Determination of Degradation Products of Doxercalciferol by Solid-Phase Extraction and Reversed-Phase HPLC

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In the current study, injectable formulations containing Doxercalciferol as the active pharmaceutical ingredient are analyzed by using gradient-elution high-performance liquid chromatography with ultraviolet detection. Various related impurities and degradants are quantified by using solid-phase extraction (SPE) for enhanced sensitivity. The assay of possible related impurities and Doxercalciferol analogues present at trace quantities is performed by using Trans-1-α-hydroxy vitamin D2 (Doxercalciferol related degradation product/Impurity B) as standard and 1-β-hydroxy vitamin D2 (Doxercalciferol related degradation product/Impurity C) as internal standards for the SPE study. The current method is shown to be stability-indicating and free from interferences from any of the formulation excipients and potential degradation products and impurities. The validated method is shown to be reproducible, accurate, sensitive and selective.

Introduction

Doxercalciferol (1α-hydroxyvitamin D2) is a synthetic analogue of vitamin D that is used for the treatment of secondary hyperparathyroidism in patients with chronic kidney disease (CKD) (1). Also, 1α-hydroxy previtamin D2 (Impurity/Degradant A), which can exist in equilibrium with the parent compound (Doxercalciferol) in post-compounding solutions, is considered to be an active analogue of Doxercalciferol and can be quantified by using the current method. High-performance liquid chromatography (HPLC) coupled with solid-phase extraction (SPE) allows for a sensitive and quantitative assay of potential impurities and degradants in formulations of Doxercalciferol (2–8). SPE in the current study is used for the preconcentration and subsequent assay of the lipophilic impurities that may be present in trace amounts. Formulations of Doxercalciferol (2 µg/mL) containing butylated hydroxytoluene (BHT; 10–20 µg/mL) are analyzed by using the current method with good precision, accuracy, sensitivity and selectivity.

Experimental

Materials and reagents

The qualified house standard Trans-1-α-hydroxy vitamin D2 (Impurity B) was used as a standard. The qualified house standard 1-β-hydroxy vitamin D2 (Impurity C) was used as the internal standard. BHT was acquired from Sigma-Aldrich (St. Louis, MO). Acetonitrile (HPLC grade) was acquired from J.T. Baker (Phillipsburg, NJ). Water (HPLC grade) was from J.T. Baker. All other reagents used in this work were of the highest purity and used as received. Waters OASIS HLB Plus SPE cartridges and reservoirs (30 cc) were used for sample loading. Test tubes (125 × 16 mm) were used for sample collection. Alltech vacuum manifold, vacuum gauge valve and glass chamber, polypropylene lid, stopcock valves and collection rack plates were used (Deerfield, IL). The placebo was prepared by mixing the following ingredients in a 1 L volumetric flask containing approximately 400 mL of water: 1.50 g of sodium chloride, 14.40 g of sodium phosphate dibasic heptahydrate, 1.80 g of sodium phosphate monobasic monohydrate, 1.10 g of disodium ethylenediaminetetraacetic acid (EDTA) dihydrate and 10.00 g of polysorbate 20. The ingredients were dissolved with the use of a stir bar. Next, 50 mL of a 0.40 mg/mL solution of BHT in ethanol were added and the contents were diluted to volume with water and mixed for an additional 5 min.

Preparation of the internal standard solution

The working internal standard solution (16 µg/mL) was prepared by diluting 8.0 mL of the 100 µg/mL stock solution prepared in methanol to 50 mL with placebo.

Working sample preparation using SPE

The required number of HLB Plus SPE cartridges (one per sample preparation) was preconditioned by using 6 mL of methanol at a rate of approximately 1 mL/min, followed by 6 mL of water at approximately 1 mL/min by using the vacuum manifold apparatus. It was ensured that the SPE cartridges did not run completely dry subsequent to the water rinse. The working sample preparation was made by premixing 25.0 mL of the Doxercalciferol formulation (20 µg/mL) with 320 µL of the internal standard solution (16 µg/mL) in a glass container. The premixed sample was quantitatively transferred into the 30 cc reservoir fitted onto the preconditioned SPE cartridge. The entire sample solution was allowed to flow through the cartridge at a rate not exceeding approximately 1 mL/min and the effluent was discarded. Each sample-loaded cartridge was rinsed with 6 mL of water at approximately 1 mL/min and the water rinses were discarded. The loaded sample was eluted from the cartridge into the collection test tube with 5.0 mL of methanol at a rate not exceeding approximately 1 mL/min. Collection was continued until no further eluate was dripping from the cartridge. The contents of each test tube were carefully vortex-mixed and subsequently transferred into an HPLC vial for analysis.

Preparation of standards of impurity B

A stock standard solution (40 µg/mL) prepared in methanol was used to prepare two intermediate stock standard solutions...
(IS1: 2.0 μg/mL and IS2: 3.2 μg/mL) with dilutions made with placebo. IS1 and IS2 were used to prepare four additional secondary intermediate stock solutions (IS3: 0.10 μg/mL, IS4: 0.16 μg/mL, IS5: 0.64 μg/mL, IS6: 1.28 μg/mL) with dilutions made with placebo. The secondary intermediate stock solutions were subsequently used to prepare the working standards without further dilution, as shown in Table I. The prepared working standards (I through N) represented approximately 0.2, 0.32, 0.6, 0.96, 1.28 and 2.56%, respectively, of Impurity Degrabadant B relative to the amount of Doxercalciferol in the 25 mL sample loaded onto the HLB Plus SPE cartridge.

**HPLC instrumentation**

The HPLC system was an Agilent 1100 or 1200 series (Agilent Technologies, Inc., Santa Clara, CA), consisting of a quaternary pump, vacuum degasser, column oven, thermostated autosampler and Agilent software (Chemstation). The detector was an Agilent 1100 diode array ultraviolet-visible (UV-VIS) detector. The analytical column was an Alltech Alltima HP C18, 150 x 4.6 mm, 3 μm.

**Chromatographic conditions**

Table II lists the chromatographic operating conditions used for the assay of Doxercalciferol-related impurities and degradants.

**Results**

Table III lists the relative retention times (RRTs) and relative response factors (RRFs) for various Doxercalciferol analogues and degradants. The RRTs were calculated relative to the retention times of Doxercalciferol as the parent compound. The RRF was calculated as the ratio of peak area and concentration of a given impurity to that of Impurity B. The RRFs for Impurities A and C were calculated to be 0.32 and 0.26, respectively.

The various Doxercalciferol impurities and analogues examined in this work are shown in Figure 1. Figures 2A and 2B depict chromatograms of pre-SPE Doxercalciferol (2.0 μg/mL) and placebo formulations. Figures 2C and 2D depict chromatograms of post-SPE working samples spiked with internal standard and impurity B.

As part of the specificity study, Figures 3A and 3B depict chromatograms of placebo (pre-SPE) and Doxercalciferol injection (2.0 μg/mL) (pre-SPE) treated with 30% hydrogen peroxide and heated at approximately 80°C for 20 min with approximately 36% degradation of Doxercalciferol. A representative regression plot for Impurity B is shown in Figure 4. The amount of analyte (Impurity B) in the standard solutions ranged from approximately 0.10 to 1.28 μg. A correlation coefficient \( R^2 \) value of ≥ 0.99 was obtained for all regression plots constructed as part of the system suitability validation experiments.

The limit of quantitation (LOQ) for Impurity B ranged from approximately 0.20% (w/w) to 0.90% (w/w) (versus amount of Doxercalciferol in the 25 mL sample). Six replicate injections of the 0.90% (w/w) Impurity B-spiked placebo solution performed as a repeatability test yielded the results shown in Table IV.

Tables V–VII list the spike recovery (accuracy) data and the corresponding reproducibility of the accuracy data (percentage recovery values), which are denoted here as precision for quantities of Impurity B (approximately 0.90–1.5% of the amount of Doxercalciferol in the working sample solution) spiked into placebo.

**Discussion**

**Optimization of elution conditions and use of SPE**

With the chromatographic conditions used in this work, baseline separation for all impurities and degradants was achieved. Despite the absence of a mass spectrometer (e.g., liquid chromatography–mass spectrometry), peak purity was ensured with high certainty using Agilent peak purity software coupled with sufficient variations in mobile phase composition and ensuring the absence of coeluting impurities/degradants.

In this work, the RRF of Impurity A, a thermal degradant that is also considered to be an active component similar to Doxercalciferol and detected in all tested samples, was calculated to be 0.32. Because of an insufficient quantity of Impurity D in the laboratory and because it was detected in trace quantities (≤ LOQ) in only a few samples tested, a value of 1.00
(versus Impurity B) was assigned as its RRF. In addition, the RRF for any possible related unknown impurity/degradant was also assigned to be 1.00 (9).

Various relatively hydrophilic excipients were removed during the SPE sample loading step and did not interfere with the quantitation of Doxercalciferol-related impurities or degradants. The SPE cartridges used in this work consisted of the C18-bonded material. The antioxidant, BHT (10–20 μg/mL) used in the formulations, elutes early and is easily quantified coupled with Doxercalciferol in pre-SPE samples by using a binary standard solution of BHT and Doxercalciferol. A gradient step post-elution of the possible known Doxercalciferol analogue (Impurity D) was used in this work to ease the detection and quantitation of possible late eluting impurities and degradants. However, no such late-eluters were detected in any of the samples examined in this work.

With the SPE sample loading and desorption procedure used in this work, a reproducible magnification factor (post-SPE/pre-SPE peak area ratio) of approximately 4 × was obtained for Doxercalciferol and its various lipophilic analogues (Impurities A, B, C and D). This involved loading replicate 25.0 mL sample volumes of Doxercalciferol injection (2.0 μg/mL) containing sufficient spiked quantities of various available analogues with subsequent elution of the adsorbed material with replicate 3.0 mL volumes of methanol. Larger and smaller (versus 3.0 mL) volumes of methanol were also tested, which, as expected, resulted in smaller and larger peak areas, respectively, for the analytes. A volume of 3.0 mL was decided upon, because this represented a sufficient sample size for HPLC analysis and ensured maximum removal of the adsorbed material from the cartridge. The preconcentration step allowed for the quantitation of Doxercalciferol-related impurities/degradants present in quantities as low as approximately 0.2% (w/w) versus the amount of Doxercalciferol present in the analyzed samples. In all formulations analyzed in this work, Impurity C was undetected as a process impurity. This, coupled with its availability in the laboratory and baseline separation from adjacent peaks, was the reason for its selection as the internal standard.

**Method validation**

The validation experiments included: specificity (forced degradation for the purpose of optimization of chromatographic separation), robustness, LOQ, limit of detection (LOD), accuracy (spike recovery), linearity, precision and intermediate precision, and those required for method transfer (linearity and precision (reproducibility)) performed by a contract laboratory. The results obtained for the various validation
experiments met the proposed criteria in the method validation protocols. The results for the critical validation experiments performed for the current method are discussed in the following sections.

**Specificity**
Pre-SPE samples were force-degraded because these contained 100% of all matrix (placebo) components that could potentially undergo degradation and generate degradant peaks. The

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**Figure 2.** Figures 2A and 2B depict chromatograms of pre-SPE Doxercalciferol (2.0 μg/mL) and placebo formulations. Figures 2C and 2D depict chromatograms of post-SPE working samples spiked with internal standard and impurity B. Representative chromatograms: pre-SPE Doxercalciferol formulation (A); pre-SPE placebo formulation (B); post-SPE Doxercalciferol formulation spiked with internal standard (C); post-SPE Doxercalciferol formulation spiked with internal standard and Impurity B (D).
Reagents and conditions used for forced degradation included: 1 N HCl, 1 N NaOH, 30% hydrogen peroxide and heat (80°C). Selectivity was ensured using the UV-VIS detector and Agilent peak purity software coupled with variations in the composition of the mobile phase and ensuring the absence of co-eluting impurities/degradants. Inspection of the chromatograms of the treated and untreated working samples and placebo solutions following the procedure outlined in the validation protocol.

**Table IV**

Repeatability of Injections for the 0.90% (w/w) Impurity B-Spiked Placebo Solution

<table>
<thead>
<tr>
<th>Area B</th>
<th>Area C</th>
<th>Area ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.83678</td>
<td>31.85889</td>
<td>0.309</td>
</tr>
<tr>
<td>10.99379</td>
<td>31.67372</td>
<td>0.347</td>
</tr>
<tr>
<td>10.25789</td>
<td>32.15884</td>
<td>0.319</td>
</tr>
<tr>
<td>10.15839</td>
<td>32.23727</td>
<td>0.315</td>
</tr>
<tr>
<td>10.4799</td>
<td>31.93768</td>
<td>0.328</td>
</tr>
<tr>
<td>10.65758</td>
<td>32.76273</td>
<td>0.325</td>
</tr>
</tbody>
</table>

Average: 0.32  
RSD (%): 4.1

**Table V**

Accuracy and Precision Results for Process Impurity B at the Level of 0.90% (w/w) versus Doxercalciferol

<table>
<thead>
<tr>
<th>B added (µg)</th>
<th>Area of B in sample</th>
<th>Area of C in sample</th>
<th>Area ratio</th>
<th>B found (µg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.439</td>
<td>13.79687</td>
<td>38.45433</td>
<td>0.359</td>
<td>0.5026</td>
<td>114.5</td>
</tr>
<tr>
<td>0.439</td>
<td>11.30288</td>
<td>33.84547</td>
<td>0.334</td>
<td>0.4673</td>
<td>106.5</td>
</tr>
<tr>
<td>0.439</td>
<td>10.3363</td>
<td>30.16476</td>
<td>0.343</td>
<td>0.4798</td>
<td>109.3</td>
</tr>
<tr>
<td>0.439</td>
<td>13.0957</td>
<td>39.80973</td>
<td>0.329</td>
<td>0.4598</td>
<td>104.8</td>
</tr>
<tr>
<td>0.439</td>
<td>10.72748</td>
<td>31.97564</td>
<td>0.335</td>
<td>0.4695</td>
<td>107.0</td>
</tr>
<tr>
<td>0.439</td>
<td>9.79663</td>
<td>28.52018</td>
<td>0.343</td>
<td>0.4810</td>
<td>109.6</td>
</tr>
<tr>
<td>0.341</td>
<td>108.6</td>
<td>3.1</td>
<td>3.1</td>
<td>108.6</td>
<td></td>
</tr>
</tbody>
</table>

Average: 3.1  
RSD (%): 108.6
indicated no chromatographic interference with the quantitation of the analytes. Figures 3A and 3B show hydrogen peroxide-treated placebo and Doxercalciferol formulations.

**Linearity**

As stated previously, the amount of Impurity B in the standard solutions ranged from approximately 0.10 to 1.28 μg, or approximately 0.2 to 2.6% of the amount of Doxercalciferol in the 25 mL of Doxercalciferol formulation loaded onto the SPE cartridge (Table I and Figure 4). Regression plots were constructed by plotting the peak area ratios of varying amounts of impurity B (approximately 0.10 to 1.28 μg) to that of 5.12 μg of Impurity C versus the amount of Impurity B (in μg) used in each standard solution.

**LOQ for impurity B and repeatability**

With the use of different HPLC–UV detectors in the authors’ laboratory and those used at the contract laboratory, coupled with an average magnification factor of approximately 4:1 for Impurity B and other analogues, the LOQ for Impurity B ranged from approximately 0.20% (w/w) to 0.90% (w/w). The repeatability of the 0.90% (w/w) level LOQ was assessed by determining the RSD of six area ratios (Impurity B/Impurity C) from replicate injections, which yielded the results shown in Table IV.

**Accuracy and precision**

The accuracy/precision of the method was assessed by determining the recovery of known trace quantities of Impurity B (0.90–1.5% of the amount of Doxercalciferol in 25 mL of the 2.0 μg/mL Doxercalciferol injection) added to the placebo. All recovery values and the corresponding precision of the recovery values expressed as RSD are reported in Tables V–VII and met the acceptance criteria, as proposed in the validation protocol.

### References

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9. B’Hymer, C.; A gradient HPLC test procedure for the determination of impurities and the synthetic precursors in 2,4-(1-hydroxy-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]-butyl)-phenyl]-2-methyl-propionic acid; *Journal of Chromatographic Science*; (2006); 44: 200–204.