HILIC–MS Determination of Genotoxic Impurity of 2-Chloro-N-(2-Chloroethyl)Ethanamine in the Vortioxetine Manufacturing Process

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Abstract

In the last decade, pharmaceutical regulatory agencies are focused on monitoring and evaluation of trace-level genotoxic impurities (GTIs) in drug substances, which requires manufacturers to deliver innovative approaches for their analysis and control. GTIs in the low p.p.m. level rising from the process of drug production have to be positively identified and quantified. Therefore, sensitive and selective analytical methods are necessary for required quantification level of these GTIs. Unfortunately, general guidance on how to develop strategy of the analysis and control of GTIs is currently missing in the pharmaceutical industry. Therefore, practical example of the analytical control of 2-chloro-N-(2-chloroethyl)ethanamine GTI in the vortioxetine (VOR) manufacturing process was demonstrated in this work. QDa mass detection with electrospray ionization in selected-ion recording mode was utilized for quantitation of GTIs. The method of hydrophilic interaction liquid chromatography coupled with mass spectrometry detection (HILIC–MS) was validated as per International Conference on Harmonization guidelines and was able to quantitate GTIs at 75 p.p.m. with respect to VOR. The HILIC–MS method was achieved using a Primesep B column (150 × 4.6 mm, 5.0 µm; Sielc, USA) using mobile phase consisting of 10 mM ammonium formate buffer pH 3.0 and acetonitrile (5 : 95, v/v) at 0.8 mL/min flow rate. The QDa mass detector was operated in the positive ion mode. Quadrupole mass analyzer was employed in selected-ion monitoring mode using target ion at m/z 142 as [M+H]+.

Introduction

The International Conference on Harmonization (ICH) Q3 guidelines summarize the requirements for identification, qualification and control of impurities in drug substances and corresponding formulated products (1, 2). The European Medicines Agency guideline on the limits of the genotoxic impurities (GTIs) (3) proposes a threshold of toxicological concern (TTC) value (1.5 µg/day) as the acceptable safe level for GTIs to ensure no significant carcinogenicity risk. While ICH Q3 guidelines provide guidance for qualification and control for the majority of the impurities, ICH M7 guidance is provided for those impurities that are DNA reactive (4). The concentration limit of GTIs in drug substances and drug products can be derived based on the maximum daily dose and the TTC concept: concentration limit (p.p.m.) = [1.5 µg/day]/[dose (g/day)]. The second possible approach is to calculate a compound-specific acceptable intake based on rodent carcinogenicity potency data such as TD50 value. This procedure is similar to that employed for derivation of the TTC (4).

Quantification limits at p.p.m. range are not achievable using conventional pharmaceutical techniques such as traditional HPLC with UV detection, where typical levels of analyte quantitation are ~0.05%. Testing and control of GTI at p.p.m. trace levels presents challenge to the pharmaceutical industry with regard to both analytical and process controls. HPLC or UHPLC coupled with tandem mass spectrometry (MS/MS) or fluorescence detector have been used to satisfy these requirements (5–8). Unfortunately, the pharmaceutical industry has no long-term experience in the routine use of these sophisticated methodologies.
within the quality control (QC) environment (9). The details of various regulations and guidances, toxicology assessment, identification of structural alerts, synthetic origins, different synthetic approaches for elimination or control and various analytical determination strategies relating to GTIs were summarized in several reviews (10, 11).

In recent years, MS has become essential for carrying out drug discovery and pharmaceutical development (12, 13). While many applications require high-resolution MS instruments, fit-for-purpose devices with lower resolution and lower cost could be sufficient for routine analytical application in pharmaceutical industry. In this study, the utilization of the recently introduced QDa mass detector as reliable support for pharmaceutical industry was investigated. Although the sensitivity is reduced relative to conventional MS instruments, it is sufficient for synthetic chemistry, where sample is generally abundant and unit mass resolution is sufficient (14). QDa mass detector is a single quadrupole type of mass spectrometer, whose dimensions are not larger than those of conventional UV–Vis detectors. QDa mass detector can measure in the mass range of \( m/z \) 30–1,250 using electrospray, both in the positive and also in the negative ionization mode. QDa mass detector is operated with a unit resolution (0.7 a.m.u.) and its sensitivity depends on the type of vacuum source used (oil rotary vacuum pump or membrane vacuum pump).

Vortioxetine (VOR) (1-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-piperazine) is a novel investigational antidepressant with multimodal activity. It has high affinity for the 5-HT transporter and moderate affinity for the 5-HT1A receptor in vitro (15–18). Brintellix is an immediate-release tablet for oral administration that contains the beta (β) polymorph of VOR hydrobromide. The recommended starting dose is 10 mg administered orally once daily without regard to meals. Recently, a new synthetic route to VOR starting from commercially available thiol (Figure 1 (I)) and 1-chloro-2-nitrobenzene (Figure 1 (II)) was developed. The key step of this synthetic strategy relies on the well-established method utilizing 2-chloro-N-(2-chloroethyl)ethanamine hydrochloride (BCEA) as a building block for construction of piperazine moieties (19–21). Thus, the thiol was submitted first to a nucleophilic substitution with the 1-chloro-2-nitrobenzene to afford the desired nitrosulphide (Figure 1 (III)), which was subsequently hydrogenated to provide the corresponding aminosulphide (Figure 1 (IV)) in excellent yield. Although a number of precedent examples are described in the literature, it was observed that a judicious choice of the reaction conditions in the last step is crucial to suppress by-products formation while still keeping reasonable yields. A rigorous inspection of this detailed reaction optimization has manifested an importance of nonbasic conditions and the presence of an iodide additive. Under optimized conditions, the aminosulphide was reacted with BCEA in the presence of 0.75 equiv. of potassium iodide (KI) in reflowing toluene yielding thus highly pure VOR. Further, BCEA is considered as potential alkylating agents that may exert genotoxic effects in bacterial and mammalian cell systems (22). Finally, BCEA is one of the nitrogen blister agents generally referred to as nitrogen mustards (23, 24).

Since BCEA is known to be genotoxic, the amount of BCEA has to be controlled rigorously in the final drug. The main objective of the current article is to demonstrate the possibility of using QDa detection for the analytical control of BCEA in the manufacturing process of VOR. If the maximum dose for VOR is 20 mg/day, then the maximum concentration limit of GTIs = [(1.5 µg/day)/(0.02 g/day)] is 75 p.p.m. in the drug substance.

**Experimental**

**Reagents and chemicals**

Acetonitrile HPLC gradient grade, methanol HPLC gradient grade (J. T. Baker, USA) and water purified by Milli-Q system (Merck/Millipore, Czech Republic) were used for preparation of samples, reference solutions and mobile phases. Ammonium formate and formic acid were of analytical grade quality (Sigma, Czech Republic). Five different batches of VOR were prepared in Zentiva k.s. (Czech Republic). The reference standard of BCEA hydrochloride was obtained from Sigma–Aldrich (Czech Republic).

![Figure 1. Reaction scheme of VOR synthesis.](https://academic.oup.com/academic.oup.com/chromsci/article-abstract/54/2/119/2754727)
Preparation of standard and sample solutions

The standards of VOR (in-house standard, purity 99.8%) and BCEA were dissolved in a mixture of acetonitrile–water (1 : 1, v/v) to obtain standard stock solution (concentration of 1.0 mg/mL). The sample solution for BCEA determination was prepared by dilution of VOR in a mixture of acetonitrile–water (8 : 2, v/v) to obtain final concentration of 1 mg/mL. The reference solution of BCEA was prepared by dilution of relevant standard stock solution to obtain final concentration of 0.075 µg/mL (the concentration of 75 p.p.m. with respect to VOR sample solution).

Instrumentation and methods

The system control, data acquisition and processing were accomplished by the Empower software (Waters, USA).

All chromatographic experiments were carried out on an Acquity UPLC H-Class system with a photodiode array detector and QDa mass detector (Waters, USA).

Four chromatographic columns were employed, namely Kinetex 2.6 uHILIC 100A, 100 × 2.1 mm, 2.6 µm (Phenomenex, USA); XBridge HILIC, 150 × 4.6 mm, 3.5 µm (Waters, Czech Republic); Primesep B column, 150 × 4.6 mm, 5.0 µm (Sielc, USA) and Obelisc R, 100 × 2.1 mm, 5 µm (Sielc, USA). The mobile phases were prepared by mixing of appropriate components in corresponding volume ratios. The buffer solutions were prepared by dissolution of adequate amounts of salts in purified water and pH was adjusted using corresponding acid (ammonium formate and/or formic acid). The injection volume was 2 µL in all the experiments. The temperature of separation was maintained at 35°C with the precision ±0.5°C.

The QDa mass detector was operated in the positive ion mode. The optimized tuning parameters were of ESI source as follows: probe (desolvation) temperature 600°C, nebulizer gas 7 MPa, cone voltage 15 V and capillary voltage 0.5 kV. Quadrupole mass analyzer was employed in selected-ion monitoring mode using target ion at m/z 142 as [M+H]+.

Results

MS conditions

Because BCEA and VOR are basic compounds, the best sensitivity was achieved using positive polarity mode detection. Figures 2A and B show typical mass spectra of BCEA and VOR at 0.8 kV capillary voltage and at 20 V cone voltage obtained in the scan mode from m/z 100 to 350. The base peak in the mass spectrum is the protonated molecular ion [M+H]+ of BCEA and VOR at m/z 142 and 299, respectively. Therefore, these ions were chosen as target ions for BCEA and VOR monitoring in the selected-ion recording (SIR) mode.

Optimization of hydrophilic interaction liquid chromatography method

The composition of the mobile phase was optimized to achieve the retention factor of VOR and BCEA k ≥ 1.0 and resolution between VOR and BCEA R ≥ 2.5. The hold-up volume was determined using toluene (injected 5 µL, 50 µg/mL in acetonitrile) with a mobile phase containing 95 volume parts of acetonitrile. The chromatographic data were calculated in agreement with European Pharmacopoeia (Ph. Eur.) (2.5).

Acceptable retention and resolution between VOR and BCEA was achieved on a Primesep B column using isocratic elution of acetonitrile and 10 mM ammonium formate buffer pH 3.0 in ratio 95 : 5 (v/v) at a flow rate of 0.8 mL/min and temperature of 35°C. The representative extracted ion chromatograms of separation of VOR and BCEA measured at the optimized hydrophilic interaction liquid chromatography (HILIC) conditions are shown in Figure 3.

Validation of chromatographic methods

The methods were validated according to ICH Q2(R1) guideline (26).

System suitability

The system suitability tests were performed before each run to assure that the analytical method could be used with a satisfactory performance (26). Repeatability of the five consecutive injections expressed as percentage relative standard deviation (%RSD) of peak area of reference solution was limited to ≤3.0%. For all the measurements performed during the validation, the RSD of peak areas for BCEA was <0.6.

Limit of detection, limit of quantification and linearity

Calculated limit of detection (LOD) and limit of quantification (LOQ) are listed in Table I.

The calibration curve was constructed ranging from LOQ to 200% of the general specification limit (from LOQ to 150 p.p.m. with regard to the concentration of VOR) by plotting the peak area of a given analyte against its concentration. The calibration equation was calculated using linear regression analysis. The parameters of calibration curve are shown in Table I.

Precision and accuracy

The accuracy of BCEA was evaluated in triplicate at four concentration levels (LOQ, 50, 75 and 100 p.p.m. regarding to the concentration of the VOR). The results are shown in Table II. The precision of BCEA determination was evaluated by analysis of three independent preparations of the five different samples under the prescribed conditions. Table III summarized the results of BCEA content in five batches of VOR.

Method selectivity

Selectivity was confirmed by successfully separating the compound of interest from other components. The method selectivity was established using analysis of blank (sample solvent) and samples spiked with other related impurities (aminosulphide and nitrosulphide). No interfering coeluting peaks in sample solvent were observed which demonstrated an adequate selectivity of both methods.

Discussion

The purpose of this study was to develop and optimize a rapid HILIC–MS method and to validate this rapid HILIC method according to the ICH guidelines.

MS conditions

Since QDa mass detector is preoptimized to work without user adjustments typical of traditional mass spectrometers can be optimized only some of its parameters. In order to achieve the highest assay sensitivity for BCEA, the optimal capillary voltage of the electrospray at 15 V cone voltage was investigated. The intensities of BCEA ions at m/z 142 were compared at the capillary voltages of 0.5, 1.0 and 1.5 kV. The result showed that the highest sensitivity was obtained using a capillary voltage of 0.5 kV.

Optimization of HILIC method

BCEA has only a weak UV absorbance in the low wavelength range (<200 nm) and HPLC with photometric detection does not allow...
sensitive determination of BCEA. On the other hand, successful approach for analysis of amino compounds by HPLC is to employ pre-column derivatization leading to hydrophobic and highly absorbing UV or fluorescence derivatives, which can be effectively separated and sensitively quantified. Unfortunately, each derivatization step is time consuming and it can be possible source of various errors. Therefore, it is advisable to avoid these approaches (27). In the most recent literature references of HPLC analysis of alkylating impurities, reversed phase modes of separation and MS detection predominates. Due to the chemical properties (pKa, log P), BCEA is an ideal...
LOD (p.p.m.) 0.3  
LOQ (µg/mL) 0.001

Correlation coefficient 0.9999
QC coefficient 0.86
LOD (µg/mL) 0.0003
LOD (p.p.m.)* 0.3
LOQ (p.p.m.)* 1.0

*With regard to the concentration VOR.

Table II. The Parameters of Regression Equation (Significance Level $P=0.95$) for Accuracy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BCEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept $a$</td>
<td>$-0.001 (±0.004)$</td>
</tr>
<tr>
<td>Slope $b$</td>
<td>1.016 (±0.057)</td>
</tr>
<tr>
<td>Correlation coefficient $R$</td>
<td>0.9919</td>
</tr>
</tbody>
</table>

Table III. The Content (p.p.m.) of BCEA Determined in Different VOR Batches

<table>
<thead>
<tr>
<th>Samples (Zentiva, Czech Republic)</th>
<th>BCEA</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 01</td>
<td>9.0</td>
<td>2.8</td>
</tr>
<tr>
<td>Batch 02</td>
<td>3.0</td>
<td>4.8</td>
</tr>
<tr>
<td>Batch 03</td>
<td>3.0</td>
<td>3.1</td>
</tr>
<tr>
<td>Batch 04</td>
<td>3.1</td>
<td>4.0</td>
</tr>
<tr>
<td>Batch 05</td>
<td>11.1</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The various stationary phases were screened under HILIC conditions for ability to separate VOR and BCEA. Various binary mixtures of 10 mM ammonium formate (pH 3.0) buffer with acetonitrile were used as mobile phases for initial screening. The flow rates on the tested analytical columns with different dimensions were optimized to achieve the same linear velocity. The low retention or no separation was obtained on Kinetex 2.6u HILIC, XBridge HILIC and Obelisc R column. The Primesep B column showed satisfactory selectivity and retention. The separation of VOR and BCEA was optimized by varying the acetonitrile content and pH of 10 mM ammonium formate buffer in the mobile phase. The influence of acetonitrile content in the mobile phase on the retention of BCEA and VOR was investigated in the range from 85 to 95% (v/v) at constant ammonium formate buffer concentration of 10 mM and at pH 3.0. The retention of BCEA and VOR significantly increased when the acetonitrile content reached ~90%. The pH buffer was varied in the range from 2.8 to 4.8. The retention and resolution of BCEA and VOR increased as the pH buffer decreased.

Validation of chromatographic methods

LOD, LOQ and linearity

LOD and LOQ were calculated based on signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a blank solution. The baseline noise was measured in the region of retention time of BCEA using chromatographic software. The linearity of the calibration curve was investigated using other statistical approaches such as the quality coefficient QC (29). If the quality coefficient QC fulfilled the criterion QC < 5%, the linearity of calibration model was demonstrated (see Table I). The calculated parameters of calibration curve indicated a satisfactory linearity.

Precision and accuracy

The determined content ($c_d$) was compared with the expected one ($c_e$) using linear regression ($c_d = a + bc_e$). The parameters of regression equation (significance level $P=0.95$) is shown in Table II. The first ($a$) and second ($b$) constants were not statistically different from zero and one, respectively.

Precision results were determined as %RSD. RSDs between 2.5 and 4.8% for a content of BCEA demonstrated good precision.

Method selectivity

The acquisition of SIR together with the retention time is the widely accepted criterion for selectivity.

Conclusion

Analysis and control of GTIs formed during drug development and manufacturing has still presented a challenge to the pharmaceutical industry. In an effort to gain control over the BCEA GTI encountered in the manufacture of VOR, HILIC–MS method was developed and validated for its testing. The validation data and criteria fully meet the requirements of regulatory agencies.

The use of the selective detection techniques such as LC–MS significantly increased the selectivity and sensitivity in comparison with the commonly used UV detection. For the determination of BCEA in VOR, a highly selective and sensitive method using HILIC–MS with an electrospray source in positive SIR mode was applied. One of the main advantages offered by the developed method is that BCEA was separated and quantified in its native form without any time-consuming derivatization step. The method does not require additional sample preparation. In addition, a simple QDa mass detector is suitable for determination of analytes at low concentration level with sufficient sensitivity and precision. The proposed method can be successfully applied for the determination of BCEA in the VOR manufacturing process.

Conflict of interest statement

None declared.

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