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Stability-indicating micellar liquid chromatography method for three novel derivatives of zidovudine in aqueous and simulated gastric and intestinal fluids matrices

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ABSTRACT

This work studies the stability of three new anti-HIV agents which were obtained by the association of zidovudine (AZT) with different amino acids, such as leucine (AZT-Leu) and valine (AZT-Val), and one with an acid group (AZT-Ac). Before commercialisation, their stability in different matrices – simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 6.8), both as the USP 32 Guideline indicates, and buffers (pH 1.2 and 6.8) – must be studied. To this end, a new stability-indicating micellar liquid chromatography (MLC) method has been optimised and validated. Measurements were based on the disappearance of reagents and the appearance of the only degradation product (AZT). This optimised and validated method used a C18 column and a mobile phase containing 0.05 M sodium dodecyl sulphate–1% (v/v)1-butanol–0.01 M NaH_2PO_4 (pH 3.0) at 30 °C, and a flow rate of 1 mL min^{-1}. Under these conditions, retention times were 1.4, 3.6, 6.3 and 9.5 min for AZT-Ac, AZT, AZT-Val and AZT-Leu, respectively. Calibrations better than 0.9995, intra- and inter-day precisions below 1.08% and good recoveries (94.47–116.52%) and robustness (RSD less that 1.08%) were obtained and were adequate to analyse the four compounds. Finally, this MLC method was applied to achieve stability studies which resulted in the evidence that all the compounds followed a pseudo-first-order kinetics, and in the determination of their kinetic constants and half-life time. A reference method, applied in the same studies, validated the MLC method reported herein.

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1. Introduction

In the last few years, biological active drugs molecules discovery has undergone important changes by employing appropriate analytical technologies and strategies, thus providing more opportunities for acquiring and integrating information to enhance discovery and success, and efficiency in developing novel chemical entities with biological activity. Generally, physicochemical properties can be used as predictors of ADMET (absorption, distribution, metabolism, excretion and toxicity) characteristics, and to reduce time, expense and the use of animals [1,2]. Earlier stability screening provides important information about possible structural modifications to improve drug molecules discovery.

The Food and Drug Administration (FDA) Guidance for Industry [3] concerning *in vivo* bioavailability and bioequivalence studies for immediate release solid oral dosage forms includes a discussion on gastrointestinal stability, along with an appropriate methodology to help classify a drug based on its intrinsic solubility, intestinal permeability and drug product dissolution. The stability of a drug substance in gastric and intestinal fluids evidences whether drug loss from the gastrointestinal tract takes place by intestinal permeation or by a degradation process in the gastrointestinal fluids prior to membrane absorption. Stability in the gastrointestinal tract may be confirmed by incubating the drug substance in gastric and intestinal fluids that are representative of *in vivo* drug exposure to these fluids; e.g., 1 h in simulated gastric fluids (SGF) and 3 h in simulated intestinal fluids (SIF). Significant degradation (>5%) of a drug assessed in this manner could suggest potential instability in the gastrointestinal tract [3]. A validated stability-indicating assay is then utilised to measure drug concentrations.

The rapid worldwide spread of acquired immunodeficiency syndrome (AIDS) has prompted intense research efforts to discover compounds that effectively inhibit the human immunodeficiency virus (HIV-1) [4–10], the aetiologic agent of AIDS [11]. Despite worldwide attempts underway to develop chemotherapeutic agents that are effective against HIV, 3′-azido-2′,3′-dideoxythymidine, or zidovudine (AZT) (Fig. 1), the first drug approved for the treatment of AIDS patients [12], is still one of

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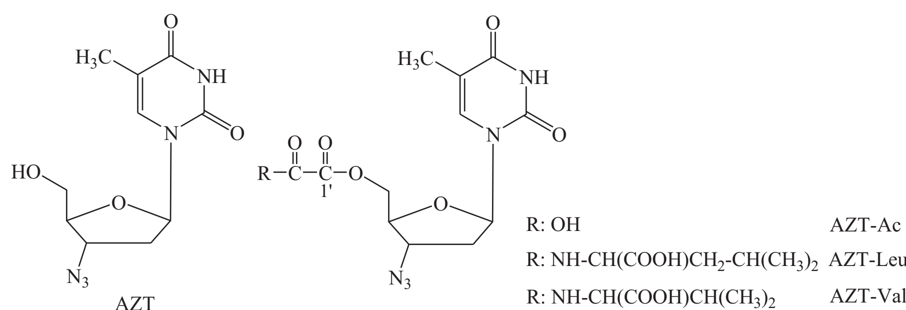

Fig. 1. Chemical structure of zidovudine (AZT) and its derivatives AZT-Ac, AZT-Leu and AZT-Val.

the most potent active agents against HIV, and is used as a primary option in AIDS treatment in combination with other HIV inhibitors. Nevertheless, utility of AZT is limited by its toxic effect on bone marrow [13], hepatic abnormalities [14], limited brain uptake [15], short half-life time in plasma [16], high susceptibility to catabolism [17], general myopathy [18], lipoatrophy [19] and the rapid progress of resistance by HIV-1 [20]. For these reasons, numerous chemical strategies have been developed by medicinal scientists to design analogues or prodrugs of AZT to increase its therapeutic efficacy [21]. Most of these have been prepared by derivatising the 5′-OH of AZT, whose mechanism of action is based on the hydrolysis and/or the enzymatic cleavage of the 5′-*O*-bonds between the drug (AZT) and its attached moiety [22].

As part of our continuing efforts to discover novel and effective antiviral agents, we have reported the synthesis and antiviral activity of novel AZT analogues at the 5′-position [23–25]. In addition, these derivatives have demonstrated bactericide effects against bacteria which can produce opportunistic infections in AIDS patients [23]. Amino acids have been employed to improve the physicochemical properties of different compounds, such as aqueous solubility [26] and intestinal permeation [27]. Among them, those associated with an acid (AZT-Ac), or with the leucine (AZT-Leu) and valine (AZT-Val) groups, have been selected because of not only their biological activity [23,25], but also their physicochemical [24] and pharmacokinetic [28–30] properties (Fig. 1). It is important to point out that AZT is the parent compound of these derivatives.

New compounds like these three novel AZT derivatives need to pass certain trials before their commercialisation, one of which is the evaluation of their stability behaviour in the gastrointestinal tract. Depending on the results, the new pharmaceutical can be formulated as an oral administration form, or it is necessary to look for alternative dosages. In addition, and according to the Guidance for Industry [3], it is necessary to employ an appropriate analytical method to correctly evaluate the intact drug molecules in the presence of their degradation products. Micellar liquid chromatography (MLC) is an alternative method to traditional high performance liquid chromatography (HPLC) [31,32], where the mobile phase is composed of a surfactant at a higher concentration than the critical micellar concentration. An organic modifier, such as 1-propanol, 1-butanol or 1-pentanol, which lower retention times and improve efficiency is usually added to the mobile phase. One of the main advantages of MLC is the possibility of quantifying drugs molecules in complex matrices without a previous extraction process [31]. In addition, MLC mobile phases are non-toxic, not flammable, biodegradable and relatively inexpensive in comparison to HPLC solvents [31,32].

Thus, this work aimed to evaluate the stability behaviour of the AZT derivatives AZT-Ac, AZT-Leu and AZT-Val, firstly in aqueous matrices and later in SGF and SIF using MLC, and then to compare the results with HPLC to establish our MLC method as a stability-indicating HPLC method.

2. Experimental

2.1. Chemicals and reagents

AZT was generously provided by Filaxis (Buenos Aires, Argentina). AZT-Ac, AZT-Leu and AZT-Val were synthesised as previously reported [23]. Monobasic potassium phosphate, sodium dihydrogen phosphate, sodium hydroxide and phosphoric acid were purchased from Anedra (San Fernando, Argentina). Sodium dodecyl sulphate (SDS) was purchased from Biopack (Zárate, Argentina). Sodium chloride was obtained from Baker Co. (New Orleans, USA). Porcine pepsin and pancreatin enzymes were American Chemical Society (ACS) reagent grade and were bought from Sigma (St. Louis, MO, USA). Dimethylsulphoxide (DMSO), hydrochloric acid, 1-butanol, all of analytical grade, were purchased from Cicarelli (San Lorenzo, Argentina). Methanol (MeOH) and tetrahydrofurane (THF) both HPLC grade, were acquired from Sintorgan (Villa Martelli, Argentina). The water used in the HPLC and MLC analyses and in all the studies was of Milli-Q grade (Millipore®), and solutions and mobile phases were filtered through Millipore filters Type FH (4.5 μm) (Millipore S.A.S., Molsheim, France) and degassed under vacuum. pH was measured using a Crison GLP 21 pHmeter (Modena, Italy).

2.2. Preparation of the SGF and SIF solutions

The SGF was prepared according to USP specifications [33]; 2.0 g of sodium chloride and 3.2 g of pepsin (obtained from porcine stomach mucosa) were dissolved in 7.0 mL of hydrochloric acid (37%) and then diluted to 1000 mL. The pH of SGF was approximately 1.2.

On the other hand, the SIF was also prepared according to the USP guide; 6.8 g of monobasic potassium phosphate were dissolved in 250 mL water, and then 77 mL of 0.2 M sodium hydroxide and 500 mL of water were added and mixed along with 10.0 g of pancreatin (obtained from porcine pancreas). The SIF solution was adjusted to pH 6.8 ± 0.1 with either 0.2 M sodium hydroxide or 0.2 M hydrochloric acid and then diluted with water to 1000 mL. To perform the stability studies in an aqueous medium at pH 1.2 and 6.8, the above-mentioned buffer components were used but without enzyme substances.

2.3. Preparation of stock and working sample solutions

The stock solutions (2×10^{-4} M) of each compound were prepared in DMSO prior to use. Then, 100 μL of the stock solution were added to a vial containing 1900 μL of buffer or matrix (gastric or intestinal fluid) to obtain the work solutions. Afterwards, the phials containing the samples were placed in a water bath at 37 °C throughout the experiment. At the appropriate time, aliquots of 200 μL were taken and added to a solution of 4800 μL of the mobile phase. Each sample was immediately stored at −18 °C until use. At this time, and after samples had reached ambient temperature,

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the solution was analysed by HPLC or MLC to determine the rate at which the parent compounds disappeared with the formation of AZT as the only degradation product. All the stock and working solutions stored at 4 °C were stable for at least 3 months, as confirmed by measuring the corresponding chromatographic signal.

2.4. Liquid chromatograph instrument

Samples were analysed using an Agilent Technologies Series 1100 liquid chromatograph system (Palo Alto, CA, USA) equipped with a quaternary pump, thermostatted autosampler (4 °C) tray and column (30 °C) compartments, and a UV–vis detector (λ = 267 nm). Instrumental control and chromatographic data acquisition were done with the Agilent ChemStation (Rev. B.03.01) software. The C18 column used was a Phenomenex Synergi 4 μ Fusion-RP 80 (4.6 mm × 250 mm) (Torrance, CA, USA).

2.5. MLC determinations

The micellar mobile phase was prepared using 50 mM SDS, which was buffered with sodium dihydrogenphosphate (0.01 M) at pH 3.0, and lastly 1-butanol was added to a concentration of 1% (v/v). Samples were analysed in 0.05 M SDS–1% (v/v)1-butanol–0.01 M NaH_2PO_4 (pH 3.0) at 30 °C, using a flow rate of 1 mL min^{-1}. Under these conditions, the retention times of AZT-Ac, AZT, AZT-Val and AZT-Leu, were: 1.4, 3.6, 6.3 and 9.5 min, respectively.

2.6. Hydro-organic determinations

The hydro-organic mobile phase [30] was prepared using 60% buffer pH 2.0 (phosphoric acid, 10 mM), 40% MeOH and 2% THF at 30 °C, and a flow rate of 1 mL min^{-1}. Under these conditions, retention times were 4.4, 5.2, 13.5 and 19.3 min for AZT-Ac, AZT, AZT-Val and AZT-Leu, respectively.

3. Results and discussion

3.1. Monitorisation of AZT

Firstly, accelerated degradation studies for AZT-Ac, AZT-Leu and AZT-Val were carried out in the acid and basic media at 70 °C for 1 h to identify their degradation products. In all cases, only AZT was detected in the reaction media [30]. Thus the development of a new stability-indicating MLC method only considers the rapid separation of the AZT-AZT-Ac, AZT-AZT-Leu and AZT-AZT-Val couples with maximum efficiency, and with adequate resolutions and asymmetry factors.

3.2. Optimisation strategy and mobile phase selection

Several studies were carried out to select efficient parameters for the stability determinations. The first experiments employed mobile phases containing 0.1 M SDS, and 4% of propanol, butanol and pentanol, buffered at pH 3. We observed how propanol yielded longer retentions times, and pentanol caused the appearance of AZT-Leu with the dead time. Thus butanol was selected for further optimisation studies into compound separation. Moreover, pH 3.0 was chosen given the good retention times, good efficiencies and minimize the analysis time.

The optimisation protocol began with the experimental design used for the drugs consisting of the five mobile phases containing SDS-butanol: four located at the corners of a rectangular factor space and the fifth in its centre. The second step involved fitting the retention data to an adequate model which, for the example considered, had four parameters. Errors below 3% were usually obtained for the prediction of the retention factors.

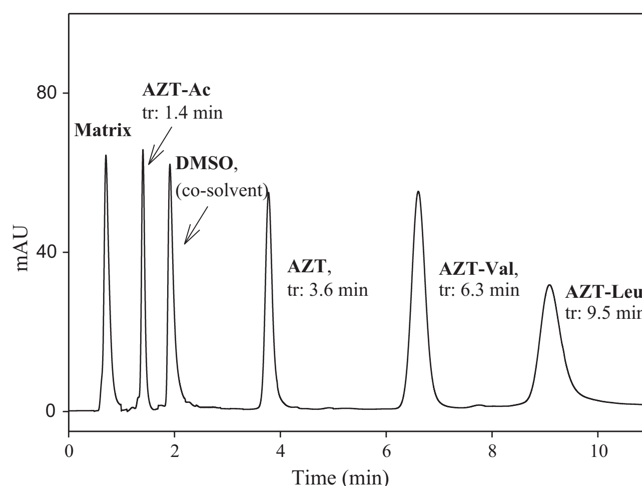

Fig. 2. Chromatogram showing the separation of AZT, AZT-Ac, AZT-Leu and AZT-Val. For details of the separation conditions see Section 2.5.

In order to find the best composition of the mobile phase, the four compounds were injected into the mobile phases at pH 3.0, which contained SDS (M)/butanol (%, v/v): 0.05/1, 0.05/3, 0.1/2, 0.15/1, and 0.15/3. Afterwards, the chromatographic parameters capacity factor (k), efficiency (N) and asymmetry factor (B/A) for the four AZT, AZT-Ac, AZT-Leu and AZT-Val in the five mobile phases were obtained. The usual behaviour in MLC with SDS was observed: (1) the retention factors decreased when the SDS and butanol concentrations increased, (2) efficiencies decreased when the surfactant concentration increased; conversely, efficiencies increased at higher modifier concentrations. The chromatographic data were used in an interpretative optimisation strategy, assisted by computer simulation, which mimics the methodology followed by experienced chromatographers with less time and effort. We selected the most convenient mobile phase with the help of the Michrom software by considering the factor of maximum resolution and the minimum analysis time [34]. This software allows the graphic observation of the changes in the chromatograms when the user progressively varies the surfactant and modifier concentrations. Using this strategy, the selected mobile phase was 0.05 M SDS–1% (v/v) 1-butanol–0.01 M NaH_2PO_4 (pH 3.0). Under these conditions, asymmetry factors were around 1.1 in all cases, and the mean dead time was 1 min. Finally, capacity factors and efficiencies (k–N) were 1.4–2500, 3.6–2000, 6.3–2150 and 9.5–1850 for AZT-Ac, AZT, AZT-Val and AZT-Leu, respectively. Fig. 2 shows the real chromatogram obtained for the separation of AZT-Ac, AZT, AZT-Val and AZT-Leu in the selected mobile phase 0.05 M SDS–1% (v/v) 1-butanol–0.01 M NaH_2PO_4 (pH 3.0). The main conclusion was that no interference appeared between AZT with AZT-Ac, and between AZT-Leu and AZT-Val, revealing that these new derivatives can be determined in the presence of their degradation product by both HPLC and MLC.

3.3. Validation method

The Food and Drug Analysis (FDA) validation guidance [35] was followed to validate the method. The parameters evaluated were: linearity, limits of detection and quantification, intra and inter-day precision, selectivity, recovery and robustness. These parameters were obtained experimentally for the MLC method developed here.

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Table 1
Calibration parameters including the slope, intercept, regression coefficients (r^2), limits of detection (LOD) and quantification (LOQ) for AZT, AZT-Ac, AZT-Leu and AZT-Val.

Compound	Slope	Intercept	r^2	LOD, M	LOQ, M
MLC					
AZT	4800200 ± 340	9.10 ± 0.68	0.9999	0.7×10^{-7}	3.2×10^{-7}
AZT-Ac	3989400 ± 305	10.97 ± 0.58	0.9998	1.0×10^{-7}	3.1×10^{-7}
AZT-Leu	3259700 ± 231	0.79 ± 0.04	0.9999	1.4×10^{-7}	4.2×10^{-7}
AZT-Val	3112100 ± 220	4.67 ± 0.46	0.9998	1.5×10^{-7}	4.5×10^{-7}
HPLC					
AZT	3602700 ± 370	−9.98 ± 3.77	0.9999	1.2×10^{-7}	3.5×10^{-7}
AZT-Ac	3098300 ± 410	−4.22 ± 1.86	0.9997	1.6×10^{-7}	4.9×10^{-7}
AZT-Leu	1465500 ± 395	7.76 ± 1.61	0.9995	3.0×10^{-7}	10.1×10^{-7}
AZT-Val	2327200 ± 215	3.41 ± 4.13	0.9998	2.0×10^{-7}	6.4×10^{-7}

Table 2
Intra- and inter-day precisions (RSD, %) at the different sample concentrations (($c_1 = 9 \times 10^{-6}$, $c_2 = 7.5 \times 10^{-5}$ and $c_3 = 1.5 \times 10^{-4}$ M)), corresponding to the studies of AZT-Ac, AZT-Leu and AZT-Val and to their only degradation product AZT.

Compound	Intra-day			Inter-day		
	c_1	c_2	c_3	c_1	c_2	c_3
MLC						
AZT	0.42	0.26	0.11	1.08 ± (0.93)	0.33 ± (0.09)	0.13 ± (0.02)
AZT-Ac	0.46	0.25	0.20	0.48 ± (0.03)	0.63 ± (0.53)	0.34 ± (0.19)
AZT-Leu	0.78	0.34	0.10	1.02 ± (0.34)	0.58 ± (0.33)	0.23 ± (0.18)
AZT-Val	0.31	0.11	0.34	0.44 ± (0.18)	0.11 ± (0.00)	0.71 ± (0.52)
HPLC						
AZT	0.44	0.04	0.05	0.48 ± (0.05)	0.08 ± (0.05)	0.18 ± (0.18)
AZT-Ac	0.52	0.30	0.02	0.78 ± (0.36)	0.41 ± (0.15)	0.04 ± (0.02)
AZT-Leu	0.45	0.39	0.26	0.50 ± (0.07)	0.43 ± (0.06)	0.31 ± (0.07)
AZT-Val	0.46	0.19	0.19	0.52 ± (0.08)	0.22 ± (0.04)	0.27 ± (0.25)

3.3.1. Calibration parameters, and limits of detection (LOD) and quantification (LOQ)

The calibration curves for AZT, AZT-Ac, AZT-Leu and AZT-Val were constructed using the areas of the chromatographic peaks (triplicate injections) obtained at ten different concentrations equally distributed in the range from 59×10^{-6} to 59×10^{-4} M. The adjusted parameters obtained are shown in Table 1 The regression coefficients (r^2) were always higher than 0.9995.

The limits of detection (LOD) and quantification (LOQ) for the four compounds were calculated with the *3s criterion* and the *10s criterion* (three and ten times the standard deviation of the lowest concentration solution included in the calibration divided by the slope of the calibration curve) using a series of 10 solutions containing a low concentration of each compound (Table 1).

3.3.2. Intra- and inter-day precisions

Intra- and inter-day precisions were determined in the method by analysing the four derivatives at three different concentrations ($c_1 = 9 \times 10^{-6}$, $c_2 = 7.5 \times 10^{-5}$ and $c_3 = 1.5 \times 10^{-4}$ M) in the SGF and SIF samples. The intra-day precision was determined by injecting these three test solutions ten times on the same day. The inter-day precision determination involve the average of ten measurements of the intra-day precision values taken on ten days over a 3-month period performed by different analysts and in different equipment at the same concentrations. The results obtained for the three matrices are similar. Table 2 presents the results for SIF, expressed as the percentage of the relative standard deviation and relative error (RSD, %) for the intra- and inter-day values. As seen, all the compounds were easily determined at the three concentration levels, and the obtained recoveries were quantitative in all cases with RSDs values lower than 1.08%. These results prove that the proposed method is suitable for the analysis of these compounds in buffer, and the SGF and SIF samples. Thus, the procedure developed can be used in the quality control, routine analyses and pharmacokinetic studies of AZT, AZT-Ac, AZT-Leu and AZT-Val.

3.3.3. Recovery studies

In a new series of experiments with AZT, AZT-Ac, AZT-Leu and AZT-Val, the three concentrations ($c_1 = 9 \times 10^{-6}$, $c_2 = 7.5 \times 10^{-5}$ and $c_3 = 1.5 \times 10^{-4}$ M, ten replicates for each standard) were spiked in the buffer and in the SGF and SIF matrices, and were determined in the proposed mobile phase. Standard solutions were processed and analysed following the above-described procedure. Relative (analytical) recovery was calculated by comparing the concentration obtained from the standard solution with the actual added amounts. The data obtained show satisfactory recoveries for the four compounds (Table 3).

3.3.4. Robustness

The robustness of the method was evaluated in terms of the SDS (0.050 ± 0.05 M), the percentage of butanol (1 ± 0.05%, v/v), pH (3.0 ± 0.1) and the flow rate (1 ± 0.05 mL min^{-1}) of the mobile phase by ten replicate injections of a standard solution at 7.5×10^{-5} M of the four compounds. The RSD (%) obtained for changes in the retention time, peak area and resolutions, corresponding to the influence of these parameters were calculated, and were found to be less than 3.2. By way of conclusion, the variations in all the

Table 3
Recovery (%) for the studied derivatives AZT-Ac, AZT-Leu and AZT-Val, as well as AZT, their only degradation product.

Compound	Recoveries		
	c_1	c_2	c_3
MLC			
AZT	116.52	102.59	98.66
AZT-Ac	96.14	99.07	94.47
AZT-Leu	100.61	100.28	103.61
AZT-Val	106.26	97.26	103.48
HPLC			
AZT	102.98	101.26	96.07
AZT-Ac	102.45	95.77	103.48
AZT-Leu	104.84	100.51	99.59
AZT-Val	101.69	103.79	98.04

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studied parameters had no significant effect on retention time, peak area or resolution, and the method proved robust using the recommended MLC method. Using the proposed method, it was possible to inject more than 500 consecutive biological samples without column damage, measured as chromatographic or pressure changes, and consequently without affecting the analytical performance.

3.4. Application of the MLC method to degradation studies of AZT-Ac, AZT-Leu and AZT-Val in SGF (pH 1.2) and in SIF (pH 6.8) using the MLC method.

Degradation of AZT-Ac, AZT-Leu and AZT-Val in the SGF and pH 1.2 solutions at 37 °C was performed. Data were adjusted to a pseudo first-order kinetics. Table 4 shows their apparent rate constants (k_{obs}) determined from the slopes of the pseudo first-order plots and their half-life times ($t_{1/2}$). In the SGF medium, the hydrolyses of AZT-Leu and AZT-Val were slightly higher than 5% after a reaction time of 1 h (AZT-Leu, 5.70%, AZT-Val, 5.73%), whereas AZT-Ac showed a major degradation process of 7.74%.

When stability was studied at pH 1.2, a degradation process lower than 5% for the amino acids derivatives was observed (AZT-Leu, 2.03% and AZT-Val 2.46%), whereas AZT-Ac showed a degradation process of 8.89%. It is possible to rationalise that pepsin affects amino acids stability with two-fold rate constants compared with AZT-Ac, which presented similar degradation constants in both media.

Table 4 also shows the apparent pseudo first-order kinetics and half-life times for the degradation of AZT-Ac, AZT-Leu and AZT-Val in simulated intestinal fluid (SIF) and phosphate buffer pH 6.8 at 37 °C, which followed a pseudo first-order kinetics.

Here it is clearly possible to note a different behaviour of the two amino acids compounds and the acid one. After a 3-h reaction time, AZT-Leu and AZT-Val in the SIF medium had undergone a complete degradation process, while degradations of 81.29% and 90.41%, respectively, were observed at pH 6.8. However, AZT-Ac displayed a degradation of 4.79% and 1.26% in the SIF and pH 6.8 media, respectively.

Table 4 reveals a significant difference in the behaviour of the studied derivatives, which can be separated into two different groups: those with an amino acids promoiety (AZT-Leu and AZT-Val) and that with an acid one (AZT-Ac).

AZT-Leu and AZT-Val were more stable than AZT-Ac in the SGF and aqueous pH 1.2 media since <5% of degradation in acid solution took place after 1 h, whereas the degradation process was over 5% in the presence of pepsin in the reaction medium; this evidences the influence of pepsin on the hydrolysis between AZT and the amino acid moieties. On the other hand, AZT-Ac exhibited greater instability than the AZT amino acid derivatives, with degradation over 5%, but without pepsin being of significant influence.

Conversely, the hydrolysis susceptibility of AZT-Leu and AZT-Val differed in the SIF and neutral media, showing $t_{1/2} \cong 1$ h in pH 6.8, while in the presence of pancreatic enzymes, $t_{1/2} = 0.22$ and $t_{1/2} = 0.04$ h for AZT-Val and AZT-Leu, respectively. AZT-Ac showed major stability in these media with a degradation process lower than 5%.

It is important to point out that all three AZT derivatives have carboxylic groups in their molecules with pK_a values of 2.18, 3.52 and 3.44 for AZT-Ac, AZT-Leu and AZT-Val [36], respectively, and that none of them had ionised forms at pH 1.2, while these groups were totally ionised at a neutral pH. Thus, instability of AZT-Leu and AZT-Val at pH 6.8 could be attributed to an intramolecular catalysis by the acid group of the amino acid moiety which, in its ionised form, could attack the C1′ carbonyl group (Fig. 1), thus making AZT a degradation product. Although the acid moiety of AZT-Ac was also ionised at pH 6.8, it could not reach the C1′ carbonyl group and no hydrolysis took place, implying major stability in this medium. This kind of intramolecular attack is widely reported [37–39].

Both MLC and HPLC were validated and in accordance with all the necessary requirements, and proved to be simple, rapid, precise and accurate as indicative analytical stability methods for such compounds. By way of example, Figs. 3a–c show the MLC chromatograms for AZT-Val in SGF (a) and AZT-Leu in SIF (b) and an HPLC chromatogram for AZT-Ac in the buffer at pH 1.2 (c). It is also important to point out that all the retention times for the four

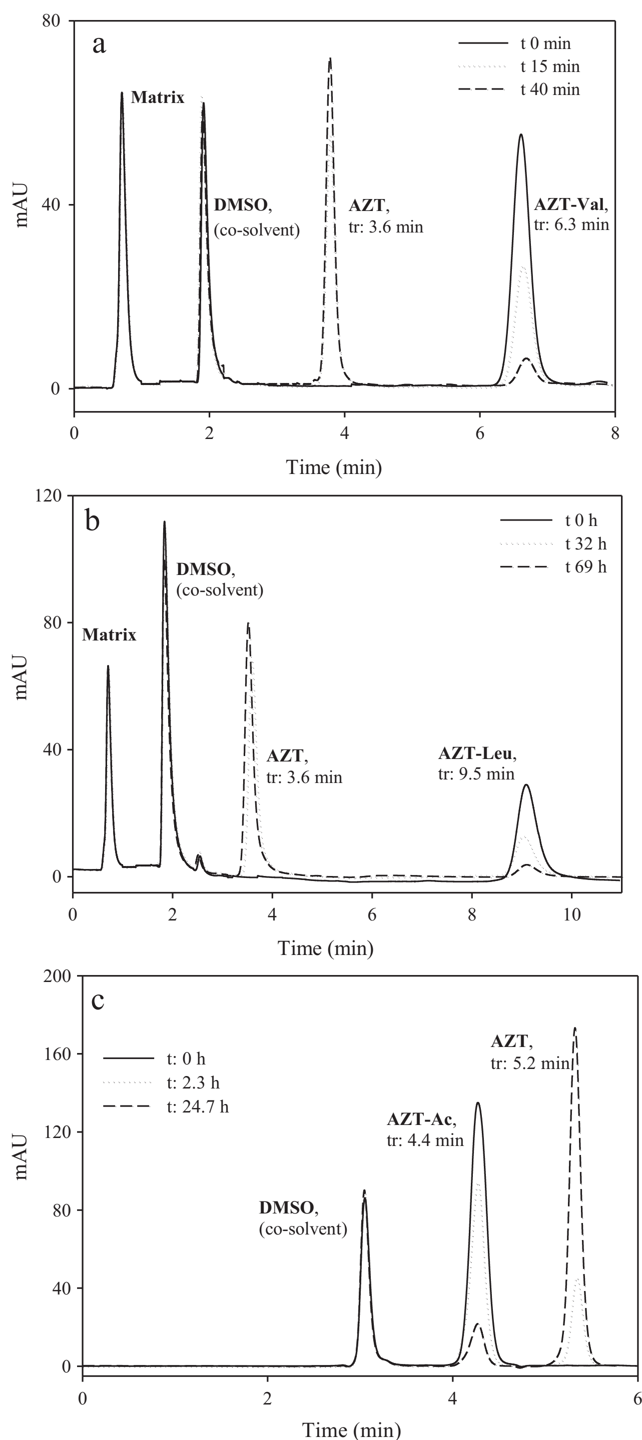

Fig. 3. Chromatograms showing: (a) AZT-Val in SGF – MLC, (b) AZT-Leu in SIF – MLC, and (c) AZT-Ac in buffer pH 1.2 – HPLC. For details of the separation conditions, see Sections 2.5 and 2.6.

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Table 4
Apparent rate constants (k_{obs}) and half-life times ($t_{1/2}$) of AZT-Ac, AZT-Leu and AZT-Val at 37 °C in gastric (SGF) and simulated intestinal (SIF) fluids, and in an aqueous solution of pH 1.2 and pH 6.8.

Medium	AZT-Ac		AZT-Leu		AZT-Val	
	k_{obs} (h^{-1})	$t_{1/2}$ (h)	k_{obs} (h^{-1})	$t_{1/2}$ (h)	k_{obs} (h^{-1})	$t_{1/2}$ (h)
SGF	0.0689 ± 0.0032	10.06	0.0475 ± 0.0035	14.59	0.0476 ± 0.0011	14.56
pH 1.2	0.0738 ± 0.0019	9.39	0.0272 ± 0.0012	25.48	0.0322 ± 0.0041	21.52
SIF	0.0090 ± 0.0015	77.02	17.6535 ± 0.7276	0.04	3.1372 ± 0.0386	0.22
pH 6.8	0.0083 ± 0.0001	83.51	0.5462 ± 0.0336	1.27	0.7682 ± 0.0846	0.90

compounds studied (AZT with AZT-Ac, AZT-Leu or AZT-Val) were shorter in MLC than in HPLC.

4. Conclusions

The significant degradation (>5%) of AZT-Ac, AZT-Leu and AZT-Val evaluated in this study may indicate potential drug instability in the gastrointestinal tract [3]. Based on these results, zidovudine derivatives are considered instable in SGF and SIF at 37 °C in reaction times of 1 h and 3 h, respectively, in accordance with the FDA/CDER's BCS Guidance [3]. These studies have demonstrated that they must be formulated in a form that differs from an oral one. In addition, these analytical methods have been validated and are in accordance with all the necessary analytical stability requirements. The MLC method does not require complex procedures such as sample extraction, and there is no need for large volumes of solvents. In addition, this method has a low degree of toxicity and entails no risk of polluting the environment by organic solvents.

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