Simultaneous Quantification of Related Substances of Perindopril Tert-Butylamine Using a Novel Stability Indicating Liquid Chromatographic Method

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Received 14 August 2014; revised 26 November 2014

A novel stability indicating gradient reverse-phased high-performance liquid chromatographic method has been developed for the quantification of impurities of perindopril tert-butyamine (PER) in pharmaceutical dosage form. Separation of the active substance and its known (Impurities B, C, D, E, F) and unknown impurities was achieved on a BDS Hypersil C18 column (250 mm × 4.6 mm, 5 µm), thermostated at 70°C, using a mobile phase comprised of aqueous solution of sodium 1-heptanesulfonate adjusted to pH 2 with perchloric acid and acetonitrile. The flow rate was maintained at 1.5 mL min−1, injection volume of 20 µL was utilized and detection of analytes was performed at 215 nm. The developed method was validated in accordance with current ICH Guidelines for all suggested parameters, including forced degradation studies and proved to be linear, accurate, precise and suitable for the impurity testing of PER, being subsequently applied during on-going stability studies of a newly developed generic formulation.

Introduction

Perindopril is a member of the angiotensin-converting enzyme (ACE) inhibitors class, which prevent conversion of angiotensin I to angiotensin II resulting in a reduction of angiotensin II in the plasma and increasing vasodilator bradykinin level, being successfully utilized in numerous cardiovascular diseases, including treatment of hypertension, heart failure and ischemic heart disease (1, 2). It acts as a prodrug, the ester being hydrolyzed to form the diacid perindoprilat, a potent, long-acting metabolite (3–5).

Oral administration of perindopril is generally via its tert-butyamine salt, however, in some countries from climatic zones III and IV, perindopril arginine is also available, which is therapeutically equivalent to the previous, but more stable (6).

ACE inhibitors in general are relatively unstable and can undergo degradation via hydrolysis of the side chain ester group, intramolecular cyclization, isomerization at chiral carbon atoms and oxidation. In the case of PER, main degradation pathways include hydrolysis of the ester group, especially in alkaline media, to form the diacid derivative, perindoprilat (Impurity B) and intramolecular cyclization, under excessive heat, to form the diketopiperazine derivate (Impurity F). Other degradants include Impurity C and D, which are epimers, formed after further hydrolytic degradation of Impurity F (5, 7, 8). Along with the mentioned degradation products, perindopril isopropyl ester (Impurity E) is also listed as specified impurity in perindopril erbumine tablets monograph of the British Pharmacopoeia (BP) (9). Chemical structure of PER and related substances are presented in Figure 1.

A literature survey reveals several methods for quantitative determination of degradation products of PER in pharmaceutical dosage forms. The official BP monograph proposes a 65-min gradient RP-HPLC method, using water adjusted to pH 2.5 with perchloric acid as mobile phase A and 0.03% solution of perchloric acid in acetonitrile as mobile phase B (9). Apart from its time consuming nature, the method did not succeed in providing adequate peak shape in the case of Impurity E.

Medenica et al. (5) described a simple isocratic HPLC method for the simultaneous determination of PER and its impurities: perindoprilat (Impurity B), Y31 (Impurity F), Y32 (Impurity C) and Y33 (Impurity D). Unfortunately, when employing the proposed method, insufficient retention of Impurity B was observed along with inadequate resolution between Impurities C, D and PER. Moreover, the method was not validated for the quantitative analysis of Impurity E and the stability-indicating nature of the technique has not been demonstrated, failing to meet current regulatory requirements. The same problems arose in the case of the microemulsion liquid chromatographic method described by members of the same research group (10).

Zaazaa et al. reported the development and validation of two stability-indicating methods (SIMs) for the impurity testing of amloclpine besylate and perindopril arginine in combined dosage forms (11). However, both TLC-densitometric and RP-HPLC methods were validated only for the quantitative determination of perindoprilat (Impurity B), but not the other specified impurities.

When developing SIMs, a clear definition needs to be made between specific and selective SIMs. As highlighted by Bakshi and Singh (12), specific SIMs are able to unequivocally determine and quantify the active pharmaceutical ingredient (API) of interest in the presence of degradation products and excipients. Selective SIMs, however, can be used to quantify both the API and its degradation products in the presence of excipients and possible additives. Thus, it is able to separate the API and all degradation products and it is also specific, i.e., can be used to quantitatively measure them. Apart from the selective SIMs, a number of specific SIMs have been developed for the quantitative determination of PER, in the presence of excipients and degradation products (13–16). However, in our case, where the quantitative assessment of PER impurities was of primary importance, these methods cannot be used successfully.

As it can be seen, none of the methods described in the literature deals with the quantitative assessment of all impurities specified in the official monograph, while successfully discriminating between them and unknown degradation products.
formed under stressed conditions. Our aim was the development and extensive validation of a rapid liquid chromatographic method, which fulfills the abovementioned criteria and meets current regulatory requirements.

**Experimental**

**Reagents and chemicals**

PER working standard (99.7% purity) was supplied by Aurobindo Pharm, Hyderabad, India. The related substances for PER (Impurities B, C, D, E and F) were purchased from Molcan Corporation, Toronto, Canada. Super-gradient grade acetonitrile, perchloric acid 70% and heptane-1-sulfonic acid sodium salt were products of VWR International, purchased through a local vendor (Bioaqua Group, Tîrgu Mureș, Romania). Ultrapure, deionized water was produced by a Millipore Synergy UV (Millipore, Molsheim, France) water system. Tablets containing 8 mg PER as well as the placebo mixture were supplied by the Production Department of Vim Spectrum Pharmaceutical Company, Corunca, Romania.

**Instrumentation and analytical conditions**

The HPLC system was a Finnigan Surveyor model (Thermo Finnigan, San Jose, USA) equipped with a quaternary pump, an autosampler with column thermostat and a photodiode array (PDA) detector. Data acquisition was performed using Chromquest 5.0 software. Separations were carried out on a Hypersil™ BDS C18 250 × 4 mm, 5 μm particle size column (Thermo Fischer Scientific, Waltham, USA), thermostated at 70°C. Mobile phase A consisted of 1.46 g heptane-1-sulfonic acid sodium salt dissolved in 1,000 mL ultrapure water, adjusted to pH 2 ± 0.05 with perchloric acid 70%, while acetonitrile was used as mobile phase B. After preparation, both mobile phases were vacuum-filtered through 0.45 μm nylon membrane filters and degassed in an ultrasonic bath, prior to use. Gradient elution was employed at a flow rate of 1.5 mL min⁻¹ according to the conditions described in Table I. Injection volume was 20 μL and detection was performed at 215 nm. In the case of forced degradation studies, in order to express peak purity values, the PDA detector was used in scan mode from 190 to 360 nm.

**Preparation of solutions**

All solutions were prepared using a solvent mixture of 67% mobile phase A and 33% acetonitrile.

**PER stock solution**

PER working standard, 8.0 mg, was accurately weighted and dissolved in 100 mL solvent mixture in a volumetric flask. Then, 5 mL of the obtained solution was further diluted to 50 mL with solvent mixture (concentration: 16 μg/mL PER).

**Impurity B, C, D, E, F stock solution**

Separate stock solutions for all impurities were prepared by weighting 4.0 mg of each impurity standard in separate volumetric flasks and dissolving them in 50 mL solvent mixture (concentration: 8 μg/mL for each impurity).

**Standard solution**

To prepare standard solution, 5 mL of PER stock solution and 5 mL of each impurity stock solution were transferred in a 50-mL volumetric flask and diluted to sign with solvent mixture (concentration 1.6 μg/mL PER, and 8 μg/mL Impurities B, C, D, E, F).

**Sample solution**

To prepare sample solution, 20 tablets, randomly taken, were finely powdered and a quantity corresponding to 80 mg PER was accurately weighed in a 100-mL volumetric flask. Then, 50 mL solvent mixture was added and the solution was sonicated for 30 min. After cooling, the solution was diluted to sign with solvent mixture (concentration: 800 μg/mL PER).

**Placebo solution**

The same amount of placebo mixture (containing lactose monohydrate, microcrystalline cellulose, magnesium stearate and colloidal silicon dioxide) as in the case of sample solution was weighed and prepared accordingly.

Prior to injection, all solutions were filtered through 0.45 μm polyamide filters (Macherey-Nagel, Düren, Germany).
Method validation

Method validation was carried out in accordance to ICH (17) and current regulatory guidelines, with respect to specificity (including forced degradation study), linearity, accuracy, precision (repeatability, system precision and intermediate precision), limits of detection and quantification. Robustness testing, including stability of analytical solutions, was also performed.

Specificity and forced degradation study

In order to test the specificity of the method the chromatograms of placebo, standard and sample solution were recorded. The placebo and tablet samples were also subjected to stress degradation studies as follows:

(a) Acidic hydrolysis: the same amount of tablet powder, respectively placebo was weighed as in the case of sample solution and treated with 3 mL HCl 1 N and HCl 0.1 N. After 3 h, solutions were neutralized with 3 mL NaOH 1 N and NaOH 0.1 N. Further preparation of the solutions was the same as in the case of the sample solution.

(b) Basic hydrolysis was carried out the same way as acidic hydrolysis, only in this case, both sample and placebo were treated with 3 mL NaOH 1 N and NaOH 0.1 N and after 3 h neutralized with 3 mL HCl 1 N and HCl 0.1 N.

(c) Oxidation with H2O2: the weighed tablet powder and placebo was treated with 3 mL H2O2 30 g/L and left for 3 h in dark. Further preparation of the solution was the same as of the sample solution.

(d) Heat stress: 3.0 g of tablet powder and placebo mixture were kept in a laboratory oven (BMT Vacucell 22, Brno, Czech Republic) at 105 °C for 3 h. After cooling, solutions were prepared from both powders in the same manner as in the case of the sample solution.

(e) Photolytic degradation: 50 mL solvent mixture was added to the weighed tablet powder and placebo. The solutions were subjected either to UV irradiation at 254 nm in a black box or natural light and left for 3 h. The obtained solutions were further treated the same as the sample solution.

Linearity and range

For all analytes, linearity was assessed over a concentration ranging from LOQ to 120% of the acceptance criteria for each specified impurity and PER, respectively, stated in the official monograph of perindopril erbumine tablets (9). The acceptance criteria were the following: max. 1.5% for Impurities B and F; max. 0.6% for Impurities C and D; max. 0.4% for Impurity E. Unknown impurities are expressed in PER and limited to max. 0.25%.

In order to construct the calibration curve, five different concentrations were prepared in duplicate, employing appropriate dilutions from stock solutions.

Accuracy

Accuracy of the method was evaluated by spiking placebo mixtures with appropriate amounts of analytes across the same range as used in linearity studies at five different concentrations, each concentration being prepared in duplicate. Recovery percentages were calculated and results were evaluated by regression analysis.

Precision

Precision was determined by means of repeatability (intra-day) and intermediate precision (inter-day and analyst variation), analyzing the same batch of generic tablets in six replicates. System precision was also evaluated employing seven consecutive HPLC injections of the standard solutions.

Sensitivity

Sensitivity of the method was determined by establishing the limit of detection (LOD) and the limit of quantification (LOQ) for PER and all specified impurities at signal-to-noise ratios of 3:1 and 10:1, respectively. Stock solutions were sequentially diluted and injected until an appropriate signal-to-noise ratio was achieved.

Robustness testing

Experimental conditions were deliberately varied and resolution between Impurity C, Impurity D and PER were monitored in order to evaluate the robustness of the developed method. Typical variations included: temperature (70 ± 5 °C), pH of mobile phase A (2.0 ± 0.1), flow rate (1.5 ± 0.1 mL min⁻¹), counter-ion concentration (1.46 ± 0.1 g heptane-1-sulfonic acid in 1000 mL mobile phase A) and HPLC column batch (batch 10,975 and 13,055), while other chromatographic conditions remained unchanged.

Because sonication was used for the preparation of sample solutions, which causes a small increase in ultrasonic bath temperature, stability of the analytes was evaluated under these conditions. Two sets of sample solutions were prepared (n = 6), one using the exact same procedure as described earlier and the other set, employing agitation instead of ultrasonication. The results were compared for possible differences in individual impurity content of the two sets.

Stability of the analytical solutions was assessed over a period of time of 24 h. Six separate sample solutions were prepared and left in capped volumetric flasks at room temperature. Determinations were made immediately, 1, 2, 3, 6 and 24 h after preparation.

Results

Method validation

The developed method was validated according to current ICH Guidelines for the following parameters: selectivity, linearity, precision, accuracy, LOD and LOQ.

Selectivity of the method

Selectivity of the method was assessed by injecting the placebo, standard and sample solution. No significant interference at the retention times of PER and specified impurities were observed (Figure 2), showing that the developed method could unequivocally determine the analytes in the presence of interfering species. In order to prove the stability-indicating nature of the method and fulfilling the requirements of ICH Stability Guidelines (18), forced degradation studies were also performed as part of the selectivity study. Summary of the forced degradation study is shown in Table II. All degradation products formed
under stressed conditions were well separated from PER and its specified impurities. Furthermore, peak purity results obtained (peak purities >0.95 for all analytes) with the PDA detector indicated identical UV spectra, confirming the homogeneity of all peaks of interest.

**Sensitivity and linearity**

The LOD and LOQ of the method were experimentally determined by sequentially diluting stock solutions. Concentrations with a signal-to-noise ratio close to 3:1 and 10:1 were taken as LOD and LOQ, respectively. Calibration curves were constructed for each of the studied analytes across the range specified, by plotting peak areas against their corresponding concentrations. Obtained data were subjected to linear regression analysis, calculating the individual slope, Y-intercept and coefficient of determination for all analytes. Summary of the results of the linearity study alongside with sensitivity analysis of the method is given in TableIII. As part of the study, y-residuals were also calculated and plotted against concentration. In all cases, residuals were randomly distributed (results not shown).

**Accuracy and precision**

The developed method showed excellent accuracy of the analytes over the concentration range studied (Table IV), individual recovery values falling between the acceptable limits of 80–120%, with mean recoveries between 93.78% (PER) and 100.80% (Impurity B).

Precision of the method was tested at three levels namely, system precision, repeatability and intermediate precision. System precision indicates the performance of the chromatographic system and it is usually part of system suitability study. Acceptable relative standard deviation (RSD) values of multiple injections of a homogenous sample are usually below 5%, for evaluating system precision in the case of impurity testing. In our case, as results show, RSD values obtained were <1% for all analytes. In the case of repeatability (intra-day precision), RSD values <0% were obtained, while intermediate precision RSD values were <15%, all fulfilling required criteria (full results of the precision study are given in the Supplementary Material, Tables SI–SIV).

**Robustness testing**

The developed method proved to be robust for all of the selected parameters. Small, but deliberate variations of temperature, pH of mobile phase A, counter-ion concentration, flow rate and HPLC column batch did not significantly alter resolution between critical peak pairs.

Comparative results indicated that possible elevation of ultrasonic bath temperature due to sonication did not affect individual impurity content of the sample solutions. No differences were found between the two sets of sample solutions, prepared by ultrasonication and agitation, respectively.

Results indicated that sample solutions remained stable for at least 6 h after preparation, when left at room temperature (see Supplementary Material, Table SV). Quantification of impurities after 24 h of preparation showed an increase in Impurity B content (difference of 6.9%). Thus, it can be concluded that samples need to be analyzed not later than 6 h after preparation.

**Discussion**

**Method development**

Our aim was to develop a method suitable for selective quantification of PER and the impurities specified in the official BP monograph in a newly developed generic formulation. The method should also be able to distinguish between the specified and
unknown impurities, formed under stressed degradation conditions in order to meet current ICH Stability Testing Guidelines (18), development of a SIM being needed for the analysis of stability samples in the pharmaceutical industry (12, 19–22).

Several of the previously described methods were evaluated in order to fulfill the abovementioned requirements. However, as described, none of the methods could be efficiently applied in our case.

Insufficient retention of Impurity B was observed in most cases along with inadequate resolution between peaks corresponding to Impurities C, D and PER. In order to increase retention, heptane-1-sulfonic acid sodium salt was added to the mobile phase, as associates of PER with the ion-pair agent showed increased interaction with the apolar surface of the stationary phase (23). Furthermore, switching from "traditional" silica-based packing to base deactivated silica containing stationary phase greatly reduced peak tailing and improved symmetry. In the case of C-terminal proline-containing peptides, like PER and its related compounds, peak broadening and peak splitting were described during the elution process, due to the existence

| Table II |
| Results of the Stress Degradation Study |

<table>
<thead>
<tr>
<th>Condition</th>
<th>Normal</th>
<th>HCl 1 N</th>
<th>HCl 0.1 N</th>
<th>NaOH 1 N</th>
<th>NaOH 0.1 N</th>
<th>H2O2 30 g/L</th>
<th>Temperature 105°C</th>
<th>UV light</th>
<th>Natural light</th>
</tr>
</thead>
<tbody>
<tr>
<td>PER</td>
<td>Area%</td>
<td>P</td>
<td>Area%</td>
<td>P</td>
<td>Area%</td>
<td>P</td>
<td>Area%</td>
<td>P</td>
<td>Area%</td>
</tr>
<tr>
<td>Specified impurities</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Impurity B</td>
<td>0.63</td>
<td>0.98</td>
<td>2.16</td>
<td>1</td>
<td>0.72</td>
<td>0.95</td>
<td>80</td>
<td>0.99</td>
<td>9.65</td>
</tr>
<tr>
<td>Impurity C</td>
<td>0.1</td>
<td>0.99</td>
<td>0.02</td>
<td>0.98</td>
<td>0.02</td>
<td>0.99</td>
<td>0.01</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>Impurity D</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.35</td>
<td>0.96</td>
<td>0.05</td>
<td>0.98</td>
<td>ND</td>
<td>0.29</td>
</tr>
<tr>
<td>Impurity E</td>
<td>0.09</td>
<td>0.99</td>
<td>0.09</td>
<td>0.99</td>
<td>0.09</td>
<td>1.00</td>
<td>0.02</td>
<td>1.00</td>
<td>0.08</td>
</tr>
<tr>
<td>Impurity F</td>
<td>0.46</td>
<td>0.99</td>
<td>0.42</td>
<td>0.99</td>
<td>0.46</td>
<td>0.99</td>
<td>ND</td>
<td>0.3</td>
<td>0.99</td>
</tr>
<tr>
<td>Unidentified impurities (RRT)</td>
<td>0.37</td>
<td>0.16</td>
<td>0.97</td>
<td>0.11</td>
<td>0.95</td>
<td>0.14</td>
<td>0.87</td>
<td>0.04</td>
<td>0.97</td>
</tr>
</tbody>
</table>

P, purity of respective peak; RRT, relative retention time (with reference to PER).

| Table III |
| Summary of the Linearity and Sensitivity Study |

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PER</th>
<th>Impurity B</th>
<th>Impurity C</th>
<th>Impurity D</th>
<th>Impurity E</th>
<th>Impurity F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range (μg mL⁻¹)</td>
<td>0.13–2.5</td>
<td>0.30–15</td>
<td>0.20–6</td>
<td>0.09–6</td>
<td>0.09–4</td>
<td>0.09–15</td>
</tr>
<tr>
<td>Slope</td>
<td>45973.1</td>
<td>53920.8</td>
<td>41597.4</td>
<td>38242.9</td>
<td>45921.5</td>
<td>36139.4</td>
</tr>
<tr>
<td>Intercept</td>
<td>304.1</td>
<td>3104.2</td>
<td>108.1</td>
<td>580.1</td>
<td>1172</td>
<td>858.2</td>
</tr>
<tr>
<td>r²</td>
<td>0.9999</td>
<td>0.9999</td>
<td>0.9999</td>
<td>0.9999</td>
<td>0.9998</td>
<td>0.9999</td>
</tr>
<tr>
<td>LOD (μg mL⁻¹)</td>
<td>0.06</td>
<td>0.12</td>
<td>0.08</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>LOQ (μg mL⁻¹)</td>
<td>0.13</td>
<td>0.30</td>
<td>0.20</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
</tr>
</tbody>
</table>

r², Coefficient of determination.

| Table IV |
| Accuracy of the Method |

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PER</th>
<th>Impurity B</th>
<th>Impurity C</th>
<th>Impurity D</th>
<th>Impurity E</th>
<th>Impurity F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean recovery (%)</td>
<td>93.78</td>
<td>100.80</td>
<td>93.81</td>
<td>95.82</td>
<td>96.56</td>
<td>98.56</td>
</tr>
<tr>
<td>SD</td>
<td>8.49</td>
<td>5.77</td>
<td>2.84</td>
<td>4.23</td>
<td>4.74</td>
<td>1.82</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>9.05</td>
<td>5.73</td>
<td>3.02</td>
<td>4.42</td>
<td>4.91</td>
<td>1.85</td>
</tr>
</tbody>
</table>

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of both cis–trans isomers (24–27). The same peak splitting was observed during method development, however, only in the case of Impurity E. Performing the chromatographic separation on higher temperatures, as others described (23, 26, 28), led to an improvement in peak shape, but did not resolve the problem completely as peak splitting was still observed. Higher organic content of the mobile phase was necessary in order to elute Impurity E as a single peak, but unfortunately, in this case Impurity B could not be retained on the column. In order to fulfill both requirements (adequate retention of Impurity B and elution of Impurity E as a single peak), a gradient program was developed, employing a mobile phase with lower organic content at first (retention of Impurity B) and then switching to higher organic content (proper peak shape for Impurity E). Using the optimized gradient, described earlier, we were able to separate PER and its related impurities in under 10 min (including re-equilibration time), with resolutions between peaks greater than 2.

**Forced degradation studies**

As described by Dugga et al. (13), PER proved to be relatively stable under acidic and oxidative degradation conditions, showing only minor degradation, mostly to Impurity B. However, when the dosage form was subjected to basic conditions, a much drastic degradation of the active substance was observed. The main degradation product was still Impurity B (80%, when PER was exposed to NaOH 1 N), with other impurities accounting for <1%.

Significant degradation of PER was also observed under thermal stress conditions. Subjected to dry heat, the active substance decomposed mainly into Impurity F (58.10%), while other specified impurities were <1%. Among all stress conditions employed, thermal degradation gave rise to the highest number of unknown impurities; however, as observed from peak purity results, none of these interfered with the detection and quantification of the specified impurities.

Under the applied conditions, PER showed only slight photolytic degradation, Impurity B being the main degradation product (1.12% in both cases).

**Conclusion**

A new SIM for impurity testing of PER in tablet dosage form was developed and validated according to current ICH Guidelines, in order to meet local regulatory requirements. The separation and quantification of PER and its impurities specified in current BP monograph were achieved in under 10 min, using a newly developed gradient elution program. The developed method resolved the unsatisfactory retention of Impurity B, co-eluted peaks of Impurities C, D and PER and also the peak splitting observed in the case of the later eluting Impurity E, observed during the development process.

Forced degradation studies were also conducted in order to challenge the method if it could differentiate between the specified and unknown impurities, formed under stressed degradation conditions and in order to meet current ICH Stability Guidelines.

The method was successfully validated according to current ICH Guidelines and also meets current national regulatory requirements.

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**Supplementary Material**

Supplementary Materials are available at *Journal of Chromatographic Science* online.

**Acknowledgments**

This article was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007–2013, project no. POSDRU/159/1.1.5/S/136893. The first author would like to thank Collegium Talentum for their financial support.

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