.4	19.5	19.2	19.6	19.5	19.4	19.4	±0.7
117–122	20.2	20.0	19.3	19.5	20.3	19.7	20.0	±2.7
122–127	6.2	6.4	6.3	6.1	6.9	5.7	6.3	±6.2

[a] Lead was determined by ETAAS applying standard addition calibration. 5 μl of 32% v/v nitric acid was added to the graphite tube before each determination.

Table 5
Measurement parameters for determination of platinum in isolated DNA samples by ETAAS with Zeeman background correction[a]

Electrothermal atomization programme						
Stage no.	Stage	Temperature (°C) start	Temperature (°C) end	Time (s) ramp	Time (s) hold	Gas flow (ml/min)
1	Dry	60	90	10	0	200
2	Dry	90	100	10	5	200
3	Dry	100	150	10	0	200
4	Ash	150	1200	10	20	100
5	Atomization	2700	2700	0	4	0
6	Clean	2800	2800	0	10	200
7	Cool	–	–	0	5	200

[a] Wavelength, 265.9 nm; spectral bandwidth, 0.40 nm; lamp current, 12.5 mA; sample volume, 10 μL. 5 μl of 32% v/v nitric acid was added to the graphite tube before each determination.

acid is used in sample pretreatment, either by diluting samples before analysis $1+1$ with 32% v/v nitric acid, or by addition of 5 μl of 32% v/v nitric acid to the graphite tube before ETAAS determination.

3.3. Calibration graph, limit of detection and repeatability of lead measurements

The calibration graph for aqueous standard solutions was linear from 3 up to 80 μg/l of lead with a correlation coefficient better that 0.999. The limit of detection (LOD), calculated on a 3 *s* basis (a value of three times the standard deviation of the blank) was found to be 1.0 μg/l of lead. The repeatability of measurement for determination of lead by ETAAS using nitric acid in sample pretreatment (5 μl of 32% v/v nitric acid added to the graphite tube before each determination) was tested on fractions eluted under the chromatographic peaks. The results for six parallel determinations are presented in Table 4. It is evident that the repeatability of measurement for lead in the samples analysed was better than $\pm$ 6%.

3.4. Optimisation of measurement parameters for direct determination of platinum in isolated DNA samples by ETAAS

The concentration of DNA in isolated DNA samples was 300–400 μg/ml. Due to the presence of DNA, these samples were highly viscosous, and they were leaked out of the graphite tube before the drying phase. In order to prevent sample leakage from the graphite tube and to reduce matrix effects, the use of nitric acid in sample pretreatment was tested for analysis of platinum in isolated DNA samples. It was found experimentally that samples can be diluted before the determination $1+1$ with 32% v/v nitric acid, or addition of 5 μl of 32% v/v nitric acid to the graphite tube before determination can be applied in the analysis. For practical reasons, 5 μl of 32% v/v nitric acid was added to the graphite tube before each determination. It was also found that when nitric acid is used in sample pretreatment, the sample matrix does not influence the sensitivity of measurement. The same results were obtained when samples were analysed against aqueous standards or a standard addition calibration was applied. The differences in results obtained by the two calibration procedures did not exceed ± 4%. For that reason determination of platinum in isolated DNA samples was performed against aqueous standards. Pyrolytically coated graphite tubes were used throughout the measurements. The measurement parameters for determination of platinum by ETAAS are presented in Table 5.

3.5. Determination of platinum in isolated DNA samples by ETAAS and accuracy checks

The concentration of platinum was determined in isolated DNA samples at different times (0–96 h) after electrochemotherapy and cisplatin treatment. Analyses were performed by ETAAS under optimised measurement conditions (Table 5). The results are presented in Table 6.

It is evident from these data that the volume of isolated DNA sample (100 μl) was adequate for reliable determination of three sample replicates by ETAAS, and that the proposed analytical procedure is convenient for determination of platinum. It should be pointed out that without use of a modifier, analyses by ETAAS were not possible due to sample leakage from the graphite tube. Use of nitric acid in sample pretreatment was proved to be an appropriate way of overcoming these interference effects. The accuracy of the procedure, applying 5 μl of 32% v/v nitric acid to the graphite tube before each determination, was checked by spiking samples with 100–500 μg/l of platinum. In performing this experiment it was not possible to spike isolated DNA samples after cisplatin or electrochemotherapy treatment, since after analysis (Table 6) the sample volume was too low. Therefore, a sample containing DNA, which was isolated from mice that were not treated with cisplatin (concentration of platinum below 5.0 μg/l, concentration of DNA 400 μg/ml) was used for spiking. Results are presented in Table 7.

Table 6
Concentrations (μg/l) of platinum in isolated DNA samples at different times after electrochemotherapy (ECT) and cisplatin (CDDP) treatment determined by ETAAS with Zeeman background correction, using aqueous standards in the calibration procedure[a]

Sample no.	Treatment	Time after treatment (h)	Pt (μg/l)	RSD (%)
1	ECT	0	505	±0.5
2	ECT	0	321	±5.0
3	ECT	0	500	±6.3
4	CDDP	0	640	±5.5
5	CDDP	0	370	±6.4
6	CDDP	4	355	±5.0
7	CDDP	4	180	±2.7
8	ECT	4	117	±1.6
9	ECT	18	470	±6.4
10	ECT	18	210	±4.8
11	CDDP	18	200	±5.0
12	CDDP	18	70.0	±0.0
13	ETC	48	170	±5.9
14	ECT	48	606	±6.4
15	ECT	48	237	±4.6
16	ECT	48	134	±6.2
17	CDDP	48	126	±3.3
18	CDDP	72	82.8	±3.3
19	ECT	72	158	±3.7
20	ECT	72	75.8	±1.0
21	ECT	72	215	±4.0
22	CDDP	96	222	±1.5
23	CDDP	96	262	±5.1
24	CDDP	96	216	±4.4
25	ECT	96	383	±1.3
26	ECT	96	236	±1.0
27	ECT	96	545	±1.0

[a] Results are expressed as the mean of three parallel determinations ± relative standard deviation of measurement. 5 μl of 32% v/v nitric acid was added to the graphite tube before each determination

Table 7
The recoveries in spiked isolated DNA sample in determination of platinum by ETAAS with Zeeman background correction, using aqueous standards in the calibration procedure[a]

Sample	Pt (μg/l)	Pt added (μg/l)	Pt found (μg/l)	Recovery %
Isolated DNA	<5.0	100 ± 1	101 ± 6	101.0
Isolated DNA	<5.0	200 ± 2	209 ± 4	104.5
Isolated DNA	<5.0	300 ± 3	296 ± 3	98.5
Isolated DNA	<5.0	400 ± 4	388 ± 4	97.0
Isolated DNA	<5.0	500 ± 5	495 ± 6	99.0

[a] Results are expressed as the mean of six parallel determinations ± standard deviation of measurement. 5 μl of 32% v/v nitric acid was added to the graphite tube before each determination.

It is evident from these data that recoveries for spiked samples lay between 97 and 104%. From these data and from the data of Table 6 it can be concluded that reliable analysis of platinum can be performed by ETAAS determination when nitric acid is used in sample pretreatment.

3.6. Calibration graph, limit of detection and repeatability of platinum measurements

The calibration graph for aqueous standard solutions was linear from 30 up to 1000 μg/l of platinum with a correlation coefficient better that 0.999. The limit of detection (LOD) calculated on a 3 *s* basis (a value of three times the standard deviation of the blank) was found to be 5 μg/l of platinum. The repeatability of measurement for determination of platinum by ETAAS using nitric acid in sample pretreatment (5 μl of 32% v/v nitric acid added to the graphite tube before each determination) was tested in five spiked isolated DNA samples. The results for six parallel determinations demonstrated that the repeatability of measurement for platinum in the samples analysed was better than ± 6% (see data in Table 7).

4. Conclusions

An analytical procedure was developed for direct determination of trace elements in various liquid biological samples by ETAAS. The use of nitric acid in sample pretreatment prevents sample leakage from the graphite tube, provides beneficial effects in the drying and ashing steps and enables reliable determination of trace elements in very complex matrices containing proteins, a high salt concentration or DNA molecules. In addition to efficient application in analysis of trace elements in spent CAPD fluids by ETAAS [18], the use of nitric acid in sample pretreatment was also found to be effective in analysis of lead in fractions eluted after gel chromatographic separation of human cerebellar nucleus dentatus supernatant and platinum in isolated DNA samples. It was demonstrated that addition of nitric acid under the recommended sample pretreatment also considerably reduced nonspecific absorption in lead determination. Depending on the concentration of biological macromolecules, samples can be diluted before analysis $1 + 1$ with 32% v/v nitric acid, or 5 μl of 32% v/v nitric acid may be added to the graphite tube before ETAAS determination. Good repeatability of measurements was obtained (RSD ± 6%) when lead and platinum were determined under optimised measurement conditions. The procedure was proved to be accurate with recoveries for spiked samples ranging from 93 to 104%. Nitric acid was found to be a better modifier than TRITON X-100. The procedure can also be applied in determination of other trace elements in eluted fractions after various chromatographic separations of liquid biological samples and in isolated DNA samples. Reliable determination of trace elements in these biological samples is of great importance in speciation studies. However, measurement parameters have to be optimised for each particular element before ETAAS determination.

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