Validation of a liquid chromatographic method for the pharmaceutical quality control of products containing elacridar

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Many anticancer drugs have an impaired bioavailability and poor brain penetration because they are substrates to drug efflux pumps such as P-glycoprotein and Breast Cancer Resistance Protein. Elacridar is a strong inhibitor of these two drug efflux pumps and therefore has great potential to improve oral absorption and brain penetration of many anticancer drugs. Currently, a clinical formulation of elacridar is unavailable and therefore the pharmaceutical development of a drug product is highly warranted. This also necessitates the availability of an analytical method for its quality control. A reverse-phase high-performance liquid chromatographic method with ultraviolet detection was developed for the pharmaceutical quality control of products containing elacridar as the active pharmaceutical ingredient. The analytical method was validated for linearity, accuracy, precision, selectivity, carry-over, stability of stock and reference solutions, stability of the final extract, stability-indicating capability and impurity testing. We found that elacridar is unstable in aqueous solutions that are exposed to light because a hydroxylation product of elacridar is formed. Therefore, sample solutions with elacridar must be protected from light.

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1. Introduction

One of the important reasons for chemotherapy failure is the fact that many anticancer agents cannot reach tumor cells in sufficient quantities. This is often the result of drug-efflux pumps such as P-glycoprotein (PgP) and Breast Cancer Resistance Protein (BCRP), which are present in the gastro-intestinal tract, at the blood-brain barrier and in tumor cells [1–4]. A drug that is a substrate for PgP and/or BCRP cannot enter the cell and therefore cannot be absorbed into the central systemic circulation, brain and tumor. Examples of anticancer drugs that are substrates of PgP/BCRP are topotecan, paclitaxel, docetaxel, erlotinib, pazopanib, imatinib and nilotinib [5,6].

Elacridar or N-(4-[(2-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)ethyl]phenyl) – 5-methoxy-9-oxo-9,10-dihydroacridine-4-carboxamide (GF120918) is an inhibitor of PgP and BCRP [7] and, as confirmed in several clinical trials, it can increase the oral bioavailability of orally administered anticancer drugs such as paclitaxel and topotecan [8–12]. Furthermore, in pre-clinical research elacridar inhibited PgP at the blood-brain barrier and consequently increased the penetration of various anticancer agents in the brain [13–21]. More clinical trials are warranted to study the boosting effect of elacridar but cannot be performed because currently there is no clinical formulation available. Therefore, we developed a tablet formulation containing 23.5 mg of elacridar as the active pharmaceutical ingredient (API). The formulation is to be used in proof-of-concept clinical studies that study the boosting effect of elacridar on various anticancer agents. An amorphous solid dispersion was made to improve the poor solubility in water [12]. In an amorphous solid dispersion the drug is molecularly dispersed into a hydrophilic amorphous polymer [22] and the presence of a hydrophilic polymer and amorphous drug particles result in improved drug solubility [23]. This new formulation also necessitated the availability of a validated analytical method for its quality control. There are currently no quality control monographs about elacridar published in the European Pharmacopoeia, United States Pharmacopoeia or Japanese Pharmacopoeia nor are there validated analytical methods for pharmaceutical quality control published in scientific literature. In this paper we describe the development and validation of a reverse-phase high-performance liquid chromatography–ultraviolet
detection (HPLC–UV) method for the pharmaceutical quality control of a drug powder, an amorphous solid dispersion and a tablet formulation containing elacridar as the API.

2. Materials and methods

2.1. Chemicals

5-Methoxy-9-oxo-9,10-dihydroacridine-4-carboxylic acid (5-MODICA) and 4-[2-(6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinolinyl)ethyl]aniline (4-DTHIA) were purchased from AvaChem Scientific (San Antonio, TX, USA). Dimethyl sulphoxide (DMSO), acetonitrile, potassium dihydrogen phosphate and sodium dodecyl sulphate (SDS) were purchased from Merck (Darmstadt, Germany). The preparation of Simulated Intestinal Fluid without pancreatic enzymes (SIFsp, pH 6.8) was according to [24]. Distilled water was from B. Braun (Melsungen, Germany). Povidone K30 was from BaSf Chemtrade (Ludwigshafen, Germany). Granulated lactose monohydrate SuperTabs 30GR was from DFE Pharma (Goch, Germany). Colloidal silicon dioxide and magnesium stearate were from Fagron (Capelle a/d Ijssel, The Netherlands). Croscarmellose sodium was from FMC (Philadelphia, USA).

2.2. Drug powder and formulated products

The drug powder was elacridar hydrochloride (purity > 99%) and was synthesized according to the procedure as described in [25]. A summary of this synthesis is displayed in Fig. 1.

The entire production process was compliant with Good Manufacturing Practices. The intermediate product was an amorphous solid dispersion. For this, a solution of elacridar hydrochloride-povidone K30-SDS (12.5:75:12.5, m/m/m) in DMSO was prepared to a total excipient concentration of 80 mg/mL. The solution was transferred to stainless steel boxes (Gastronorm 1/9, The Netherlands). DMSO was removed by lyophilization and this was performed according to a process earlier used by den Brok et al. [26] in a Lyovac GT4 (GEA Lyophil, Hürth, Germany). The intermediate product was a yellow powder stored in a glass bottle with an air-tight polypropylene screw cap in the dark at 2–8 °C in a desiccator.

The final drug product was a tablet with 23.5 mg elacridar (25 mg elacridar hydrochloride). For this, a powder mixture of intermediate product-granulated lactose monohydrate-croscarmellose sodium-anhydrous colloidal silicon dioxide-magnesium stearate (30:63:5:1:1, m/m/m/m/m) was weighed in a 2 L stainless steel box and mixed in a Turbula Mixer T10B (Willy A. Bachofen, Muttenz, Switzerland) for 30 min. The powder mixture was then pressed on an eccentric tablet press (Korsch, EKO, Berlin, Germany) and tablets were sealed in aluminium blisters and stored at –20 °C in the dark until analysis.

2.3. Sample preparation

Stock solutions contained 188 μg/mL elacridar (200 μg/mL elacridar hydrochloride) in DMSO and were stored in polypropylene tubes in the dark at ≤ 20 °C.

Calibration standards (CAL, 1–20 μg/mL elacridar hydrochloride) were prepared on the day of analysis from a stock solution and diluted to the desired concentration in SIFsp-DMSO (33:67, v/v). Quality control standards (QC, 1–20 μg/mL elacridar hydrochloride) were diluted to the desired concentration in SIFsp-DMSO (33:67, v/v) from a separately prepared stock solution.

For the preparation of reference solutions (10 μg/mL elacridar hydrochloride), two separately prepared stock solutions were diluted in water-DMSO (20:80, v/v). Reference solutions were freshly prepared for every analytical batch.

For the quality control of drug powder and intermediate product, an amount equivalent to 23.5 mg elacridar (25 mg elacridar hydrochloride) was dissolved in 50 mL DMSO by using a shaker. 0.200 mL of this solution was added to 7.800 mL of DMSO in a polypropylene tube. A volume of 2.000 mL water was added and homogenized. For the quality control of the final drug product, tablets in blister package (stored at –20 °C) were placed in the dark for 1.5 h in a desiccator to prevent the adsorption of water when the tablets reach ambient temperature. Subsequently, the tablet was pulverized with a mortar and pestle and dissolved in 50 mL DMSO. The
solution was centrifuged at 3000 rpm for 15 min and 0.200 mL of the supernatant was added to 7.800 mL of DMSO. Then 2.000 mL of water was added to the polypropylene tube and the solution was homogenized.

Aqueous samples were protected from light by transferring them to amber-colored autosampler vials immediately after preparation and by storing vials at 2 – 8 °C until and during analysis. Samples were analyzed directly after preparation.

2.4. Instruments

The HPLC–UV system consisted of an 1100 Series binary HPLC pump Model G1312A, 1100 series G1367A autosampler and 1100 series G1314A UV detector (Agilent Technologies, Santa Clara, CA, USA). The column was a Waters Symmetry end-capped C-18 deactivated silica column (150 mm x 4.6 mm, 3.5 μm). The eluent was an isocratic mixture of ammonium acetate (pH 5.0, 120 mM); acetonitrile (52.5:47.5, v/v) at a flow of 0.5 mL/min and ambient column temperature. Quantification was executed at 259 nm. The runtime was 10 min, sample injection volume was 10 μL and the autosampler temperature was 5 ± 1 °C.

2.5. Method validation

Validation of the HPLC–UV method was based on the procedure as published by the ICH guideline on validation of analytical procedures [27]. Pre-defined acceptance criteria are shown in Table 1.

2.5.1. Linearity

CALs were prepared according to Section 2.3, and analyzed in duplicate and in three different analytical batches. Least-squares linear regression was applied on the concentration versus peak area plot and the correlation coefficient (R) was calculated. Deviations from linear fit were established by comparing the back-calculated concentrations with the nominal concentrations of the calibration standards.

| Validation parameter | Conditions | Matrix | n | Nominal concentration (μg/mL) | Measured concentration (μg/mL) | Pre-defined criteria | Result |
|----------------------|------------|--------|---|------------------------------|-------------------------------|---------------------|--------|
| Linearity            | Inter-run  | SIFsp-DMSO (33:67, v/v) | 36 | 1.00–20.09 | 1.01–20.27 | R ≥ 0.995 | R: 1.000 |
|                      |            |        | 6  | 1.00  | 1.01  | R ≤ 3% | 3.00% |
|                      |            |        | 6  | 3.35  | 3.35  | R ≤ 3% | 3.00% |
|                      |            |        | 6  | 8.37  | 8.24  | R ≤ 3% | 3.00% |
|                      |            |        | 6  | 13.39 | 13.27 | R ≤ 3% | 0.90% |
|                      |            |        | 6  | 16.74 | 16.78 | R ≤ 3% | 0.24% |
|                      |            |        | 6  | 20.09 | 20.27 | R ≤ 3% | 0.90% |
| Accuracy             | Inter-run  | SIFsp-DMSO (33:67, v/v) | 10 | 1.00  | 1.00  | R ≤ 4% | 0.00% |
|                      |            |        | 10 | 3.33  | 3.38  | R ≤ 4% | 1.50% |
|                      |            |        | 10 | 8.33  | 8.27  | R ≤ 3% | 0.50% |
|                      |            |        | 10 | 16.66 | 16.60 | R ≤ 3% | 0.36% |
|                      |            |        | 10 | 19.99 | 20.25 | R ≤ 3% | 1.30% |
| Precision            | Inter-run  | SIFsp-DMSO (33:67, v/v) | 10 | 1.00  | 1.00  | RSD ≤ 4% | 1.50% |
|                      |            |        | 10 | 3.33  | 3.38  | RSD ≤ 4% | 1.94% |
|                      |            |        | 10 | 8.33  | 8.27  | RSD ≤ 3% | 0.26% |
|                      |            |        | 10 | 16.66 | 16.60 | RSD ≤ 3% | 0.28% |
|                      |            |        | 10 | 19.99 | 20.25 | RSD ≤ 3% | 0.51% |
| Selectivity          | Blank tablet | Water-DMSO (20:80, v/v) | 3  | –     | –     | No peaks at tR elacridar | No peak detected |
| Carry-over           | CAL 16.74 μg/mL, then followed by matrix solution | SIFsp-DMSO (33:67, v/V) | 4  | –     | –     | ≤ 20% of the lower limit of quantification | No peak detected |
| Stability of stock solution | –20 °C, dark | DMSO | 5  | 201.78 | 199.58 | Bias ≤ 3% | –1.09% |
|                      | 11 months  |        |    | 200.40 | 197.66 | Bias ≤ 3% | –1.37% |
|                      | 21 months  |        |    | 201.78 | 203.84 | Bias ≤ 3% | 1.02% |
| Stability of reference solution | 4 days, 2-8 °C, dark daylight, 24 h | DMSO | 5  | 10.09 | 10.11 | Bias ≤ 3% | 0.20% |
| Stability of final extract | 7 days, 2-8 °C, dark | SIFsp-DMSO (33:67, v/v) | 2  | 1.00  | 1.03  | Bias ≤ 4% | 3.00% |
|                      |            |        | 2  | 3.35  | 3.41  | Bias ≤ 4% | 1.79% |
|                      |            |        | 2  | 8.37  | 8.25  | Bias ≤ 3% | –1.43% |
|                      |            |        | 2  | 13.39 | 13.12 | Bias ≤ 3% | –2.02% |
|                      |            |        | 2  | 16.74 | 16.83 | Bias ≤ 3% | 0.54% |
|                      |            |        | 2  | 20.09 | 20.65 | Bias ≤ 3% | 2.75% |

RSD = relative standard deviation, Dev = deviation from linear fit.

* Blank tablet contains the same ingredients and in the same proportions as in an elacridar solid dispersion tablet except elacridar hydrochloride.
2.5.2. Accuracy

QC samples were analyzed in five-fold and on two different occasions. For each batch freshly prepared QCs and CALs were used. Concentrations in the QC samples were calculated by least-squares linear regression. The bias was calculated by dividing the difference between the measured concentration and the nominal concentration by the nominal concentration. Intra-run accuracy was obtained by calculating the average bias of five analyzed QCs per analytical batch per concentration level. Inter-run accuracy was obtained by calculating the average bias of all analyzed QCs on the two different analysis occasions per concentration level.

2.5.3. Precision

QC samples that were used to assess the accuracy were also used to determine the intra-run precision ( repeatability [27]) and inter-run precision (intermediate precision [27]). For intra-run precision the relative standard deviation (RSD) of the measured concentration of each QC per analytical batch per concentration level was calculated. For inter-run precision the RSD of the measured concentration of QCs of the two different analytical batches per concentration level was calculated.

2.5.4. Selectivity

Three “blank tablets” which contained all ingredients of the final drug product except the drug powder were used. Each blank tablet was processed as described in Section 2.3 and analyzed immediately after preparation.

2.5.5. Carry-over

After analysis of the upper limit of quantification (calibration standard containing 16.7 μg/mL) blank matrix samples (SIFsp: DMSO (33:67, v/v)) were analyzed. The procedure was repeated twice and performed on two different analysis days.

2.5.6. Stability of stock and reference solutions

Stock solutions were analyzed after 11 months and 21 months of storage at –20 °C and after 24 h at room temperature in indoor natural daylight. Stock solutions were prepared according to Section 2.3 and analyzed in five-fold. The concentration was measured using a reference solution prepared from a fresh stock solution. Reference solutions were stored for 4 days in the dark at 2–8 °C and quantified using freshly prepared reference solutions. The bias was calculated by the same formula as described in Section 2.5.2.

2.5.7. Stability of the final extract

Calibration standards were analyzed at t = 0 and after 7 days of storage in the dark at 2–8 °C and quantified by least-squares linear regression using freshly prepared CALs. The bias and RSD were calculated by the same formula as described in Section 2.5.2.

2.5.8. Stability-indicating capability

Reference solutions were prepared according to Section 2.3 and exposed to various stress factors: 1 M sodium hydroxide, 1 M hydrochloric acid or 25% hydrogen peroxide. Duplicate samples of each type of stress factor were prepared and one sample was stored in the dark and the other sample was stored for 24 h in indoor natural daylight. Samples were processed and analyzed immediately after preparation and after 24 h.

2.5.9. Impurity test

5-MODICA (10 μg/mL in water-DMSO (20:80, v/v)) and 4-DTHIA (10 μg/mL in water-DMSO (20:80, v/v)) were used to assess the ability of the analytical method to separate impurities related to the drug powder. Samples were measured on an HPLC system coupled to a photo diode array detector (Ultimate 3000 Series, Thermo Scientific, Waltham, MA, USA). Eluent, column, flow rate and injection volume were equal to those described in Section 2.4. Ultra-violet and visible light (UV–vis) spectra were recorded from 200 nm to 800 nm.

2.6. Application of the HPLC–UV method

The HPLC–UV method was used to assess the content, purity and dissolution of the drug powder, intermediate product and final drug product. To determine content and purity samples were prepared according to Section 2.3 and were quantified using reference solutions.

Dissolution was tested in a European Pharmacopoeia dissolution tester (Erweka, Heusenstamm, Germany) with a type II paddle at 100 rpm [28]. SIFsp (pH 6.8, 37 °C) [24] was the dissolution medium. The final drug product was placed in a vessel with
500 mL SIFsp. Duration of the dissolution test was 4 h and samples were taken at 0, 15, 30, 45, 60, 90, 120, 180 and 240 min. 1 mL of each sample was directly filtrated through a 0.45 μm PVDF filter (Darmstadt, Germany) and diluted with 2 mL DMSO. CALs and QCs were freshly prepared according to Section 2.3. Samples were processed immediately after collection, protected from light by transferring them to amber-colored autosampler vials and stored at 2–8 °C until and during analysis.

2.7. Characterization of the degradation product

Two reference solutions were prepared according to Section 2.3. One reference solution was stored for 4 days in indoor natural daylight at 15–25 °C and the other was stored for 4 days in the dark at 15–25 °C. Samples were analyzed on an HPLC system coupled to a LTQ XL Iontrap (Thermo Scientific, Waltham, MA, USA) operating in the negative ionization mode. The eluent was ammonium acetate (pH 5.0, 20 mM)-acetonitrile (65:35, v/v) and the flow was 0.5 mL/min. The column was a Waters Symmetry C-18 (150 mm x 4.6 mm, 3.5 μm) at ambient temperature. Samples were stored in a dark autosampler at 5 ± 1 °C. 10 μL of sample solution was injected and the run time was 45 min.

3. Results and discussion

3.1. Liquid chromatography method development

The literature currently describes at least five HPLC methods for elacridar quantification [18,29–32]. One of them used an HPLC–UV method with isocratic eluent ammonium acetate (pH 5.0, 200 mM)-acetonitrile-methanol (57.2:35:7.8, v/v/v) and a retention time of 11 min [31]. The concentration of ammonium acetate was lowered to 120 mM and acetonitrile was used as modifier to improve the peak shape. Using this eluent the retention time was around 7 min and the total run time was 10 min.

The original HPLC–UV method detected at 227 nm because in this method a wavelength was required that could also detect two other analytes (paclitaxel and docetaxel) [31]. For the development of an HPLC–UV for elacridar quality control, the detection wavelength was changed to 259 nm because the signal-to-noise ratio of elacridar was approximately 34 times higher than at 227 nm.

3.2. Method validation

The results for linearity, accuracy, precision, selectivity, carry-over, stock and reference solution stability and stability in the final extract are shown in Table 1. Linearity, inter-run accuracy and inter-run precision complied with the criteria. Intra-run accuracy/precision criteria were the same as inter-run accuracy/precision and were also fulfilled. No other components of the final drug product eluted at the retention time of elacridar and there was no carry-over. Stock solutions were stable at −20 °C for at least 21 months and for 24 h at room temperature in light, reference solutions could be stored at 2–8 °C in the dark for at least 4 days and final extracts were stable for at least 7 days at 2–8 °C in the dark.

The stability-indicating capability of the analytical method was
studied by exposing reference solutions to light, sodium hydroxide, hydrogen peroxide or hydrochloric acid. Fig. 2A shows a chromatogram of a reference solution that was stored for 24 h in the dark or in indoor natural daylight. In reference solutions that were stored in the light two peaks were detected after the deadtime: one peak corresponded to elacridar and the other peak eluted approximately after 6 min. The sum of the peak areas at 6 min and elacridar peak area equaled the area of an elacridar peak from a freshly prepared reference solution (Fig. 2A). According to Fig. 3 the degradation product developed predominantly in neutral solutions and in solutions exposed to sodium hydroxide and was less prominent in solutions that were exposed to hydrogen peroxide or hydrochloric acid. This suggested that the unknown peak was a light-induced degradation product of elacridar. The UV–vis absorption spectra of elacridar and the degradation product are shown in Figs. 2B and Fig. 2C, respectively. The degradation product contained an extra absorption maximum at 312 nm, indicating that the chromophore of elacridar was altered. The degradation product was also detected in formulation sample solutions with water-DMSO and SIFsp-DMSO that were stored for 24 h in the light.

For the impurity test two elacridar-related impurities (5-MODICA and 4-DTHIA) were analyzed and their chromatograms are shown in Fig. 4A and Fig. 4B, respectively. 5-MODICA and 4-DTHIA did not elute at the retention time of elacridar and therefore the analytical method passed the impurity test.

### 3.3. Application of the HPLC–UV method

The HPLC–UV method was successfully validated in order to analyze the purity, content and dissolution of the drug powder, intermediate product and final drug product. As an example, the content in a batch final drug product was 99.5% ± 2.0% and the purity was 100.0% ± 0.0% after one week of storage at –20 °C.

An example of the dissolution profile of the final drug product and a crystalline physical mixture (elacridar hydrochloride-PVPK30-SDS (1:6:1, m/m/m) are shown in Fig. 5. The low dissolution in crystalline physical mixture was caused by the low solubility of crystalline elacridar in water as previously reported [18]. The solid dispersion tablet significantly increased the dissolution of elacridar; however, after 60 min the concentration decreased due to recrystallization.

### 3.4. Characterization of the unknown degradation product

To characterize the degradation product, MS and MS\(^2\) spectra were obtained. The eluent of the validated HPLC–UV method was not MS-compliant because it contained an ammonium acetate concentration that induced ion suppression. Therefore, the ammonium acetate concentration in the eluent was lowered to 20 mM. Additionally, the acetonitrile content in the eluent was lowered to 35% to improve the separation between elacridar and the degradation product. Fig. 6 shows the HPLC–MS chromatograms of a reference solution that was stored for 4 days in the dark (Fig. 6A) or 4 days in indoor natural daylight (Fig. 6B). Elacridar (parent ion m/z 562) eluted at 31 min and was detected in both samples; however, the peak height was decreased in the solution that was stored in indoor natural daylight. The degradation product (parent ion m/z 578) eluted at 27 min and was only detected in the reference solution that was stored in indoor natural daylight. The 16 amu mass increase in the degradation product suggested hydroxylation of elacridar. The MS and MS\(^2\) spectra of elacridar are shown in Fig. 7A and they confirmed the identity of elacridar. The MS and MS\(^2\) spectra of the degradation product are shown in Fig. 7B. Only product ions in the 5-methoxy-9-oxo-9,10-dihydroacridine-4-carboxyl-ethylbenzenamine moiety were detected and in this part of the molecule no fragments with an increase of 16 amu were found. This indicates that the hydroxyl group was probably bound to the dimethoxyisoquinyl moiety of the molecule.

In the HPLC–MS chromatogram of the solution that was stored in light, an ion of mass m/z 562 was detected at 27 min (Fig. 6B) and at 31 min (elacridar). Although the mass of this ion was equal to that of the parent ion of elacridar this ion was not associated...
with elacridar. An isotope of ion \( m/z \) 560 which was formed after in-source fragmentation of \( m/z \) 578 (loss of water) explains the chromatographic peak detected at \( m/z \) 562 at the retention time of the degradation product. This effect could not be avoided by changing in-source ionization settings or by switching to positive ionization mode. The loss of a water molecule due to in-source fragmentation is common and was previously reported by us for ecteinascidin-743 which was a compound where a hydroxyl group coupled to a carbon atom next to an aliphatic amine group was lost in electrospray mode [33]. A similar structure is present in the degradation product.

To conclude, the fact that the degradation product elutes before elacridar, that it is 16 amu heavier than elacridar and that it only occurs in samples that are stored in indoor natural daylight conforms that it is caused by light-induced hydroxylation of elacridar. The proposed structure of the degradation product is hydroxylated elacridar and its chemical structure is presented in Fig. 7B.

4. Conclusion

An HPLC–UV method was developed and validated for the pharmaceutical quality control of a drug powder, an amorphous solid dispersion and a tablet formulation with elacridar as the API. The HPLC–UV method can be used to analyze the content, purity and dissolution. Light induces elacridar hydroxylation in aqueous samples and therefore light protection is required.

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