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CONDUCTOMETRY, SPECTROPHOTOMETRY AND MASS SPECTROMETRIC INVESTIGATION OF Mg(II) AND Ca(II) COMPLEXES WITH AN ANTIRETROVIRAL DRUG, ZIDOVUDINE

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ABSTRACT. Metal ions play a key role in living systems and are used to perform different physiological activities in our body. Complexation of drugs with metal ions is known to influence the bioavailability and other pharmacokinetic properties of drugs. In the present work, complexation of two essential cations, Mg^{2+} and Ca^{2+} with zidovudine (AZT), the first approved antiretroviral drug for human immunodeficiency virus (HIV) has been studied by conductometry and spectrophotometry. The plots of molar conductance versus the mole ratio of [AZT]/[M^{2+}], Job's method of continuous variation and mole ratio method showed the stoichiometry to be 1:1 (M^{2+}:AZT) for both the cations. The metal ion-AZT complexes formed through the nitrogen of the azide group were further ascertained by liquid chromatography-mass spectrometric analysis. The formation constants of the complexes as evaluated by the conductance method and the Rose-Drago method were in good agreement. The values of thermodynamic parameters (ΔH and ΔS) for the formation of complexes were obtained from the Van't Hoff plots. The results revealed that both the complexation reactions were spontaneous, endothermic and entropy stabilized.

KEY WORDS: Zidovudine, Conductometry, Spectrophotometry, Liquid chromatography-mass spectrometry, Formation constant, Thermodynamic parameters

INTRODUCTION

The complexation study of biologically important medicinal agents or drug molecules with different metal ions found in body fluids has become an important area of research in drug design and development. In almost all biological processes and for operating normal physiological activities, the response of metal ions (either as isolated ions or in clusters) ranges from deficiency to toxicity [1]. Electron rich drug molecules combine with metal ions to give "metal-based drugs" or "elemental medicines", having unique therapeutic applications [2-4]. Metal ions perform range of functions such as iron containing protein; the haemoglobin binds with the oxygen to carry this important molecule to body tissues [2]. Zinc finger, a small structural motif can act as Zn^{2+} sensors to regulate transcriptional activation domain function [5]. Calcium is an integral part of bones and other metal ions such as copper, iron, manganese and zinc are part of metalloenzymes which perform diverse chemical reactions [6]. Thus, the biological activity of drug-metal ion binding can affect the stability of the drug and its functions in the body by changing its bioavailability, pharmacokinetic and pharmacodynamics [7].

Human immunodeficiency virus (HIV), the pathogenic retrovirus responsible for the acquired immunodeficiency syndrome (AIDS) causes symptoms and infections ensuing from the specific damage to the immune system [8]. In order to treat retroviruses or to maintain HIV at a low level in the body, antiretroviral drugs are used which belong to the following classes: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase

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inhibitors (NNRTIs), protease inhibitors (PIs), entry inhibitors and integrase inhibitors [9]. Zidovudine (azidothymidine, AZT) is the first clinically successful NRTI, which acts as chain terminator of viral DNA [10]. In AZT, the azido group is responsible for its antiviral action [11]. In the highly active antiretroviral therapy (HAART), AZT is combined with other drugs belonging to the class of NRTIs, NNRTIs and PIs in order to avert the transformation of HIV into an AZT-resistant form [12, 13]. Unfortunately, there are some adverse effects associated with AZT therapy such as bone marrow suppression, nausea, myalgia, insomnia and haematological toxicity which results in macrocytic anemia and granulocytopenia [10, 14]. To reduce the burden of associated adverse effects of AZT, use of magnesium supplements has been shown to offer protective effects against AZT-induced oxidative stress, cardiac pathology, systemic inflammation [15], oxidative vascular pathogenesis and nephrotoxicity in rats [16, 17] and also attenuated high cholesterol-induced atherogenesis in a rabbit model [18]. Similarly, bone alterations have been observed in HIV-infected patients undergoing HAART therapy, in which bone lesion or bone demineralization takes place due to lack of calcium [19, 20]. This suggests that increasing Ca intake during treatment may slow down the process of bone loss [21]. Thus, it is essential to investigate the effect of metal ions on the therapeutic efficacy and the mode of action of antiretroviral drugs.

Based on the available literature and outcomes thereof, it can be effectively surmised that the interaction of AZT with essential metal ions has shown potential biological activity than uncomplexed drug. Thus, such interactions can contribute in reducing the magnitude and impact of this dreaded disease worldwide. An extensive literature survey revealed very few studies on the interaction/binding of metal ions with AZT. Complexation of AZT with Fe^{2+} cation has been studied by polarography and amperometry [22]. Physicochemical and microbial studies have been reported for Ni^{2+}-AZT complex in solid and aqueous phase [23]. Singh *et al*. [24] have presented a combined experimental and theoretical, molecular and vibrational spectroscopic study of Fe^{3+}-AZT complex. In our previous report we had evaluated the interactions of AZT with Fe^{3+}, Co^{2+}, Cu^{2+} and Zn^{2+} cations in methanol by conductometry and spectrophotometry [25]. As part of our on-going research into drug-metal ion interaction study, in the present work we report Mg^{2+} and Ca^{2+} binding with AZT using two simple and versatile techniques like spectrophotometry and conductometry [26]. The stoichiometry of the complexes were determined from the molar conductance-mole ratio plots, Job's method of continuous variation, mole ratio method and confirmed by liquid chromatography-mass spectrometric (LC-MS) analysis. Formation constants of the complexes were evaluated by conductometry and three spectrophotometric methods. The thermodynamic parameters of complexation were also estimated to gain more insight into the nature and strength of binding.

EXPERIMENTAL

Materials and solutions

The reference standard of zidovudine (99.33%) was purchased from Clearsynth Labs Pvt. Ltd (Mumbai, India). Analytical grade nitrate salts of metal ions, $Mg(NO_3)_2 \cdot 6H_2O$ and $Ca(NO_3)_2 \cdot 4H_2O$ were procured from Central Drug House (P) Ltd. (New Delhi, India) with a purity ≥ 99.6%. HPLC grade methanol and acetonitrile were obtained from Mallinckrodt Baker, SA de CV (Mexico State, Mexico). Analytical grade acetic acid was purchased from Merck Specialties India Ltd. (Mumbai, India). Water was prepared using a water purification system from Millipore Milli-Q (Bangalore, India). All standard stock solutions of AZT and metal ions were prepared by accurately weighing known amounts of the compounds in methanol having conductivity less than 1.0×10^{-7} S cm^{-1}.

Instrumental measurements and conditions

The conductance measurements were carried out using 856 Conductivity Module with touch control from METROHM AG (Herisau, Switzerland). A dip-type conductivity cell with a cell constant of 0.59 cm^{-1} was used. The cell constant of the conductivity cell was determined by measuring the conductivity of aqueous potassium chloride solutions of different concentrations [27]. The titration was carried out in a thermostatically controlled water bath maintained at the desired temperature, with an accuracy of ± 0.01 ^{o}C. Shimadzu UV-1700 double beam spectrophotometer (Kyoto, Japan) with matched 10 mm quartz cells was used for spectrophotometric measurements. The wavelength accuracy was kept within ± 0.5 nm and a bandwidth of 1 nm was used. The spectra were recorded with a scan speed of 400 nm min^{-1}. Shimadzu UV PC software version 2.0 was used for data processing. Sartorius GD503 (Bradford, MA, USA) analytical balance having a readability of 0.0001 g was employed for weighing of samples.

Chromatographic analysis of M^{2+}-AZT complexes was carried out on Shimadzu LC-VP HPLC (Kyoto, Japan) using ACE C18 (100 × 4.6 mm, 5 µ) analytical column (Advanced Chromatography Technologies, Aberdeen, Scotland), maintained at 35 ^{o}C in a column oven. A mobile phase consisting of acetonitrile: 0.5% (v/v) acetic acid in water (80:20, v/v) was delivered at a flow rate of 1.0 mL min^{-1} under isocratic conditions. The autosampler temperature was maintained at 5 ^{o}C and the pressure of the system was kept at 1200 psi. Mass spectral analysis was performed on a triple quadrupole mass spectrometer MDS SCIEX API-2000 (Toronto, Canada), equipped with electro spray ionization operating in the negative ionization mode. The optimized parameters set were, turbo heater temperature: 450 ^{o}C; ion spray voltage: 4500 V; entrance potential: -8 V; declustering potential: -27 V; curtain gas pressure: 32 psig; Gas 1 (nebulizer gas): 35 psig; and dwell time: 200 ms. Analyst classic software version 1.5.1 was used to control all parameters of HPLC and MS.

General procedure for conductometry, spectrophotometry and LC-MS analysis

In order to determine the formation constants (log K_f), 25 mL (2.0×10^{-4} mol L^{-1}) solutions of metal nitrates in methanol were placed in specially designed water jacketed titration cell. To maintain a constant temperature it was connected to a thermostated circulator water bath and the conductance of the solution was measured. A 2.0×10^{-3} mol L^{-1} solution of AZT was added in a stepwise manner using a pre-calibrated micro-burette and the conductance of the solution was measured after each addition and stirring the mixture at the desired temperature. Addition of the AZT solution was continued until its concentration was four times that of the metal ion. The same procedure was followed at 25, 35 and 45 ^{o}C, and the conductivity data was used for the calculation of the formation constant of the complexes at the desired temperatures. The conductivity data was analyzed by a non linear regression analysis using IBM® Software SPSS Statistics 20 from IBM Corporation (USA) for 1:1 complex formation.

Molar ratio method [28] and Job's method of continuous variation [29] were used to determine the stoichiometry of metal ion-AZT complexes. For mole ratio method, a series of methanolic solutions containing fixed amounts of cations (2.0×10^{-4} mol L^{-1}) and varying concentration of AZT were prepared. For Job's method, the absorbance of series of solutions containing metal ions (2.0×10^{-4} mol L^{-1}) and AZT (2.0×10^{-4} mol L^{-1}) in different mole fractions but constant total concentration was measured. Prior to spectrophotometric measurement all the solutions were equilibrated at 25 ^{o}C at their respective wavelength maxima.

For LC-MS analysis, standard solutions of AZT and metal ions (2×10^{-4} mol L^{-1} each) in methanol were prepared and mixed in 1:1, 1:2 and 1:3 (M:AZT) stoichiometry amounts. Aliquots of 10 µL were then injected into the chromatographic system and the mass spectra were recorded from *m/z* 50-400.

RESULTS AND DISCUSSION

Conductometric study

Conductometry is a simple and an inexpensive tool for precise measurement of conductivity of solutions. It gives a useful measure of stabilities of the resulting complexes and also the stoichiometry [26]. In the present work, the interaction/binding between Mg^{2+}, Ca^{2+} cations and AZT was studied by measuring the changes in molar conductance and the accompanying changes in the thermodynamic parameters like free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) in methanol. The changes in molar conductance (Λ_M) versus [AZT]/[M^{2+}] were recorded at three different temperatures as shown in Figure 1. As evident from these figures that there is a constant decrease in the molar conductance of the solutions for both the cations. This clearly indicates that the complexes formed were less mobile compared to the free solvated cations. Further, the corresponding slopes of these plots change at a point where the [AZT]/[M^{2+}] mole ratio is 1, indicating formation of 1:1 stoichiometry of the complexes. Additionally, the Λ_M for the complexation reactions increases with increase in temperature but with no apparent change in the curvature of the plots. This can be related to the fact that at higher temperature, the complexed species [M^{2+}-AZT] were even less solvated and thus Λ_M increases in methanol.

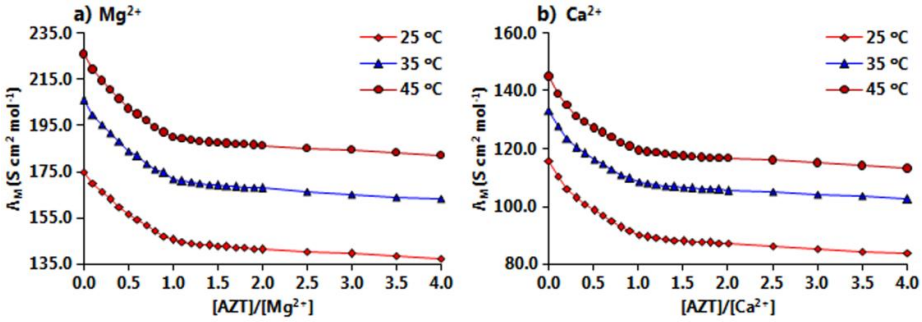

Figure 1. Molar conductance vs. mole ratio [AZT]/[M^{2+}] plots for (a) Mg^{2+} and (b) Ca^{2+} in methanol at 25 ºC, 35 ºC and 45 °C.

The formation constant values for both the cations were evaluated using a nonlinear least-squares curve fitting program from the corresponding molar conductance vs. mole ratio plots at three different temperatures using the following expression

$$K_{M-AZT} = \frac{\Lambda_{M^{2+}} - \Lambda}{\Lambda - \Lambda_{M-AZT}[AZT]} \quad (1)$$

where $\Lambda_{M^{2+}}$, Λ_{M-AZT} and Λ represent the molar conductivity of the metal nitrate salt solution before the addition of AZT, the complexed species and that observed during the titration, respectively. The formation constant values for both the cations at different temperatures are shown in Table 1.

Table 1. Formation constants for M^{2+}-AZT complexes at different temperatures by conductometry.

Temperature (^oC)	Log K_f ± SD (n = 3)	
	Mg^{2+} (0.72 Å)	Ca^{2+} (1.00 Å)
15	3.35 ± 0.02	2.32 ± 0.02
25	3.37 ± 0.03	2.33 ± 0.02
35	3.43 ± 0.03	2.39 ± 0.02
45	3.46 ± 0.03	2.41 ± 0.04

Values in parenthesis are ionic radii.

It is evident from the results that the stability of Mg^{2+}-AZT is greater compared to Ca^{2+}-AZT complex. The probable structure of the complex is illustrated in Figure 2. Das and Pitre [22] have shown that the complexation takes place through the N-atom of the azide group based on IR spectral data of Fe^{2+}-AZT complex. The influence of temperature on log K_f values implies that the stability of the complexes increases with increase in temperature.

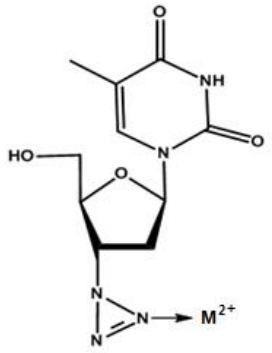

Figure 2. Probable structure of zidovudine-Mg^{2+}/Ca^{2+} complexes in methanol.

In order to have a better understanding of the thermodynamic behavior of M^{2+}-AZT complexes, it is useful to consider the contribution of enthalpy (ΔH) and entropy (ΔS) to these reactions in methanol. The thermodynamic data for both the cations are summarized in Table 2.

Table 2. Gibbs free energy, enthalpy and entropy for M^{2+}-AZT complexes in methanol by conductometry (n = 3).

Thermodynamic parameters at 25 ^oC	Cation	
	Mg^{2+}	Ca^{2+}
ΔG ± SD (kJ mol^{-1})	-19.2 ± 0.16	-13.3 ± 0.11
ΔH ± SD (kJ mol^{-1})	8.73 ± 0.26	6.73 ± 0.30
ΔS ± SD (J mol^{-1} K^{-1})	93.9 ± 1.33	67.3 ± 2.6

The negative value for free energy (ΔG) indicates that the complexation process is spontaneous. The values for thermodynamic quantities ΔH and ΔS were evaluated from the corresponding ln K_f vs temperature plots by applying a linear least square analysis according to the Van't Hoff equation [30] as shown below,

$$2.303\log K_f = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (2)$$

The plots of ln K_f vs. 1000/T for both the complexes were linear as evident from Figure 3. The values of ΔH and ΔS were computed from the slopes and intercepts of these plots. The thermodynamic data given in Table 2 shows that both the cation complexes are enthalpy destabilized and entropy stabilized. However, the unfavorable contribution of enthalpy was adequately compensated by the magnitude of TΔS values, which favors the process of complexation.

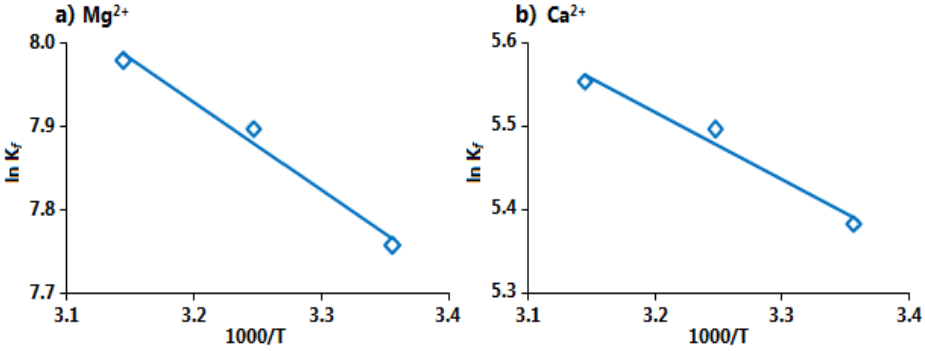

Figure 3. Van't Hoff's plot of ln K_f vs. 1000/T for the metal complexes with zidovudine (a) Mg^{2+} and (b) Ca^{2+} in methanol.

Spectrophotometric measurements

The absorption spectra of AZT and its Mg^{2+} and Ca^{2+} complexes in methanol are shown in Figure 4. A wavelength shift of 12 nm for Mg^{2+}-AZT complex (λ_{max} 221 nm) and 7 nm for Ca^{2+}-AZT complex (λ_{max} 216 nm) was observed from the wavelength maxima of AZT (λ_{max} 209 nm). The mole ratio plots of absorbance vs. [AZT]/[M^{2+}] at respective wavelength maxima are presented in Figure 5.

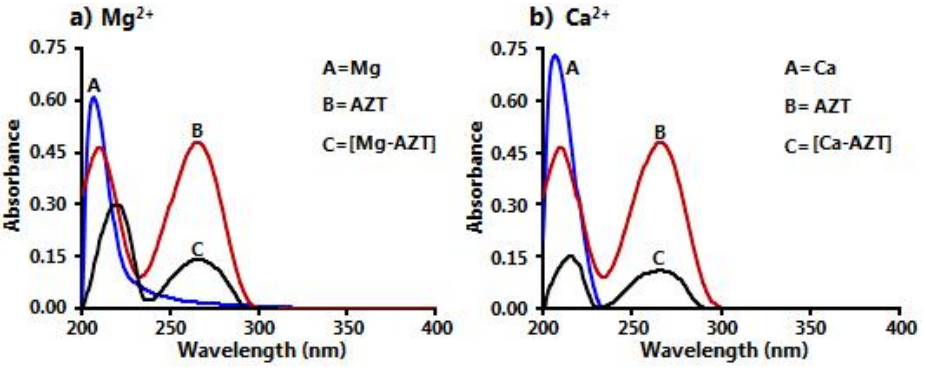

Figure 4. Absorption spectra of (a) Mg^{2+} salt solution, zidovudine and their complex, (b) Ca^{2+} salt solution, zidovudine and their complex in methanol.

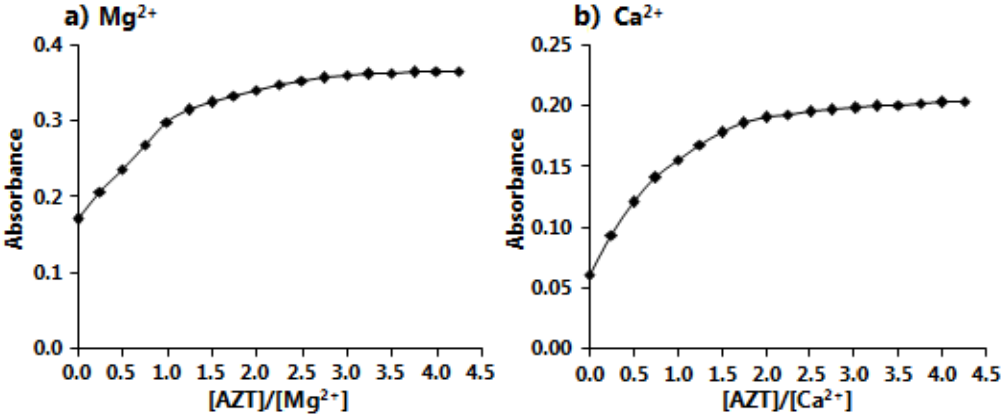

Figure 5. Mole ratio [AZT]/[M^{2+}] plots of (a) Mg^{2+} and (b) Ca^{2+} in methanol at wavelength maxima of 221 nm (Mg^{2+}-AZT complex) and 216 nm (Ca^{2+}-AZT complex), respectively.

It can be observed that initially (in the absence of AZT) there is some absorbance due to the metal ion solutions as they have wavelength maxima (Mg^{2+}: λ_{max} 205 nm; Ca^{2+}: λ_{max} 206 nm) close to their complexed species. Further, as the [AZT]/[M^{2+}] ratio increases, there is an increase in absorbance until this ratio reaches 1:1. Beyond this point the absorbance starts to level off for both the cations. This indicates that the stoichiometry of the complexes formed is 1:1 (M^{2+}:AZT). These findings were further confirmed using Job's method of continuous variations. The change in absorbance at the corresponding wavelengths was recorded for a set of solutions in which the total concentration of AZT plus cation was constant while their individual molar concentration varied from one solution to another. Further, as AZT has a wavelength maxima (209 nm) close to that of Mg^{2+}-AZT complex (λ_{max} 221 nm) and Ca^{2+}-AZT complex (λ_{max} 216 nm) there was some contribution towards absorbance even at zero concentration of the cations. Figure 6 shows that the change in absorbance maxima at AZT mole fraction of around 0.50. This confirms formation of relatively stable metal ion-AZT complexes with 1:1 stoichiometry.

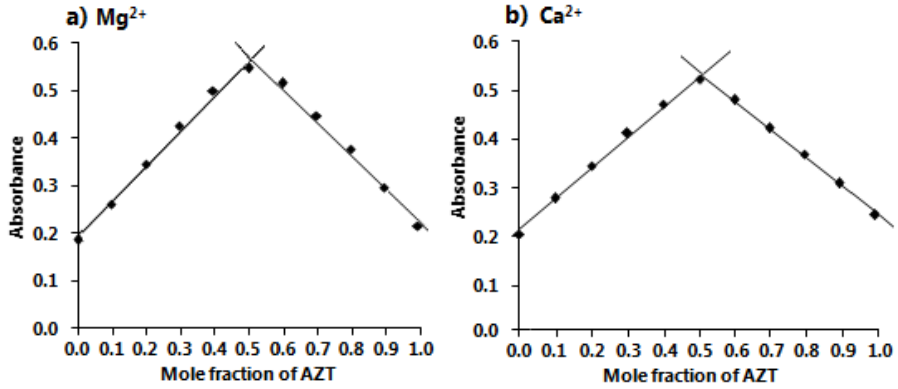

Figure 6. Job's plots for the complexation of zidovudine with (a) Mg^{2+} and (b) Ca^{2+} cations in methanol at wavelength maxima of 221 nm and 216 nm, respectively.

The formation constants (K_f) values for M^{2+}-AZT complexes were determined using the Rose-Drago method for UV-Visible spectrophotometric measurements as described by Hirose [30], employing equation 3,

$$\frac{1}{K} = \frac{A_{obs} - \epsilon_M \cdot [M]_t - \epsilon_{AZT} \cdot [AZT]_t}{\epsilon_c - \epsilon_M - \epsilon_{AZT}} - ([M]_t + [AZT]_t) + \frac{\epsilon_c - \epsilon_M - \epsilon_{AZT}}{A_{obs} - \epsilon_M \cdot [M]_t - \epsilon_{AZT} \cdot [AZT]_t} \cdot [M]_t \cdot [AZT]_t \quad (3)$$

where, ϵ_c, ϵ_M and ϵ_{AZT} are the molar absorptivity of the complex, metal ion and AZT respectively; A_{obs} is the absorbance value for the solution of standard mixtures containing $[M]_t$ and $[AZT]_t$ (total concentration of the cation and the drug respectively). This equation is widely used as the only assumption for this method is that there can be at the most only two absorbing species which obey the Beer's Law.

For comparison purpose, the formation constant of the complexes was also evaluated by two other methods, the Job's method and mole ratio method, as studied previously for drug-metal ion complexation [31, 32]. For Job's method the following equation was used,

$$K_{AZT} = \frac{[A_2/A_1]}{[1 - A_2/A_1] \times [C_{AZT} - C_M \times A_2/A_1]} \quad (4)$$

where, A_1 = absorbance at break point, A_2 = actual absorbance, C_{AZT} = concentration of AZT, C_M = concentration of metal ions (Mg^{2+}/Ca^{2+}).

The equation used for the mole ratio method for 1:1 complexation is as follows,

$$K_{AZT} = \frac{[A/\varepsilon b]}{[C_M - A/\varepsilon_\lambda b] \times [C_{AZT} - A/\varepsilon b]} \quad (5)$$

where; ε_λ = molar absorptivity, b = path length and A = absorbance at peak point.

The mean values of formation constants (average of three determinations) for both the cations obtained from Rose-Drago method, Job's method and mole ratio method were in close agreement (Table 3). Further, the results obtained by conductometric titration are also comparable with the spectrophotometric methods. In all the methods, the stability of [Mg^{2+}-AZT] was higher compared to [Ca^{2+}-AZT] complex. Perhaps this could be the reason that Ca^{2+} cation is not preferred for concomitant use as a supplement with NRTIs and is mainly prescribed with PIs, primarily to reduce PIs induced diarrhea [33].

Table 3. Formation constants for M^{2+}-AZT complexes at 25 °C by spectrophotometry and conductometry.

Cation	λ_{max}	Log K_f ± SD (n = 3) at 25 °C			
		Spectrophotometry			Conductometry
		Rose-Drago method	Job's method	Mole ratio method	
Mg^{2+}	221.0	3.39 ± 0.01	3.38 ± 0.02	3.37 ± 0.05	3.37 ± 0.03
Ca^{2+}	215.0	2.35 ± 0.03	2.32 ± 0.03	2.36 ± 0.03	2.33 ± 0.02

LC-MS analysis

LC-MS analysis of metal ion-drug complexes showed clear evidence of 1:1 (M:AZT) stoichiometry. Figure 7(a-c) presents Q1 full scan mass spectra in the range of *m/z* 50-400 amu for AZT and its complexes with Mg^{2+} and Ca^{2+} cations in the negative ionization mode. Figure

7a shows the deprotonated precursor ion at *m/z* 266.0 for AZT and its major product or daughter ion at *m/z* 126.0, which corresponds to the fragment obtained after elimination of 4-azido-5-(hydroxymethyl)oxolan-2-yl moiety from the pyrimidine base structure. In Figure 7b and c the precursor ion peaks can be attributed to Mg^{2+}-AZT and Ca^{2+}-AZT complex at *m/z* 290.3 and *m/z* 306.7, respectively. In addition, the peaks corresponding to deprotonated AZT and its major product ion can also be observed in these figures albeit with much reduced intensities.

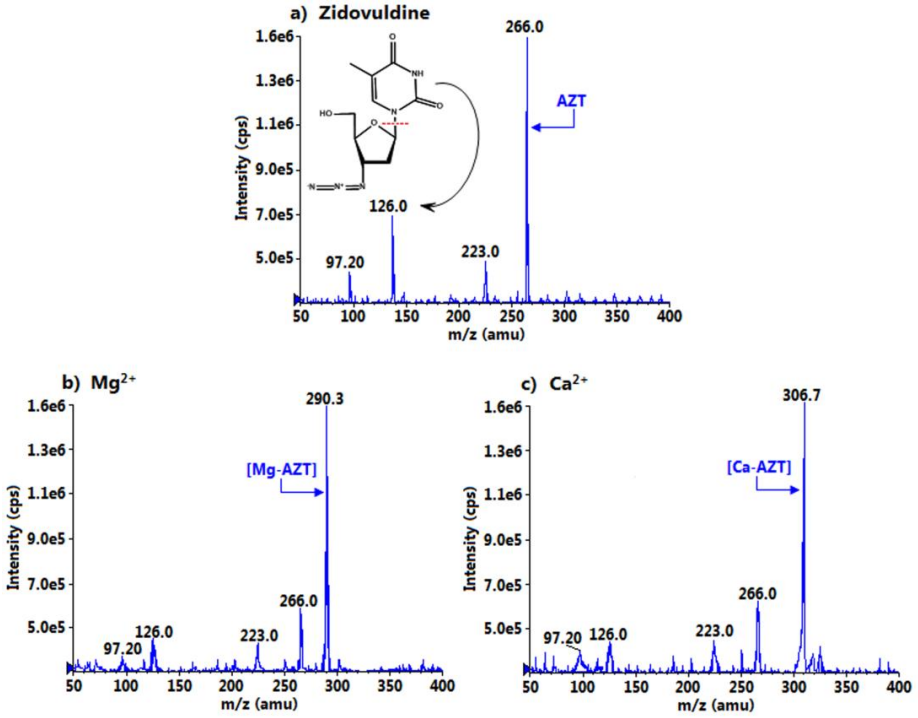

Figure 7. Q1 mass spectra of (a) zidovudine and its complexes with (b) Mg^{2+} and (c) Ca^{2+} cations.

CONCLUSION

In the present work we have successfully demonstrated the binding ability of AZT with Mg^{2+} and Ca^{2+} cations by conductometry and spectrophotometric techniques. The study revealed that [Mg^{2+}-GMP] complex has higher stability compared to [Ca^{2+}-GMP] in methanol. Further, the 1:1 stoichiometry of the complexes formed is confirmed by LC-MS analysis in addition to Job's method and mole ratio method. The thermodynamic data showed that the process of complexation was endothermic and that the cation complexes were enthalpy destabilized and entropy stabilized. However, the higher magnitude of $T\Delta S$ values compared to ΔH showed an enthalpy-entropy compensation effect which favoured complex formation. Finally, such studies may be useful to provide further insight into the role and impact of metal ions in the field of medical science.

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