Original Article

A novel micro-extraction strategy for extraction of bisphosphonates from biological fluids using zirconia nanoparticles coupled with spectrofluorimetry and high performance liquid chromatography

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ABSTRACT

Extraction of bisphosphonates from biological fluids is important and time consuming step in sample preparation procedure. This paper describes a simple and green sample preparation technique for dispersive micro solid phase extraction (DMSPE) of alendronate sodium (ALS) from urine and serum samples prior to direct spectrofluorimetry (DSFL) and high performance liquid chromatography with fluorescence detection (HPLC-FLD), respectively. The DMSPE strategy is based on the selective chemisorption of ALS on zirconia nanoparticles (ZNPs) as an adsorbent followed by derivatization of the eluted analyte using o-phthalaldehyde (OPA) in the presence of 2-mercaptoethanol (2ME) at basic medium to form fluorescent species. The chemical and instrumental influencing parameters on DMSPE and measuring methods were optimized for the efficient extraction and determination of ALS. The presented methods were capable of extracting ALS from human urine and serum samples and determining over the wide ranges of 5–1000 and 5–2500 $\mu g\,L^{-1}$ with limits of detection (LOD) of 1.5 and 1.4 $\mu g\,L^{-1}$ for DSFL and HPLC methods, respectively. The relative recoveries for the three spiked standard levels of ALS in urine and serum samples ranged from 89.0% to 107.0%, and the intra-day relative standard deviations (%RSDs) were in the range of 2.9–7.9%.

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1.   Introduction

ALS (Sodium [4-amino-1-hydroxy-1-(hydroxy-oxido-phos-phoryl)-butyl] phosphonic acid trihydrate) is a widely used therapeutic drug in treatment of diseases involving calcium metabolism such as osteoporosis or Paget's disease and belongs to bisphosphonate family as the first-line treatment [1]. The chemical structure of this compound is depicted in Fig. S1. Pharmacokinetic studies of this drug have shown low oral absorption (approximately 1%) and the urinary excretion of unchanged drug as major route of elimination due to its high polar and hydrophilic nature [2,3].

Generally, for separation of bisphosphonates from biological samples suitable clean up, extraction and pre-concentration procedures are required even in chromatographic methods [4,5]. Up to our knowledge, a time consuming, long and tedious treatment process for extraction of bisphosphonates from biological samples including repeated co–precipitation reaction in several steps with calcium phosphate and consequent removal of calcium ions on various SPE cartridges, as the sole extraction technique has been frequently used in all previously reported works [1,4–10].

Extraction methodologies based on using a solid phase are the most important separation and preconcentration strategies because of their simplicity, flexibility to choose the solid phase, high pre-concentration factor, low consumption organic solvents, and low extraction time for sample preparation, especially when nanoparticles are used as adsorbent [11]. However, new miniaturized approaches such as liquid phase micro extraction (LPME) [12], micro solid phase extraction (MSPE) [13], DMSPE [14] and, solid phase micro extraction (SPME) [15] present more advantages of suitability for analysis of trace amounts of analyte, small sorbent consumption, and lower usage of toxic organic solvents.

On the basis of our experiences with the zirconia based materials [16–18] and on the literature data [19–26], they have shown strong surface complexing ability for selective adsorption of phosphate groups with fast kinetics and also high physicochemical stability against acids, alkalis, oxidants and reductants. Their non-toxicity, high thermal resistance and very low solubility in water are other superior features of these materials [27]. Using ZNPs can markedly enhance adsorption capacities of phosphate groups due to unique properties of nano-scaled materials such as high surface area-to-volume ratio and homogeneous distribution in solution [28]. Presence of two phosphate groups in ALS molecule makes it a good candidate for selectively extraction from biological matrices by ZNPs.

Various analytical methods for determination of bisphosphonates in pharmaceuticals and biological fluids have been reviewed by Zacharis et al. [29]. Generally, analysis of these compounds is problematic due to their ionic nature and lack of a suitable absorbing chromophore. Some current analytical approaches involve extensive analyte derivatization procedures and there is no doubt that derivatization of compounds which possess primary amine group in their structures with OPA is the method of choice in terms of simplicity, sensitivity, selectivity due to minimizing work, time and cost of analytical procedure [30–33], although,

instability of these isoindole derivatives is the main drawback of this method [34]. It is well known that OPA reacts with the compounds containing primary amino groups via its aldehyde groups in the presence of 2ME as a suitable nucleophilic-agent to produce 2-hydroxyethylsulfanyl-N-substituted isoindols which exhibit high fluorescence efficiency [35,36] (Fig. S2).

To date, Reversed-phase HPLC after pre-column derivatization is the most frequently employed method for ALS determination in biological materials due to its high sensitivity and excellent selectivity [4–9,37,38]. Other techniques such as capillary electrophoresis [39,40], indirect post-column fluorescence detection after ion chromatographic separation [41], fluorimetric detection method based on kinetic measurement of the fluorescence decrease of an Al^{3+}-morin complex [42], and anodic stripping voltammetry [43] have also been reported for ALS assay in biological samples.

In this work, the utility of ZNPs for selective extraction of ALS from human urine and serum samples by DMSPE method has been investigated for the first time. In addition, DSFL has been developed and validated for the determination of ALS in human urine samples. In both DSFL and HPLC-FLD techniques, pre-column derivatization of ALS was performed via its ability to form fluorescent species in the presence OPA/2ME reagent.

2.   Experimental

2.1.   Materials and solutions

ALS standard was a kind gift from Arasto chemical company-Iran. ZNPs (ZrO_2, 30–60 nm) were obtained from Inframat Advanced Materials (Farmington, CT, USA). OPA was obtained from Sigma. 2ME, tri-chloroacetic acid (TCA), acetonitrile (ACN), and borax salt ($Na_2B_4O_7.10H_2O$) were purchased from Merck. All other solvents and chemicals used were of analytical grade. A 100 mg L^{-1} stock solution of ALS was prepared from Alendronate Sodium tri-hydrate salt. This solution was kept in a dark cold place (4 °C). The working solutions were prepared daily by serial dilution. OPA/2ME reagent was prepared daily by dissolving 5 mg OPA and 25 μL 2ME (OPA/2ME molar ratio: 0.01) in 0.05 mol L^{-1} NaOH solution and diluting with the same NaOH solution to 5 mL in a volumetric flask. In case of the HPLC method, the preparation of OPA/2ME reagent was performed in borate buffer. A 0.025 mol L^{-1} borate buffer solution was prepared using borax salt and adjusting in pH 9. pH adjustments were performed using NaOH and HCl solutions. Deionized water was used throughout in the experiments.

2.2.   Instrumentation

The spectrofluorimetric measurements were performed on a Termo SCIENTIFIC – LUMINA (America) Fluorescence spectrometer. The chromatographic separations and assays were carried out using an Agilent model 1260 (Agilent Technologies, USA) HPLC equipped with fluorescence detector. The separation was performed on Eurosphere 100 C8 (Knauer) analytical column (4.6 mm × 250 mm, 5-Micro). Data collection and integration were performed with Chemstation (Agilent

Technologies) software. A pH-meter (WTW model 720, Germany) was used for pH adjustments. Solvent degassing was performed by an ultrasonic bath (Elma, Germany). All sample solutions were mixed and stirred using and A TAITEC vortex (Japan) and a Heidolph magnetic stirrer (Germany). The syringe filter (0.2 μm, Sartorius) was also used for the separation of ZNPs from the sample solution.

2.3. Extraction and determination procedure

The analytical procedure was planned in two steps: extraction of ALS using ZNPs in the first step and determination of the analyte based on formation of a fluorescent derivative with OPA/2ME using spectrofluorimetric and HPLC-FLD methods in the second step. The DMSPE procedure was carried out in a batch process. A 5 mL standard solution containing appropriate concentration of ALS solution was stirred at 250 rpm with 7 mg ZNPs for 2 min at pH 7. After filtration of the solution using syringe filter, the collected nanoparticles on filter were washed with three portion of 2 mL deionized water. In order to removal ALS from ZNPs, the syringe filter was eluted with 500 μL of 0.5 mol L^{-1} NaOH solution followed by 100 μL deionized water. For the derivatization of ALS, the obtained solution was mixed with 400 μL of OPA/2ME reagent. The fluorescence of resulting solution was immediately monitored by spectrofluorimeter and HPLC-FLD with detector set at an excitation (Ex) wavelength of 335 nm and emission (Em) wavelength of 470 nm. A blank solution was also run under the same conditions without adding the analyte. A schematic illustration of the analytical procedure is shown in Fig. 1.

2.4. Extraction and determination procedure for human urine and serum real samples

To extract the analyte from urine samples, 200μL of the spiked urine sample containing appropriate amounts of ALS, was transferred to a 5 mL volumetric flask. After addition of 7 mg ZNPs and adjusting pH at 7, the solution was diluted with deionized water to the 5 mL and stirred at 250 rpm for 2 min. The solution was then filtered using syringe filter and washed with three portion of 2 mL deionized water. Desorption of analyte from ZNPs was conducted using 500 μL of 0.5M NaOH and then 100 μL distilled water. Derivatization procedure was similar to the aforementioned method (Section 2.3). The fluorescence of the resulting solution was immediately monitored by spectrofluorimeter for quantification of ALS.

The serum samples were taken from Blood Transfusion organization (Ahvaz, Iran). 1 mL spiked serum sample was mixed with 0.4 g TCA in order to precipitate the real sample proteins. The obtained suspension was vortexed and centrifuged for 5 min at 10,000 rpm. The supernatant was separated and diluted in a 5 mL volumetric flask with deionized water. DMSPE and derivatization procedures were similar to the method described in section 2.3, except the syringe filter was eluted with 500 μL of 0.5 mol L^{-1} NaOH solution followed by 100 μL borate buffer (pH 9). From the resulting solution containing ALS derivative 50 μL was immediately injected into the HPLC.

2.5. Chromatographic conditions

An isocratic elution system was employed using a mixture of acetonitrile and water with the ratio of 10:90 (v/v) as mobile phase. The flow rate was 1.0 mL min^{-1} and the injection volume was 50 μL. The detector was set at 335 and 470 nm for Ex and Em wavelengths, respectively.

3. Results and discussion

3.1. Fluorescence emission spectrum of ALS/OPA/2ME derivative

In order to obtain optimum excitation and emission wavelengths, Em spectrum for 1 mL of 250 μg L^{-1} ALS consisting 400 μL OPA/2ME derivatization reagent was recorded in various Ex wavelengths in the range of 300–380 nm. The maximum fluorescence intensity was obtained at 335 nm (Ex) and 470 nm (Em) (Fig. S3). The selected slit width used for both Ex and Em wavelengths was 10 nm.

3.2. Optimization of the extraction conditions

The optimum conditions for pH, adsorbent amount, concentration and volume of eluent, and contact time were investigated for extraction step of the proposed method. However, the effects of time in the self-fluorescence of OPA/2ME reagent and volume of this reagent in the formation of ALS derivative
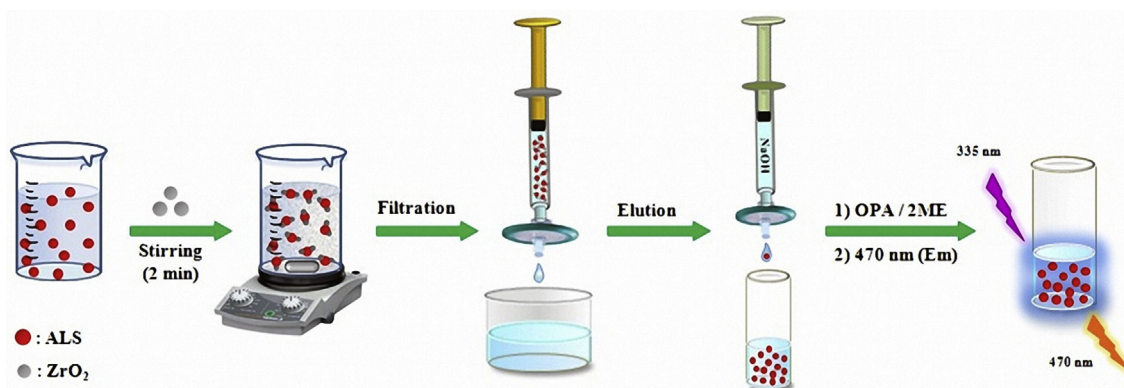

Fig. 1 – A schematic illustration of the analytical procedure.

were studied in derivatization step. Furthermore, the stability of derivatized ALS against time was investigated in order to obtain the optimum time of measurement. In all optimization procedures the fluorescence of ALS derivative was determined with the spectrofluorimeter. The obtained data were the averages of three analyses.

3.2.1. Effect of pH

The influence of pH on the adsorption of ALS on ZNPs was studied by applying the extraction and desorption procedure to the analyte solutions (100 μg L^{-1}) that their pHs were adjusted to values ranging from 1 to 10. As shown in Fig. 2a, although, the maximum adsorption of ALS on ZNPs was obtained at pH 7, in the whole range of the pH, specially, between 4 and 8 values, few changes in fluorescence intensity of the solution was observed. Also, the results showed that in both high and low pHs ALS adsorption was slightly diminished. A possible explanation for this phenomenon might be that at more acidic solution (pH < 3), although the surface of ZNPs is positively charged by protons, the phosphoric groups in ALS molecule are protonated due to its pKa_1 (2.72) and therefore, less attraction exists between ALS and positively charged surface of ZNPs. On the other hand, at higher pH values (pH > 8) and existence of plenty of hydroxide ions, the surface of ZNPs is covered by these ions and sorption of the phosphate groups of ALS containing negative charges decreases due to the their dissociation constants of ALS ($pKa_2 = 8.73$, $pKa_3 = 10.5$ and $pKa_4 = 11.6$) and strong competition between these groups and hydroxide ions. Taken together, both ion exchange mechanism (chemisorption) and electrostatic forces are responsible for interaction between ZNPs and the phosphate groups in ALS molecule [20,44,45]. The results obtained from the present research support the idea that inherent attraction between phosphate groups of ALS and ZNPs via ion exchange is a dominate process and therefore, in all pHs the adsorption of phosphate groups occurs [19]. However, at very low and high pHs this attraction and therefore, adsorption efficiency slightly decreases because of the existence of repulsive forces.

3.2.2. Effect of amount of ZNPs

To achieve the acceptable efficiency for ALS extraction, the amount of ZNPs in DMSPE procedure ranging between 1 and 50 mg was optimized. As depicted in Fig. 2b the fluorescence intensity grows up to 7 mg of the adsorbent, because the higher mass of adsorbent means that more surface area is available and more sorption occurs. However, it is somewhat surprising that in higher amounts of ZNPs the adsorption of the analyte drops significantly. The observed phenomena accords with our earlier observations and might be due to high surface area of ZNPs and the small volume of desorbing solvent [28,46].

3.2.3. Effect of contact time

The influence of contact time on sorption process was assessed by mixing the 100 μL^{-1} solution of ALS with 7 mg ZPNs at pH 7 in different time intervals ranging 2 min to 2 h. The illustrated results in Fig. 2c revealed that the adsorption rate was high and reached equilibrium in <2 min and such a rapid adsorption might be due to the chemical binding between ALS and ZNPs as discussed before [47].
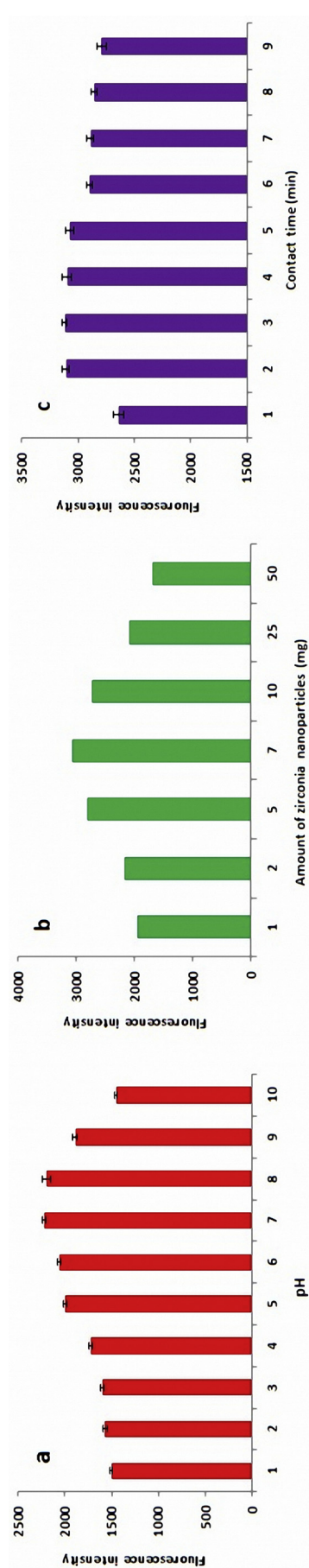

Fig. 2 – Effects of (a) pH, (b) amount of ZNPs, and (c) contact time on the adsorption of ALS on ZNPs (initial conditions: 5 mL of ALS solution, 100 μg mL^{-1}; 5 mg ZNPs; 10 min contact time).
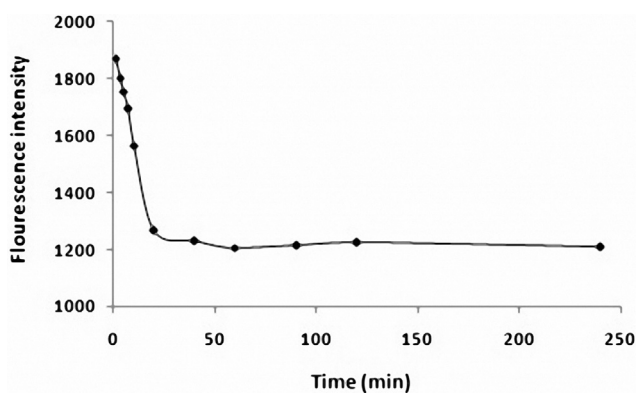
Fig. 3 — Effect of time in the self-fluorescence of OPA/2ME (conditions: 5 mg OPA; 25 μL 2ME; final volume 5 mL; borate buffer solution at pH 9 as diluent).
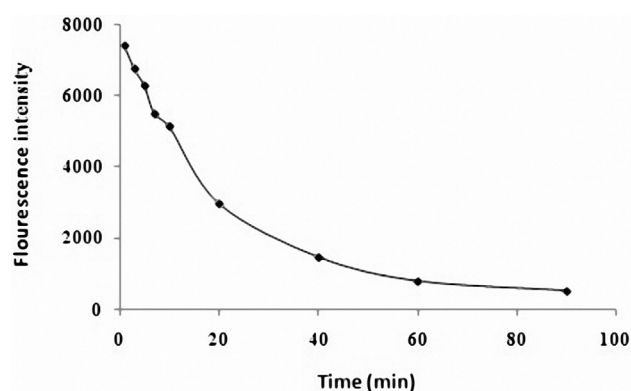

Fig. 4 — The Stability of derivatized ALS (conditions: 5 mg OPA; 25 μL 2ME; final volume 5 mL; borate buffer solution at pH 9 as diluents; 60 min incubation time in room temperature for OPA/2ME reagent; 5 mg L^{-1} ALS concentration).

3.2.4. Desorption conditions

Effect of the concentration and volume of the eluent for desorbing of ALS from the adsorbent was studied in order to recover the analyte. It was seen that in high pHs (≥9) the adsorption of ALS on ZNPs decreased (section 3.2.1). Therefore, NaOH solution was chosen as desorbing reagent to remove ALS from ZNPs. The effect of the concentration and volume of the eluent are illustrated in Figs S4 and S5. As the results show, 500 μL of 0.5M NaOH solution exhibits the best fluorescence intensity, thus these conditions were selected and used in the subsequent experiments.

3.3. Optimization of the derivatization conditions

3.3.1. Effect of time in the self-fluorescence of OPA/2ME

In contrary to some literature data, OPA/2ME reagent showed a considerable blank fluorescence value in this study [32]. In order to decrease the background emission of the derivatization reagent, the fluorescence of solution containing OPA/2ME was recorded at different time intervals ranging from 1 to 240 min. The illustrated results in Fig. 3 revealed that the emission remained almost low and constant after 60 min, and the fluorescence stability was achieved for the reagent. It was expected that more reliable data could be obtained after that time. Thus, daily prepared OPA/ME reagent was used after 60 min in all experiments. It is worthwhile to mention that the subtraction of the blank value from the fluorescence emission of the sample solution is necessary in spectrofluorimetry.

3.3.2. The stability of derivatized ALS

It has been proven that aforementioned fluorescent isoindole derivatives are somewhat unstable products [5,32,33]. They degrade via two ways: hydrolysis in aqueous solution and attack by excess OPA [33,48]. To achieve the robust method in determination of ALS and optimum time of measurement, the changes in fluorescence of OPA/2ME/ALS system showing instability of isoindole product, was followed as a function of time over the range of 0–90 min. As seen in Fig. 4, the fluorescence of the sample solution decreased continually and reached to its minimum value in 90 min. These results indicated that acceptable analytical sensitivity and precision could be obtained by careful timing of the reaction. Therefore, the time immediately after mixing of OPA/2ME with the analyte (T = 0), when the fluorescence of the analyze solution is maximum, was chosen for measuring by both spectrofluorimetry and HPLC-FLD.

3.3.3. Effect of OPA/2ME reagent volume

The volume of derivatizing reagent is a crucial parameter with an important effect on the fluorescence of sample solution. Moreover, in a microextraction method the volume of reagents is taken as small as it can be in order to achieve the highest possible enrichment factor and the least toxic effects for the environment. To investigate the effect of the volume of derivatizing reagent, experiments involving different volumes of OPA/2ME reagent (100–400 μL) were performed with the analytical procedure. The results showed that by increasing the volume of derivatizing reagent the fluorescence of analyte solution was increased rapidly (Fig. S6). As a result in higher volumes of the reagent, high analytical signals could be obtained. Thus, 400 μL was chosen as optimum volume of the reagent.

Table 1 — Analytical features for determination of ALS by the proposed methods.

Method	LOD[a]	LOQ[b]	LDR[c]	External calibration	R^{2d}	In real matrix calibration	R^2
DSFL	1.5	5.1	5–1000	y = 4.05 x + 171.43	0.9989	y = 1.00 x + 147.81	0.9967
HPLC-FLD	1.4	4.8	5–2500	y = 0.10 x + 0.56	0.9982	y = 0.07 x + 0.34	0.9991

[a] Limit of detection (μg L^{-1}).
[b] Limit of quantification (μg L^{-1}).
[c] Linear dynamic range (μg L^{-1}).
[d] Regression coefficient.

Table 2 – Relative recoveries and RSDs for ALS determination on three spiked standard levels in deionized water.

ALS	DSFL				HPLC			
Added[a]	Found[b]	Recovery[c]	RSD[d]		Found	Recovery	RSD	
			Intra-day (n = 5)	Inter-day (n = 3)			Intra-day (n = 5)	Inter-day (n = 3)
5.0	5.3	106.6	7.9	8.5	4.5	90.0	7.7	8.2
50.0	46.1	92.2	5.6	6.7	53.1	106.2	5.3	7.1
500.0	488.6	97.7	3.1	5.0	513.5	102.7	2.9	4.3

[a] Spiked concentrations of ALS ($\mu g\,L^{-1}$).
[b] Recovered concentrations of ALS ($\mu g\,L^{-1}$).
[c] Relative recovery (%).
[d] Relative standard deviations (%).

Table 3 – Determination of ALS content and recovery tests in two urine and serum samples with the proposed methods (n = 3).

Sample	Added ($\mu g\,L^{-1}$)	Found ($\mu g\,mL^{-1}$)[a]	Relative recovery (%)
Urine 1	10	8.9 ± 0.7	89.0
	100	102.5 ± 5.3	102.5
	500	496.0 ± 9.1	99.2
Urine 2	10	10.7	107.0
	100	99.4 ± 4.2	99.4
	500	510.5 ± 8.6	103.1
Serum 1	10	9.1 ± 0.5	91.0
	100	95.9 ± 8.7	95.9
	500	493.7 ± 8.3	98.7
Serum 2	10	9.4 ± 0.6	94.0
	100	92.8 ± 6.9	92.8
	500	506.5 ± 9.2	101.3

[a] $\bar{x} \pm s$ (n = 3).

3.4. Analytical performance and method validation

To evaluate and validate of the proposed methods, the analytical features of these methods such as linear range of the calibration line, LOD, LOQ, accuracy and precision were determined. Under optimum conditions for extraction and derivatization, the calibration lines were constructed by plotting the fluorescence intensities in DSFL and peak areas in HPLC-FLD methods as a function of ALS concentration in $\mu g\,L^{-1}$ at eight selected concentration levels. In both methods the calibration curves were plotted using least squares linear regression method. The external calibration curves with wide linear ranges and satisfactory correlation coefficients ($R^2 \geq 0.9967$) were observed for both methods. LOD and LOQ of DSFL method were determined based on three and ten times the standard deviation of the blank (3Sb, 10Sb), respectively. For HPLC-FLD method, LOD and LOQ values were defined as a signal-to-noise ratio (S/N) of 3 and 10 by injecting a series of standard samples with known concentration, respectively (Table 1). The fluorescence and peak area measurements of the analyte solutions containing the quality control (QC) amounts of ALS were interpolated from the calibration curve on the same day to obtain concentrations of the analyte. In order to evaluate the intra- and inter-day precisions of the developed methods which show the precision and accuracy terms, five similar experiments were performed for the QC samples at concentration levels of 5, 50 and 500 $\mu g\,L^{-1}$ on the same day and on three consecutive days. The given data in
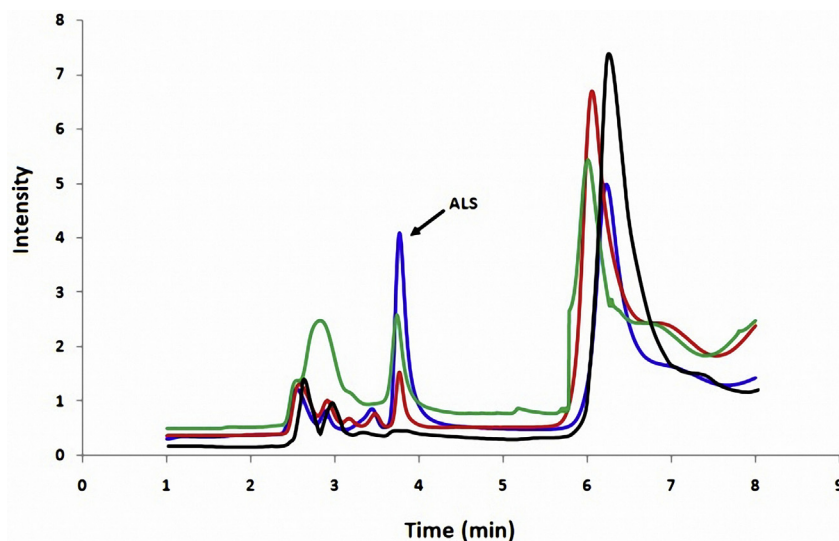

Fig. 5 – The chromatograms of the blank solution and the spiked serum samples with ALS at 50, 100, and 500 $\mu g\,L^{-1}$ concentration levels.

Table 4 – Comparison of the proposed methods with some of the methods reported in literature for determination of ALS.

Method	Sample	Linear range (mg L^{-1})	LOD (µg L^{-1})	LOQ (µg L^{-1})	RSD (%)	Ref.
HPLC-FLD	Urine	0.305–0.300	NR[a]	3.5	<8	[4]
Spect.[b]	Tablet	14–60	NR	14,000	0.37–1.6	[5]
HPLC-UV	Tablet	10–60	90	300	0.76–1.55	[5]
HPLC-FLD	Urine	0.002–0.032	0.2	0.6	1.8–8.3	[5]
HPLC-FLD	Urine	0.005–0.5	NR	NR	<15	[6]
HPLC-FLD	Human plasma	0.001–0.1	NR	1	<15	[7]
HPLC-FLD	Urine-Tablet	0.025–1.49	~6	NR	<2.95	[9]
CE[c]	Urine	2.5–250	775	2325	1.93–3.64	[39]
ISFL[d]	Human serum	0.250–1.250	NR	62.5	97.8–102.6	[42]
DPASV[e]	Human plasma-Tablet	0.096–0.288	8.6	29	1.67	[43]
DSFL	Urine	0.005–1	1.5	5.1	3.1–7.9	This work
HPLC-FLD	Human serum	0.005–2.5	1.4	4.8	2.9–7.7	This work

[a] Not reported.
[b] Spectrophotometry.
[c] Capillary electrophoresis.
[d] Indirect spectrofluorimetry.
[e] Differential pulse anodic stripping voltammetry.

Table 2 revealed that the RSD results ranged from 2.9% to 7.9% for analysis of intra-day spiked samples, indicating the suitable reproducibility of the both proposed methods. The inter-day precision results for both techniques were in acceptable range between 4.3% and 8.5%.

3.5. Real sample assay

The suggested analytical procedure was applied to analyze ALS in three urine and three serum samples. At first, to eliminate the possible matrix effects and obtain the more accurate results the matrix matched calibration curve using solutions consist of urine and serum samples fortified for all levels of the external standard curve were constructed and their analytical merits of figures were compared with that of obtained with standard solutions (Table 1). The LOQs and LDRs of the matrix matched calibration graphs in urine and serum real samples were same as their external standard curves. However, the intercepts and slopes of two standard curves for each analytical technique were slightly different. Hence, the matrix matched calibration graphs were used to obtain the accurate recovery data. To examine the reliability and accuracy of the methods, different amounts containing low, middle, and high concentrations of ALS (10, 100, and 500 µg L^{-1}) were spiked into the serum and urine samples at optimum conditions. The obtained analytical data are given in Table 3. The extraction recoveries from urine and serum real sample solutions were in the range of 89.0–107.0% showing the capability of the proposed method to ALS assay in these samples with good accuracies. The typical chromatograms of a serum samples after being spiked with ALS at 50, 100, and 500 µg L^{-1} are shown in Fig. 5.

4. Conclusions

In conclusion, a novel DMSPE methodology has been introduced for extraction of ALS from human serum and urine samples using ZNPs which allows the development of simple, rapid, low cost and eco-friendly procedure in comparison to laborious conventional method. Up to our knowledge, this is the first time ZNPs was applied as solid phase for extraction of bisphosphonates from biological matrices. In the presented method the chemisorption reaction reaches equilibrium in short time which has the advantage of saving time. ZNPs showed a strong affinity toward the phosphate groups on ALS molecule, which provided selectivity and sensitivity of the method. Besides, to the best of our knowledge, DSFL of bisphosphonates after derivatization has not been reported yet for biological materials. Combination of DMSPE using ZNPs with DSFL and HPLC-FLD techniques for determination of ALS in urine and serum samples has merits of simplicity, selectivity and sensitivity. A comparison between these methods and the other reported works in determination of ALS is presented in Table 4. As can be seen, the characteristics of the presented methods are better or comparable to some of the previously reported studies. Finally, these extraction/determination methodologies might be used for the other members of NH$_2$-containing bisphosphonates in various biological matrices.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jfda.2018.03.005.

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