A SIMPLE AND SENSITIVE STABILITY-INDICATING UHPLC-DAD METHOD FOR THE DETERMINATION OF CEFETAMET PIVOXIL HYDROCHLORIDE

PIOTR GARBACKI1*, JUDYTA CIELECKA-PIONTEK1, PRZEMYSŁAW ZALEWSKI1, IRENA OSZCZAPOWICZ1 and ANNA JELIŃSKA1

1 Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Poznan University of Medical Sciences, Grunwaldzka 6, 60-780 Poznań, Poland
2 Department of Modified Antibiotics, Institute of Biotechnology and Antibiotics, Starościńska 5, 02-516 Warszawa, Poland

Abstract: A fast and sensitive UHPLC-DAD method was developed and subsequently validated for determination of cefetamet pivoxil hydrochloride in the presence of its degradation products. The chromatographic separation was carried out on a Waters Acquity BEH C18, (2.1 × 100 mm, 1.7 µm) column. The mobile phase was composed of 0.1% formic acid and acetonitrile (40:60, v/v) at the flow rate 0.7 mL/min. The detection wavelength was 265 nm and the temperature was 30°C. Cefetamet pivoxil hydrochloride was susceptible to degradation under the influence of sodium hydroxide, hydrochloric acid and in the conditions of increased temperature and relative humidity. However, it was stable after irradiation, in increased temperature in dry air and in the presence of oxidizing agent. The developed UHPLC-DAD method was linear over the concentration range of 10–240 µg/mL (r² = 0.9999; n = 12). The obtained RSD values were less than 2%, demonstrating that the described procedure is precise. The accuracy was also confirmed (mean recoveries were 97.79–102.08%). Under applied chromatographic conditions LOD and LOQ values were 2.08 mg/mL and 6.29 mg/mL, respectively. The proposed method was successfully applied in determination of cefetamet pivoxil hydrochloride in aqueous solutions as well as in the solid state.

Keywords: UHPLC-DAD, cefetamet pivoxil hydrochloride, stability

Cefetamet pivoxil hydrochloride ((6R,7R)-7-[[(2-amino-4-thiazolyl)-(methoxyiminoo)acetyl]-amino]-3-methyl-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid, (2,2-dimethyl-1-oxo-propoxy) methyl ester hydrochloride) (CPH) (Fig. 1), is an oral third-generation cephalosporin. The active form of CPH - cefetamet is formed after oral administration by hydrolysis carried out by nonspecific mucosal esterases (1). CPH represents excellent microbiological in vitro activity against wide range of Gram-positive as well as Gram-negative respiratory pathogens such as Streptococcus pneumoniae, Haemophilus influenzae, β-hemolytic streptococci, Neisseria gonorrhoeae, and Enterobacteriaceae (2). Thanks to its significant stability against β-lactamases, corresponding with the presence of α-methoxyimino group, CPH is administered in the treatment of otitis media, pneumonia and in pharyngotonsilitis (3). It is also applied in the pharmacotherapy of both upper and lower respiratory tract and severe urinary tract infections, in the group of children and elderly patients (1, 4).

The structure determining the bactericidal activity of cephalosporins, which is generally considered as their most unstable domain, is β-lactam moiety. Moreover, it is widely reported that degradation products of β-lactam antibiotics are mostly responsible for side effects of this group of drugs. It was proved that CPH, similarly to other cephalosporins, is susceptible to degradation in

Figure 1. Chemical structure of cefetamet pivoxil hydrochloride

* Corresponding author: e-mail: pgarbacki@ump.edu.pl; phone: +48 61 854-66-49
aqueous solutions (5) and in the solid state (6). Therefore, fast, sensitive and accurate analytical assays appropriate for determination of active pharmaceutical ingredient (API) in the presence of its related products are expected. Although, many chromatographic methods for the determination of CPH in the bulk substance (7), in pharmaceutical dosage forms (7, 8) and in biological samples (9, 10) have been reported, they are based on mobile phases (e.g., phosphate buffers) which do not allow the transfer to LC-MS/MS technique.

All drugs during stability studies should be determined using stability-indicating methods (SIAMs) recommended by International Conference of Harmonization (ICH) guidelines (Q1A–R2) (ICH Q2B, validation of analytical procedures, methodology) (11). The stress tests are carried out to evaluate the influence of degrading factors on the stability of API in the solid state (at increased temperature, humidity and after irradiation) and in solutions (the impact of increased temperature, oxidizing agent, pH and buffers).

The aim of this work was to develop fast, sensitive and accurate UHPLC-DAD method suitable for determination CPH in the presence of its degradation products.

EXPERIMENTAL

Materials

Cefetamet pivoxil hydrochloride was received from the Institute of Biotechnology and Antibiotics in Warsaw, Poland. All other chemicals were purchased from Merck KGaA (Darmstadt, Germany). High-quality pure water was prepared using the Millipore purification system (Millipore, Molsheim, France, model Exil SA 67120).

Chromatographic conditions

Chromatographic separation was carried on a Thermo Scientific UHPLC-UltiMate 3000 system. As a stationary phase Waters Acquity BEH C18, (2.1 × 100 mm, 1.7 µm) column was used. The mobile phase composed of 60 volumes of 0.1% formic acid and 40 volumes of acetonitrile. The flow rate was 0.7 mL/min and the wavelength of DAD detector was set at 265 nm. Separation was carried out at 30°C. The injected volume was 5 µL. The components of mobile phase and sample solutions were filtered through 0.2 µm nylon membranes.

Preparation of stock solutions

Stock solutions were prepared by dissolving 5.0 mg of CPH powder in small amount of methanol and later diluted to desired volume (25.0 mL) in distilled water. The final concentration 0.2 mg/mL was achieved. Stock solutions were stored in darkness at 4°C and proved stable during the time of the study.

Validation methodology

The proposed UHPLC procedure was validated according to the International Conference on Harmonization Guidelines (ICH Q2B, validation of analytical procedures, methodology) (11). The method
A simple and sensitive stability-indicating UHPLC-DAD method for... 623

was validated with respect to selectivity, linearity, precision, accuracy, LOD, LOQ and robustness.

Selectivity studies
The selectivity of proposed chromatographic method was evaluated for non-degraded and for degraded CPH samples in aqueous solutions (acidic, basic, oxidative and thermal hydrolysis) as well as in the solid state (degradation under the influence of increased humidity, temperature and radiation). Photodiode array detection was carried out to evidence the selectivity of the procedure and to evaluate the homogeneity of CPH peaks.

Linearity
The linearity was confirmed by preparing twelve standard solutions of CPH in the concentration range 10.0-240.0 µg/mL (5-120% of the target concentration of CPH samples during forced degradation studies). Each standard solution was injected in triplicate to evaluate the reproducibility of detector response at each concentration level.

Precision
The precision of proposed analytical procedure was confirmed in relation to repeatability (intra-day) and intermediate precision (inter-day). In order to evaluate repeatability of the method six samples were determined during the same day for three concentration levels of CPH (160, 200 and 240 µg/mL). Inter-day precision was investigated by comparing the results obtained on two different days.

Accuracy
The determination of the accuracy of the developed UHPLC-DAD method was carried out by recovering CPH from the placebo. The recovery test was performed for three concentration levels of CPH: 160, 200 and 240 µg/mL. Each of abovementioned solutions was injected six times.

Limits of detection (LOD) and quantification (LOQ)
The LOD and LOQ were calculated from the regression equation of the CPH: LOD = 3.3 (Sy/a), LOQ = 10 (Sy/a), where Sy is a standard error and a is the slope of the corresponding calibration curve.

Robustness
The robustness of the method was determined after changing the following experimental conditions: the composition of the mobile phase (concentration of acetonitrile in the range 38–42%, concen-
tation of formic acid in the range 0.05-0.15%), the mobile phase flow rate (flow rate in the range 0.68-0.72 mL/min), wavelength of absorption (265 ± 5 nm), temperature (30 ± 2°C). For each parameter change its influence on the retention time ($t_R$), resolution (RS), area (A) and asymmetry of the peak was evaluated.

**Procedure for forced degradation study**

**Degradation in aqueous solution**

The degradation studies of CPH in aqueous solutions were carried out in the following conditions: in water at 343 K, in the solution of hydrochloric acid (0.1 mol/L) at 343 K, in the solution of sodium hydroxide (0.1 mol/L) at ambient temperature. Solutions were obtained by dissolving 5.0 mg of CPH in small amount of methanol and later diluted with either distilled water, hydrochloric acid or sodium hydroxide to achieve the concentration 0.2 mg/mL. At specified times, samples of the reaction solutions (1.0 mL), except the sodium hydroxide, were instantly cooled with a mixture of ice and water.

**Thermal degradation**

In order to achieve the thermal degradation in the solid state 5.0 mg samples of CPH were weighted in 5.0 mL vials and placed in heat chamber at 343 K in desiccator containing saturated solution of inorganic salt – sodium chloride (~76 % RH) and in a sand bath (dry air conditions). At specified time intervals, determined by the rate of degradation, the vials were removed, cooled to room temperature and their contents were dissolved in small amount of methanol and later diluted with distilled water to obtain the concentration 0.2 mg/mL.

**Oxidative degradation**

To perform oxidative degradation, 5.0 mg samples of CPH were accurately weighted and dissolved in small amount of methanol and then diluted with either 0.3, 1.2 or 3% of a H$_2$O$_2$ solution to achieve the concentration 0.2 mg/mL. The abovementioned solutions were kept at ambient temperature.

**Radiolytic degradation**

Five mg of samples of CPH were weighed in 5.0 mL vials and closed with a plastic stopper. The samples in the vials were exposed to irradiation in a linear electron accelerator LAE 13/9 (electron beam 9.96 MeV and current intensity 6.2 1 A) till they absorbed a dose of 25 and 400 kGy. The vials were removed and their contents were dissolved in small amount of methanol and later diluted with distilled water to obtain the concentration 0.2 mg/mL.

**RESULTS AND DISCUSSION**

It was observed that the most satisfactory chromatographic parameters for determination of CPH in the presence of its degradation products were achieved using C18 1.7 µm column as a stationary phase and a mixture of 0.1% formic acid with acetonitrile (40 : 60, v/v) at the flow rate 0.7 mL/min as a mobile phase. The retention time of CPH was 1.570 min (Fig. 2), which is greatly shorter than values presented in available HPLC procedures (6.26-9.93 min) (5, 7). The purity of the peak and the asymmetry were 99.8% and 1.34, respectively. The selectivity studies, performed for non-degraded and degraded samples, confirmed that proposed UHPLC method is suitable for determination of CPH in the presence of its degradation products. The peak of CPH demonstrated satisfactory symmetry and was clearly separated from the peaks originating from degradants (Fig. 2). It was also observed that peak purity values were more than 98.5% for CPH at 265 nm, which proves that the degradation products were not interfering with the main peak (Table 1).

The calibration plot of developed chromatographic procedure is linear in the concentration range 10-240 µg/mL. The calibration curve was described by the equation $y = ac + b$ given in Table 2. The $b$ value, calculated from equation $y = ac + b$, was significant because it was higher than the critical value $t_b = b/S_b$. The intra- and inter-day precision was determined at three levels of initial concentration of CPH during stability studies: 80% (c = 320 µg/mL), 100% (c = 400 µg/mL) and 120% (c = 480 µg/mL).

<table>
<thead>
<tr>
<th>Spiked concentration (µg/mL)</th>
<th>Intra-day precision RSD (%)</th>
<th>Inter-day precision RSD (%)</th>
<th>Recovery RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>160</td>
<td>0.13</td>
<td>0.09</td>
<td>102.08</td>
</tr>
<tr>
<td>200</td>
<td>0.43</td>
<td>0.14</td>
<td>99.42</td>
</tr>
<tr>
<td>240</td>
<td>0.60</td>
<td>0.13</td>
<td>97.79</td>
</tr>
</tbody>
</table>
The percentage RSD values were between 0.14-0.43%, demonstrating that developed UHPLC method is precise. Recovery values obtained from the recovery test showed that applied chromatographic procedure is accurate and reproducible. LOD and LOQ values were 2.08 mg/mL and 6.29 mg/mL, respectively. All of abovementioned validation parameters are collected in Tables 2 and 3.

The robustness studies were carried out to evaluate the influence of slight alternations of the following parameters: concentration of components and flow ratio of the mobile phase, detection wavelength and temperature, on the chromatographic separation. The results are collected in Table 4. It was proved that the changes of abovementioned parameters, except the concentration of acetonitrile, did not significantly affect the retention time, resolution, area and asymmetry of the CPH peak. Modification of the content of organic modifier in the mobile phase resulted in the changes of peak retention times.

**Results of forced degradation experiments**

According to R. Sehrawat et al., 20-80% of the investigated substance should be degraded during stability studies to confirm that proposed method is suitable to indicate stability (12). The literature reports the significant susceptibility of compounds containing β-lactam moiety to degradation caused by physical and chemical factors (13-15). CPH, similarly to other cephalosporins, was found to be vulnerable to decomposition in the presence of acids, bases and in conditions of increased temperature and relative humidity. The main degradation products had retention times of about 0.373-0.553 min (Fig. 2). As it was expected, CPH rapidly hydrolyzed in the presence of 0.1 mol/L NaOH (about 1.2% of the initial amount of CPH remained after 1 min). CPH was also degraded in the solution of hydrochloric acid (0.1 mol/L) at 343 K. It was noticed that the degradation rate of CPH in the solid state was strongly determined by the relative humidity. The percentage of remained drug stored in dry air at 343 K for 14 days was about ten times higher than in the case of sample exposed to the increased humidity at the same temperature for 10 days. The similar retention times of degradation products observed on chromatograms obtained for samples in the solid state as well as in solutions allow to suggest that in both cases the hydrolysis of amide bond in β-lactam structure occurs. The drug was also found to be fairly resistant to oxidative stress conditions. It was also noticed that the stability of CPH depends on the concentration of hydrogen peroxide. Cefetamet pivoxil hydrochloride was not found to be susceptible to radiolytic degradation (about 1.5 to 12.5% of CPH was decomposed). Despite the significant loss of the content of CPH during forced degradation experiments, no additional peaks on chromatograms were observed what can be associated with the fact that no degradation products containing chromophore structure were formed. However, structures which are the result of intermolecular interactions (characteristic to all cephem derivatives) can be noticed. The results of forced degradation experiments on CPH under various stress conditions are summarized in Table 1.

---

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$t_r$</th>
<th>RS'</th>
<th>A</th>
<th>Peak asymmetry$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal</td>
<td>1.573</td>
<td>4.92</td>
<td>38.8514</td>
<td>1.34</td>
</tr>
<tr>
<td>ACN = 38%</td>
<td>1.887</td>
<td>4.91</td>
<td>39.4674</td>
<td>1.34</td>
</tr>
<tr>
<td>ACN = 42%</td>
<td>1.330</td>
<td>4.37</td>
<td>38.4332</td>
<td>1.32</td>
</tr>
<tr>
<td>Formic acid = 0.05%</td>
<td>1.653</td>
<td>5.11</td>
<td>37.7831</td>
<td>1.25</td>
</tr>
<tr>
<td>Formic acid = 0.15%</td>
<td>1.527</td>
<td>5.27</td>
<td>39.4010</td>
<td>1.31</td>
</tr>
<tr>
<td>$f = 0.68$ mL/min</td>
<td>1.623</td>
<td>5.06</td>
<td>40.2544</td>
<td>1.30</td>
</tr>
<tr>
<td>$f = 0.72$ mL/min</td>
<td>1.527</td>
<td>5.16</td>
<td>37.7802</td>
<td>1.30</td>
</tr>
<tr>
<td>$\lambda = 260$ nm</td>
<td>1.570</td>
<td>5.21</td>
<td>40.1812</td>
<td>1.36</td>
</tr>
<tr>
<td>$\lambda = 270$ nm</td>
<td>1.577</td>
<td>5.02</td>
<td>37.0306</td>
<td>1.35</td>
</tr>
<tr>
<td>$T = 28^\circ$C</td>
<td>1.600</td>
<td>5.11</td>
<td>39.0548</td>
<td>1.38</td>
</tr>
<tr>
<td>$T = 32^\circ$C</td>
<td>1.543</td>
<td>5.03</td>
<td>38.6818</td>
<td>1.32</td>
</tr>
</tbody>
</table>

$^a$ Peaks are separated to baseline if resolution is > 1.5; $^b$ peak asymmetry < 1.5 indicates symmetry of peak.
CONCLUSIONS

Developed UHPLC-DAD method is suitable for the determination of CPH in the presence of its degradation products. The proposed chromatographic procedure demonstrates satisfying validation parameters such as: specificity, linearity, precision, accuracy and robustness. Thanks to the short time of analysis, the elimination of compounds containing ion pairs from the mobile phase and possibility to transfer to LC-MS technique the method meets the criteria of modern analytical approaches and can be used for routine quality control and stability indicating studies on CPH.

Declaration of interest

The authors declare that there are no conflicts of interest.

REFERENCES


Received: 24. 11. 2014