A Simple and Rapid LC-MS/MS Method for Therapeutic Drug Monitoring of Lenalidomide

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Abstract
Immunomodulatory drugs lenalidomide (LENA) and pomalidomide (POMA) are synthetic compounds derived by modifying the chemical structure of thalidomide to improve its potency and reduce its side effects. LENA is used as a treatment for myeloma and blood disorders called myelodysplastic syndromes. The maximum clinical dose of LENA for some haematological cancers is generally ≤25 mg. Therapeutic drug monitoring (TDM) is important for the individualization of drug dosage. A highly sensitive and high performance liquid chromatography tandem mass spectrometry (HP LC–MS/MS) assay was developed and validated for the quantification of LENA in human plasma. LENA was extracted from human plasma by liquid-liquid extraction by ethyl acetate and analysed using a reversed phase isocratic elution on a Poroshell 120 EC-C18, (4.6 - 50 mm, 2.7µm) column. A. 0.1% formic acid: methanol (10:90% v/v), was used as mobile phase and detection was performed by triple quadrupole mass spectrometry LC–MS/MS using jet stream electrospray ionization in negative mode. POMA was used as the internal standard (IS). Analyte and IS were detected by tandem mass spectrometry using multiple reaction monitoring (MRM) of precursor–product ion transitions with 0.100 s dwell time, at m/z 258.0 > 213.0 for LENA and m/z 272.0 > 161.0 for POMA. The calibration curves were consistently accurate and precise over the concentration range of 20 to 1000 ng/mL in plasma for LENA. This novel LC–MS/MS method competes with all the regulatory requirements and shows satisfactory accuracy and precision. It is sufficiently sensitive for the performance of pharmacokinetic, bioequivalence and TDM studies in humans.

Keywords: Lenalidomide, LC-MS/MS, Therapeutic drug monitoring.

Introduction

B-cell malignancies of the plasma cell cause multiple myeloma which is the second most common haematological malignancy. LENA (Fig. 1) is one of the novel compounds which is used for the treatment of this disease, it is based on the molecular structure of thalidomide and has an improved immunomodulatory effect and safety profile [1]. Thalidomide is a known human teratogen that causes severe life-threatening human birth defects. Use of LENA during pregnancy may cause anomaly or embryo-fetal death. LENA (3-(4-amino-1,3-dihydro-1-oxo-2H-isindol-2-yl)-2,6-piperidinedione is an oral immunomodulatory drug (IMiD), with antineoplastic, antiangiogenic and anti-inflammatory properties [2]. It has been used for the treatment of transfusion-dependent anemia due to lower intermediate-risk myelodysplastic syndromes (MDS) associated with a deletion 5q cytogenetic abnormality with or without additional cytogenetic abnormalities and in combination with dexamethasone for the treatment of multiple myeloma (MM) patients who have received at least one prior therapy [3]. LENA has a short half-life (3–4 h) and does not accumulate in plasma upon repeated dosing. Its pharmacokinetics are
consistent across patient populations, regardless of the type of hematologic malignancy [4]. Studies have shown that LENA may work through various mechanisms in different hematologic malignancies. These mechanism involved direct cytotoxicity as well as through indirect effects on tumor immunity. Thus the differential efficacy noted with LENA therapy among various disease states can possibly be explained individual's immune status and disease specific pathophysiology [5].

Figure 1. Chemical structure of Thalidomide (a), Lenalidomide (b), Pomalidomide (c)

The aim of TDM of LENA is to individualize therapeutic regimens for maximizing patient benefit. It is unnecessary to employ TDM for the majority of medications, and it is used mainly for monitoring drugs with narrow therapeutic ranges, drugs with marked pharmacokinetic variability, medications for which target concentrations are difficult to monitor, and drugs known to cause therapeutic and adverse effects [9]. The results from this trial demonstrated that daily doses of LENA equal to or greater than 75 mg can result in significant myelosuppression. Although thrombocytopenia was also observed, it was the severity of neutropenia induced by the treatment that proved dose limiting [10]. The maximum approved therapeutic dose of LENA is 25 mg daily for patients with RRMM (relapsed and refractory multiple myeloma) and 10 mg daily for patients with MDS. The maximum clinical dose of LENA for other hematological cancers is generally ≤25 mg. In terms of pharmacokinetic disposition, LENA undergoes minimum metabolism, and 82% of the LENA dose is eliminated unchanged via urinary excretion. Renal impairment reduces the elimination of LENA, thereby increasing drug concentration in plasma. As patients with MM or MDS often have compromised renal function due to their age or comorbidities, plasma LENA exposure is generally higher in these patients than in young patients.
Among the currently available bio-analytical techniques LC-MS/MS has gained a considerable attention in recent years and has been emerged as the preeminent analytical tool for pharmaceutical and biomedical analysis because of its high speed, low time consumption, better resolution and better sensitivity.

The present study describes the development and validation of an HPLC method coupled with HPLC-MS/MS for the determination of LENA in human plasma for TDM study. The proposed method is simple, highly sensitive and it has high-throughput and reproducible.

Materials and Methods

Chemicals and reagents

LENA working standard was certified to contain 100.30% purity for LENA. Internal standard was certified to contain 99.70% purity for POMA. LC-MS Hyper grade methanol was used as an organic solvent and obtained from Merck (Darmstadt, Germany). Standard preparation and further dilution was made by ethanol. The HPLC grade Merck (Darmstadt, Germany) ethyl acetate was used in liquid-liquid extraction. Ultrapure water was obtained from a Milli-Q water system (Millipore, Barnstead). HPLC grade formic acid used in mobile phase preparation was supplied from Merck (Darmstadt, Germany).

Chromatographic conditions

Agilent 1260 Infinity Liquid Chromatography system and Triple Quadrupole QQQ-6460 detector were used in this study. Analytical separation was carried out on a Poroshell 120 EC-C18, (4.6 - 50 mm, 2.7µm) chromatographic column and maintained at 30°C. The LC mobile phase was consisted of methanol: 0.1% formic Acid (90:10%, v/v). The flow rate of the analytical method was 0.8 mL/min. The injection volume was 3.0 µL and the analysis runtime was 2.0 min.

Mass spectrometry conditions

Detection was carried out Agilent 6460 triple quadrupole MS/MS combining with Agilent Jet Stream Electrospray ionization (AJS-ESI)
operated in the negative ion mode. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of m/z 258.0 > 213.0 for LENA and m/z 272.0 > 161.0 for POMA, with the dwell time of 100 ms. Ultrapure (99.999%) nitrogen gas was used in the collision cell for generating product ions and fragments. The optimized conditions were as follows: nebulizer pressure, drying gas temperature, sheath gas temperature, drying gas flow, sheath gas flow, capillary, and nozzle voltage. The MRM transitions and the related optimized fragmentor voltage were 140 V for LENA and 120 V for POMA. Collision energy were 11 V for analyte and 14 V for the IS.

**Preparation of solutions**

Standard stock solutions of LENA 1.0 mg/mL (w/v) and POMA (IS) 1.0 mg/mL (w/v) were separately prepared in 5 mL volumetric flasks with methanol. Working solutions for calibration and quality control samples were prepared from the stock solution by diluting with methanol. Spiked plasma calibration standard solutions and quality control sample solutions were prepared at concentrations of 20, 50, 100, 200, 500, 1000 ng/mL and 30, 250, 750 ng/mL, respectively. IS working solution (50.0 µg/mL) was prepared by diluting by the stock solution.

**Plasma sample preparation**

A simple and rapid extraction procedure was developed which provides a consistent matrix composition between samples and minimizes the potential for concentration dependent variation in protein binding. 500 µL of plasma samples were taken into a polypropylene tubes and 100 µL of IS added. Sample preparation involved a liquid-liquid extraction with ethyl acetate. 1.0 mL of ethyl acetate was added to each tube and vortexed for 2.5 min at 2800 rpm. Then samples were centrifuged at 5000 rpm for 10 min at 20°C, 0.75 mL of supernatant layer was transferred to the vial and analyzed immediately.

**Method validation**

**Selectivity**

The analytical method should be able to differentiate the analyte(s) of interest and IS from endogenous components in the matrix or other components in the sample. Selectivity should be proved using at least 6 individual sources of the appropriate blank matrix, which are individually analysed and evaluated for interference.

The selectivity of the method towards endogenous plasma matrix components and component medications was assessed in human blank plasma.

**Linearity**

Linearity demonstrates the accurate measurement of concentrations of spiked samples. For this purpose, plasma samples spiked at 6 concentration levels (20-50-100-200-500-1000 ng/mL) were extracted. For the quantification of the samples a calibration curve was constructed by spiking blank plasma at 6 concentration levels (20-1000 ng/mL).

Calibration curves were constructed using matrix matched calibration standard solutions by plotting the peak area of the quantitative ion of each analyte versus concentrations. The determination coefficients (r²) were consistently greater than 0.999 during the course of validation.

**Accuracy and precision**

Accuracy was assessed on samples spiked with known amounts of the analyte, the quality control samples (QC samples). The QC samples were spiked independently from the calibration standards, using separately prepared stock solutions. Accuracy was determined by analysing 6 samples per level at a minimum of 3 concentration levels which are covering the calibration curve range.

Precision was demonstrated for the LOQ, low, medium and high QC samples, within a single run and between different runs, i.e. using the same runs and data as for the demonstration of
accuracy. Precision is expressed as %CV (coefficient of variation) for 18 determinations. Intra assay precision and accuracy of LENA were calculated at 30 ng/mL, 250 ng/mL and 750 ng/mL levels for the six replicates, each of the same analytical run. Inter assay precision and accuracy was calculated after the replicates in three different analytical runs.

**Matrix effect**

Liquid chromatography coupled to mass spectrometry (LC–MS) is a powerful tool for bioanalysis. Reliable bioanalysis requires the characterization of the matrix effect, i.e. influence of the endogenous or exogenous compounds on the analyte signal intensity.

Matrix effect is evaluated using pool plasma from different sources to prepare samples. For analyte and the IS, the matrix factor (MF) are calculated in each matrix, by calculating the ratio of the peak area in the presence of matrix (measured by analysing blank matrix spiked with analyte at a concentration of quality control samples after extraction), to the peak area in absence of matrix (pure solution of the analyte). The IS normalised MF is calculated by dividing the MF of the analyte by the MF of the IS.

Extracted and aqueous samples were compared to determine the MF for analyte and IS. IS normalized MF for individual lot is also determined. The calculated MF of all LQC (low quantity concentration) and HQC (high quantity concentration) samples must be within 85–115% of their nominal concentration.

**System suitability**

System suitability tests are an integral part of GC and LC methods. These tests are used to verify that the chromatographic system is adequate for the intended analysis. The tests are based on the concept that the equipment, electronics, analytical operations, and samples analyzed constitute an integral system that can be evaluated as such. RS (resolution) is a function of the number of theoretical plates, N (number of theoretical plates) (also referred to as efficiency) and \( k' \) (capacity factor).

**Results and Discussion**

The analysis was performed in the mass spectrometer using an electrospray (ESI) in the negative ionization mode. The reliability of the method was assessed on the basis of linearity, sensitivity, selectivity, precision and accuracy. On the basis of the results obtained for the above parameters, the combination of mobile phase, flow rate and detector parameters were selected for validation. Out of several tried combinations, results suggested a combination of 0.1% formic acid: methanol (10:90% v/v) ratio of mobile phase, flow rate 0.800 mL/min which resulted in a retention time of 0.568 min for LENA and 0.543 min for the IS. LC-MS/MS operation parameters were carefully optimized for the determination of LENA. Both analyte and IS were detected by tandem mass spectrometry using MRM of precursor–product ion transitions with 0.100 s dwell time at m/z 258.0 > 213.0 for LENA and m/z 272.0 > 161.0 for POMA. The mass spectrometer was tuned initially in both positive and negative ionization modes for LENA. It was observed that the signal intensity of negative ion was much higher than that of positive ion. Parameters, such as capillary and nozzle voltage, desolvation temperature, ESI source temperature and flow rate of desolvation gas and cone gas, were optimized to obtain the optimum intensity of molecules of LENA and IS for quantification.

The optimized mass analyzer conditions were as follows; nebulizer pressure 45 psi, drying gas temperature 320°C, sheath gas temperature 250°C, drying gas flow 5 (L/min), sheath gas flow 11 (L/min), capillary 3500 V and nozzle voltage 500 V. The MRM transitions and the related optimized fragmentor voltage were 140 V for analyte and 120 V for POMA. Collision energy were 11 V for analyte and 14 V for the IS.

MRM is a highly specific and sensitive mass spectrometry technique that can selectively quantify compounds within complex mixtures. This technique uses a triple quadrupole MS that firstly targets the ion corresponding to the
compound of interest with subsequent fragmentation of that target ion to produce a range of product ions. One (or more of these fragment product ions can be selected for quantification purposes. Only compounds that meet both these criteria, i.e. specific precursor ion and specific product ions corresponding to the mass of the molecule of interest are isolated within the mass spectrometer. Mass spectrometry MRM mode also provides us to analyze compounds with the same retention time (See Fig. 2 and Fig. 3).

**Figure 2.** Overlaid chromatograms of lenalidomide and pomalidomide (IS) with using MRM mode

**Figure 3.** Extracted chromatograms of lenalidomide and pomalidomide (IS) with using MRM mode

**Figure 4.** Chromatogram of blank plasma
The selectivity was carried out using six different individual sources and analyzed using the proposed extraction method. The plasma batch did not show any interferences at the retention times of LENA and POMA. Representative chromatogram of blank plasma is given in Fig. 4.

The calibration curve regression \( r^2 \) for lenalidomide was found to be 0.99976. Coefficient of determination \( r^2 \) for validation was greater than 0.99 which provide the requirements of bioanalytical method validation guideline. Calibration curve’s equation is \( y = 0.001x - 0.0173 \). Chen et. al. investigated plasma pharmacokinetic parameters of LENA in volunteers. The Cmax was found 390 ng/mL for young adults and 568 ng/mL for older adults in the clinical trial [4]. For the propose of Cmax value must be in the range of calibration curve. Linear concentration range of LENA was found to be accurate and precise from 20 to 1000 ng/mL. LOD and LOQ values were determined as 4.8 ng/mL and 14.5 ng/mL, respectively.

Two ultra-fast HPLC-MS/MS methods have been developed for the determination of LENA in plasma in the literature. While our linear range values are similar, there are differences in LOQ values due to ultra-fast HPLC and LC methods [2, 17].

Accuracy and precision of LENA were calculated at 30 ng/mL, 250 ng/mL and 750 ng/mL levels for the six replicates. Coefficients of variation results were 4.60 %, 3.75% and 1.65%, respectively whereas accuracies were 103.02 ± 3.02, 99.06 ± 0.94 and 101.01 ± 1.01, respectively in human plasma (Table 1). Values are within the acceptance limit of ±15%. The results indicate that the method has good precision and accuracy.

Table 1. Lenalidomide accuracy and precision in QC samples.

| Conc. (ng/mL) | Accuracy ( % Error) | Precision ( % CV) |
|--------------|---------------------|------------------|
| 30           | 103.02 ± 3.02       | 4.60             |
| 250          | 99.06 ± 0.94        | 3.75             |
| 750          | 101.01 ± 1.01       | 1.65             |

The calculated MF coefficients of variation was 11.67% within 85–115% of their nominal concentration. Determination of matrix effect by the slope of the calibration curve is given in Fig. 5. The method showed that no matrix effect was found for human plasma for TDM routine analysis.

System suitability parameters found as specified in guideline. Theoretical plate number (N) was calculated as 15700, capacity factor (k') 9.8, resolution (Rs) 14, tailing factor (T) 1.1 for LENA. Detailed parameters are given in Table 2.

Table 2. ICH guideline system suitability parameters, limits and results.

| Parameters                  | Limits    | Results |
|-----------------------------|-----------|---------|
| Capacity factor (k')        | > 2       | 9.80    |
| Injection accuracy (RSD)    | ≤ %1, n ≥ 5 | 0.49   |
| Resolution (Rs)             | > 2       | 14      |
| Tailing factor (T)          | ≤ 2       | 1.1     |
| Number of theoretical plates (N) | > 2000 | 15700   |

For LENA, TDM aims to enhance drug efficacy, reduce toxicity or assist with diagnosis. Despite its apparent advantages, it has also limitations. Some large hospitals have services which provide support with drug monitoring and interpretation of results. Unless TDM is being used to forecast a dose or there are concerns about toxicity, samples should be taken at steady state (4-5 half-lives after starting therapy). TDM can optimise patient management and improve clinical outcomes [9].
Conclusion

A novel simple, economical, high-throughput and highly sensitive HPLC-MS/MS method was successfully developed and validated for the determination of LENA in human plasma. The method involved simple one step protein precipitation method for plasma sample preparation and short runtime (2.0 min) for analysis. The proposed method could be practical and reliable for pharmacokinetic and TDM study for LENA in human.

References

1. J. B. Bartlett, K. Dredge and A. G. Dalgleish, Nat. Rev. Cancer, 4 (2004) 314. doi: 10.1038/nrc1323.
2. M. S. Hasnain, S. Rao, M. K. Singh, N. Vig, A. Gupta, A. Ansari, P. Sen, P. Joshi and S. A. Ansari, Analyst, 138 (2013) 1581. doi: 10.1039/c2an36701g.
3. N. Chen, L. Wen, H. Lau, S. Surapaneni and G. Kumar, Cancer Chemo. Pharma., 69 (2012) 789. doi: 10.1007/s00280-011-1760-3.
4. N. Chen, S. Zhou and M. Palmisano, Clin. Pharma., 56 (2017) 139. doi: 10.1007/s40262-016-0432-1.
5. V. Kotla, S. Goel, S. Nischal1, C. Heuck, K. Vivek, B. Das and A. Verma, J. Hematol. Oncol., 2 (2009) 36. doi:10.1186/1756-8722-2-36
6. C. Hiemke, N. Bergemann, H. W. Clement, A. Conca, J. Deckert, K. Domschke, G. Eckermann, K. Egberts, M. Gerlach, C. Greiner, G. Gründer, E. Haen, U. Havemann-Reinecke, G. Hefner, R. Helmer, G. Janssen, E. Jaquenoud, G. Laux, T. Messer, R. Mössner, M. J. Müller, M. Paulzen, B. Pfuhlmann, P. Riederer, A. Saria, B. Schoppek, G. Schoresantisits, M. Schwarz, M. Silva Gracia, B. Stegmann, W. Steimer, J. C. Stingl, M. Uhr, S. Ulrich, S. Unterecker, R. Waschgler, G. Zernig, G. Zurek and P. Baumann, Pharmacopsychiatry, 51 (2018) 9. doi:10.1055/s-0043-116492
7. C. Bardin, G. Veal, A. Paci, E. Chatelut, A. Astier, D. Leveque, N. Widmer and J. Beijnen, Eur. J. Cancer, 50 (2014) 2005. doi:10.1016/j.ejca.2014.04.013
8. L. K. Johnson-Davis and G. A. McMillin, Chapter3 in Personalized Immunosuppression in Transplantation, Role of Biomarker Monitoring and Therapeutic Drug Monitoring, (2016) 57, M. Oellerich and A. Dasgupta (Eds). doi:http://dx.doi.org/10.1016/B978-0-12-800885-0.00003-5
9. R. A. Ghiculescu, Aust. Prescr., 31 (2008) 42. doi: 10.18773/austprescr.2008.025.
10. R. A. Sharma, W. P. Steward, C. A. Daines, R. D. Knight, K. J. O’Byrne, A. G. Dalgleish, Eur. J. Cancer., 42 (2006) 2318. doi:10.1016/j.ejca.2006.05.018.
11. A. P. G. Rodríguez, Adv. Ther., 28 (2011) 1. doi: 10.1007/s12325-010-0104-8.
12. B. S. Sastry, S. Gananadhamu, S. V. S. G. Prasad and K. V. G. Raju, Int. J. Pharm. Tech. Res., 1 (2009) 416. http://sphinxsai.com/PTVOL3/PT=5%20GANA%20NADHAM%20(416-419).pdf
13. I. A. Darwish, N. Y. Khalil, A. H. Bakheit and N. Z. Alzoman, Chem. Cent. J., 6 (2012) 118. doi:10.1186/1752-153X-6-118.
14. B. G. López, A. P. Pitarch, M. A. M. Gómez, B. P. Oltra, M. C. Martí and M. M. Sanjuán, J. Lab. Automation, 21 (2016) 806. doi:10.1177/2211068216636570
15. Q. Liu, K. L. Farley, A. J. Johnson, N. Muthusamy, C. C. Hofmeister, K. A. Blum, L. J. Schaaf, M. R. Grever, J. C. Byrd, J. T. Dalton and M. A. Phelps, Ther. Drug Monit., 30 (2008) 620. doi:10.1097/FTD.0b013e318185813d.
16. L. Anderson and C. L. Hunter, Mol. Cell Proteomics, 5 (2006) 573. doi:10.1074/mcp.M500331-MCP200
17. P. Ranganathan, V. Gunasekaran, I. Singhvi and M. J. Ansari, Saudi J. Biol. Sci., 26 (2019) 1843. doi:10.1016/j.sjbs.2018.02.006