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Short communication

Development of a liquid chromatographic assay for an anti-HIV tablet containing lamivudine, zidovudine and TMC278.HCL

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ABSTRACT

A liquid chromatographic method was developed to analyse a tablet containing three anti-human immunodeficiency virus (HIV) compounds: lamivudine, zidovudine and a compound with the code name TMC278.HCl. Due to the presence of UV absorbing chromophores in the three active components, a single LC method with UV detection was developed. A Hypersil BDS C_{18} column was used as stationary phase and the assay was performed with gradient elution using mobile phases containing acetonitrile, 0.2 M potassium dihydrogen phosphate and water. The sample pretreatment is performed by treating the formulation with dimethyl sulfoxide–water (1:1) followed by filtration. After method development, the influence of the different chromatographic parameters on the separation, the interference of other active compounds and excipients, the repeatability and the linearity were investigated. The method was shown to be robust, selective, linear and repeatable. Finally, the content of the compounds in the tablet was determined.

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1. Introduction

Zidovudine (3′-azido-3′-deoxythymidine) was the first drug to be approved for treatment of HIV infection. However, zidovudine and other antivirals show decrease in efficacy during long term monotherapy partly due to development of resistance. It was found that lamivudine (2′-deoxy-3′-thiacytidine) has synergetic action in combination with zidovudine. It has been demonstrated that combination therapy including three drugs has a more potent antiviral effect than combinations of only two [1–3]. Zidovudine and lamivudine are synthetic nucleoside analogs (see Fig. 1a and b). They interfere with reverse transcriptase activity by competing with the natural substrates and incorporating into viral deoxyribonucleic acid (DNA) to act as chain terminators in the synthesis of proviral DNA [4]. TMC278 (4-[[4-[[4-(2-cyanoethenyl)-2,6-dimethylphenyl]-amino]-2-pyrimidinyl]-amino]-benzonitrile) is a diarylpyrimidine non-nucleoside reverse transcriptase inhibitor (NNRTI). In this formulation it is used as the hydrochloric acid salt (HCl) (see Fig. 1c). Like with other NNRTIs, TMC278 interacts directly with a hydrophobic allosteric binding site at the HIV-1 reverse transcriptase enzyme and therefore interferes in a non-competitive fashion with the binding of the normal substrates [5–8].

Simple liquid chromatography with ultraviolet detection (LC-UV) methods were described for the quantification of lamivudine in biological fluids [9–12]. Later, several LC–mass spectrometry (MS) methods were developed to improve the selectivity and sensitivity of the analysis. Kathryn et al. described a first LC–MS method for the simultaneous quantification of lamivudine and zidovudine in human serum [13]. With the increase of the usage of mass spectrometry, several methods were published to quantify zidovudine [14–16], sometimes combined with the simultaneous determination of lamivudine in biological fluids [17–19]. However, most LC–MS methods were described to quantify other antiretroviral agents consisting of either lamivudine or zidovudine [20–27]. Only, Aymard et al. determined 12 antiretroviral agents including lamivudine and zidovudine using LC-UV [28]. The European Pharmacopoeia describes LC-UV methods for zidovudine and lamivudine bulk samples [29]. The United States Pharmacopeia has monographs for lamivudine and zidovudine drug substances and for zidovudine drug products [30]. Most of the literature reports on the LC analysis of biological samples.

In this study, an LC-UV assay method was developed for tablets containing lamivudine, zidovudine and TMC278.HCl. After development, the LC method was checked for robustness, selectivity, linearity and repeatability.

2. Experimental

2.1. Instrumentation

The liquid chromatographic system from Dionex (Germering, Germany) consisted of a P680 HPLC pump, an ASI-100 automated

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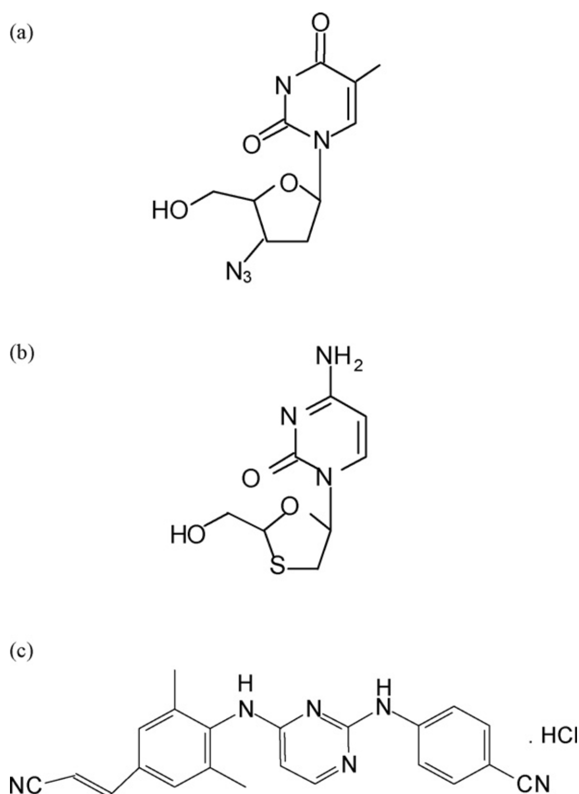

Fig. 1. Chemical structures of (a) zidovudine, (b) lamivudine and (c) TMC278.HCl.

sample injector and a UVD 170U detector. Hypersil BDS C_{18} (250 mm × 4.6 mm) 5 μm, 120 Å (Thermo Electron Corporation, Kleinostheim, Germany) was used as the stationary phase. The temperature of the column, immersed in a water bath, was maintained using a Julabo EM heating circulator (Seelbach, Germany). Chromeleon software (Dionex) was connected to the detector to record the signals.

2.2. Reagents and samples

Potassium dihydrogen phosphate, HPLC grade acetonitrile (ACN) and dimethyl sulfoxide (DMSO) were obtained from Acros Organics (Geel, Belgium). A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to further purify demineralised water. Lamivudine and zidovudine were obtained by extraction from commercial samples. TMC278.HCl and excipients were obtained from Tibotec bvba (Mechelen, Belgium). Tablets containing lamivudine (150 mg), zidovudine (300 mg) and TMC278.HCl (55 mg) were prepared by the Laboratory for Pharmacotechnology and Biopharmacy, Katholieke Universiteit Leuven (Leuven, Belgium).

2.3. Sample preparation

Weigh an amount of tablet mixture corresponding to 0.055 mg/ml of lamivudine, 0.11 mg/ml of zidovudine and 0.02 mg/ml of TMC278.HCl in a 100.0 ml volumetric flask. Add 50 ml of DMSO and sonicate for 5 min. Add 45 ml of water, let cool to room temperature and make up to volume with water. Shake and filter the solution using an 0.2 μm nylon filter.

2.4. Reference solution preparation

Weigh reference substances of lamivudine (22.0 mg), zidovudine (22.0 mg) and TMC278.HCl (20.0 mg) into 3 volumetric flasks of 20.0 ml, 20.0 ml and 50.0 ml, respectively. Dissolve in DMSO and make up to volume with the same solvent. Pipette 1.0 ml, 2.0 ml and 1.0 ml of the lamivudine, zidovudine and TMC278.HCl solutions, respectively, into a 20.0 ml volumetric flask. Add 6 ml of DMSO, 8 ml of water, let cool to room temperature, make up to volume with water and shake. This solution is further referred to as reference solution.

3. Results and discussion

3.1. Method development

Method development was started using gradient elution with mobile phases containing ACN:0.2 M KH_2PO_4:water; A: (10/5/85, v/v/v) and B: (70/5/25, v/v/v); column: Hypersil BDS C_{18}, 250 mm × 4.6 mm, 120 Å, 5 μm; column temperature: 30 °C; injection volume: 20 μl; flow rate: 1.0 ml/min; detection: UV at 270 nm. The linear gradient starts at 0 min and reaches to 100% B in 4 min. With this gradient application, lamivudine eluted along with the system peak. However, the remaining two active components were well separated. So, to slow down the system, an isocratic step was added from 0 to 2 min. For the accurate determination of the contents, there should be no interference from degradation products. The selectivity of the method towards degradation compounds was examined on 6-month-old solutions. These solutions were prepared at pH 2.0 and 9.0 by dissolving the active components separately according to the procedure specified in 2.4, using 0.02 M phosphate buffer instead of water, and stored at 40 °C. After 6 months, there was no degradation of the active components at pH 2.0 and 9.0. Further, these solutions were heated at 100 °C during 15, 30 and 60 min. At pH 9.0 and 60 min, lamivudine and TMC278.HCl degraded. Degradation products from lamivudine eluted along with the system peak, while the degradation product from TMC278.HCl, eluted very closely to zidovudine. So, further optimization of the method was necessary. Different ACN concentrations in mobile phase B were tried out. Lowering the ACN concentration in the mobile phase B resulted in an increase of the run time, but not in a significant improvement in selectivity. Even though at low ACN concentrations high column temperatures increase the selectivity, temperature was kept constant at 30 °C to extend the column life time. Different gradient times were tested and final conditions were selected as shown in Table 1. Typical chromatograms of an anti-HIV tablet containing the three compounds and of degraded reference solutions are shown in Fig. 2a and b, respectively.

3.2. Robustness

For the robustness test, a reference solution was prepared as prescribed in Section 2.4, using 0.02 M phosphate buffer pH 9.0 instead of water, and heated at 100 °C for 30 min to generate the degradation products. The resulting solution was stored at −20 °C to avoid further degradation. The effects and interactions between four chromatographic parameters were examined using a

Table 1
Chromatographic conditions.

Time (min)	%A (v/v)	%B (v/v)
0–2	100	0 (isocratic)
2–7	0	100 (gradient)
7–10	0	100 (isocratic)
10–10.5	100	0 (gradient)
10.5–13	100	0 (isocratic)

Mobile phase: ACN:0.2 M KH_2PO_4:water, A (10/5/85, v/v/v) and B (70/5/25, v/v/v); column: Hypersil BDS C_{18}, 250 mm × 4.6 mm, 120 Å, 5 μm; column temperature: 30 °C; injection volume: 20 μl; flow rate: 1.0 ml/min; detection: UV at 270 nm.

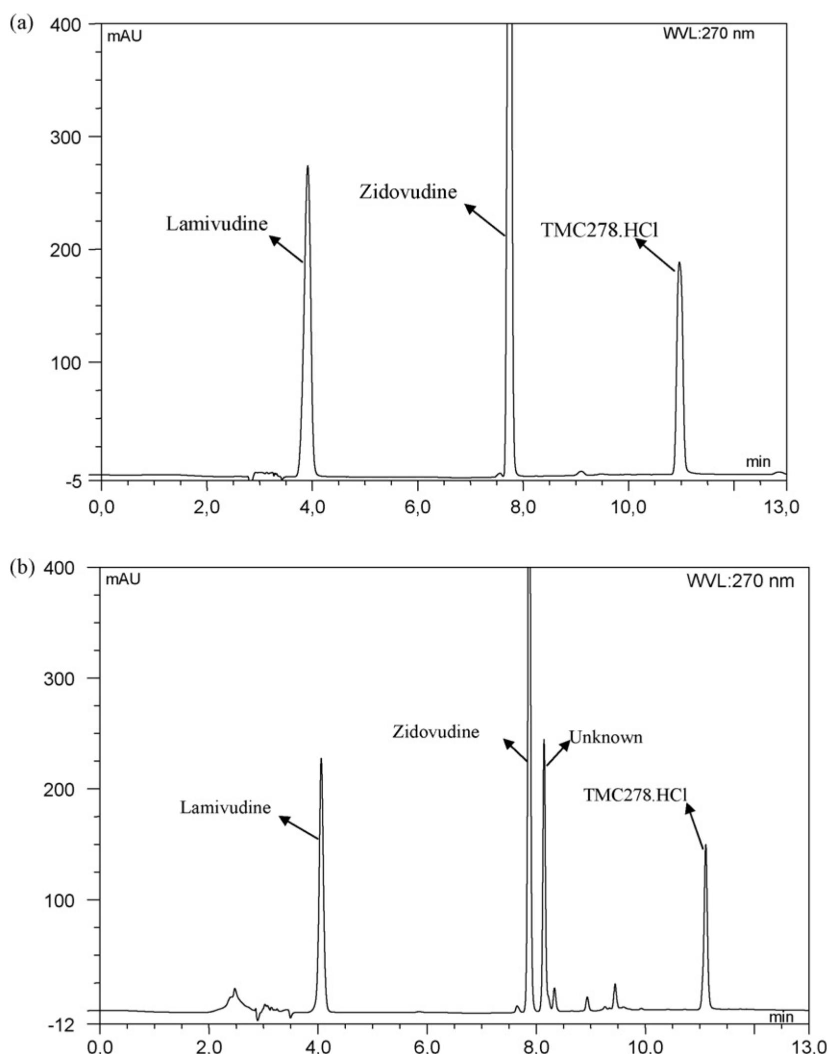

Fig. 2. Typical chromatogram of (a) the anti-HIV tablet containing lamivudine, zidovudine and TMC278.HCl, and (b) a degraded reference solution used in the robustness test. See Table 1 for experimental conditions.

central composite design. Modde 5.0 software (Umetrics AB, Umea, Sweden) was used to investigate the responses. $2^k + 2k + n = 27$ experiments were carried out where k and n were the number of factors ($k = 4$) and central points ($n = 3$), respectively. Table 2 shows the lower (−1), central (0) and higher (+1) values for the chromatographic parameters studied.

The resolution between the critical pair zidovudine and the degradation peak (UNK) of TMC278.HCl (Fig. 2b) was taken as response variable. In the regression coefficient plot of Fig. 3, single coefficients describe the quantitative effect of a factor, crossproducts the interaction between factors and squared coefficients the non-linear effects. The mean effect of a factor is denoted by a bar and the two-sided 95% confidence limits by an error line. A regression coefficient (effect) smaller than the error line interval shows that the variation in the response caused by changing that variable is smaller than the experimental error.

Table 2
Lower, central and upper values used in the factorial analysis of the chromatographic parameters.

	Lower value (−1)	Central value (0)	Upper value (+1)
ACN% in mobile phase A (ACN A)	9	10	11
ACN% in mobile phase B (ACN B)	65	70	75
0.2 M KH_2PO_4% in mobile phase (0.2 M PP)	4	5	6
Column temperature (°C) (Temp)	25	30	35

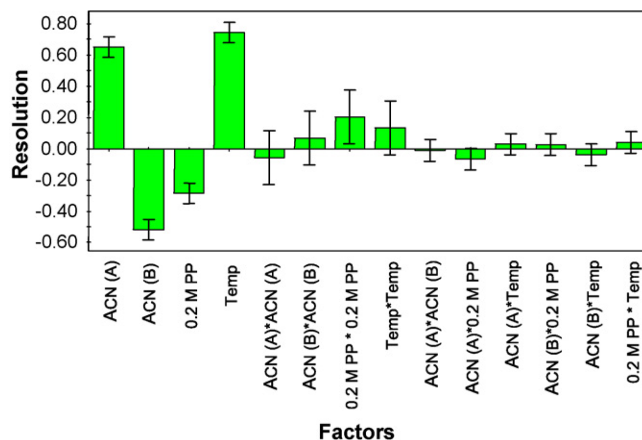

Fig. 3. Regression coefficient plots for the effects of the parameters on the resolution between zidovudine and UNK.

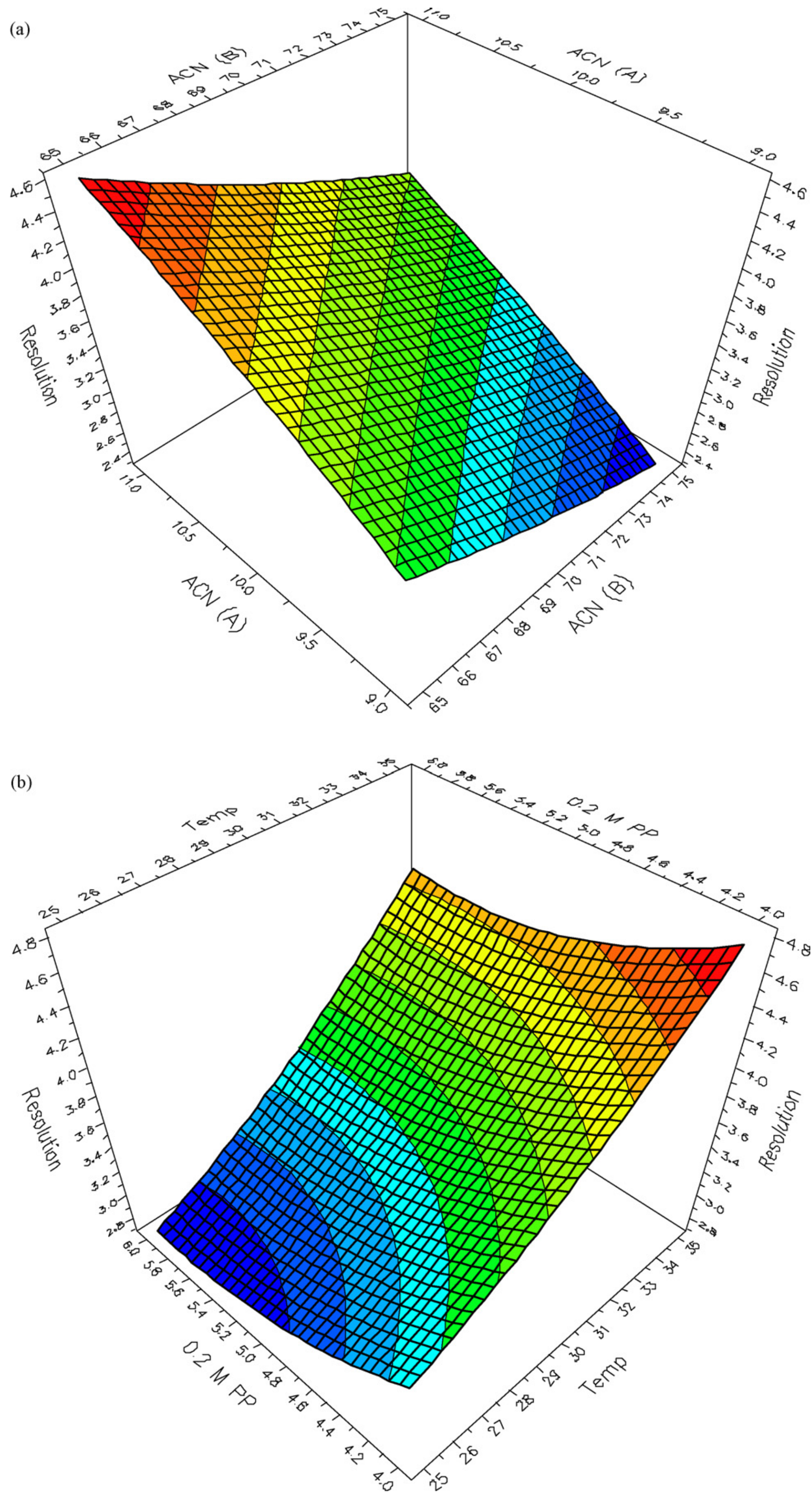

Fig. 4. Response surface plots illustrating the effects of (a) ACN (A)–ACN (B) and (b) Temp–0.2 M PP (B) on the resolution. The other factors were kept constant at central values.

Table 3
Linearity and repeatability data.

	Repeatability for 100% ($n=6$), R.S.D. (%)	Linearity			
		Range, x (%) ($n=21$)	R^2	y	$S_{y,x}$
Lamivudine	0.2	10–125	0.999	$0.41x+0.01$	0.21
Zidovudine	0.1	10–125	0.999	$0.66x+0.79$	0.82
TMC278.HCl	0.2	10–125	0.999	$0.25x+0.16$	0.23

R.S.D.: relative standard deviation; range: percentage range studied; n: number of injections; R^2: coefficient of determination; y: peak area; x: concentration (%); $S_{y,x}$: standard error of estimate.

Table 4
Mean percentage content of lamivudine, zidovudine and TMC278.HCl in 5 individual tablets.

Compound	Tablet 1 % (R.S.D.)	Tablet 2 % (R.S.D.)	Tablet 3 % (R.S.D.)	Tablet 4 % (R.S.D.)	Tablet 5 % (R.S.D.)	Mean % (R.S.D.)
Lamivudine	96.4 (0.4)	95.4 (0.2)	95.9 (0.2)	99.4 (0.2)	96.4 (0.2)	96.7 (1.6)
Zidovudine	95.1 (0.6)	93.4 (0.1)	92.2 (0.1)	99.5 (0.2)	95.2 (0.3)	95.3 (3.0)
TMC278.HCl	97.0 (0.4)	93.7 (0.3)	91.9 (0.2)	97.9 (0.5)	95.0 (0.3)	95.1 (2.6)

The results show that the concentration of ACN (A) and temperature have a significant positive effect, while ACN (B) and 0.2 M PP buffer have a significant negative effect. A positive effect means that an increase of the factor value also increases the response studied. A negative effect means that an increase of the factor value causes a decrease of the response studied. In all cases examined, there is sufficient separation between the peak pair studied. The interactions had no important influence and the non-linear effects were not significant. Fig. 4a and b shows the response surface plots for the resolution between zidovudine and UNK as a function of the percentages ACN in mobile phase A and B and the temperature and the 0.2 M PP in mobile phase, respectively. The other factors were kept constant at their central values. Further optimization was not considered necessary.

3.3. Quantitative aspect of compounds

The linearity of the method was tested at 10%, 25%, 50%, 60%, 80%, 100% and 125%. The 0.055 mg/ml, 0.110 mg/ml and 0.020 mg/ml concentrations of lamivudine, zidovudine and TMC278.HCl, respectively, were considered as 100%. The reference solution (125%) was prepared in an analogous way as in Section 2.4. The remaining solutions were prepared by diluting the 125% solution. Each solution was injected 3 times. The repeatability was tested by injecting 6 times the 100% solution. Results are shown in Table 3.

3.4. Quantification of the actives

Quantification of lamivudine, zidovudine and TMC278.HCl was performed on 5 individual tablets. The tablets were crushed and from each tablet, a test solution was prepared according to the procedure specified in the sample preparation. Next, the solutions were analysed 6 times. Table 4 shows the results. All the mean percentage contents of the active compounds are above 95%.

4. Conclusion

A liquid chromatographic method was developed for the simultaneous determination of lamivudine, zidovudine and TMC278.HCl in tablets. The method is robust, selective, linear, repeatable and easy to perform.

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