Micellar electrokinetic chromatography method development for determination of impurities in Ritonavir

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ABSTRACT

Ritonavir is a synthetic peptidomimetic human immunodeficiency virus (HIV) protease inhibitor employed in the treatment of AIDS since 1996. Synthetic precursors are potential impurities in the final product. In the present work a micellar electrokinetic chromatography (MEKC) method for the separation of Ritonavir from three available synthetic precursors was developed. The optimized separation is performed in a background electrolyte composed of sodium tetraborate (pH 9.6; 15 mM) containing sodium dodecylsulfate (30 mM) and acetonitrile (18%, v/v). Mass spectrometry was used to confirm the identity of the tested substances. Good repeatability was observed for migration time (RSD about 0.4%) and peak area (RSD about 0.8%). The limits of detection (LOD) obtained allow the determination of two of the impurities at levels as low as 0.005% m/m, and one at a level of 0.3% m/m.

1. Introduction

One of the major problems in the treatment of AIDS is its causative agent's elevated rate of mutation, which has led to several virus strains resistant to the anti-HIV drugs developed from the mid 80s. HIV seropositive individuals acquired extended life expectation only after the introduction of the currently adopted highly active antiretroviral therapy (HAART), in 1996. HAART was possible due to the development of various anti-HIV drugs, aiming the viral proteins reverse transcriptase, protease, and later integrase. This therapy is a combination of at least three drugs belonging to at least two different classes of antiretroviral agents. Typically these classes are two nucleoside analogue reverse transcriptase inhibitors (NRTIs) plus either a protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI).

Ritonavir (RTV), a peptidomimetic HIV-protease inhibitor, is the active substance in the anti-HIV drug Norvir© (Abbott Laboratories), approved by the FDA in 1996 [1]. RTV is no longer considered the most efficient PI for anti-HIV treatment, but its association with

other PIs, such as Lopinavir (LPV) in Kaletra® has an important use. Several PIs, including LPV, are mainly metabolized by the oxidase CYP3A4, from the cytochrome P450 enzymatic complex, while RTV is a potent CYP3A4 inhibitor. The use of RTV in sub-therapeutic doses with other PIs is referred as RTV-boosted therapy [2].

Liquid chromatography (LC) is the pharmacopoeial separation technique of choice for the assay and impurity test of RTV, and official methods are available for this purpose [3–6]. There are published capillary electrophoresis (CE) methods dealing with the separation and determination of RTV and other anti-HIV drugs, mainly applied to the determination of serum drug levels [7–12]. However, the application of CE in the determination of related substances and/or impurities in RTV samples was not yet reported.

CE and LC should not be considered as competing techniques, but rather as complementary. Since the separation principles are different for the mentioned techniques, also distinct analytical information can eventually be obtained from each of the approaches, providing a more comprehensive sample analysis. Lately, a rising number of CE methods applied to impurities analysis in pharmaceuticals are available in the literature [13–16], giving rise to the publication of a review discussing about 150 publications from the period between 1980 and 2007 on this subject [17].

Synthetic precursors are potential impurities in the final product. Many steps are necessary to obtain RTV, but the final steps comprise the sequential coupling of two activated ester "wings" to the amino groups of the core (see Fig. 1). RTV and LPV share a common core and two different wings. Several synthesis pathways are described for LPV/RTV-core [18–20]. The RTV/LPV core

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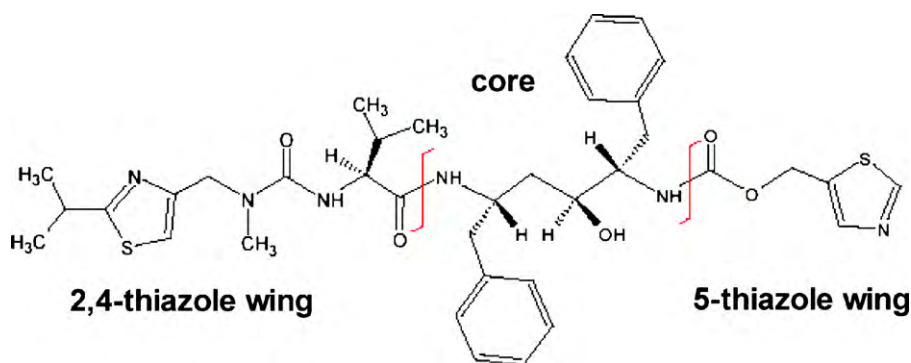
Fig. 1. (A) Ritonavir chemical structure. Two markers are used to indicate the 2,4-thiazole wing, the molecule core and 5-thiazole wing. (B).

is commercially available already Boc-protected (Boc-core, where Boc stands for tert-butyloxycarbonyl group) on one of its amino groups for the sequential binding of the wings [20]. One of the wings of RTV is 5-hydroxymethylthiazole (5-thiazole wing). The other wing is 2-isopropyl-4(methylaminomethyl)thiazole-carbonyl-L-valine (2,4-thiazole wing).

In this work a MEKC method is presented and discussed aiming the separation and determination of the Boc-core, 5-thiazole wing and 2,4-thiazole wing in RTV samples.

2. Materials and methods

All chemicals used were of analytical grade. Sodium tetraborate decahydrate, sodium dodecylsulfate (SDS), sodium cholate (SC), were purchased from Acros Organics (Geel, Belgium). Boric acid and sodium hydroxide were obtained from Riedel-de Haën (Seelze, Germany) and acetic acid and cetyltrimethylammonium bromide (CTAB) from MERCK (Darmstadt, Germany). Vanillic acid was purchased from Sigma–Aldrich (Seelze, Germany). Methanol, 2-propanol, acetone and acetonitrile were purchased from Fischer Chemicals (Leicester, UK). RTV bulk sample, 2,4-thiazole wing, 5-thiazole wing and Boc-core were donated by WHO (World Health Organization, Geneva, Switzerland).

All solutions were prepared with ultrapure MilliQ-water (Millipore, Milford, MA, USA) and filtered with a 0.2 μm nylon filter (Euroscientific, Lint, Belgium). The pH value of buffers was measured and adjusted with the aid of a pH-meter Metrohm 691 (Herisau, Switzerland), prior to addition of organic solvent, when applicable. Tetraborate buffers with pH values equal to or higher than 9.2 were prepared with sodium tetraborate, and the pH was adjusted with solutions of sodium hydroxide. Tetraborate buffers with pH below 9.2 were prepared with boric acid (with the molarity corrected for tetraborate) and the pH was adjusted in the same way.

During method development, sample stock solutions were individually prepared at a concentration of 1 mg ml^{-1} in methanol–water (50:50, v/v) and stored at 7 °C, for at most 1 week.

Optimized solutions were prepared as follows: BGE/sample diluent stock solution is composed of sodium tetraborate (30 mM) buffer containing SDS (60 mM); BGE is prepared by mixing 50 parts of the described stock solution with 18 parts of acetonitrile and 32 parts of water; sample diluent is prepared by mixing 25 parts of the described stock solution to 50 parts of acetonitrile and 25 parts of water.

CE experiments were performed on a P/ACE™ MDQ equipment with diode array detector (DAD) and the data acquisition was done by means of 32 Karat™ 4.0 software (both Beckman-Coulter, Fullerton, CA, USA). Uncoated fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). New capillaries were conditioned at 30 °C by rinsing with 1 M NaOH

(5 min), 0.1 M NaOH (30 min) and water (5 min). Daily conditioning was performed at a temperature of 30 °C by rinsing with water (5 min), 0.1 M NaOH (10 min), water (2 min) and running buffer (10 min). Between each run the capillary was rinsed at 30 °C with BGE (3 min). All the rinsing procedures were performed by applying a pressure of 138 kPa. The inlet/outlet vials were replaced every 3 runs. Acetone (0.2%, v/v) was used as neutral marker to identify the electroosmotic flow (EOF) migration time in different BGEs.

Mass spectrometry (MS) experiments were performed on an ion trap LCQ (Thermo – Finnigan, San Jose, CA, USA).

3. Results and discussion

3.1. Method development – separation BGE and sample preparation

3.1.1. Surfactant selection

RTV is a weak base, with pKa values of 1.76 and 2.56 [21] and is practically insoluble in water (around 0.4 μg ml^{-1}), freely soluble in methanol, sparingly soluble in acetone and very slightly soluble in acetonitrile. Considering these characteristics, initial experiments, based on CZE, were performed in an acidic BGE (ammonium formate – 15 mM; pH 3.0) containing a considerable amount of methanol (25%, v/v) to improve RTV solubility. In these conditions separation was observed, but the combination of low pH and high viscosity, due to methanol in the BGE, yielded a very slow electroosmotic flow (EOF), and the separation of all compounds required about 45 min, with broad peaks.

MEKC is a useful technique for the analysis of neutral and/or more hydrophobic substances [22,23]. Initially, three surfactant systems were screened, namely CTAB (20 mM), SDS (20 mM) and a mixed system composed of SDS-SC (10 mM each). All three systems were buffered with sodium tetraborate (15 mM; pH 9.2). The baseline and signal-to-noise ratio (S/N) observed for CTAB were far worse than in the SDS and SDS-SC systems. Between these last two systems, peak shape and S/N were slightly better for SDS alone, although the separation observed in the mixed system was better, since Boc-core and RTV were not resolved with SDS alone. Taking into account that sensitivity is crucial in impurity analysis, SDS was chosen and efforts were done to improve the separation in this system.

3.1.2. Buffer system

Three different buffering systems were investigated, namely sodium acetate (15 mM; pH 4.8), sodium phosphate (15 mM; pH 7.0) and sodium tetraborate (15 mM; pH 9.2). Although acetate has the largest migration window among the tested buffers, most of the substances co-migrate just after the EOF, indicating low interaction with the micelles. Moreover, the baseline was not good in proximity of the peaks. In phosphate and tetraborate systems all
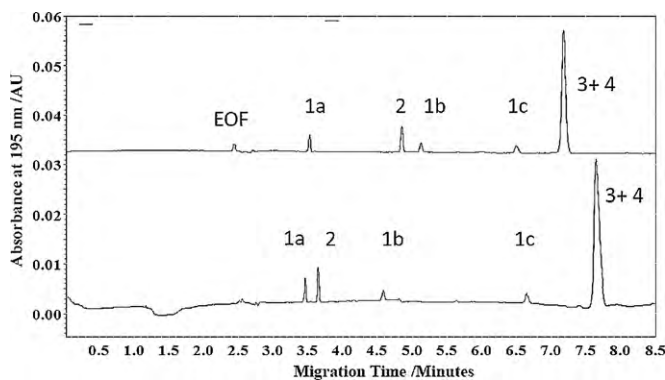
Fig. 2. Electropherogram showing the separation of RTV from its impurities using different buffer systems. CE: capillary, uncoated fused silica 75 μm i.d., 375 μm o.d., 60 cm long (50 cm effective); voltage, 27 kV; temperature, 28 °C; detection, UV 195 nm; injection, inlet pressure 3.45 kPa for 5 s; BGE (top trace) 15 mM sodium tetraborate (pH 9.2), SDS 20 mM; (bottom trace) 15 mM sodium phosphate (pH 7.0), SDS 20 mM; sample dissolved in methanol–water (50:50, v/v): 2,4-thiazole wing (peaks 1a, 1b and 1c), 5-thiazole wing (peak 2), Boc-core (3) and RTV (4), 0.025 mg ml^{-1} each.

peaks were resolved but Boc-core and RTV (see Fig. 2). Overall the peaks were better resolved in tetraborate, and further optimization was carried out with this buffer. Three peaks were observed for 2,4-thiazole wing samples; two of these peaks presented good linearity in function of concentration while the intensity of the third peak (peak 1c, Fig. 2) was not related to sample concentration, and is considered a system peak. Another observation considering the tested substances concerns the yellow color observed when the 5-thiazole wing sample is dissolved in alkaline pH (above 7). Indeed, 5-thiazole wing solution shifts from transparent to yellow and vice-versa depending upon the pH, which indicates ionization of this substance at high pHs, which is not consistent with the chemical structure shown in Fig. 1. MS was used to identify the actual structures of the tested substances (see Section 3.2).

3.1.3. Selection of organic modifier

The possible usefulness of organic modifiers was evaluated. The presence of a certain amount of polar solvents in MEKC can effectively change the separation pattern for several reasons, such as by changing the EOF velocity, changing the structure of the micelles, altering the micelle: monomer equilibrium of the surfactant [22,23]. Additionally, the solubility of hydrophobic substances
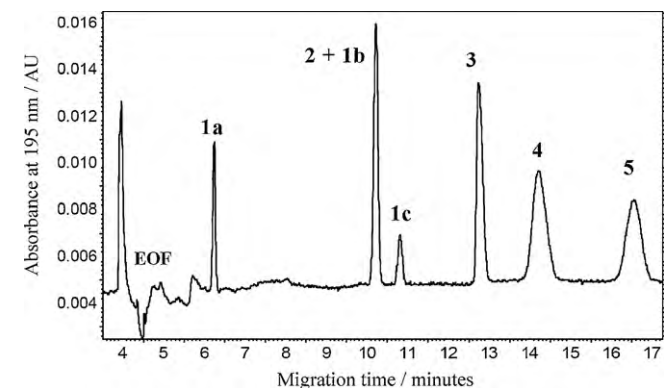

Fig. 3. Electropherogram showing the separation of RTV, impurities and the internal standard. CE: capillary, uncoated fused silica 75 μm i.d., 375 μm o.d., 60 cm long (50 cm effective); voltage, 27 kV; temperature, 28 °C; detection, UV 195 nm; injection, inlet pressure 3.45 kPa for 5 s; BGE, 15 mM sodium tetraborate (pH 9.6), SDS 30 mM, acetonitrile 18% v/v; sample dissolved in BGE: 2,4-thiazole wing (peaks 1a, 1b and 1c), 5-thiazole wing (peak 2), Boc-core (peak 4) and RTV (peak 5), 0.04 mg ml^{-1} each. Vanillic acid (peak 3) is used as internal standard (0.01 mg ml^{-1}).

in the aqueous phase is improved, so that the separation of more apolar substances is made possible by promoting their solubilization (partition) outside the micelles. The possibility to increase the solubility of RTV by using organic solvents is also advantageous since higher amounts can be injected, thus leading to lower LODs for the impurities.

According to the data available concerning RTV solubility, as reported in the introduction, methanol would be the right choice over acetonitrile for RTV solubilization. Both solvents are often used in MEKC [23], but the mixture methanol–water increases analysis time due to its high viscosity and consequent reduction of EOF velocity. In order to evaluate the possibility of using acetonitrile as modifier, the solubility of RTV in mixtures of water–acetonitrile was assessed. Table 1 shows an estimation of the solubility values in some solvent mixtures at different proportions. One can observe that the mixture water–acetonitrile is able to solubilize RTV in high amounts, although pure acetonitrile is not a good solvent for RTV. Therefore, acetonitrile should not be ruled out as a possible additive.

The effect of acetonitrile and 2-propanol on the separation was investigated. Differences in selectivity were observed depending on the organic modifier used. With acetonitrile as modifier, sharper and better-shaped peaks were obtained. Additionally, RTV is migrating as the last peak, which is an advantage since higher amounts of RTV must be injected as test solution, and eventually, a large RTV peak migrating just before Boc-core could overlap with it. However, 5-thiazole wing co-migrates with one peak of 2,4-thiazole wing in the acetonitrile BGE. The equipment used is equipped with DAD, and 5-thiazole wing is the only substance which absorbs visible radiation (maximum at 400 nm), so the proportion of 2,4-thiazole wing and 5-thiazole wing can be obtained from the co-eluted peak by means of multiple wavelength detection and further mathematical calculation. Thus, acetonitrile was adopted for the continuation of the method development.

3.1.4. Concentration of organic modifier

Higher amounts of acetonitrile would improve RTV solubility, allowing the injection of more concentrated RTV samples and consequently leading to lower LOD of the impurities. The performance of separations with different concentrations of acetonitrile (from 15 to 35%, v/v) in the BGE was investigated. It was observed that concentrations of acetonitrile higher than 20% (v/v) lead to a complete modification of the separation profile; the migration times of Boc-core and RTV decrease drastically and these two substances migrate closer to the EOF and are not well resolved from some of the other peaks, depending on the exact amount of solvent. This effect of acetonitrile is probably due to the breakup of the micelles, decreasing significantly the interaction of RTV and Boc-core with SDS, although interaction of monomeric units of surfactant with separands is still a valid approach in CE.

Surfactants are able to solubilize hydrophobic substances, and the SDS concentration was optimized, with values comprised between 20 and 50 mM. It was observed that higher concentrations of SDS enable to use more concentrated RTV solutions. However, increased SDS concentration leads to extended analysis time and higher separation currents. A BGE containing SDS (30 mM) and acetonitrile (18%, v/v) showed a good compromise between RTV solubility and analysis time. The pH was further optimized within the sodium tetraborate buffering range. Finally, a value of 9.6 was selected as optimum, since an increase of the pH value leads to higher resolution, but also to an increased analysis time. The separation voltage was optimized by plotting a curve of the generated current in function of the applied voltage. The linear range (where Ohm's law is valid) is the working range and the maximum voltage value in this range (27 kV) was adopted as the optimal separation voltage. To correct injection volume imprecision common in CE, an
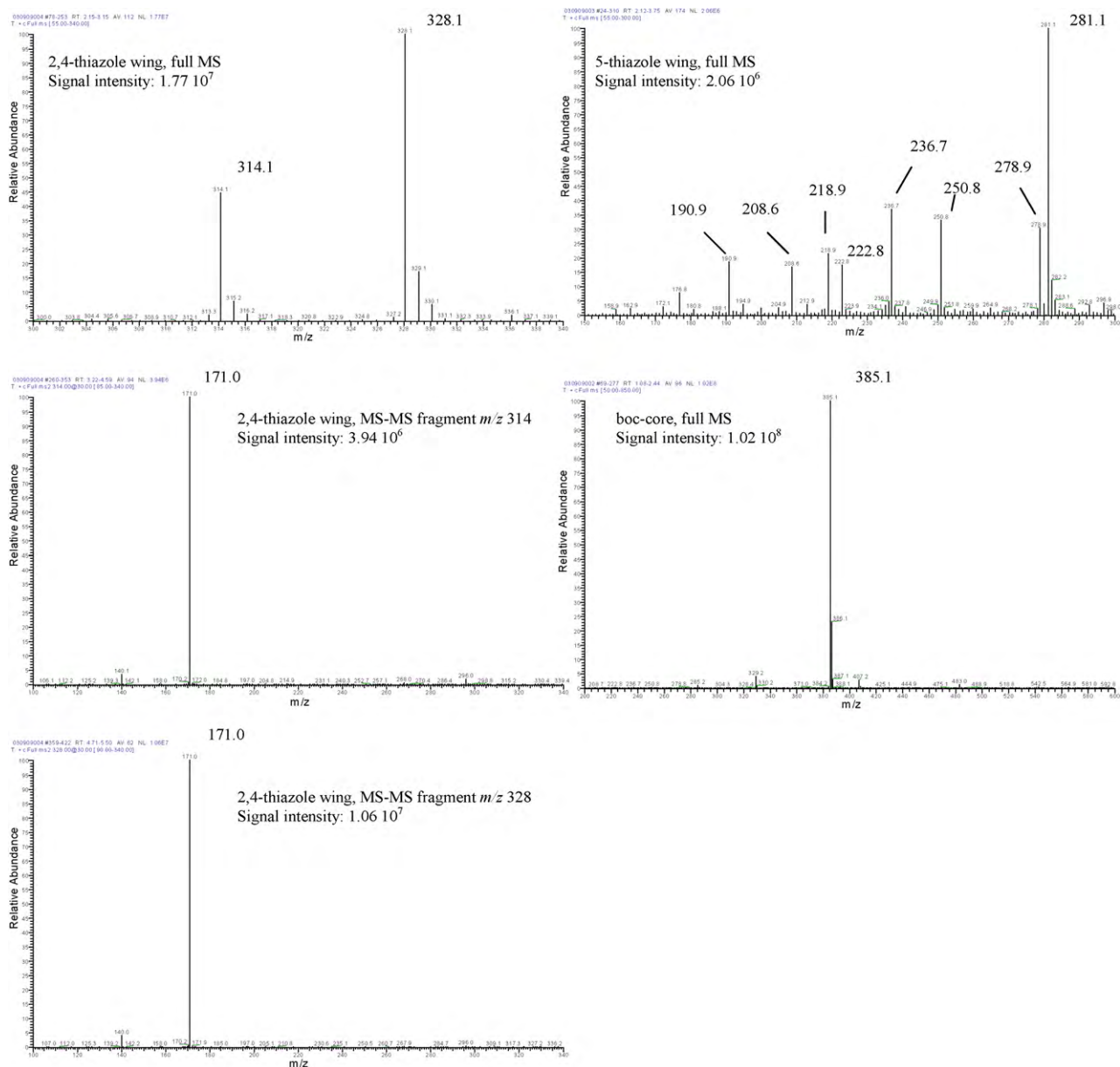
Fig. 4. Mass spectra used for confirmation/identification of the chemical structure of RTV synthetic precursors.

internal standard was adopted, namely vanillic acid. Fig. 3 shows a separation with the optimized parameters.

3.1.5. Sample dilution

As discussed along the previous parts of this work, the solubility of RTV is an important issue for the determination of its impurities. High concentrations of acetonitrile and SDS increase the solubility of RTV. Unfortunately, SDS concentration is limited in view of analysis time and Joule heating, and high concentra-tions of acetonitrile deteriorate the separation profile. However, the sample could be dissolved in a different solution than the BGE.

Sample size was determined by dissolving RTV in a concentra-tion of $20\,mg\,ml^{-1}$ with the sample diluent solution (described in Section 2) and performing sequential dilution down to $1.25\,mg\,ml^{-1}$. The highest concentration which could be injected without disturbing the baseline and causing RTV peak deformation was found to be $5\,mg\,ml^{-1}$.

Table 1
Solubility of Ritonavir in water–organic solvent mixtures.

Water (%, v/v)	Methanol (%, v/v)	Acetonitrile (%, v/v)	Solubility ($mg\,ml^{-1}$)
30	0	70	>30
50	0	50	>21
55	0	45	20
45	55	0	10.9

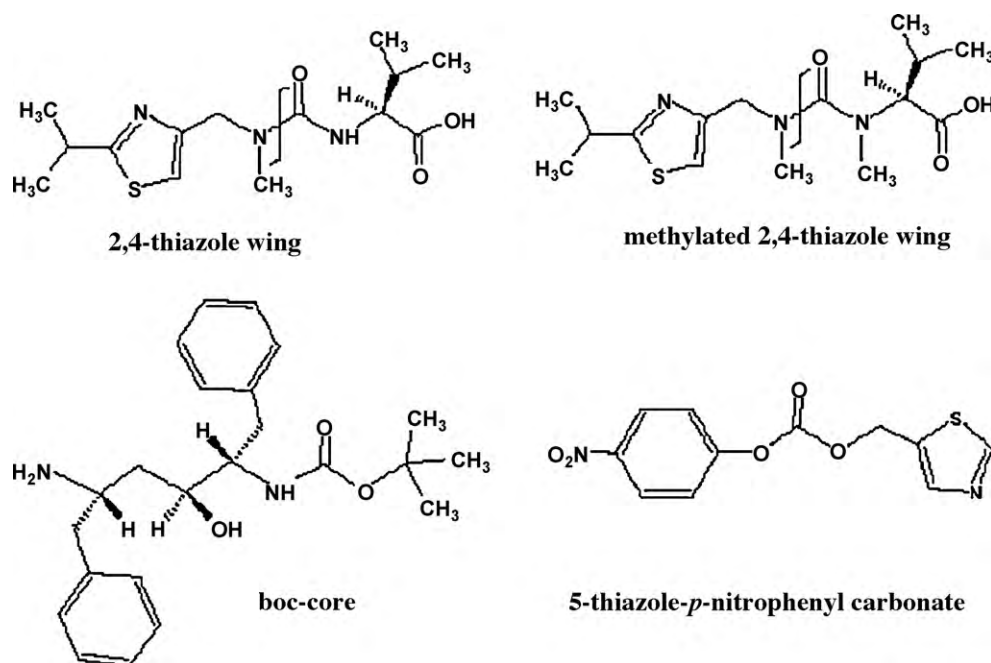Estimation performed at room temperature, approx. $23\,°C$.

Fig. 5. Chemical structures of Ritonavir chemical precursors 5-thiazole wing, 2,4-thiazole wing and boc-core. The methylated variation of 2,4-thiazole wing is also displayed. The two 2,4-thiazole wing variants are displayed with a marker to show the main fragmentation.

The injection of a solution containing RTV (5 mg ml^{-1}), 2,4-thiazole wing, 5-thiazole wing and Boc-core (all at 0.05 mg ml^{-1} – 1% m/m) was performed to verify if separation was still possible in such conditions. It was observed that the Boc-core peak did not show up. It was found that Boc-core cannot be dissolved with the proposed diluent solution, even in conditions where RTV is absent.

The maximum possible sample size for determination of Boc-core was investigated in a similar way as described above, but replacing sample diluent solution by BGE and starting with RTV at a concentration of 1 mg ml^{-1}. Dilution in steps of 0.1 mg ml^{-1} was adopted in place of sequential dilution. The maximum RTV concentration that can be obtained in BGE is 0.7 mg ml^{-1}.

3.2. Mass spectrometry identification of the tested substances

To confirm the actual identity of the tested substances and to elucidate the reason for the appearance of two peaks for 2,4-thiazole wing and of the yellow color presented with 5-thiazole, ESI-MS was performed, and most relevant mass spectra are displayed in Fig. 4. RTV was used for tuning the MS equipment, and as expected the observed m/z base peak was 721 ([RTV + H]$^+$). Boc-core presented a base peak with m/z 385 ([boc-core + H]$^+$), confirming a RTV-core with a protective boc group attached. Concerning 2,4-thiazole wing two main m/z peaks were observed; the expected m/z value 314 ([2,4-thiazole wing + H]$^+$) and a m/z value 328. Fragmentation of both ions led to the same MS–MS pattern, with a high intensity fragment of m/z 171, suggesting that both structures share a representative common part of their structures. The difference 328 − 314 suggested a substitution of one hydrogen atom by a methyl group. In 2,4-thiazole wing there are two most probable places for such methylation: the nitrogen atom or the carboxylic acid from the valine molecule present in 2,4-thiazole wing. The methyl ester is less stable in the aqueous environments in which the majority of the experiments were performed in this work, which suggests that the methyl group is bound to the amide group.

The ionization behavior of 5-thiazole wing is similar to the ionization of nitrophenols, considering the observed color change with pH. The esterification of 5-hydroxymethyl thiazole with p-nitrophenyl chloroformate produces an activated intermediate for coupling of 5-thiazole wing with boc-core and is a described synthetic approach [20]. The attachment of a nitrophenyl group to 5-thiazole wing was confirmed by MS, with the observation of a base peak at m/z 281 ([5-thiazole-p-nitrophenyl carbonate + H]$^+$). It was thus shown by MS that the 5-thiazole wing was present in the form of a derivative. Fig. 5 shows the actual chemical structures of the synthetic RTV precursors used in this work.

3.3. Determination of the relative response factor

In cases where the peak area is considered for the impurity determination it is necessary to know the response factor of the analytes considering the chosen detection method. In the present case, DAD is used. First, the absorbance spectrum of RTV and its synthetic precursors was obtained in order to determine the best wavelength. UV wavelengths with significant absorbance are listed in Table 2.

One can see from Table 2 that maximum sensitivity for all substances is obtained at 195 nm. Other values listed in Table 2 are helpful for identification of the peaks when using DAD. Another absorbance maximum (not listed in Table 2) was observed in the visible region, at 400 nm, for 5-thiazole wing, as previously described in this text.

Samples were prepared containing RTV and boc-core, RTV and 5-thiazole wing and RTV and 2,4-thiazole wing at concentrations of 0.020 and 0.040 mg ml^{-1} for each substance. The relative response

Table 2
UV normalized absorbance values for Ritonavir and its impurities.

	Wavelength (nm)					
	195	205	210	230	240	265
Ritonavir	100	52	41	17	17	0
Boc-core	100	58	52	0	0	0
5-Thiazole wing	100	63	63	39	37	36
2,4-Thiazole wing	100	66	55	26	26	3

Note: Tabled absorbance values should not be compared between different substances.

Table 3
Relative response factor for impurity area normalization relative to RTV area.

	Wavelength (nm)		
	195	210	240
Relative response factor			
Boc-core/RTV	1.21 ($\pm$0.06)	1.11 ($\pm$0.05)	–
5-Thiazole wing/RTV	0.43 ($\pm$0.02)	0.46 ($\pm$0.03)	1.29 ($\pm$0.02)

Table 4
Linearity evaluation of Ritonavir and its impurities.[a]

Substance	Range (μg ml^{-1})	Regression equation[b]	R^2
RTV	13–400	$y = 0.0601x + 0.358$	0.9994
Boc-core	10–60	$y = 0.0780x - 0.153$	0.9993
5-Thiazole wing	1–40	$y = 0.0366x + 0.016$	0.9997
2,4-Thiazole wing[c]	1–40	$y = 0.0431x - 0.009$	0.9996
2,4-Thiazole wing[d]	5–40	$y = 0.0122x + 0.0016$	0.9998

[a] Six concentration points injected in triplicate, unless stated otherwise.
[b] Where y and x represent corrected peak area divided by internal standard corrected peak area and concentration expressed in μg ml^{-1}, respectively.
[c] First migrating peak.
[d] Second migrating peak; four concentration points injected in triplicate.

Table 5
Sensitivity evaluation for the determination of Ritonavir impurities.

	Boc-core[a]	5-Thiazole wing[b]	2,4-Thiazole wing[b,c]
LOD (μg ml^{-1})	2.04	0.21	0.27 and 1.38
LOD (% m/m)	0.29	0.004	0.005 and 0.026
LOQ (μg ml^{-1})	6.80	1.0	1.0 and 4.6
LOQ (% m/m)	1.0	0.013	0.017 and 0.08
RSD at LOQ (%)	17.8	10.5	17.5

[a] Considering a sample containing 0.7 mg ml^{-1} of RTV.
[b] Considering a sample containing 5 mg ml^{-1} of RTV.
[c] Values given are for peak 1a and 1b, respectively.

factors impurity/RTV were determined by dividing the corrected peak area values (area/migration time) of the RTV peak by the corrected area of the respective impurities. The obtained relative response factor values for boc-core and 5-thiazole wing at different wavelengths are listed in Table 3. However, the 2,4-thiazole wing response factor could not be calculated because the proportion of the two substances in the sample is unknown.

3.4. Linearity, repeatability and sensitivity

Table 4 shows linearity obtained for RTV and its impurities. For the determination of the linearities vanillic acid was used as internal standard (10 μg ml^{-1}) and a linear expression was obtained. Table 5 shows limits of detection (LOD) and quantification (LOQ), determined at the most sensitive wavelength (195 nm). For calculation, corrected peak areas were used. LOD and LOQ values are calculated as 3 and 10 times the S/N. Boc-core LOD and LOQ expressed as percentage are calculated considering a sample containing 0.7 mg ml^{-1} of RTV, while 5-thiazole wing and 2,4-thiazole wing LOD and LOQ are expressed as percentage versus a sample containing 5 mg ml^{-1} of RTV. Good sensitivity was also observed for 5-thiazole wing at 400 nm, with LOD and LOQ in the order of 0.75 μg ml^{-1} (0.015% m/m) and 2.5 μg ml^{-1} (0.05% m/m), respectively. The values displayed in Tables 4 and 5 were determined by the injection of each substance separately (plus internal standard).

Repeatability was evaluated by injecting six times a sample containing 0.2 mg ml^{-1} of RTV; RSDs for migration time and corrected peak area are 0.4 and 0.8%, respectively.

4. Conclusion

A repeatable method for the determination of the three last precursors in RTV synthesis was developed. The optimized BGE is composed of sodium tetraborate (15 mM; pH 9.6), SDS (30 mM), acetonitrile (18%, v/v). Total separation time is about 30 min, including capillary pre-washing prior injection. Good repeatability was obtained. Low levels of the studied impurities can be detected, especially for RTV wings. Solubility data and UV spectral information were obtained and MS identification was performed.

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