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Short communication

Evaluation of an International Pharmacopoeia method for the analysis of ritonavir by liquid chromatography

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ABSTRACT

A gradient LC method for the determination of related substances in ritonavir (RTV) has been recently published in the International Pharmacopoeia. The method uses a base-deactivated reversed-phase C18 column kept at a temperature of 35 °C. The mobile phases consist of acetonitrile, phosphate buffer pH 4.0 and water. UV detection is performed at 240 nm. A system suitability test (SST) is described to govern the quality of the separation.

Since no brand names of columns are mentioned in pharmacopoeial texts, analysts often have problems to select a suitable stationary phase which is only described in general terms. So, the separation towards RTV components was investigated on 18 C18 columns and correlation was made with the column classification system developed in our laboratory. The method was further evaluated using a Hypersil BDS C18 column (25 cm × 4.6 mm i.d.), 5 μm, which was also used for the development of the method. A central composite design was applied to examine the robustness of the method. The method shows good precision, linearity, sensitivity and robustness. Four commercial samples were examined using this method.

1. Introduction

Ritonavir (RTV) is a selective, competitive and reversible inhibitor of the human immunodeficiency virus (HIV) protease enzyme. It is widely used in the treatment against the acquired immune deficiency syndrome (AIDS) and prescribed in combination with other antiretroviral drugs. The chemical structures of RTV and two of its related substances are shown in Fig. 1.

So far, two LC methods for stability indicating analysis were published for RTV soft gelatin capsules [1,2]. LC methods for the assay and purity control of RTV drug substance have been published in the Indian Pharmacopoeia (IP), International Pharmacopoeia (Ph. Int.), United States Pharmacopeia (USP) and Pharmeuropa [3–6]. The Ph. Int. prescribes potentiometric titration for the assay of RTV. The LC method described in the USP for the assay and purity control of RTV was adapted into Pharmeuropa and separates about 20 impurities.

The objective of this study was to evaluate the LC method of the Ph. Int. monograph. Since no brand names are mentioned in the monograph, the suitability of a set of 18 columns, all complying the prescriptions of the monograph, was investigated towards the separation of RTV and its impurities. It was checked whether a relation could be made with the column classification system developed in our laboratory [7–9]. Such a study can also be useful for the LC analysis of other pharmaceuticals described in pharmacopoeial texts. Since the approach was similar as for some other antiretrovirals, more information can be found in the literature [10–12].

The selectivity, limit of detection, limit of quantification, linearity, repeatability and intermediate precision were examined on a Hypersil BDS C18 column.

2. Experimental

2.1. Reagents and reference substances

HPLC gradient grade acetonitrile (ACN) and sulphuric acid were purchased from Fisher Scientific (Leicester, UK), phosphoric acid from Riedel-de Haën (Seelze, Germany), sodium phosphate monobasic dihydrate and sodium phosphate dibasic anhydrous from Sigma–Aldrich Chemie (Steinheim, Germany) and sodium hexanesulphonate (SHS) from Acros Organics (Geel, Belgium). RTV commercial sample, crude sample, 2,4-thiazole wing and

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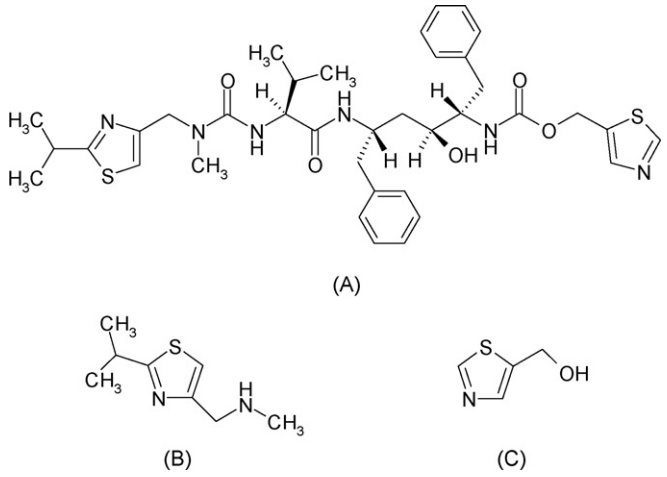

Fig. 1. Chemical structures of RTV (A) and two of its related substances: 2,4-thiazole wing (B) and 5-thiazole wing (C).

5-thiazole wing were donated by the WHO (World Health Organization, Geneva, Switzerland). Commercial samples of RTV were also obtained from different manufacturers.

2.2. Preparation of standard solutions

For the investigation of the separation of RTV and its impurities on the set of 18 reversed-phase columns and for the robustness study, a spiked crude RTV sample solution was prepared by dissolving 1.5 mg of 2,4-thiazole wing and 0.5 mg of 5-thiazole wing in 50 ml of ACN. 10 ml of this solution was transferred to a 50 ml recipient containing 25 mg of crude RTV sample. The volume was made up with mobile phase A. The spiked solution was stored in the refrigerator. For purity control, RTV sample solution was prepared at a concentration of 0.5 mg/ml (100%) and dilutions were made to obtain 0.5 μg/ml (0.1%) solutions. Fresh solutions were made for quantification experiments.

2.3. Instrumentation and liquid chromatographic conditions

LC equipment I (LaChrom, Merck Hitachi, Darmstadt, Germany) consisted of an L-7100 pump, an L-7200 autosampler, an L-7400 UV detector set at a wavelength of 240 nm and a D-7000 interface. EZChrome Elite 4.0 (Merck Hitachi) software was used for data acquisition. The column was kept in a water bath at 35 °C and the temperature was controlled using an EC Julabo thermostat (Seelbach, Germany). A Hypersil BDS C18 column (25 cm × 4.6 mm i.d.), 5 μm (Thermo Hypersil-Keystone, Cheshire, UK) was used for the validation work. The flow rate was 1.0 ml/min and the injection volume 20 μl.

For the intermediate precision study, analyses were carried out by another analyst using a new Hypersil BDS C18 column and LC equipment II (LaChrom Elite, Merck Hitachi) consisting of an L-2130 pump, an L-2200 autosampler and an L-2400 UV detector. Other conditions were identical.

2.4. Mobile phases

The mobile phases consisted of ACN, phosphate buffer pH 4.0 and water: A (35:28:37 v/v/v) and B (70:28:2 v/v/v). The phosphate buffer pH 4.0 was prepared by dissolving 7.8 g of sodium phosphate monobasic dihydrate and 1.88 g of SHS in 800 ml of purified water, adjusting the pH to 4.0 with phosphoric acid (105 g/l) and diluting to 1000 ml with purified water. The gradient applied is: 0–20 min, 70% A and 30% B; 20–30 min, linear increase of mobile phase B from 30% to 100%; 30–40 min, 100% B; 40–45 min, return to the initial conditions; 45–50 min, re-equilibration with 70% A and 30% B. For some columns prolonged equilibration (up to 90 min instead of 50) was necessary to obtain repeatable results.

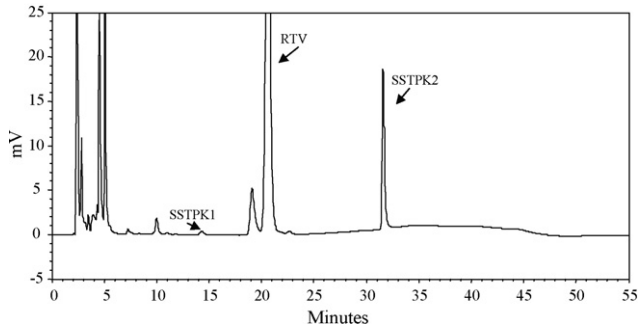

Fig. 2. Typical chromatogram of SST solution, prepared by mixing 5.0 ml of a 0.5 mg/ml RTV solution with 1.0 ml of sulphuric acid (475 g/l) and by heating carefully in a boiling water bath for 60 min, using a Hypersil BDS C18 column (25 cm × 4.6 mm i.d.), 5 μm. 1.0 ml of SST solution was neutralized with 1.0 ml of 1 M disodium hydrogen phosphate solution before the injection.

2.5. System suitability test

According to the Ph. Int. monograph, a system suitability test (SST) solution was prepared by mixing 5.0 ml of a 0.5 mg/ml RTV solution with 1.0 ml of sulphuric acid (475 g/l) and by heating carefully in a boiling water bath for 20 min.

The resolution between the peak due to RTV (retention time about 22 min) and the peak with a retention time of about 0.8 relative to the RTV peak (SSTPK1) should not be less than 3.5 (SST1). Moreover, the resolution between the principal peak and the peak with a retention time relative to the principal peak (SSTPK2) of about 1.5 should not be less than 9.0 (SST2).

In order to improve the decomposition, prolonged heating (up to 60 min) was necessary to obtain the required height of SSTPK1. 1.0 ml of SST solution was neutralized with 1.0 ml of 1 M disodium hydrogen phosphate before injection. A typical chromatogram of the SST solution obtained on a Hypersil BDS C18 column is shown in Fig. 2.

2.6. Selection of a set of C18 columns

The RTV monograph prescribes the use of a base-deactivated reversed-phase C18 column (25 cm × 4.6 mm i.d.), 5 μm. The monograph LC method has been developed using a Hypersil BDS column. The manufacturer claims that this stationary phase is both base-deactivated and end-capped. Therefore, it was decided to examine the separation on a set of 18 columns, available in our laboratory and which are at least either base-deactivated or end-capped (the latter were included to check their performance). The columns were chosen based on a column ranking system developed in our laboratory [7–9]. A list of columns examined in this study is shown in Table 1. These columns were re-characterized prior to this investigation as they had been used for analysis already (the values given on the website have been obtained for new columns).

The SST solution, a commercial sample solution and a spiked sample solution were used to investigate the influence of different stationary phases on the separation.

Table 1
List of C18 columns (250 mm × 4.6 mm i.d.), 5 μm involved in this study with their characteristics provided by the manufacturer and listed by increasing *F*-values

Column number	*F*-value	Name of the column	Manufacturer/supplier	Pore diameter (Å)	End-capped	Base-deactivated	Opt	SST1 (>3.5)	SST2 (>9)	CRF
1	0.000	Hypersil BDS C18	ThermoQuest	130	+	+	No	9.1	19.3	1.00
2	0.460	ACE C18	Advanced Chrom. Tech./Achrom	100	+	+	No	DC	DC	0.31
3	0.462	Hypersil ODS C18	ThermoQuest	120	+	–	Yes	8.4	18.6	1.00
4	0.483	DCscovery C18	Supelco	180	+	–	Yes	DC	3.9	0.42
5	0.545	Validated C18	PerkinElmer	100	+	–	Yes	DC	DC	0.00
6	0.874	Symmetry	Waters	100	+	–	Yes	DC	DC	0.00
7	1.396	Nucleosil HD	Macherey-Nagel/Filter Service	100	+	–	Yes	DC	DC	0.13
8	1.865	Purospher Star	Merck	80	+	+	Yes	DC	DC	1.00
9	2.240	Brava BDS	Alltech	145	+	+	Yes	17.1	19.8	0.00
10	2.534	Kromasil EKA	Akzo Nobel/SerCoLab	100	+	–	Yes	DC	DC	1.00
11	3.016	Supelcosil LC-18 DB	Supelco	120	–	+	Yes	DC	6.6	0.00
12	3.325	Alltima C18	Alltech	120	+	+	Yes	DC	DC	0.09
13	5.775	Apex ODS II	Jones Chromatography/Sopachem	100	+	–	Yes	DC	DC	0.00
14	6.244	Purospher	Merck	80	+	–	Yes	DC	DC	1.00
15	10.337	Platinum C18	Alltech	100	+	+	Yes	DC	DC	0.00
16	11.715	LiChrospher	Merck	100	–	+	Yes	DC	DC	0.00
17	12.207	Platinum EPS C18	Alltech	100	–	+	Yes	DC	DC	0.00
18	16.409	Apex Basic	Jones Chromatography/Sopachem	100	+	+	Yes	DC	DC	0.00

Column 1 (Hypersil BDS C18) is taken as reference column (*F* = 0). CRF, chromatographic response function; SST, system suitability test; Opt, organic modifier in mobile phase A optimized; DC, difficult to calculate.

3. Results and discussion

3.1. Column differentiation based on the SST

It was decided to optimize the amount of organic modifier in mobile phase A so that RTV was eluted between 19 and 24 min. Some typical chromatograms are shown in Fig. 3. The SST results for all 18 columns are shown in Table 1. A change in elution order or coelution was observed on several columns. This may easily lead to inappropriate peak identification.

According to the requirement of the Ph. Int. monograph, 15 columns did not reach the SST. Only columns 1, 3 and 9 have resolutions more than 3.5 and 9, respectively, for SST1 and SST2. Problems were mainly observed in the determination of SST1 since SSTPK1 could hardly be identified. When SST and CRF are more closely examined, it can be observed that the SST criterion does not always give the required information, as shown in Table 1. Columns 8, 10 and 14 give baseline separation for all the peaks, but according to the SST they do not comply. On the other hand, column 9, which complies according to SST, gave poor overall separation (CRF = 0).

3.2. Correlation between the column classification and the separation of RTV

As in previous studies, three arbitrarily chosen ranges of *F*-values were distinguished for the columns in Table 1: $F < 2$, $2 < F < 6$ and $F > 6$ [7–12].

Three out of eight columns with $F < 2$ gave baseline separation for all peaks (CRF = 1.00). Two columns have CRF = 0. Columns 2, 4 and 7 have CRF values of 0.31, 0.42 and 0.13, respectively, due to the partial separation of peaks 6, 7, 8 and 9. When *F* is between 2 and 6, one out of five columns give CRF = 1.00, one column gave a CRF close to 0 (0.09) while other columns gave CRF = 0 mainly due to coelution of peaks 6, 7, 8 and 9. For columns with $F > 6$ the probability to separate RTV from its impurities clearly decreases: only one of five columns gave complete separation (CRF = 1.00), while for the other columns CRF = 0 because several peaks are coeluted.

3.3. Method validation

3.3.1. Robustness study

The influence of four (*k*) chromatographic parameters on the separation was investigated using the Hypersil BDS C18 column. The parameters examined were the amount of ACN in mobile phase A, the amount of SHS, the pH of the buffer in the mobile phases and the column temperature (°C). Their effects on the resolution of different peak pairs 2–3, 6–7, 7–8, 8–9, 9–10, SSTPK1–RTV and RTV–SSTPK2 were evaluated by means of a central composite face centered design using Modde 5.0 software (Umetrics, Umea, Sweden). Each of these parameters was investigated at three levels: ACN (34–36 volumes), temperature (33–37 °C), amount of SHS (1.78–1.98 g) and the pH (3.9–4.1). It was observed under the conditions examined that the amount of ACN in the mobile phase has the most important effect on the resolution of peak pairs 2–3, 7–8, SSTPK1–RTV and RTV–SSTPK2. The pH of the buffer mainly influences peak pairs 7–8, SSTPK1–RTV and RTV–SSTPK2. Temperature and SHS were found to have only slight effects. No important interactions were found.

3.3.2. Quantitative aspects

3.3.2.1. Precision, linearity and sensitivity. The method precision was assessed using multiple preparations of a single commercial

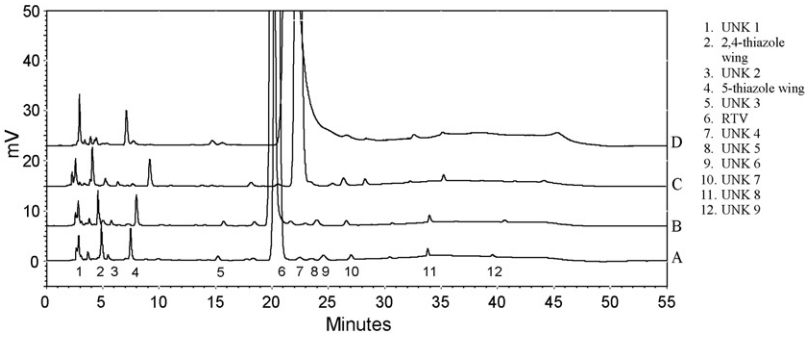

Fig. 3. Overlay of chromatograms for purity control obtained on different columns for a spiked RTV sample. (A) Hypersil BDS; $F = 0.000$; CRF = 1.00 (column 1), (B) ACE; $F = 0.460$; CRF = 0.31 (column 2), (C) Alltima; $F = 3.325$; CRF = 0.09 (column 12) and (D) Apex basic; $F = 16.409$; CRF = 0.00 (column 18).

Table 2
Intermediate precision data for RTV and some of its impurities

Level (%)	5-Thiazole 0.34	Unknown 2 0.46	RTV 99.12	Unknown 8 0.08
% R.S.D. ($n = 9$)				
Day 1	3.28	3.40	2.96	5.21
Day 2	2.64	2.81	1.93	10.79
Day 3	3.38	2.81	2.76	14.60
% R.S.D. ($n = 27$)				
Days 1–3	5.77	5.06	4.66	11.44
% R.S.D. ($n = 9$)				
Day 4	8.26	9.31	3.23	19.07
% R.S.D. ($n = 18$)				
Days 3–4	10.43	10.96	8.12	17.39

Table 3
Purity control of RTV samples

Impurities	Sample no. 1	Sample no. 2	Sample no. 3	Sample no. 4
Sum of impurities (%)	0.07	0.06	1.07	0.55
Number above 0.1%	0	0	4	2
Number above 0.2%	0	0	2	0
Number above 0.3%	0	0	2	0

sample. Three different preparations of the same commercial RTV sample, each 0.5 mg/ml, were analyzed in triplicate on a single day. New preparations were made and analyzed on each of three successive days. An intermediate precision study was performed as described in [10–12]. The results are summarized in Table 2.

The LOQ for RTV was 0.05% ($n = 6$; R.S.D. = 8.3%) and the LOD was 0.015% (0.5 mg/ml = 100%, 20 μl injected). The linearity was checked by analyzing RTV in the concentration range of 0.05–125%. The following results were found: $y = 43033x + 5434$; $r^2 = 0.999$ and $S_{y,x} = 39353$, where y = peak area, x = concentration of RTV solution (%).

3.3.2.2. Analysis of commercial samples. The Ph. Int. sets the limit for any individual impurity not to be more than 0.3% and the sum of impurities not to be more than 1.0% in bulk samples. Four RTV samples were analyzed for related substances and results obtained are summarized in Table 3. Sample 3 does not comply. All the impurities are expressed as RTV, using a 0.5% dilution (10.0 μg/ml) as reference.

4. Conclusions

Depending on the column used, the gradient LC method proposed in the Ph. Int. shows a good separation of RTV from the impurities present in bulk drug substances. The robustness study indicated that the amount of ACN and pH of the buffer should be monitored carefully. The SST requirements of the Ph. Int. monograph do not always give the required information. Only the Hypersil BDS column complies for SST and the required separation (CRF = 1). For other columns, it was found that, with the increase of the F-values, the probability of finding a suitable column decreased. The use of chromatographic parameters to describe a stationary phase can help a lot in the selection of a suitable column.

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