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Research Article

Microextraction Methods for Preconcentration of Aluminium in Urine Samples

Farzad Farajbakhsh[1,2], Mohammad Amjadi[2], Jamshid L. Manzoori[3], Mohammad R. Ardalan[4], Abolghasem Jouyban[5,6*]

[1]*Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*
[2]*Department of Analytical Chemistry, Faculty of Chemistry, Tabriz University, Tabriz, Iran*
[3]*Department of Chemistry, Tabriz Islamic Azad University, Tabriz, Iran*
[4]*Chronic Kidney Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*
[5]*Pharmaceutical Analysis Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran*
[6]*Kimia Idea Pardaz Azarbayjan (KIPA) Science Based Company, Tabriz University of Medical Sciences, Tabriz, Iran*

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ABSTRACT

Background: Analysis of aluminium (Al) in urine samples is required in management of a number of diseases including patients with renal failure. This work aimed to present dispersive liquid-liquid microextraction (DLLME) and ultrasound-assisted emulsification microextraction (USAEME) methods for the preconcentration of ultra-trace amount of aluminum in human urine prior to its determination by a graphite furnace atomic absorption spectrometry (GFAAS).
Methods: The microextraction methods were based on the complex formation of Al^{3+} with 8-hydroxyquinoline. The effect of various experimental parameters on the efficiencies of the methods and their optimum values were studied.
Results: Under the optimal conditions, the limits of detection for USAEME-GFAAS and DLLME-GFAAS were 0.19 and 0.30 ng mL^{-1}, respectively and corresponding relative standard deviations (RSD, n=5) for the determination of 40 ng mL^{-1} Al^{3+} were 5.9% and 4.9%.
Conclusion: Both methods could be successfully used to the analysis of ultra trace concentrations of Al in urine samples of dialysis patients.

Introduction

Aluminum (Al) is a non-essential, toxic metal to humans which are frequently exposed by drinking water and food intakes. Some widely used pharmaceutical products, such as antacids contain Al compounds, and are administered for the treatment of peptic disorders.[1] This element has been involved as a causative factor in some diseases, particularly in chronic renal failure.[2]

Al binds rather weakly to plasma components, maximizing its facility to be transferred to binding sites within tissues. It has been estimated that 45–75% of intravenously injected ^{26}Al is excreted in the first 24 h after injection.[3] Since only 0.5% of injected Al remains in human plasma one day after injection, it quickly transfered to different tissues. Al accumulates in all tissues of mammals, preferentially in kidney, liver, heart, bone and brain.[4] Some studies suggest that Al may be accumulated in the brain via different routes, interfere with the normal activities of nervous system and is considered as a possible cause in some diseases such as; renal osteodystrophy, Parkinson and Alzheimer diseases.[5,6] Therefore, monitoring the levels of Al in biological fluids is a valuable tool in biomedical investigations. Concerning these demands, it is essential to establish simple, rapid, sensitive and environment-friendly methods for quantification of Al at trace levels in biological samples.

Graphite furnace atomic absorption spectrometric (GFAAS) method is a suitable technique for the determination of Al because of its high sensitivity, precision, selectivity, and versatility. However, it is generally impossible to determine trace Al in biological samples directly because of the presence of interfering species from the matrix, or the very low concentration of the analyte. So developing new, sensitive and selective preconcentration and separation techniques is still necessary.

Recent investigations are focused on the development of efficient, economical, and miniaturized sample preparation methods leading to the development of different liquid phase microextractions such as single drop microextraction, hollow fiber liquid phase microextraction and dispersive liquid–liquid microextraction (DLLME).[7] In DLLME method, an appropriate mixture of the extraction solvent and the dispenser solvent is injected into the aqueous solution using a syringe to form a cloudy mixture. The cloudy

***Corresponding Author:** Abolghasem Jouyban, E-mail: ajouyban@hotmail.com

state results from the formation of fine droplets of the extraction solvent that disperse in the sample solution. The cloudy solution is centrifuged and the fine droplets are sedimented at the bottom of the conical test tube. Determination of analytes in the collected organic phase can be performed by instrumental techniques. Simplicity of the operation, speed, low sample volume, low cost, acceptable recovery and high enhancement factor are some advantages of DLLME.[8,9] DLLME is widely applied for environmental water samples but rarely applied for the analysis of trace elements in complex matrices such as biofluids.

A few papers have reported the application of DLLME in urine samples. Fuh and co-workers[10] developed DLLME combined with liquid chromatography electrospray tandem mass spectrometry for the extraction and determination of 7-aminoflunitrazepam in urine samples. In a subsequent study, Xiong et al.[11] proposed a DLLME method combined with HPLC-UV for determining of three psychotropic drugs in urine samples.

Recently, a novel type of liquid-liquid microextraction methods has been developed based on the application of ultrasonic radiation as an efficient tool to facilitate emulsification phenomenon and accelerate the mass transfer process between two immiscible phases. Regueiro et al.[12] reported the application of ultrasonic radiation as an alternative for the dispersion of the extraction solvent in the aqueous solution and named the procedure ultrasound-assisted emulsification microextraction (USAEME).

The aims of this study were to compare the efficiencies of DLLME and USAEME for the extraction of Al from human urine samples and its determination by GFAAS method. The effects of various experimental parameters, such as the kind and volume of extraction and dispersive solvent, extraction time, sample solution pH, salt effect, sample volume, centrifugation time and speed were studied and optimized systematically. The advantages and disadvantages of both methods for the GFAAS analysis of Al were also discussed.

Materials and Methods

Reagents, solutions and Instrumental

A CTA-3000 atomic absorption spectrophotometer (Chemtech limited Co. UK) with a self-reversal background correction and a graphite furnace atomizer system was used. An Al hollow-cathode lamp was used as radiation source at 309.3 nm. The optimum operating parameters for GFAAS are given in Table 1. All chemicals used were of analytical-reagent grade and solutions were prepared with high purity deionized water (Ghazi Pharmaceutical Co, Tabriz, Iran). Stock standard solution of Al^{3+} at a concentration of 1000 mg L^{-1} was obtained from the National Institute of Standards. Working standard solutions were obtained by appropriate dilution of the stock solution.

A 0.5 mol L^{-1} solution of 8-hydroxyquinoline (HQ or oxine) was prepared by dissolving appropriate amounts of absolute acetonitrile from the commercially available product (99%, analytical grade, Scharlau, Barcelona, Spain). All other chemicals, such as chloroform, carbon tetrachloride, dichloromethane, dichloroethylene and chlorobenzene, used as extraction solvents; ethanol, methanol, acetone, and acetonitrile, used as disperser solvents, HCl (37%), NaOH (99% purity) and sodium nitrate were purchased from Merck (Darmstadt, Germany). A pH-meter (model 744, Metrohm, Switzerland), a centrifuge (MSE Micro Center MSB010.CX2.5, Sanyo, Japan) and a 32 kHz and 350 kW ultrasonic water bath (Alex Machine, Turkey) were employed.

Table 1. Operating parameters for GFAAS.

Parameter	Value
Lamp current (mA)	6.0
Wavelength (nm)	309.3
Slit (nm)	0.4
Ar flow rate (mL min^{-1})	200 (stopped during atomizing)
Sample volume (µL)	20
Temperature program	
Drying 1	90 °C (ramp 10 s, hold 5 s)
Drying 2	140 °C (ramp 10 s, hold 10 s)
Ashing	1500 °C (ramp 10 s, hold 15 s)
Atomizing	2400 °C (ramp 0 s, hold 3 s)
Cleaning	2500 °C (ramp 1 s, hold 2 s)

Analytical procedure

All experiments and optimizations were performed in urine. Since urine has a variable matrix, individual sample-based standards give the most accurate results.

Dispersive liquid–liquid microextraction (DLLME) procedure

The urine sample was spiked with appropriate amount of Al and 10 mL of sample was transferred into a centrifuge tube and 3% w/v of sodium nitrate was added. The pH of solution was adjusted by adding NaOH or HCl. Then 50 µL of the chelating solution was added to the sample, vortexed and let to react for 30 min at room temperature with occasional mixing. Then, 600 µL of acetonitrile (as disperser solvent) containing 120 µL of chloroform (as extraction solvent) was injected rapidly into the sample solution by using a 2 mL syringe. A cloudy mixture was formed in the test tube. In this step, the $Al–HQ_3$ is extracted into the very fine droplets of chloroform in few seconds. After centrifugation for 5 min at 5000 rpm, the extraction solvent was sedimented in the bottom of the conical test tube. 20 µL of the extract was introduced into the GFAAS by manual injection.

Ultrasound-assisted emulsification microextraction (USAEME)

Similar to the previous section sample solution was prepared and tube was immersed into an ultrasonic water bath. Then 120 µL chloroform, as the extraction

solvent, was slowly injected into the biological sample by a home-designed syringe. During 4 min sonication process at 32 kHz with power of 350 kW at 25±3 °C, the solution became turbid due to the emulsification of fine chloroform droplets in the aqueous solution. The analyte was then extracted into the fine droplets of chloroform. The formed emulsion was then centrifuged for 5 min at 5000 rpm. Subsequently, the organic phase was collected and 20 µL was injected into the GFAAS.

Results and Discussion

DLLME and USAEME combined with GFAAS were developed and optimized for the extraction and determination of Al in urine samples. To obtain high extraction efficiency, the influence of different factors affecting extraction conditions, such as type of extraction and disperser solvents and their volumes, pH of sample solution, salt effect, extraction time and centrifugation time were investigated.

Optimization of the complex formation/extraction conditions

Time required for complex formation

The rate of the Al–HQ_3 chelation reaction is moderately influenced by the matrix of the reaction solution. In aqueous solutions the reaction requires about 20 min to be completed. Once a maximum value was reached, absorbance remained unchanged for 24 h; longer periods were not studied. For urine samples a longer reaction time was allowed for the complex formation (studied range, 5–120 min). According to Figure 1, 40 min was selected as appropriate time for complex formation.

Effect of pH

The effect of pH on the extraction of Al^{3+} ions was studied within the range of 4.0-9.0 by adding appropriate volumes of HCl or NaOH solution to the samples. As shown in Figure 2, the signal of Al improved with the increasing of pH from 4.0 to 6.0, and remained unchanged between 6.0 and 8.0. The reduced analytical signal at pH>8.0 could be due to the hydroxide formation of Al ions, resulting in decreased concentration of free Al^{3+} ions in the sample solution. Thus, in order to maintain a constant working pH that allows complex formation and stability, pH was adjusted at 6.5 in subsequent experiments.

Effect of other parameters

The effects of centrifugation time and speed, on the analytical responses were also investigated. Based on the obtained results, 5 min, 5000 rpm were selected as optimum centrifugation time and speed, respectively.

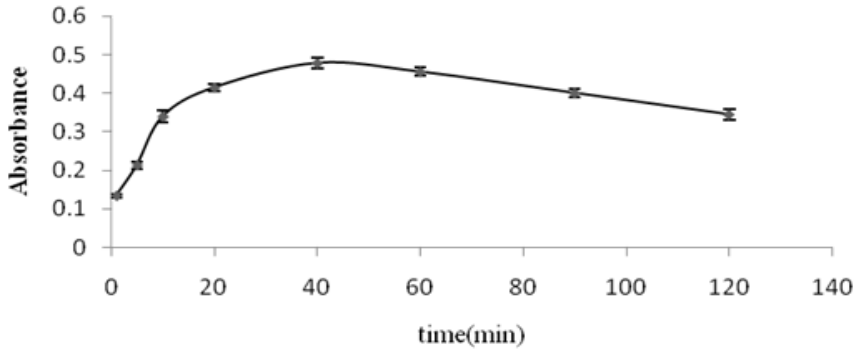

Figure 1. Effect of time on the complex formation of aluminum-oxine (for DLLME and USAEME). Conditions: 10 mL of solution containing 40 ng $mL^{-1}Al^{3+}$; 50 µL of oxine 0.5 mol L^{-1}, 600 µL of acetonitrile containing 120 µL of chloroform, 3% $NaNO_3$ (w/v) centrifugation time 5 min, pH=6.5.

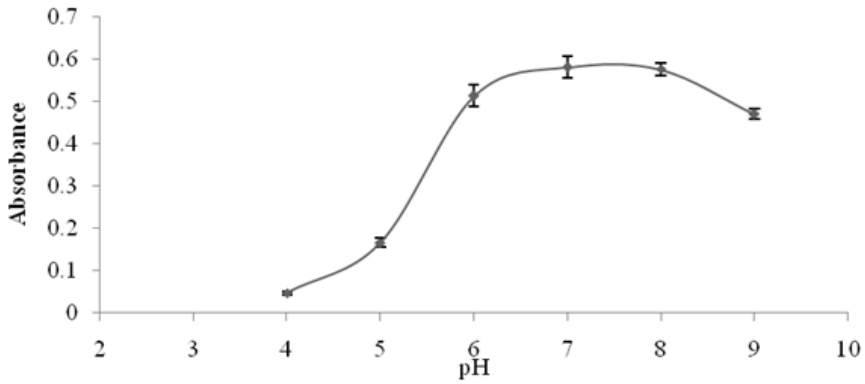

Figure 2. Effect of pH on the complex formation of aluminum-oxine (for DLLME and USAEME). Conditions: 10 mL of solution containing 40 ng $mL^{-1}Al^{3+}$; 50 µL of oxine 0.5 mol L^{-1}, 600 µL of acetonitrile containing 120 µL of chloroform, 3% $NaNO_3$ (w/v) centrifugation time 5 min.

Optimization of the USAEME operation parameters

Effect of type and volume of extraction solvent

The type of extraction solvent is very important in obtaining satisfactory extraction efficiency because the physico–chemical properties of the solvents determine the emulsification phenomena and the extraction efficiency. The extracting solvent should be able to form a cloudy mixture in the aqueous phase. In addition it must have, high extraction capability for the compounds of interest, low water solubility and compatibility with the analytical instrumentation.[13] Extraction of Al was carried out by different extracting solvents such as $CHCl_3$, CCl_4 and CH_2Cl_2 from aqueous solutions. Dichloromethane was completely dissolved in the ultrasonic process. This behavior was also observed by Ma et al.[14] Therefore, this solvent was excluded for further optimization. The extraction of Al^{3+} was quantitative by both chloroform and carbon tetrachloride (98%), however, chloroform was chosen as a solvent for further study because of its less toxicity.[15]

During USAEME process, extraction volume was an essential factor which could influence the formation of cloudy state and also determine enrichment performance. To examine the effect of extraction solvent volume, different volumes of $CHCl_3$ were added to 10 mL aqueous phase. The results are shown in Figure 3. As can be seen, the percent of extraction efficiency increases with the increase of $CHCl_3$ volume in the range of 70-200 µL, after that it decreased slowly when the volume was continuously increased. Therefore, 120 µL was chosen, in order to achieve a higher enrichment factor and lower limit of detection.

Effect of extraction time

The duration of ultrasonication possesses an important role in the dispersion of organic phase, which affects the extraction efficiency of the analytes.[16] Efficient sonication makes the extraction solvent disperse finely into the aqueous solution and is resulted in an excellent cloudy mixture. So, the effect of sonication time was evaluated in the range of 0.0 to 16.0 min (see Figure 4). It was seen that the extraction efficiency reached a maximum at 4 min and remained constant after this time. Therefore, 4 min was chosen for further studies.

Effect of salt addition

For investigating the influence of ionic strength on the performance of USAEME, sodium nitrate varying from 0 to 10% w/v was added, while other experimental conditions were kept constant. Increasing the sodium nitrate concentration had no significant effect on extraction factor, perhaps because of the two opposite effects of salt addition in USAEME of Al: one involves increasing the volume of the organic phase, which decreases the enrichment factor, and the other is the salting-out effect that increases the enrichment factor.

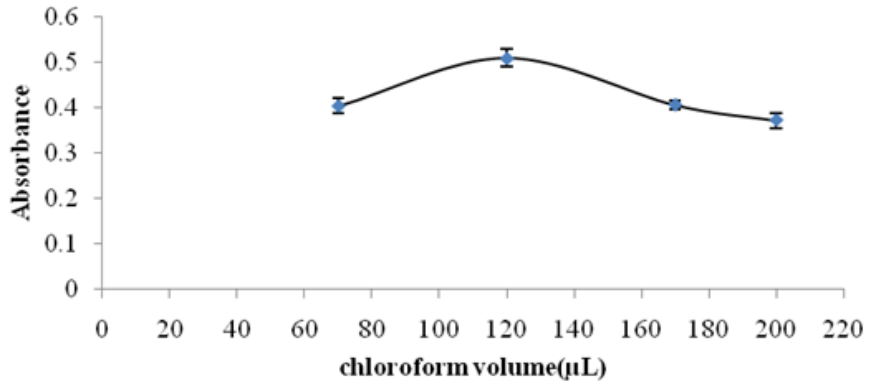

Figure 3. Effect of the extraction solvent volume on the analytical signals in USAEME method; Conditions: 10 mL of solution containing 40 ng $mL^{-1}Al^{3+}$, 50 µL of oxine 0.5 mol L^{-1}, pH=6.5, complex formation time: 0 min, centrifugation time 5 min.

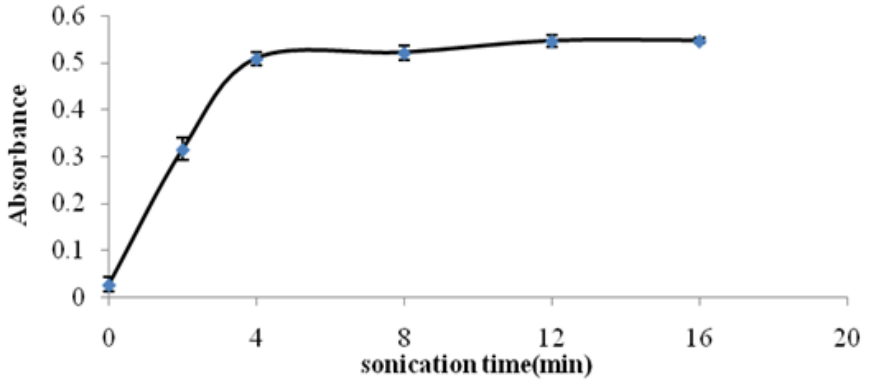

Figure 4. Effect sonication time at USAEME. Conditions are the same as in Figure 3.

Optimization of the DLLME operation parameters

Effect of type and volume of extraction solvent

The selection of an appropriate extraction solvent is a key parameter for DLLME procedure. Hence, several organic reagents including dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), carbon tetrachloride (CCl_4), dichloroethylene ($C_2H_2Cl_2$) and chlorobenzene (C_6H_5Cl) were investigated.

600 µL of acetonitrile containing different volumes of the extraction solvent was rapidly injected into the sample solution. Here, 65, 68, 200, 70 and 120 µL of C_6H_5Cl, $C_2H_2Cl_2$, CH_2Cl_2, CCl_4 and $CHCl_3$ were used, respectively. When C_6H_5Cl, $C_2H_2Cl_2$, CCl_4 were used as extraction solvent, white lipid phase was sedimented in the bottom of the conical test tube, probably due to the co-sedimentation of the matrices (such as carbamide and uric acid) in urine at high pH values. Whereas, with CH_2Cl_2 and $CHCl_3$ as extraction solvent, the cloudy state was formed and a sedimented droplet of extract was obtained on the bottom of test tube after centrifugation. The results are shown in Figure 5. $CHCl_3$ was selected as extraction solvent.

In order to optimize the extraction solvent volume, different volumes (70, 120, 170 and 200 µL) of $CHCl_3$ were added to 600 µL of acetonitrile and the resulting mixtures were subjected to the same DLLME procedures. Figure 6 indicates that the absorption signal increased by increasing the volume of the chloroform up to 120 µL. At higher volumes of extraction solvent, the ratio of the dispersive to extraction solvent decreased which probably lowered the number of formed droplets and thereby decreased the efficiency of extraction. Based on these observations, a volume of 120 µL was used for further experiments.

Effect of salt addition

The effect of salt addition was studied by using $NaNO_3$ in the concentration range of 0–10% (w/v). It was found that the extraction efficiency of DLLME significantly increased with increasing sodium nitrate concentration up to 3% (w/v), and then decreased remarkably. This behavior could be attributed to the fact that salt addition can enhance the extraction efficiency by salting-out effect, but may also prevent the formation of cloudy state at higher concentrations which causes a decrease in the sedimented phase volume. Hence, 3% $NaNO_3$ (w/v) was used in the subsequent experiments for method validation.

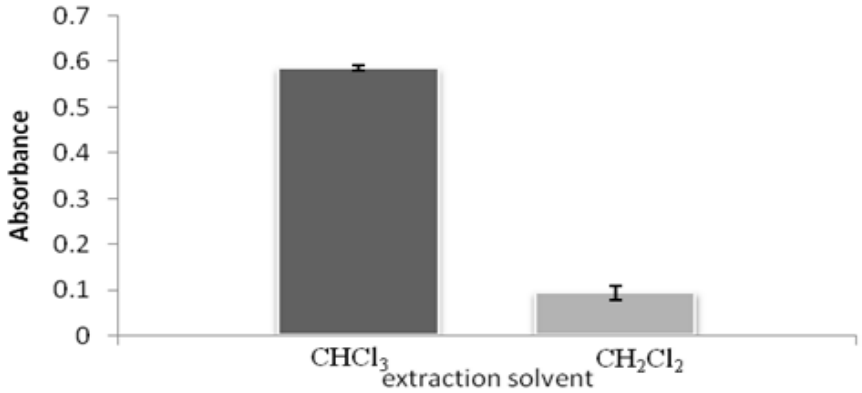

Figure 5. Effect extraction solvent in DLLME. Conditions: 10 mL of solution containing 40 ng $mL^{-1}Al^{3+}$; 50 µL of oxine 0.5 mol L^{-1}, 600 µL of acetonitrile, 3% $NaNO_3$ (w/v), centrifugation time 5 min, pH=6.5.

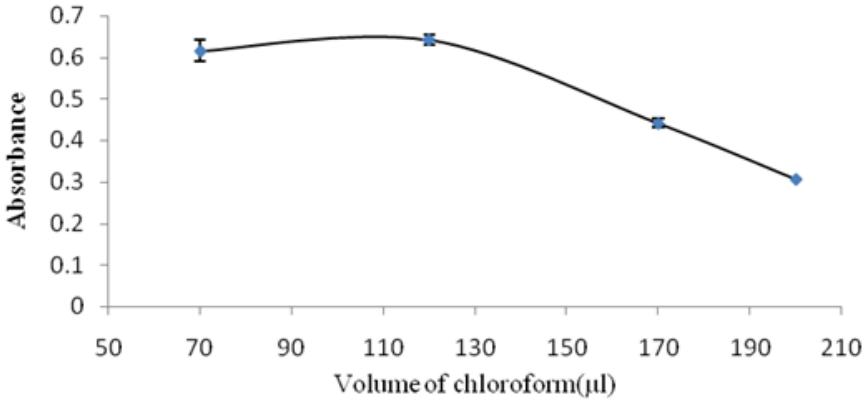

Figure 6. Effect of extraction solvent ($CHCl_3$) volume on extraction efficiency in DLLME. Conditions: 10 mL of solution containing 40 ng $mL^{-1}Al^{3+}$; 50 µL of oxine 0.5 mol L^{-1}, 600 µL of acetonitrile, 3% $NaNO_3$ (w/v), centrifugation time 5 min, pH=6.5.

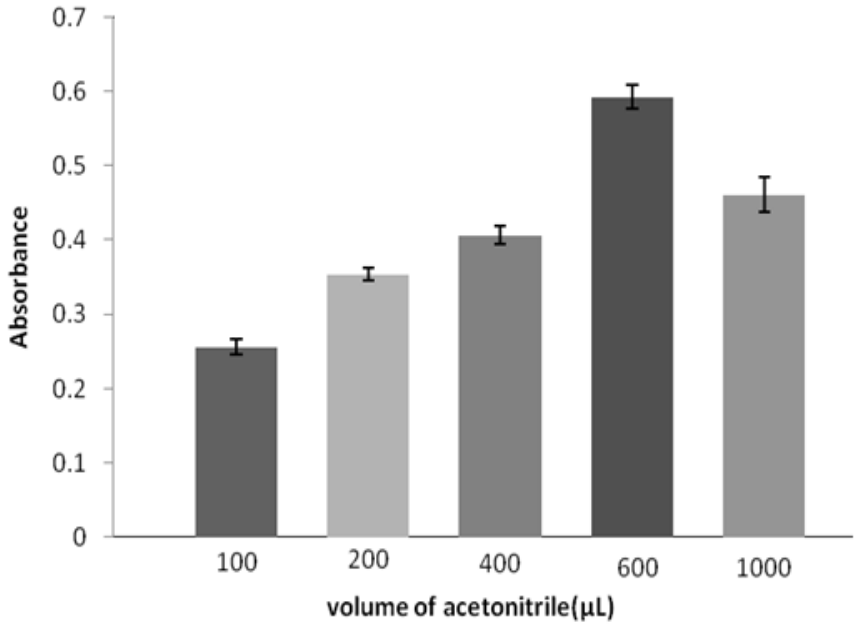

Figure 7. Effect of disperser solvent (acetonitrile) volume on extraction efficiency. Conditions: 10 mL of solution containing 40 ng $mL^{-1}Al^{3+}$; 50 µL of oxine 0.5 mol L^{-1}, 120 µL of chloroform as extraction solvent, 3% $NaNO_3$ (w/v), centrifugation time 5 min, pH=6.5.

Analytical performance of the USAEME and DLLME procedures

The figurers of merit in the USAEME and DLLME methods including precision, linear dynamic range, limit of detection, enrichment factor and extraction recovery for the Al^{3+} from 10 mL of aqueous solutions were investigated under optimum conditions.

Precision

The repeatability expressed as relative standard deviation (RSD, n=5) for replicated determination of aluminum was 5.9% and 4.9% for Al concentration level of 40 ng mL^{-1} for the USAEME and DLLME methods, respectively. The repeatability of the DLLME method was found to be better than that of USAEME.

Linear dynamic range

Calibration graphs were obtained by the preconcentration of a series of 20 solutions according to the USAEME and DLLME procedures. The response function of the USAEME and DLLME methods was linear in the ranges of 1–60 and 1–70 ng mL^{-1} with correlation coefficients of 0.9974 and 0.9948, respectively. The regression equations were Y = 0.009 CAl + 0.076 and Y = 0.006 CAl + 0.046, where Y was the relative peak area, and CAl was Al concentration in ng mL^{-1} for the USAEME and DLLME, respectively. Good linearity was obtained for Al using both extraction techniques. However, the dynamic range for the DLLME method was a little broader compared to the USAEME method.

The enrichment factor was obtained 53 and 35 by using calibration curve in USAEME and DLLME methods respectively.

Limit of detection

The limits of detection (LODs) of the USAEME- and DLLME-GFAAS techniques using a sample volume of 10 mL, based on the signal-to-noise ratio (S/N), were 0.19 and 0.30 ng mL^{-1}, respectively. It was clear that the LOD of the USAEME method was better than that of the DLLME method, indicating that the USAEME enabled a higher enrichment of the analyte.

The results of our study were compared with other reports used different analytical method for determining of aluminum in biological samples as listed in Table 2.

Interference study

The influence of foreign ions which can potentially interfere with determination of Al by the presented method in urine was examined. In this experiment, 10.0 mL of solutions containing 40 ng mL^{-1} of Al^{3+} and added interfering ions were treated according to the recommended procedure. The results are given in Table 3. Tolerable limit was defined as the highest amount of foreign ions that produced an error not exceeding 5%. As can be seen in Table 3, most of ions did not interfere in the extraction of Al3+ ion. The high selectivity of the method may be due to the use GFAAS technique. The most remarkable interference was caused by ions which may form complex with the chelating agent under the experimental conditions, as well as ions which may form strong anionic complexes with Al3+ such as SO_4^{2-} and PO_4^{3-}. The other anions such as Cl^-, I^-, NO_3^-, NO_2^-, citrate and urea do not interfere significantly in the determination of Al^{3+}.

Table 2. Results of comparison between different analytical methods for determining of aluminum in biological samples.

Method	Separation	%RSD	LOD	Ref
Spectrofluorimetry	Solid-phase extraction	<10%	25 µg/L	17
Flame atomic absorption spectrometry	-	5.1%	1 µg/L	18
Spectrofluorimetry	Solvent extraction	8.7%	2.2 µg/L	19
Atomic absorption spectrometry	Ultrasonic-assisted ionic liquid-based microextraction	1.7%*	0.66 µg/L	20
GFAAS	USAEME	5.9%	0.19 ng/mL	This work
GFAAS	DLLME	4.9%	0.30 ng/mL	This work

*%RSD is calculated in drinking water

Table 3. Effect of interferent ions on the extraction of 40 ng mL^{-1} Al^{3+}.

Coexisting ions	Amount of interferent (µg mL^{-1})
Na^+, K^+, NO_3^- , I^-	40000
Zn^{2+}, Mg^{2+}, Ca^{2+}, NO_2^-	20000
Cd^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+}, NH_4^+	9000
Oxalate, urea	6000
Cu^{2+}, citrate, Hg^{2+}, Pb^{2+}	2000
PO_4^{3-}, F^-, SO_4^{2-}	1000
Fe^{3+}	400

Sample		Sample result		Spiked recovery		
	volunteer	Total Al^{3+} ng mL^{-1}		Added Al^{3+} ng mL^{-1}	Recovery(%)	
		DLLME	USAEME		DLLME	USAEME
Peritoneal dialysis urine	1	14.2±0.1	14.9±0.6	5.0	96.0±3.4	97.1±2.4
	2	12.0±0.5	12.8±0.8	5.0	97.2±4.1	95.3±4.5
	3	27.4±0.4	28.3±0.6	5.0	95.3±2.8	98.2±3.8
	4	17.6±0.3	18.2±0.5	5.0	94.2±3.2	96.3±4.9
	5	55.7±0.6	56.4±0.6	5.0	96.6±4.2	94.4±4.2
Hemodialysis urine	1	14.3±0.7	15.1±0.3	5.0	95.1±3.8	94.2±5.1
	2	19.8±0.6	18.9±0.4	5.0	94.3±4.1	95.4±3.6
	3	23.6±0.7	23.0±0.5	5.0	97.5±3.6	95.2±4.8
	4	30.0±0.9	31.0±0.4	5.0	95.4±4.2	96.5±3.9
	5	17.2±0.8	16.7±0.7	5.0	96.6±3.7	97.1±4.1

Application of USAEME and DLLME methods to dialysis pat ients urine samples

To demonstrate the applicability of the techniques, the procedure was applied for the analysis of hemodialysis and peritoneal dialysis urine samples. The sample donors signed a written consent form approved by the ethics committee of Tabriz University of Medical Sciences.

The urine from dialysis patients were collected in disposable polyethylene containers and kept at 4 ºC before analysis. In order to reduce the matrix effect; the urine sample was diluted to 1:1, using deionized water. 100 µL of HNO_3 10% was added to each sample. The precipitate was filtered and a 5 mL of an aliquot of the clear supernatant urine sample was placed in a test tube and diluted to 10 mL with deionized water diluted. The extraction was performed by general procedure described in experimental. The extract was injected into GFAAS. Then the sample was spiked with low concentrations of Al (5 ng mL^{-1}) and extracted, and injected in the usual manner. The results are shown in Table 4. As can be seen, the presence of major endogenous components, coexisting drugs and their metabolites in urine samples has no obvious influence on the determination of Al under the selected conditions and the proposed method has a good selectivity for the analysis of analytes. It should be added that using above discussed microextraction methods could act as a preconcentration method or even as a clean up technique without significant enrichment of the target analytes in the sample solution.[21] As has been discussed in a recent review article,[22] these methods could also be used in preconcentration and/or clean up of chiral analytes in biological samples.

Conclusion

Two microextraction methods were developed for the determination of Al in urine samples. Both methods are simple, rapid, relatively cheap and efficient. In addition, only very small amounts of organic solvent are necessary for analysis and the techniques are environmental friendly with a high enrichment factor. Using these methods, the interface between extraction solvent and aqueous phase is infinitely large. Therefore, the transfer of Al from the aqueous phase to

the extraction solvent is fast. Compared to the USAEME method, the DLLME technique requires simpler laboratory equipment since an ultrasonic bath is needed for the USAEME method. The DLLME method exhibits a broader linear dynamic range, better repeatability and faster extraction times. On the other hand, the organic solvent consumption for the USAEME method is less than the DLLME method because the DLLME technique requires a dispersion solvent.

Conflict of interests

The authors claim that there is no conflict of interest.

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