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Renewal of an old European Pharmacopoeia method for Terazosin using modeling with mass spectrometric peak tracking

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ABSTRACT

An older method for terazosin was reworked in order to reduce the analysis time from 90 min (2 × 45 min) to below 5 min. The method in European Pharmacopoeia (Ph.Eur.) investigates the specified impurities separately. The reason of the different methods is that the retention of two impurities is not adequate in reversed phase, not even with 100% water. Therefore ion-pair-chromatography has to be applied and since that two impurities absorb at low UV-wavelength they had to be analyzed by different method than the other specified impurities. In our new method we could improve the retention with pH elevation using a new type of stationary phases available for high pH applications. Also a detection wavelength could be selected that is appropriate for the detection and quantification of all impurities.

The method development is the bottleneck of liquid chromatography even today, when more and more fast chromatographic systems are used. Expert knowledge with intelligent programs is available to reduce the time of method development and offer extra information about the robustness of the separation. Design of Experiments (DoE) for simultaneous optimization of gradient time (t_G), temperature (T) and ternary eluent composition (t_C) requires 12 experiments. A good alternative way to identify a certain peak in different chromatograms is the molecular mass of the compound, due to its high specificity. Liquid Chromatography–Mass Spectrometry (LC–MS) is now a routine technique and increasingly available in laboratories. In our experiment for the resolution- and retention modeling the DryLab4 method development software (Version 4.2) was used. In recent versions of the software the use of (*m*/*z*)-MS-data is possible along the UV-peak-area-tracking technology. The modelled and measured chromatograms showed excellent correlations. The average retention time deviations were ca. 0.5 s and there was no difference between the predicted and measured $R_{s,crit}$ −values.

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1. Introduction

Lloyd Snyder and his group started resolution- and retention modeling for isocratic methods in 1986 and later for gradient methods. They focused on the resolution of the least separated "critical" peak pair, $R_{s,crit}$; it was their analytical target profile (ATP) [1,2]. Since in HPLC separations, the concentration of the organic eluent content (%B), gradient time (t_G), pH, ternary composition (B1:B2) (f.e., AcN:MeOH) and also instrument factors, such as temperature, dwell volume, column dimensions, etc. affect selectivity, further adjustments on the software (DryLab) were necessary [3,4]. It became clear, that in a given Design of Experiment (DoE), peaks are moving, therefore the so called peak tracking process had to be better understood [5,6]. Furthermore since the column performance depends on many parameters, which had to be controlled, when a robust method is required [7,8].

After the peak tracking process, based on comparison of peak areas was working properly, the optimization using 2 measured parameters was developed, and experimentally verified in several papers [9–11]. Difficult samples could be separated and robust methods for pharmaceutical products like steroids were developed [12].

A great step forward to the development of more robust methods was the evolving of a 3-dimensional resolution map, the Cube. This can work with 3 measured parameters (tG, T, pH or tC) and calculate 8 other parameters, to deliver transparent way why methods might fail due to peak movements [13–15].

Retention modeling is important not only for small molecules, but increasingly for macromolecules like proteins and monoclonal antibodies [5,8,16,17].

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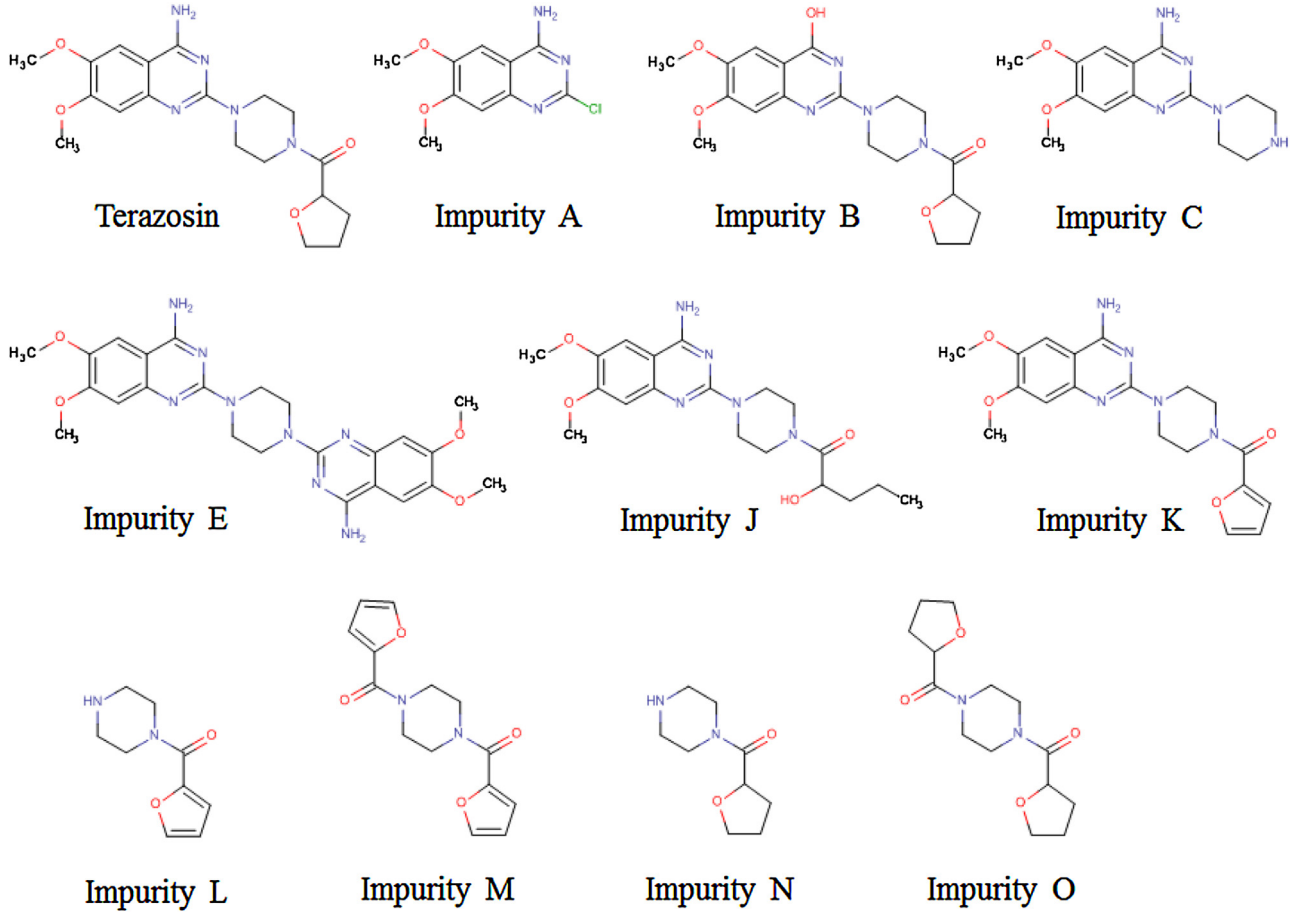


Fig. 1. Terazosin and its specified impurities.

The European Pharmacopoeia (Ph.Eur) contains two HPLC methods with long analysis time of 2 × 45 = 90 min for the determination of the specified impurity of terazosin active pharmaceutical ingredient (API) [18]. Since regulatory agencies increasingly request the application of Quality by Design (QbD) principles in liquid chromatography method development [19], these methods were reworked. The goal was to model the changes that can help for the better column selection and to reduce the analysis time, using a similar process, which was already published 3 years ago [20]. Nowadays the pharmaceutical industry advocates the tasks about the better understanding of the chromatographic processes based on computer supported modeling, which was recently demonstrated by J. Fekete, R. Kormány, Sz. Fekete [20–22] and J. Kochling [23].

In another case study, an old Ph.Eur. method, the run time of which was 160 min, was reworked. It was easier to make a new method using DryLab® 4, than revalidate the Ph.Eur. method. The method development strategy has been published in the paper of A. Schmidt and I. Molnár [24]. The analysis was reduced from 160 to less than 3 min using method modeling.

The peak tracking process, the handling of a DoE is still the bottleneck for modeling of HPLC methods for many laboratory workers, who work with this growing field for the first time [25–43,49–53]. In this area more progress can be expected in the near future. Improving communication between applicants and regulatory agencies is very important [41–46]. It is worth mentioning, that the separation of new natural macromolecular drugs such as recombinant proteins, monoclonal antibodies and antibody-drug-conjugates (ACD's) have been successfully modeled recently [47,48,54,55].

The novelty of the present paper is the improved modeling in UHPLC method development with a better peak tracking enabled by peak identification using molecular weight information for easier peak alignment.

2. Experimental

2.1. Chemicals

Eluents: The mobile phase was a mixture of purified water, acetonitrile (AcN) and methanol (MeOH). Each eluent contained 0.1 v/v% cc. ammonium hydroxide (NH_4OH) solution, which provided a slightly alkaline eluent (pH of 0.1% NH_4OH in water is 10.7). Under these circumstances the components were in ion suppressed form so their retention was larger than at lower pH. AcN (gradient grade), MeOH (gradient grade) and NH_4OH-solution (analytical grade) were purchased from Merck (Darmstadt, Germany). For measurements, water was prepared freshly, using ELGA Purelab UHQ water (ELGA, Lane End, UK).

The sample contained 5 mg/mL terazosin and 7.5 μg/mL specified Ph.Eur. impurities (see Fig. 1). They were purchased from European Directorate for the Quality of Medicines & HealthCare (EDQM). Sample solvent was MeOH.

2.2. Equipment, software

UPLC™ measurement was performed using a Waters Acquity UPLC™ system equipped with binary solvent delivery pump, an auto sampler, a photo diode array (PDA) detector, QDa mass detector and Empower3 software (Waters, Milford, USA). Detection was

Table 1
Calculated exact masses and pK_a values.

Name	Exact mass[a]	pK_a[a]
Terazosin	387.19	7.24
Impurity A	239.05	2.94
Impurity B	388.17	4.90
Impurity C	289.15	8.72
Impurity E	492.22	7.54
Impurity J	389.45	7.24
Impurity K	383.16	7.24
Impurity L	180.09	7.82
Impurity M	274.10	–
Impurity N	184.12	7.82
Impurity O	282.16	–

[a] Determined by MarvinSketch software.

performed at 220 nm and cone voltage of QDa mass detector used in positive mode was 50 eV.

The UPLC™ system injector contained 1 μL loop and the volume of detector flow cell was 500 nL. The dwell volume of the system was measured to be 0.125 mL.

UPLC method development and method modeling was performed by using DryLab® 4, v.4.2.1.3 optimization software (Molnár-Institute, Berlin, Germany).

Determination of exact masses and pK_a values were identified by MarvinSketch v.6.0.2 software (ChemAxon, Budapest, Hungary).

2.3. Preliminary experiments, stationary phase

The most important step during liquid chromatography method development is the selection of the right stationary phase. The studied components are basic molecules with high pK_a values (see Table 1). Therefore a pH resistant stationary phase is necessary. Acquity CSH C18 column (Waters, Milford, USA) is a good choice for investigation of basic compounds, due to its special manufacturing technology. This stationary phase is pH resistant between 1 < pH < 11 and below 45 °C [56]. The dimension of the chosen column was 50 × 2.1 mm with 1.7 μm particle size for the UPLC™ system. The maximum pressure during the measurements was ca. 800 bar, due to the higher viscosity of the water-methanol eluent system.

2.4. Design of experiments (DoE)

For the simultaneous optimization we used a simple, but very efficient DoE consisting of gradient time (t_G), temperature (T) and ternary eluent composition (t_C). It required 12 experiments, as illustrated in Fig. 2 [46]. For the better handling, the experiments were numbered from 1 to 12. The mobile phase A consisted of 0.1 v/v% ammonium hydroxide in water, mobile phase B1 was acetonitrile, B2 was methanol and their 50:50% mixture with 0.1 v/v% ammonium hydroxide. Two linear gradients, tG1 with 3 min and tG2 with 9 min, (corresponding to a factor 3 difference) and a gradient range of 10–>90% B (=B1 + B2), were carried out at two column temperatures: 20 and 40 °C. The flow rate was 0.5 mL/min. The injection volume was 0.2 μL.

2.5. Sample preparation

To achieve the appropriate signal-to-noise ratio of impurities, the concentration of the API was 1 mg/mL. Using the UPLC system with columns of 50 × 2.1 mm size, the 1 μL sample injection is common for the suitable efficiency. This injection volume cannot be applied in every situation. In our case the solubility of the sample at the starting composition (water:MeOH)(80:20)(V:V) was not adequate. Furthermore, the components 6. and 11. precipitated with

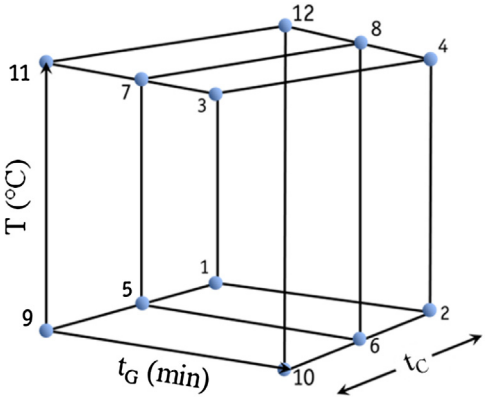


Fig. 2. Design of Experiments (DoE), used to obtain the 3-D-models. The experiments 1, 2, 5, 6, 9 and 10 were carried out at the low temperature, (i.e. 20 °C), the experiments 3, 4, 7, 8, 11 and 12 at the high temperature (i.e. 40 °C). The experiments 1, 3, 5, 7, 9 and 11 were carried out with a steep gradient (i.e. t_G = 3 min), the flat gradient experiments 2, 4, 6, 8, 10 and 12 (i.e. t_G = 9 min). The ternary composition (t_C) of eluent B (the ratio of AcN vs. MeOH) could be 100% AcN with run 1, 2, 3 and 4, it could be AcN/MeOH = 50/50 v/v% with run 5, 6, 7, and 8 and it could be 100% MeOH with runs 9, 10, 11 and 12.

time (Fig. 3a vs. b). Methanol is a good solvent for terazosin, but in this case the peak of component 1. splitted, as shown in Fig. 3b.

This problem can be solved by injecting a smaller volume. Using a loop with 1 μL volume in the UPLC system allows injecting 0.2 μL with the "partial loop with needle overfill" (PLUNO) injection mode. This solution requires greater sample concentration to maintain the quantity of the injected sample. 1 mL of methanol dissolves 5 mg of terazosin, so the amount of the injected sample is the same as before. In addition the previous problems were solved (Fig. 4).

Flow-through-needle (FTN) injection mode is also feasible. It is an important information because nowadays many UHPLC instruments apply FTN injection mode and not off-line loop samplers.

2.6. Effect of mobile phase pH

During the investigation of the pH-dependence of the method, it occurred, that there was no retention time change between pH = 10–11 (Fig. 5a–c). However the retention time of Impurity B, which contains a phenolic OH-group, increased with decreasing pH. At pH = 9 the retention time of Impurity B increased so much that it eluted after Impurity M and Impurity C and coeluted with Impurity A. (Fig. 5d). Under pH = 9 the form of Impurity M continuously deteriorated, and the retention of Impurity N and Impurity L were decreasing (Fig. 5e–f). For pH adjustment under pH 10 ortho-phosphoric acid was used. The 0.1% NH_4OH-solution was titrated, until we received the desired pH value. To achieve the pH 11-solution we had to prepare and use a 0.4 v/v% NH_4OH-solution.

Summarizing, the use of 0.1 v/v% NH_4OH-solution was optimal, because it ensured, that the pH value was above 10 and it could be taken in water-organic mixtures without any precipitation. The preparation of the eluent is extremely simple: add 500 μL of 25 v/v% ammonium hydroxide in 500 mL eluent. The pH value of the 0.1 v/v% NH_4OH-solution is 10.7.

3. Results and discussion

3.1. Mass spectrometry supported peak tracking

Chromatographic modeling, including the possibility to calculate a model chromatogram, requires a decent peak tracking. This means, we need to know, where each peak elutes in a chromatogram in all basic runs, which are created and used to calculate a separation model. This can be done by comparing the peak areas,

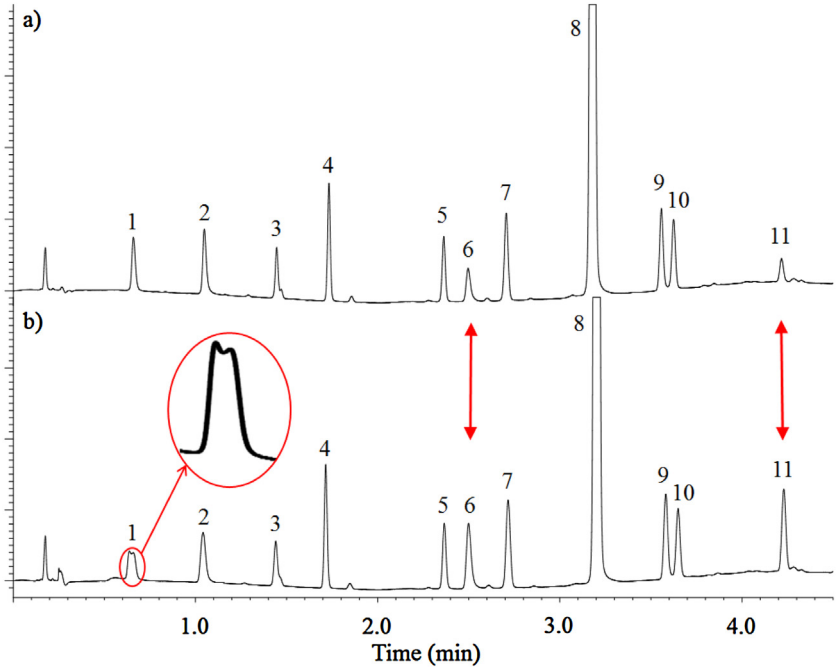


Fig. 3. 1 μL sample injection dissolved in the weak solvent A (a) and in pure MeOH (b).

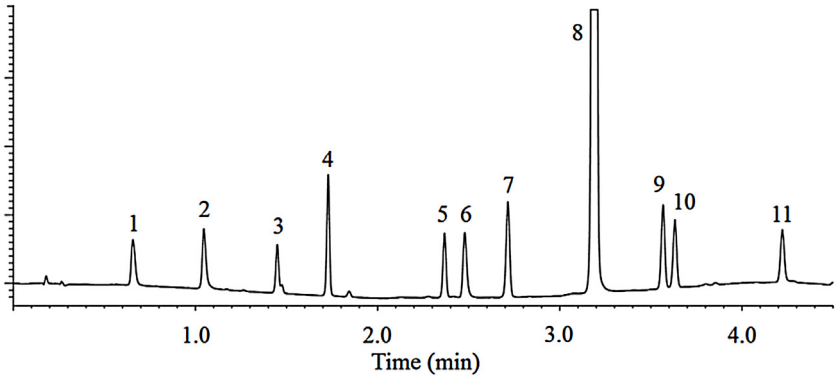


Fig. 4. Chromatogram using a sample injection of 0.2 μL.

because peak areas are expected to remain constant in a tG-T-model, so far the sample, the injected sample amount, the flow-rate and detection wavelength are remaining constant.

This procedure reaches its limitations, when the peaks have similar areas, or if the integration leads to stronger variation of peak areas, which often occurs with very small peaks. In case of similar peak areas UV-spectra might support the correct peak assignments.

A good alternative to find a certain peak in different chromatograms is the property of the molecular mass of the compound, due to its high specificity. Liquid Chromatography–Mass Spectrometry (LC–MS) is now a proven technique and more and more available in HPLC laboratories. The main disadvantage of using MS spectra for peak tracking may be that not all compounds will give a suitable MS signal under different elution conditions (see Fig. 6).

The ideal case to use MS data for peak tracking would be, if all sample components and their masses are known. If we have unknown components, but have retention times from UV detection, we can look for molecular masses under each peak, which follows the typical peak shape, increasing at the peak start, going through a maximum and decreasing to baseline at the tail of the peak. This mass then would belong to that certain peak and we can look for this mass in the other chromatograms of the DoE. It is therefore advisable to allow entering the molecular mass into the peak tracking data table. The latest DryLab® 4 is offering both: UV peak area – ***and*** molecular mass values for tracking peak movements. In this way the automation of the complex task "Peak Tracking" is becoming easier possible.

3.2. Calculation of a 3D-Critical resolution Space(CRS) called also method operable design region (MODR)

In Fig. 7c we can see, that at 100% MeOH the system has a range where $R_{s,crit} > 1.5$, but the system is not suitable at AcN-MeOH mixture and AcN-rich eluents. With 100% AcN the resolution between Imp M, Imp C and Imp A is small ($R_{s,crit} < 1.5$) and the peak elution order is altered. In case of AcN-MeOH mixture the peak pair Imp J-Imp K is exhibiting partial overlap and the retention of peak pair Imp B-Imp O is changed. Using 100% MeOH as eluent B, we achieve a much better separation of the critical impurity components. In this case we can see, that methanol is the better solvent compared with acetonitrile or ternary mixtures of AcN with MeOH, as suggested in the Ph.Eur. method.

The best Working Point (WP) was: $t_G = 6$ min (10–90% B), $T = 30\,^{\circ}C$, t_C = 100% MeOH + 0.1% cc. NH_4OH-solution in water. The

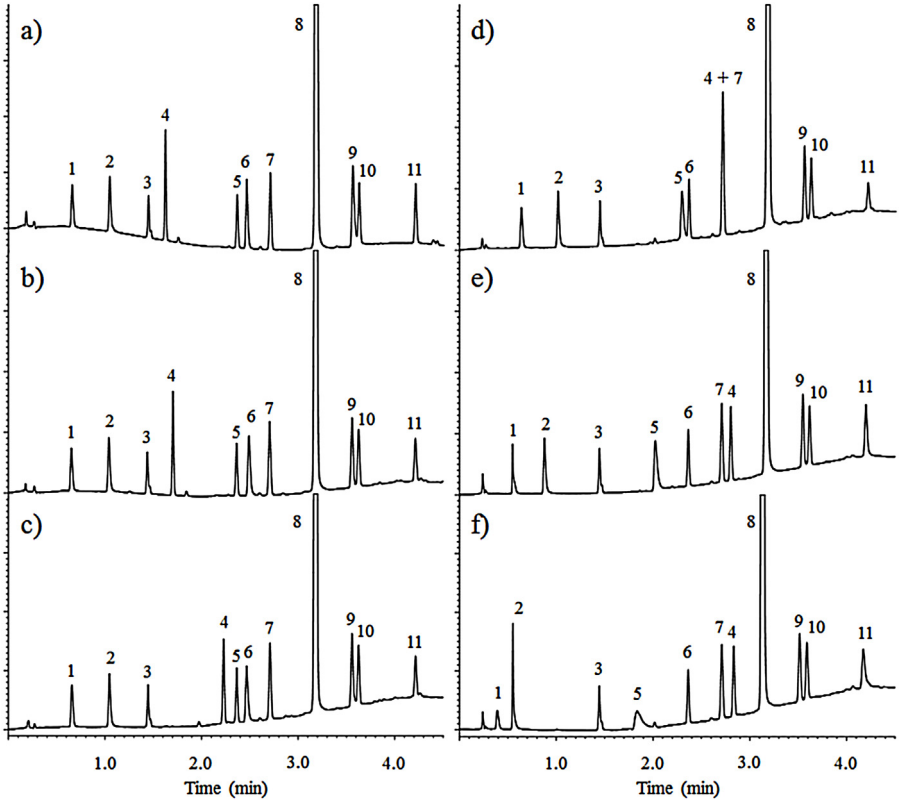


Fig. 5. Effect of mobile phase pH. a) pH = 11.0; b) pH = 10.7 (0.1 v/v% NH_4OH-solution); c) pH = 10.0 d) pH = 9.0; e) pH = 8.0; f) pH = 7.0

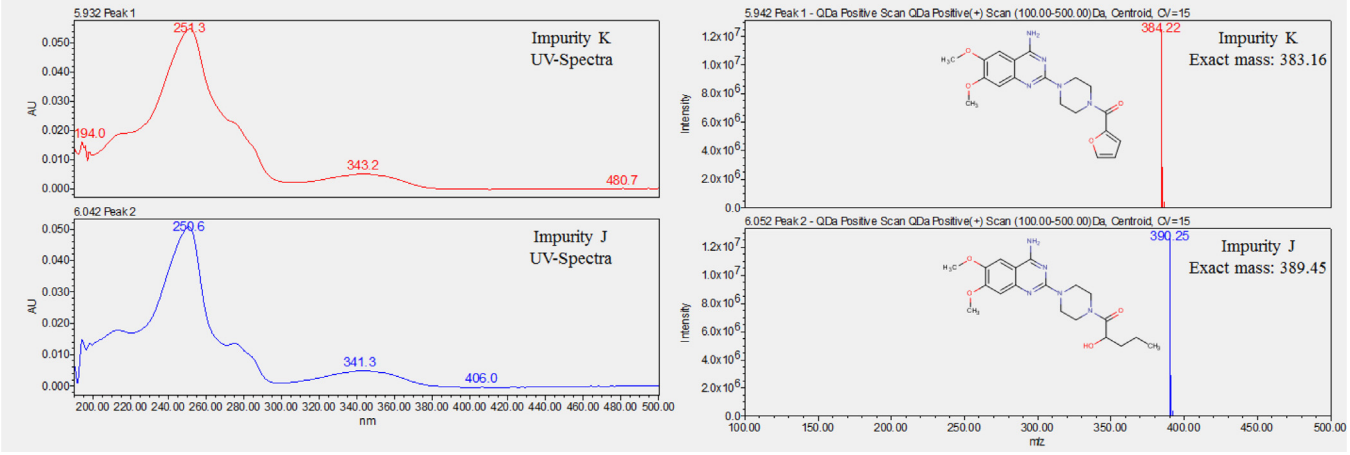


Fig. 6. UV- and mass spectra of Impurity K and Impurity J. In this case the peak areas of Impurity K and Impurity J and also the UV spectra are very similar so there is not much chance to differentiate them from each other. A great help is here to be able to use the *m*/*z* values which are different enough and allow a unique identification of these impurities.

Table 2
Predicted and experimental retention times and measured masses.

Peak Number	Name	Predicted t_R (min)	Experimental t_R (min)	Measured mass
1	Impurity N	0.65	0.66	185.09
2	Impurity L	1.00	1.05	181.03
3	Impurity O	1.44	1.45	283.12
4	Impurity B	1.71	1.73	389.21
5	Impurity M	2.36	2.37	275.07
6	Impurity C	2.47	2.48	290.13
7	Impurity A	2.70	2.72	240.00
8	Terazosin	3.18	3.19	388.20
9	Impurity K	3.56	3.57	384.22
10	Impurity J	3.62	3.63	390.25
11	Impurity E	4.21	4.22	493.29

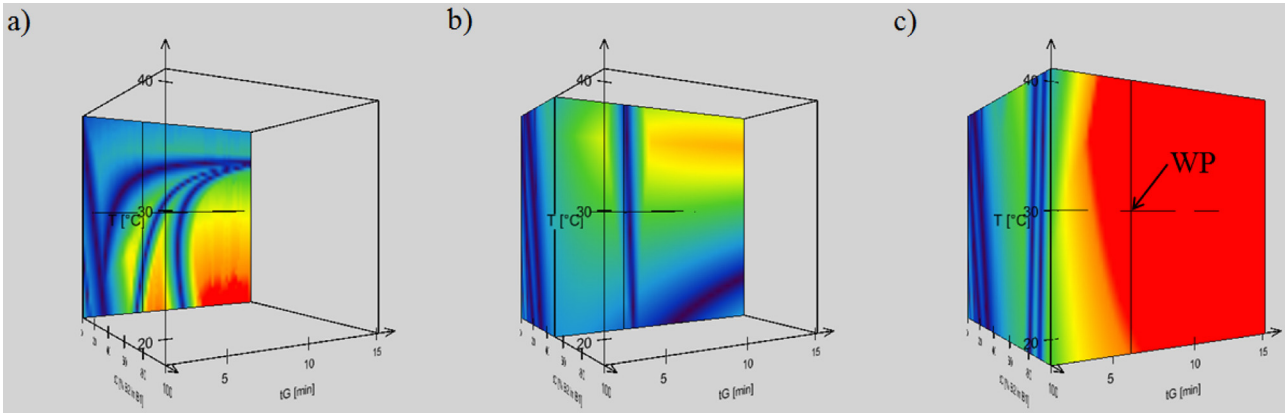


Fig. 7. 3D-critical resolution Cube with different organic modifiers, a) B1: 100% AcN, b) (B1:B2)(50:50)(AcN:MeOH)(V:V), c) B2: 100% MeOH. Red colors mean regions above $R_{s,crit} > 1.5$ (baseline resolution of the critical peak pair), blue colors indicate coelution ($R_{s,crit} = 0$) of the closest ("critical") peak pair. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

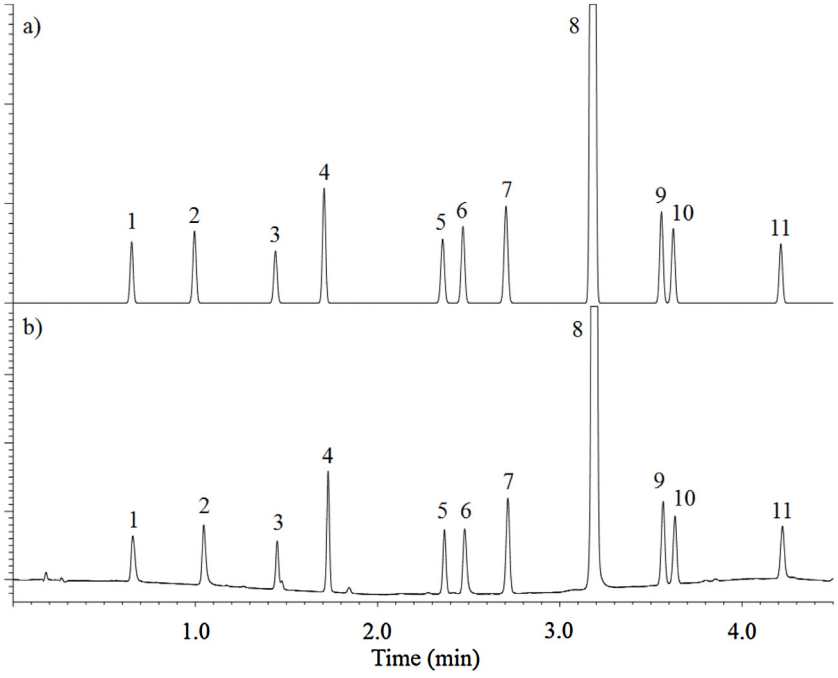


Fig. 8. Predicted a) and experimental b) chromatograms. $R_{s,crit} = 1.82$ between Imp K and Imp J in predicted chromatogram and $R_{s,crit} = 1.82$ also between Imp K and Imp J in experimental chromatogram.

gradient steepness is 13.33%B/min. With this steepness we can reduce the analysis time to 4.5 min, the last component is eluted at 4.2 min (Table 2). The final eluent composition is at 70%B.

Fig. 8 shows the a) predicted and b) the measured chromatograms. Table 2 shows that correlation between modelled and measured retention times was excellent. The average deviation of the retention times is 0.5 s, as long as the $R_{s,crit}$ in both cases is 1.82. This precision is revolutionary in separation modeling.

4. Summary

In this work the goal was to actualize the method described in the European Pharmacopoeia (Ph.Eur.) for the related substances measurement of terazosin. Since the original description contained two 45 min (=90 min) long HPLC runs, it was time to update the old method to a more current and rapid one.

At the beginning the best ratio between the injected sample solvent and the sample concentration was determined. The 1 μL injection volume was not sufficient, because in the weak solvent the sample could not be completely dissolved and in MeOH the shape of the first peak was deteriorated.

The solution was to inject the least possible volume, but in this case it was necessary to increase the sample concentration, to achieve a reasonable signal to noise ratio.

Also the pH of the mobile phase influenced the success of the separation. Decent chromatograms were achieved above pH = 10, but for this a pH-stable stationary phase was needed, like Acquity CSH C18 hybrid column, 50 × 2.1 mm 1.7 μm.

For resolution- and retention modeling the DryLab4 method development software (Version 4.2) was used. In recent versions of the software the use of (*m*/*z*)-MS-data is possible along the UV-peak-area-tracking-technology. The modelled and measured chromatograms showed excellent correlations. The average retention time deviations were 0.5 s. There was no difference between the $R_{s,crit}$ −values.

At the end of the method development only one method with 4.5 min analysis time was achieved instead of two methods with $2 \times 45 = 90$ min run time. In this way an old pharmacopoeia method was rejuvenated. The fact, that DryLab modeling was involved makes the method adjustment more flexible since alteration of the variables in the Design Space is not considered to be a "Change". This means, after the adjustment of the "set point" (odder working point) inside of the MODR, the revalidation of the method is not necessary.

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