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Short communication

Development and validation of a stability indicating method for seven novel derivatives of lamivudine with anti-HIV and anti-HBV activity in simulated gastric and intestinal fluids

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ABSTRACT

A simple micellar liquid chromatography (MLC) method has been developed and validated for use in stability indicating studies of lamivudine and its carbonate derivatives with proved activity against human immunodeficiency and hepatitis B viruses (HIV and HBV, respectively), in simulated gastric (SGF) and intestinal (SIF) fluids samples. The optimized method involves a C18 column thermostated at 30 °C, UV detection at 272 nm, a flow rate of 1.0 mL min^{-1} and a micellar mobile phase composed by 0.15 M sodium dodecyl sulphate (SDS) – 4% (*v/v*) 1-butanol – 0.01 M KH_2PO_4–Na_2HPO_4 (pH 7), using zidovudine (AZT) as internal standard. Validation under Food and Drug Administration (FDA) guideline of the analytical parameters include: linearity ($r^2 > 0.9996$), LODs (1.6×10^{-7}–6.9×10^{-6} M) and LOQ (1×10^{-5} M), intra (0.02–1.48%) and inter-day precision (0.04–1.66%) expressed as relative standard deviation (R.S.D.), and robustness parameters (less than 1.98%). Using this method, recoveries ranging from 92.9 to 119% were obtained for the eight substances. Thus, this method provides a simple, sensitive, accurate and precise assay for the determination of all compounds that can be readily adaptable to routine use by clinical laboratories with standard equipment. In addition, we evaluated the stability of carbonates of lamivudine in buffer pH 1.2 and 6.8; SGF (pH 1.2) and SIF one (pH 6.8), all as indicated in United States Pharmacopeia (USP) 32. Finally, this chromatographic method was applied to stability studies which resulted in all the compounds following a pseudo-first-order kinetics, and in the determination of its kinetic constant and half-life time.

1. Introduction

As the world enters the third decade of the acquired immunodeficiency syndrome (AIDS) epidemic, this pandemic has rapidly grown into the fourth leading cause of mortality globally [1]. Introduction of highly active antiretroviral therapy (HAART), which employs a combination of nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and/or protease inhibitors (PIs), has significantly improved the treatment of HIV/AIDS [2–5]. Lamivudine (3TC), 2′,3-deoxy-3′-thiacytidine, is a deoxycytidine analogue belonging to the class of drugs called NRTIs, used in the treatment of AIDS and Hepatitis B, which etiologic agents are (human immunodeficiency) HIV and hepatitis B (HBV) viruses, respectively [6]. This drug has been shown to be somewhat less toxic than other antiviral drugs but present resistance within a few weeks of treatment. To overcome this problem, we used a 5′-*O*-carbonate substitution strategy by linking different aliphatic alcohols on the side chains 5′-*O* position of 3TC, leading to those associate with methanol (3TC-Metha), ethanol (3TC-Etha), *n*-propanol (3TC-Pro), *n*-butanol (3TC-Buta), *n*-pentanol (3TC-Penta), *n*-hexanol (3TC-Hexa) and *n*-Octanol (3TC-Octa) (Fig. 1); demonstrating to exhibit important biological activity against HIV and HBV [7,8].

The purpose of stability indicating tests provides evidence on how the quality of a drug substance or drug product varies with time, under the influence of different environmental factors, such as temperature, humidity and light, as well as to establish a retest period for the drug substance or expiration date for the drug product, and recommend storage conditions. Stability in the gastrointestinal tract may be confirmed incubating the drug substance in simulated gastric and intestinal fluids that are representative of *in vivo* drug exposure to these fluids; e.g., 1 h in SGF and 3 h in SIF. Significant degradation (>5%) of a drug assessed in this manner could suggest potential instability in the gastrointestinal tract [9]. Then, a validated stability-indicating assay must be employed to measure disappearing intact drug concentrations in the above

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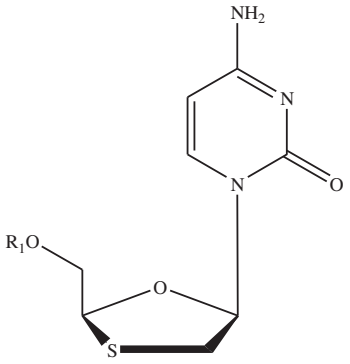

Compound	R
3TC	$R_1 = H$
3TC-Metha	$R_1 = C(O)OCH_3$
3TC-Etha	$R_1 = C(O)OCH_2CH_3$
3TC-Pro	$R_1 = C(O)O(CH_2)_2CH_3$
3TC-Buta	$R_1 = C(O)O(CH_2)_3CH_3$
3TC-Penta	$R_1 = C(O)O(CH_2)_4CH_3$
3TC-Hexa	$R_1 = C(O)O(CH_2)_5CH_3$
3TC-Octa	$R_1 = C(O)O(CH_2)_7CH_3$

Fig. 1. Chemical structure of the lamivudine derivatives studied.

mentioned matrices. The main goals of analytical validation methods are to assure repeatability, reliability and suitability for the purpose for which it was planned [10–13].

Micellar liquid chromatography (MLC) allows the analysis of drug molecules from complex matrices without the aid of the extraction procedures [14]. So, it reduces the cost and the time of the analysis runs, increases sample throughput, and decreases error sources due to the minimized risks of losses and chemical changes in the analyte, because of the reduced number of steps. In addition, MLC has proved to be a useful technique in the determination of diverse groups of drugs in different complex matrices [15].

Thus, the purpose of this work was to develop and validate the MLC procedure not only for screening but also for stability study in aqueous, SGF and SIF matrices of the above mentioned seven carbonates of lamivudine.

2. Experimental

2.1. Chemicals and reagents

3TC and AZT (zidovudine) were generously provided by Filaxis (Buenos Aires, Argentina). All the carbonates of Lamivudine were synthesised as previously reported [7]. Monobasic potassium phosphate, sodium dihydrogen phosphate and sodium hydroxide were purchased from Anedra (San Fernando, Argentina). 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-propanol, 1-butanol, all of analytical grade, were purchased from Cicarelli (San Lorenzo, Argentina). Methanol (MeOH) HPLC grade, was 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.

2.2. Equipment and software

The HPLC analysis were carried out in an Agilent Technologies Series 1100 apparatus (Palo Alto, CA, USA) equipped with a quaternary pump, a thermostatted autosampler tray and column compartments, and a UV–vis detector ($\lambda = 272$ nm). Instrumental control and chromatographic data acquisition were done with the Agilent ChemStation (Rev. B.03.01) software. A Crison GLP 21 (Barcelona, Spain) equipped with a combined Ag/AgCl/glass electrode was used to measure the experimental pH of the solutions.

2.3. Chromatographic conditions

A reversed-phase Kromasil C_{18} analytical column (Scharlab, Barcelona, Spain); 4.6 mm × 250 mm, 5 μm particle size) was used to separate the studied compounds. The flow rate was 1 mL min^{-1}, the injection volume was 20 μL and the temperature was maintained at 30 °C. The micellar mobile phase used was 0.15 M SDS, 4% (*v/v*) 1-butanol and was buffered with disodium hydrogen phosphate at pH 7.0. Before the analyses, the mobile phase was filtered through a 0.45 μm filter (Millipore), and then vacuum degassed.

2.4. Mobile phase and standard solutions preparation

The micellar mobile phase was prepared by weighing the appropriate amounts of SDS and disodium hydrogen phosphate. These reagents were dissolved in ultrapure water. Then, the pH was adjusted as required. Finally, 4% (*v/v*) 1-butanol was added to achieve the desired concentration of the organic solvent and water was added up to the volumetric flask mark-up.

Stock solutions of 3TC derivatives (2×10^{-2} M) and AZT (2.6×10^{-2} M) used as internal standard were prepared in DMSO prior to use. Purity of the standards was higher that 98.5%. Standard solutions of these compounds were prepared by diluting 100 μL of the corresponding stock solution with 1900 μL of corresponding medium. Then, the vials 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 off and 10 μL of AZT and 1790 μL of the mobile phase were added to obtain work solutions. Each solution was stored at −20 °C until it was used, and was shown to be stable for at least 1 month. At this time and after samples had reached ambient temperature, the solution was analysed by MLC, thus 3TC was detected as the only product in the reaction media, which is also the parent compound of these prodrugs. The SGF was prepared according to USP specifications [16].

3. Results and discussion

3.1. Identification of degradation products

Firstly, accelerated degradation studies for these compounds were carried out in the acid and basic media at 70 °C for 1 h to identify their degradation products. In all cases, only 3TC was detected in the reaction media.

3.2. Mobile phase selection

The mobile phase selection was based on the resolution of compounds and suitable analysis time. The first experiments employed mobile phases containing 0.05 M SDS, and 1% (*v/v*) of

1-propanol, 1-butanol or 1-pentanol. Elution strength of these modifiers increase in the order propanol, buntanol, pentanol, according to the long of its chain. Experimental data indicates that propanol yielded longer retentions times and, 1-pentanol, overlaps some of the. Thus butanol was selected for further optimisation. On the other hand, pH 7.0 provide adequated chromatographic parameters, and was selected in front to more acidic media that could reduce the life of the column.

In order to find the best composition of the mobile phase, all the compounds were injected into the respective mobile phases at pH 7.0, containing the surfactant SDS (M) and 1-butanol (%, *v/v*) as modifier with the following ratio: 0.05/1, 0.05/7, 0.10/4, 0.15/1, and 0.15/7. Afterwards, the chromatographic parameters, retention factor (k), efficiency (N) and asymmetry factor (B/A) for all compounds in the five mobile phases were obtained. 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 criteria of maximum resolution and efficiency combined with minimum analysis time [17]. 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 0.15 M SDS – 4% (*v/v*) 1-butanol – 0.01 M Na_2HPO_4 (pH 7.0). Fig. 2a shows the real chromatogram obtained for the separation of 3TC, its derivatives, AZT (as internal standard) and DMSO (as co-solvent) in the selected micellar mobile phase. Fig. 2b shows a blank. The main conclusion was that no interference appeared among all compounds.

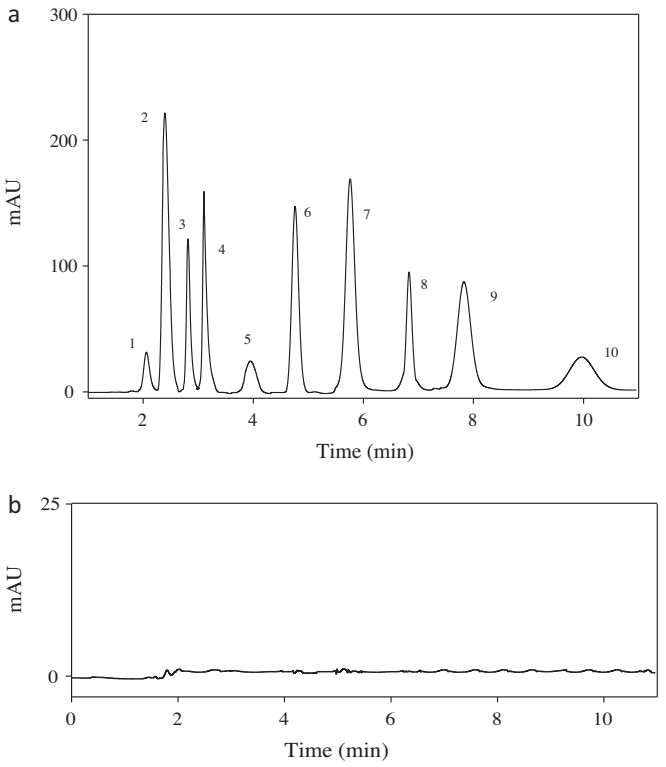

Fig. 2. (a) Chromatogram showing the separation of DMSO ((1) co-solvent), 3TC (2), AZT ((3), internal standard), 3TC-Metha (4), 3TC-Etha (5), 3TC-Pro (6), 3TC-Buta (7), 3TC-Penta (8), 3TC-Hexa (9) and 3TC-Octa (10); with retention times: 2.0; 2.4; 2.8; 3.1; 4.0; 4.8; 5.8; 6.8; 7.8 and 10.0, respectively and (b) blank chromatogram.

3.3. Validation method

This methodology has been validated according to the FDA validation guidance [10] which includes linearity, limits of detection (LOD) and quantification (LOQ), precision and accuracy, selectivity, recovery and robustness. It should be pointed out that the values of the studied parameters did not depend on the matrix evaluated.

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

The calibration curves for 3TC and its carbonates were constructed using the relation of areas of the chromatographic peaks (triplicate injections) and internal standard obtained at ten different concentrations equally distributed in the range from 1.0×10^{-5} to 5.0×10^{-4} M. To study the variability of the calibration parameters, curves were obtained for 5 days over a 2 month period for a different set of standards. The slopes and intercepts were determined by the least-squares linear regression analysis method. The adjusted parameters obtained are shown in Table 1. The regression coefficients (r^2) were always higher than 0.9996.

The LOD for the compounds were calculated with the *3s criterion* (three 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). The LOQ as *10s criterion* also appears in Table 1.

3.3.2. Intra- and inter-day precisions

To assess intra-day precision, also known as within-day repeatability, three aliquots of each drug ($c_1 = 2.0 \times 10^{-5}$, $c_2 = 7.5 \times 10^{-5}$ and $c_3 = 5.0 \times 10^{-4}$ M) were prepared and analysed. The intra-day repeatability was determined by injecting these three solutions ten times on the same day. The study inter-day repeatability involve the average of ten measurements of the intra-day values taken on ten days over a 3-month period performed by different analysts and instrumentation. RSDs values, lower than 1.66%, indicate that the proposed method is useful for the routine analyses of the 3TC derivatives studied in buffer, SGF and SIF matrices.

3.3.3. Recovery studies

Recoveries of 3TC and its carbonates were determined by spiking in buffer, SGF and SIF matrices the three concentrations

Table 1
Calibration parameters including the slope ($b \pm s_b$), intercept ($a \pm s_a$), regression coefficients (r^2), limits of detection (LOD, *3s criterion*) and quantification (LOQ, *10s criterion*)[a] for 3TC and its derivatives.

Compound	Slope	Intercept	r^2	LOD, M	LOQ, M
3TC	0.754 ± 0.013	0.030 ± 0.050	0.9996	2×10^{-6}	6×10^{-6}
3TC-Metha	0.605 ± 0.009	0.025 ± 0.027	0.9997	6×10^{-6}	2×10^{-5}
3TC-Etha	0.217 ± 0.006	0.005 ± 0.005	0.9996	2×10^{-6}	6×10^{-6}
3TC-Pro	0.531 ± 0.005	−0.004 ± 0.004	0.9999	1×10^{-7}	3×10^{-7}
3TC-Buta	0.685 ± 0.025	0.025 ± 0.030	0.9996	1×10^{-6}	3×10^{-6}
3TC-Penta	0.631 ± 0.008	−0.005 ± 0.007	0.9999	7×10^{-7}	2×10^{-6}
3TC-Hexa	0.521 ± 0.029	0.012 ± 0.020	0.9998	4×10^{-6}	1×10^{-5}
3TC-Octa	0.291 ± 0.009	0.006 ± 0.007	0.9996	5×10^{-6}	1×10^{-5}

Table 2
Apparent rate constants (k_{obs}), half-life times ($t_{1/2}$) and % of degradation (3 h) of 3TC derivatives at 37 °C in simulated intestinal fluid (SIF).

Compound	k_{obs} (h^{-1}) SIF	$t_{½}$ (h) SIF	% degradation
3TC	Stable	Stable	Stable
3TC-Metha	0.036	19.40	9.97
3TC-Etha	0.040	17.33	10.86
3TC-Pro	0.075	9.24	19.26
3TC-Buta	0.065	10.66	17.90
3TC-Penta	0.041	16.90	11.30
3TC-Hexa	0.340	2.04	57.50
3TC-Octa	0.194	3.57	44.44

($c_1 = 1.0 \times 10^{-5}$, $c_2 = 5.0 \times 10^{-5}$ and $c_3 = 5.0 \times 10^{-4}$ M, ten replicates for each standard) and were processed and analysed following the procedure described above. The data obtained showed satisfactory recoveries (93–118%).

3.3.4. Robustness

The robustness of the method was examined by replicate injections ($n = 6$) of a standard solution at 7.5×10^{-5} M with slight modifications to the chromatographic parameters (surfactant concentration, percentage of 1-butanol, pH and flow rate). The R.S.D. (%) obtained changing the retention time, peak area and resolutions were calculated, and were found to be less than 1.98% (Table S1, Supplementary Material). In conclusion, variations in all the studied parameters had no significant effect on retention time, peak area or resolution, and the method proved to be robust using the recommended MLC method.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2013.01.027.

3.4. Application of the MLC method to degradation studies of 3TC derivatives in buffers (pH 1.2 and pH 6.8), SGF and SIF

3TC derivatives prodrugs have not shown degradation neither in SGF matrix nor in buffers pH 1.2 during 1 h and pH 6.8 during 3 h, which is in accordance with those of chemical stability previously reported at these pHs media [18], besides demonstrating that pepsin, a component of SGF medium, does not influence over degradation process of these carbonates. Related to SIF matrix, carbonates follow a pseudo first order kinetic which observed rate constants (k_{obs}) and half-times ($t_{½}$) summarized in Table 2. This fact means that pancreatin, a component of SIF medium; affect the stability of all 3TC carbonates giving 3TC as only degradation product.

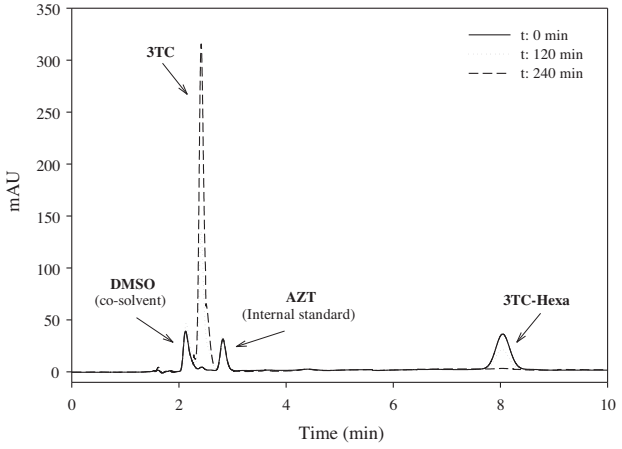

Fig. 3. Chromatograms showing the retention time of DMSO (co-solvent), 3TC (only degradation product), AZT (internal standard) and 3TC-Hexa, in SIF at different times.

It is important to point out that pancreatin is a matrix suggested by the USP and it is constituted by a series of enzymes such as amylase, lipase and protease which present diverse functions and catalytic sites [18]. These features could lead to particular behavior of each derivative in SIF medium and could explain the lack of relationship of the carbonates between $t_{½}$ in SIF and indicatives physicochemical properties of a drug molecule such as lipophilicity [18] (r^2 0.625) and water solubility at 37 °C (r^2 0.569) [19].

MLC were validated in accordance with all the necessary requirements, and proved to be simple, rapid, precise and accurate as indicative analytical stability method for such compounds. For example, Fig. 3 shows the MLC chromatograms for 3TC-Hexa in SIF medium at different times.

4. Conclusions

Stability studies are important features of prodrug screening. Prodrugs usually need to have acceptable chemical stability, but cleave to active drugs molecules under specific enzymatic conditions. The significant degradation (>5%) of 3TC derivatives evaluated in this study could indicate potential instability in the gastrointestinal tract [9], and it is possible to infer that, via oral administration, before intestinal permeation a high percentage of 3TC, the active drug, could be present. To resolve this trouble problem different alternative could be employed such as complex formation between drugs and excipients that often leads to stabilization of drugs. For example, stabilization by formation of inclusion complexes with cyclodextrins, or incorporation into liposomes, micelles or emulsions, could be considered [20].

In addition, these analytical techniques have been validated and accord to all the 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. Besides, this method has a low degree of toxicity and entails no risk of polluting the environment by organic solvents.

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