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HPLC method development, validation, and impurity characterization of a potent antitumor indenoisoquinoline, LMP776 (NSC 725776)

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Abstract

An HPLC method for the assay of a DNA topoisomerase inhibitor, LMP776 (NSC 725776), has been developed and validated. The stress testing of LMP776 was carried out in accordance with International Conference on Harmonization (ICH) guidelines Q1A (R2) under acidic, alkaline, oxidative, thermolytic, and photolytic conditions. The separation of LMP776 from its impurities and degradation products was achieved within 40 min on a Supelco Discovery HS F5 column (150 mm × 4.6 mm i.d., 5 μm) with a gradient mobile phase comprising 38–80% acetonitrile in water, with 0.1% trifluoroacetic acid in both phases. LC/MS was used to obtain mass data for characterization of impurities and degradation products. One major impurity was isolated through chloroform extraction and identified by NMR. The proposed HPLC assay method was validated for specificity, linearity (concentration range 0.25–0.75 mg/mL, $r = 0.9999$), accuracy (recovery 98.6–100.4%), precision (RSD = 1.4%), and sensitivity (LOD 0.13 μg/mL). The validated method was used in the stability study of the LMP776 drug substance in conformance with the ICH Q1A (R2) guideline.

Keywords

LMP776; NSC 725776; Forced degradation; HPLC validation; Impurity and degradation product; characterization; NMR

1. Introduction

DNA topoisomerases regulate the topological issues associated with DNA replication, transcription, recombination, and chromatin remodeling by introducing temporary single- or double-strand breaks in the DNA. In addition, these enzymes fine-tune the steady-state level of DNA—supercoiling to facilitate protein interactions with the DNA and to prevent excessive deleterious supercoiling [1]. The nuclear DNA topoisomerase 1 (TOP 1) is a recognized target for ovarian, lung, and colorectal cancer therapy [2].

The alkaloid camptothecin (CPT) is specifically targeted at the nuclear DNA TOP 1 from which the potent anticancer agents, irinotecan and topotecan, are derived [2]. However, these drugs suffer from structural instability (lactone moiety), and the TOP 1 cleavage complexes they induce are rapidly reversible. To overcome these limitations, the new class of non-CPT TOP 1 inhibitor called indenoisoquinolines has been synthesized, developed, and tested for their ability to inhibit TOP 1 and tumor growth [3]. Indenoisoquinolines have better chemical stability than their camptothecin counterparts. LMP776 and LMP400 (NSC 743400) are two representative compounds. In vitro, they induce TOP 1 cleavage at unique genomic positions and cause cell cycle arrest in both S and G (2)-M phases [4]. Protein-linked DNA breaks were observed in cells treated with nanomolar concentrations of LMP776 and LMP400, and both produced TOP 1 cleavage complexes with better stability even after removal of drug (1 μ M) than when induced by CPT or SN-38 (the active metabolite of irinotecan) [3–5]. They have demonstrated activity against camptothecin-resistant cell lines and produce DNA-protein crosslinks, which are resistant to reversal. They also show low or no resistance to cells overexpressing the ATP-binding cassette (ABC) transporter, ABCG2, and multidrug resistance (MDR-1) gene defects [3–5]. In addition to their effects on TOP 1, LMP776 and LMP400 may also exert part of their antitumor effect through antiangiogenesis [6]. As a result of their favorable pharmacological characteristics, LMP776 and LMP400 are promising anticancer drug candidates. They are currently being studied in a Phase I clinical trial in patients with relapsed solid tumors and lymphomas [7].

To support clinical development, LC MS/MS assay methods were validated for quantitation of LMP776 and LMP400 in human, rat, and dog plasma [8,9].

In spite of considerable numbers of publications describing the synthesis, efficacy, and mechanism of indenoisoquinolines, information about HPLC analysis and impurity characterization is lacking in the literature.

The goal of this work was to develop and validate a stability-indicating HPLC method for LMP776, a leading compound of its kind, in accordance with International Conference on Harmonization (ICH) guideline Q2 (R1), and to identify the impurities present in the drug substance, as well as degradation products observed in stress testing. The study was carried out with HPLC, LCMS, and NMR spectrometry. The validated HPLC method was used in the stability study of the LMP776 drug substance in conformance with the ICH Q1A (R2) guideline.

2. Materials and methods

2.1. Chemicals and reagents

LMP776 (NSC 725776) was provided by NCI (Bethesda, MD, USA). Chloroform (CHCl_3), trifluoroacetic acid (TFA), deuterated water (D_2O), and chloroform-*d* (CDCl_3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile (ACN) and hydrogen peroxide (H_2O_2) 30% solution were purchased from Mallinckrodt (Paris, KY, USA). Water was purified through a Millipore Super-Q Pure Water System (Waltham, MA, USA). Solutions of hydrochloric acid (HCl) and sodium hydroxide (NaOH) were prepared from Dilute-it Analytical Concentrate (J.T. Baker, Phillipsburg, NJ, USA).

2.2. HPLC

An Agilent 1100HPLC system (Wilmington, DE, USA) equipped with a solvent degasser, quaternary pump, autosampler, and a diode array detector was used in the study. Agilent ChemStation for LC 3D (Rev. A. 10.01) software was used for instrument operation control and data collection. The HPLC was performed on a Supelco, Discovery HS F5 column (5 μ , 150 $\times$ 4.6 mm I.D., St. Louis, MO, USA). The column was held at ambient temperature (23 $\pm$ 1 $^{\circ}$ C). The mobile phase was a combination of solvent A (0.1% TFA in water, v/v) and solvent B (0.1% TFA in ACN, v/v). The following gradient program was used: 0–20 min, 38% solvent B and 62% solvent A; 20–25 min, linear gradient to 80% solvent B and 20% solvent A; 25–30 min, hold at 80% solvent B and 20% solvent A; 30.1–40 min, re-equilibrate at 38% solvent B and 62% solvent A before the next injection. The injection volume was 5 μ L. For all gradient segments, the elution flow rate was 1.0 mL/min, and the detection wavelength was set at 270 nm.

LC/MS was performed on an Agilent LC/MS system consisting of an Agilent 1200 binary LC pump, a temperature-controlled autosampler, a photo diode array (PDA) UV detector, and a 6530 Accurate Mass Q-TOF mass spectrometer (Wilmington, DE, USA). The mass spectrometer was equipped with a JetStream® ESI probe operating at atmospheric pressure. The ESI source parameter settings were: mass range m/z 100–1000, gas temperature 350 $^{\circ}$ C, gas flow 10 L/min, nebulizer 50 psi, sheath gas temperature 400 $^{\circ}$ C, sheath gas flow 12 L/min, capillary voltage (Vcap) 3500 V, nozzle voltage 500 V, fragmentor 200 V, skimmer 65 V, octopole RF (OCT 1 RF Vpp) 750 V. Tandem mass spectrometry was performed using ramped collision energy at a slope of 3 and an offset of 10. The LC conditions used for identification of impurities and decomposition products of LMP776 were the same as those described above.

A second LC/MS system was used in some of the work for impurity D identification (Fig. 3). It was a ThermoQuest system consisting of a Surveyor LC pump, autosampler, PDA UV detector, and a Finnigan LCQ-DUO ion trap mass spectrometer (San Jose, CA, USA). The mass spectrometer was used with an electrospray ionization (ESI) probe at atmospheric pressure in positive ion mode, and signals in the mass range of m/z 200–2000 were collected. The LC conditions used in the LC/MS system were the same as those used in the Agilent 1100 LC-UV system except that the flow rate was reduced, and the gradient time and injection volume were increased to gain ESI–MS sensitivity. The following gradient program was used: 0–40 min, 38% solvent B and 62% solvent A; 40–50 min, linear gradient to 80% solvent B and 20% solvent A; 50–60 min, hold at 80% solvent B and 20% solvent A; 60.2–80 min, re-equilibrate at 38% solvent B and 62% solvent A before the next injection. The injection volume was 10 μ L. For all gradient segments, the elution flow rate was 0.5 mL/min.

2.3. Environmental chambers

The forced degradation study under UV and visible light was carried out in the ES 2000 Environmental Chamber (Environmental Specialties, Inc., Raleigh, NC, USA), equipped with a cool white lamp (8.0 klx) and a UV-A lamp (14.00 W/m²), in conformance with the

ICH Q1B option 2 for photostability testing. Temperature and humidity conditions were set at 25 °C/60% RH.

The LMP776 drug substance stability study was conducted with two ES 2000 environmental chambers set at 25 °C ± 2 °C/60% RH ± 5% RH, and 40 °C ± 2 °C/75% RH ± 5% RH in conformance with the ICH Q1A(R2) guideline.

2.4. Sample preparation

The HPLC test solutions were prepared by dissolving 1 mg of the drug substance in 2 mL of ACN/H₂O(1:1) with 0.1% TFA to obtain a 0.5-mg/mL solution. Although LMP776 is soluble at 0.5 mg/mL in ACN/H₂O (1:1), precipitation was observed after storage at room temperature for 20 h. TFA was needed to keep the compound soluble in the test solution.

The calibration standards were prepared by dissolving proportional amounts of the standard in 2 mL of ACN/H₂O (1:1) with 0.1% TFA to obtain five solutions at the concentrations of 0.25, 0.38, 0.50, 0.63, and 0.75 mg/mL.

The forced degradation samples were prepared as shown in Table 1. A stock solution was made by dissolving the LMP776 solid in ACN/H₂O (1:1, v/v) with 0.1% TFA at 1 mg/mL. The solutions for the forced degradation samples were prepared by 1:1 mixing of the stock solution and the various reagents; they were then treated with heat for a period of time (Table 1). The solid forced degradation samples were prepared by heating the solid sample or by exposing it to UV and cool white light in an environmental chamber (Table 1), and then dissolving in ACN/H₂O (1:1, v/v) with 0.1% TFA at ~0.5 mg/mL. The forced degradation samples were divided for HPLC/UV and LC/MS analysis.

For extraction of impurity **D**, approximate 200 mg of LMP776 (lot U/D7) was weighed into a 100-mL volumetric flask, and CHCl₃ was added to the mark. The solution was sonicated a few minutes at a time to avoid generating extra heat for a total of about 10 min and then left at room temperature overnight. The solution was then poured through a filter paper, and the filtrate was dried by a forced stream of air. The residue was then washed into a vial with about 5 mL of CHCl₃, and the resulting solution was similarly air dried. One mL of deuterated chloroform (CDCl₃) was added to dissolve the residue with sonication. The solution was filtered and split for NMR and LC/MS experiments. A blank solution was also prepared using CHCl₃ and the same extraction procedure. An ¹H NMR spectrum was obtained from the blank solution.

3. Results and discussion

3.1. HPLC method development and validation

The chemical structure of LMP776 (Fig. 1) contains a basic moiety of imidazole, and a hydrochloric salt was prepared to improve the compound's solubility for pharmaceutical use. Reversed-phase liquid chromatography with TFA as a weak ion-pairing agent is commonly used for analysis of basic compounds. A low-pH (pH <3) mobile phase would fully protonate the compound, resulting in a substantial retention time that would not be affected

by small variations in pH. The low-pH mobile phase also suppresses the silanols in the stationary phase and minimizes the tailing of basic compounds.

Method development was first started with a conventional C18 reversed-phase column and ACN/water mobile phase containing 0.1% TFA. The compound can be sufficiently retained but with excessive tailing (tailing factor > 3). Given the aromatic nature of the compound, a phenyl column was attempted. Tailing was slightly reduced on the phenyl column, yet impurities and degradation products were not well resolved. Because the compound possesses a multi-aromatic electron rich system, a pentafluorophenyl stationary phase was considered as a potential alternative since it may offer complementary interaction through its electron-deficient sites. Thus, a Discovery HS F5 column was selected to improve the method. The peak shape was dramatically improved on the HS F5 column (tailing factor 1.1). Forced degradation of the drug substance was performed (Section 2.4), and the gradient program was optimized to separate all impurities and degradation products.

The optimized LC condition (Section 2.2) was validated in accordance with the current ICH guideline Q2 (R1). Fig. 2a is a typical HPLC assay chromatogram with the system suitability data presented on the figure.

The method's specificity was verified by a UV peak purity check (Agilent ChemStation® software). Resolution of the peaks preceding and following the active drug in all forced-degradation samples was greater than 3.0.

Linearity of the method was demonstrated by standard curve in the range of 0.25 – 0.75 mg/mL (50–150% of the target assay concentration). The sample peak area (A, mAU) versus drug concentration (C, mg/mL) was analyzed by linear least square regression, which resulted in $A = 25778 C - 287.48$ with an excellent correlation coefficient ($r = 0.9999$).

Accuracy and intra-day precision were established by evaluating recoveries, and RSD values obtained with three test solutions each at concentrations of 0.25, 0.50, and 0.75 mg/mL corresponded to 50%, 100%, and 150% of the target assay concentrations, respectively. Recovery was calculated by comparing the theoretical concentration calculated from the calibration curve and the nominal concentration. The accuracy results showed recoveries between 98.6% and 100.4%. Precision was validated to be no greater than 1.4% RSD (intra-day). Inter-day precision was determined by repeat injection of one standard solution at 0.5 mg/mL on three separated days while the solution was kept at room temperature. The peak area RSD% was no greater than 1.07%. The detail accuracy and precision validation data are provided in Supplementary Tables 1 and 2. The LOD and LOQ were shown to be 0.13 µg/mL and 0.43 µg/mL, respectively, using the criteria of signal to noise (S/N) >3 for LOD and S/N >10 for LOQ. The sample solution stability was tested and shown to be stable at room temperature for 3 days with 100.1% recovery.

3.2. Characterization of impurities and degradation products

3.2.1. Characterization of impurities/degradation products by LC/MS—Fig. 2 shows chromatograms of the LMP776 drug substance. In a pilot developmental lot (U/D6), four impurities (A–D) were greater than 0.1% (Fig. 2a and b). Forced degradation was

performed with this lot. The compound was stable when it was stressed with acid, base, dry heat, and light exposure under the conditions described in Table 1. No major degradation products were observed. However, in H₂O₂ solution, degradation products **E**, **F**, and **G** were observed (Fig. 2c). In four subsequent lots synthesized at a different facility, impurity **D** became prominent with a peak area percentage that varied between 1.7–2.7%. Fig. 2d shows a chromatogram of lot U/D7, which contained 2.7% of impurity **D**.

LC/MS was performed to characterize the major impurities and degradation products. Mass and UV spectra were obtained through LC with high-resolution MS and PDA detection and are provided in Supplementary Figs. 1–2. Table 2 gives a summary of the major impurities and degradation products along with their mass and UV features. Based on the high-resolution accurate mass data and UV data, the proposed chemical structures of the major impurities and degradation products are provided in Fig. 1.

Impurities **A**, **B**, **C**, and **D** are present in the bulk drug substance. They are likely the synthetic by-products formed during the manufacturing process. The synthetic route of LMP776 is described in the cited references [5,10], and the key steps are summarized in Fig. 3 in Supplementary data. Impurity **A** may have been generated when intermediate 15 and imidazole were treated with K₂CO₃ at 100 °C for 4 h. Decomposition of imidazole in base at high temperature provided instead the primary alkyl amine (Impurity **A**). Impurity **B** may have been generated when intermediate 14 went through the intramolecular Friedel-Crafts acylation, in a non-selective manner, to give the alternative regioisomer, intermediate 15a, which was converted by subsequent steps to impurity **B**. Impurity **B** has a UV spectrum substantially different from that of LMP776, indicating the conjugation systems in the two molecules are not identical (Supplementary Fig. 2). Impurity **C** is the chlorine-substituted LMP776 which was likely generated during the treatment of intermediate 14 with excessive thionyl chloride (SOCl₂). The resultant Cl-containing intermediate 15b underwent the same chemical transformations as LMP776 to yield impurity **C**. Impurity **D** may have arisen when intermediate 14 was treated with SOCl₂ to form intermediate 15. The bromoalkyl side chain in 15 may have been substituted with chlorine in the presence of a large excess of SOCl₂.

MS/MS was performed to further characterize impurities and degradation products. The available MS/MS spectra are given in Supplementary Fig. 4. MS/MS fragment interpretations are given in Supplementary Fig. 5a and b. A common fragment of *m/z* 392.1129 was observed in LMP776 and impurities **A** and **B**, representing the loss of the imidazole group on the alkyl side chain. Similarly, impurity **C** has a major fragment of *m/z* 426.0793, also resultant from the loss of imidazole on the side chain. This fragment indicates that the chlorine substitution is not on the imidazole but on the indenoisoquinoline nucleus. Impurity **C** and degradation product **F** have the same rough mass (*m/z* 494) but differ in accurate mass. It was the accurate mass data that enabled the differentiation of a chlorine-substituted **C** and a dihydroxyl-substituted **F**. The HPLC retention times also appear to support the identities of **C** and **F** as the hydrophobic **C** eluted late at 24.7 min, whereas the hydrophilic **F** eluted early at 3.8 min.

3.2.2. Identification of impurity D—As impurity **D** was prominent in multiple synthetic lots, it was crucial to establish its definitive identity. MS data indicated impurity **D** was

possibly a compound with a chlorine substituted for imidazole in LMP776 (Fig. 1). This proposed structure has an inde-noisoquinoline skeleton identical to the active drug LMP776. We opted to use NMR spectral data to identify impurity **D**, but it had to be isolated from the active drug in order to obtain meaningful spectra because the active drug's signals are more prominent than those of the impurity when it co-exists with large amounts of the parent compound.

The common way to isolate impurities from the active drug is by preparative HPLC. However, this process is time-consuming because it requires collecting HPLC fractions and removing the solvents. At the same time, large quantities of solvents will be needed.

Considering the active drug is a hydrochloric salt and the proposed impurity **D** would be a non-polar lipophilic compound, we utilized their solubility differences to design the isolation process. As an HCl salt, the active drug LMP776 would not be soluble in chloroform. Therefore, impurity **D** was isolated from LMP776 lot U/D7 by chloroform extraction as described in the Section 2.4. The drug substance lot U/D7 contained 2.7% of impurity **D** by the peak area% in its chromatogram. The extracted amount of impurity **D** from a 200-mg of lot U/D7 material was estimated to be 3–5 mg, and this was dissolved in 1 mL of CDCl₃ (3–5 mg/mL) for ¹H NMR analysis. LC/MS was performed on the extract to verify the identity of the isolate. The retention time, UV, and mass spectra of the main component in the CHCl₃ extract were identical to those obtained from peak **D** in the original drug substance sample solution (Fig. 3). The chromatogram showed that the extracted material from LMP776 lot U/D7 drug substance contained mainly impurity **D**. ¹H NMR, correlation spectroscopy (COSY), heteronuclear multiple-quantum correlation (HMQC), and heteronuclear multiple-bond correlation spectroscopy (HMBC) data were obtained from the CHCl₃ extract. Supplementary Fig. 6 shows the ¹H NMR spectra of the isolate. A few signals marked with “*” are artifacts from the blank. The COSY, HMQC, and HMBC spectra are shown in Supplementary Figs. 7–9. The NMR data are consistent with the proposed structure of impurity **D** as indicated by the signal assignments given in Supplementary Figs. 6, 8 and 9. The ¹H NMR data obtained were also found to be consistent with the values reported in the literature [5]. Based on the LC/MS, NMR data and the reported values from the reference, the identity of impurity **D** in LMP776 was definitively established as 6-(3-chloro-1-propyl)-5,6-dihydro-5,11-dioxo-2,3-dimethoxy-8,9-methylenedioxy-11*H*-indeno[1,2-*c*]isoquinoline.

3.3. Stability study

The validated method was used in the stability study of the LMP776 drug substance (lot U/D7). The assay, purity, and water content results are given in Supplementary Table 3. The stability results indicate that the compound is chemically stable at all tested conditions and time points.

4. Conclusion

An HPLC assay method has been developed and validated for LMP776. The HPLC method separates LMP776 from its impurities and forced degradation products. Identities of the impurities and degradation products have been elucidated by MS and UV spectral data. One

major impurity was isolated and identified by NMR. The impurity was isolated by chloroform extraction. The isolation method utilized the solubility difference between LMP776 and the impurity and obviated the need for tedious and time-consuming preparative HPLC work. It also demonstrated a good alternative method in the field of impurity isolation for identification. The major impurity was identified to be 6-(3-chloro-1-propyl)-5,6-dihydro-5,11-dioxo-2,3-dimethoxy-8,9-methylenedioxy-11*H*-indeno[1,2-*c*] isoquinoline. The assay method has been validated to be specific, linear ($r = 0.9999$), accurate (recovery 98.6–100.4%), precise (RSD = 1.4%), and sensitive (LOD = 0.13 $\mu\text{g/mL}$). The validated method was used in the stability study of the LMP776 drug substance in conformance with the ICH Q1A (R2) guideline. The stability results indicate that the compound is chemically stable at 25 °C/60% RH for at least 12 months, and at 40 °C/75% RH for at least 6 months.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jpba.2016.02.036>.

Fig. 1.
Structure of LMP776 and proposed structures of its impurities and degradation products.

Fig. 2.

Chromatograms of LMP776.

- (a) 0.5 mg/mL U/D6 in ACN/H₂O (1:1) with 0.1% TFA, full scale.
- (b) 0.5 mg/mL U/D6 in ACN/H₂O (1:1) with 0.1% TFA, expanded scale.
- (c) 0.5 mg/mL U/D6 in ACN/H₂O (1:1) with 0.1% TFA and 5% H₂O₂ (1:1), heated at 80°C for 2 h, expanded scale.
- (d) 0.5 mg/mL U/D7 in ACN/H₂O (1:1) with 0.1% TFA, full scale.

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Fig. 3. Comparison of peak **D** in LMP776 lot U/D7 and in CHCl_3 extract.(a) Chromatogram of LMP776 lot U/D7, 1 mg/ml in $\text{ACN}/\text{H}_2\text{O} \times 10 \mu\text{l}$, detected at UV 270 nm.(b) Chromatogram of the CHCl_3 extract from LMP776 lot U/D7 in $\text{CDCl}_3 \times 2 \mu\text{l}$, detected at UV 270 nm.(c) UV of peak **D** from chromatogram (a).(d) MS of peak **D** from chromatogram (a).(e) UV of peak **D** from chromatogram (b).(f) MS of peak **D** from chromatogram (b).

Table 1

Forced-degradation condition.

Sample Description	Degradation Condition
Stock solution	1 mg/mL in ACN/H ₂ O(1:1) with 0.1% TFA
Diluent ^a	ACN/H ₂ O (1:1) with 0.1% TFA, 80 °C, 2 h
Acid ^a	0.1 N HCl, 80 °C, 2 h
Base ^a	0.1 N NaOH, 80 °C, 2 h, neutralize with HCl
Oxidation ^a	5% H ₂ O ₂ , 80 °C, 2 h
Solid dry heat	Solid heated at 80 °C, 24 h
Solid photo	UV 14 h, cool white light 168 h
Solid photo control	UV 14 h, cool white light 168 h, wrapped in foil

^aThese samples were 1:1 (v/v) mixture of the stock solution and the specified reagents.

Table 2

Summary of chromatographic and spectral data for impurities and degradation products.

Peak Label	RT (min)	λ_{max} , nm	Measured m/z	Calculated m/z	Error (ppm)
F	3.8	240/266/302	494.1572	494.1558	2.83
G	4.1	258/374	424.1508	424.1503	1.18
A	10.0	270/288	409.1388	409.1394	-1.47
B	10.6	260/304	460.1508	460.1503	1.09
E	12.4	262/306/328	431.39 ^a	431.1449	N/A
LMP776	15.1	270/288	460.1511	460.1503	1.74
C	24.7	268/288	494.1114	494.1113	0.20
D	25.9	268/288	428.0901	428.0895	1.40

^a Accurate mass data not available for peak **E**.