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A Validated Uplc-Ms/Ms Method for the Quantification of Cyclophosphamide in Human Plasma: Application to Therapeutic Drug Monitoring in Cancer Patients

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Abstract:

A rapid, simple, sensitive, and highly specific method has been developed for determination of Cyclophosphamide in human plasma. It is in a class of medication called alkylating agents used to treat various forms of cancer. The analytical procedure involves a liquid-liquid extraction method using Cyclophophamide-D8 as an internal standard. Chromatographic separation was carried out on a reversed phase Agilent Zorbax, XDB (150mm x 4.6mm, 5µm) column. Cyclophosphamide and Cyclophophamide-D8 were detected with proton adducts at (262.90/141.60) and (270.90/148.60) respectively in multiple reaction monitoring (MRM) positive mode. The method was linear over the concentration range of 20–15000 ng mL−1. The lower limit of detection (LLOD) Cyclophosphamide was 20 ng mL−1. The

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method was shown to be precise with the average within-run and between-run variations of 2.78 to 11.13% and 2.82 to 10.70%, respectively. The mean recoveries of Cyclophophamide from human plasma by the developed method were 100.1-101%. This validated method has been successfully applied for routine therapeutic drug monitoring of Cyclophosphamide in cancer patients.

Key Words: Cancer, Cyclophosphamide, UPLC–MS/MS, human plasma, Liquid-liquid extraction & therapeutic drug monitoring.

INTRODUCTION:

The most frequently used alkylating agents are the nitrogen mustards. Cyclophosphamide is one among which exerts its effects through the alkylation of DNA. The drug is not cell-cycle phase-specific and metabolizes to an active form capable of inhibiting protein synthesis through DNA and RNA crosslinking [1] [2]. It has been shown that Cyclophosphamide is highly effective in the treatment of malignant lymphomas, multiple myeloma, leukemias, and carcinoma of the breast. Cyclophosphamide is effective alone in susceptible malignancies. However, it is more effective when applied with other antineoplastic drugs. Monitoring the concentration of Cyclophosphamide in the blood and plasma of patients can direct dose adjustments to achieve optimum therapy with minimum adverse reactions. [3]. Cyclophosphamide produces less gastrointestinal and hematopoietic toxicity than other alkylating agents [4]. The drug is predominantly activated by liver cytochrome P450 enzymes. [5, 6] The efficacy and toxicity of many anticancer drugs are related to parent drug and/or metabolite concentrations. [7] Therefore, a profound knowledge of the monitoring of the drug is essential. Furthermore, since anticancer agents often have narrow therapeutic windows, drug monitoring routines in clinical settings may be required for safe and efficacious therapeutic use. [8]

Till date, various methods were reported to estimate the Cyclophosphamide using various chromatographic techniques such as high performance liquid chromatography-ultra violet detector (HPLC-UV) [9-13], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [14-22], gas chromatography-mass spectrometry (GC-MS) [23-24]. However, these methods have experienced some shortcomings that limited their applications to high sample throughput or monitoring drug concentration in humans. For example, several methods based on HPLC have long analytical run time >5 min with high flow rate of >0.5ml min-1 which

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consume more solvent and the cost of the method increases. The sensitivity is relatively low and even large amount of plasma have been used. Moreover, no publication has described the quantitative analysis of Cyclophosphamide using ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS-MS) utilizing Cyclophosphamide D8 as IS which plays a important role in enhancing the accuracy of the method by minimizing the matrix effect [25].

UPLC–MS-MS has been evaluated as a faster and more efficient analytical tool compared with current chromatography. In the present study, we developed a UPLC–MS-MS method for the determination of Cyclophosphamide using Cyclophosphamide D8 as an internal standard (IS). This new method has been fully validated in terms of selectivity, linearity, lower limit of quantification (LLOQ), accuracy, precision, stability, matrix effect and recovery. It has been successfully applied in a TDM study conducted in cancer patients.

2. MATERIALS AND METHODS:

Chemicals and reagents

The reference standards of Cyclophosphamide (N,N-bis(2-chloroethyl)-2-oxo-1,3,2λ^5-oxazaphosphinan-2-amine) (Figure 1) & Cyclophosphamide D8 as internal standard (IS) 2-(bis(2-chloroethyl-1,1,2,2-D4)amino)-1,3,2-oxazaphosphinane 2-oxide, (Figure 2) were commercially procured from Clearsynth Labs Limited (Mumbai, India). HPLC-graded acetonitrile, water, formic acid were purchased from Merck (Mumbai, India).

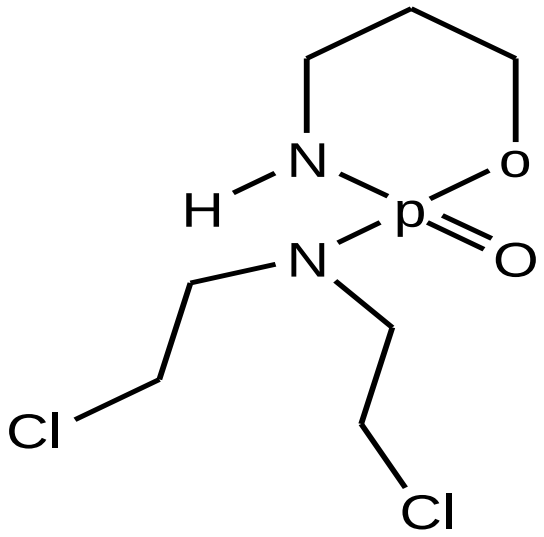

Figure 1: Structure of Cyclophosphamide

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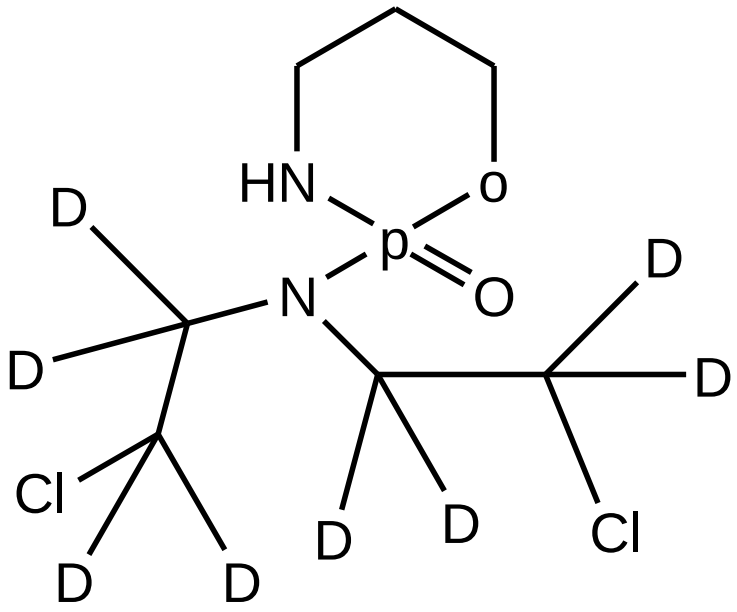

Figure 2: Structure of Cyclophosphamide D8

Instrument

Waters Quattro Premier LC-MS/MS with Acquity UPLC System from Waters Corporation (Hyderabad, India) equipped with a vacuum degasser, gradient binary pump, autosampler, column oven with ESI mode of MRM employed to analyze the samples. Centrifuge apparatus (Remi, Mumbai, India), Sonicator (Sharp Analytical, Hyderabad, India), Rotary shaker (Vibramax, Germany), Analytical balance (Metler Toledo, Mumbai, India), Pipettes- (Accupipet, West Bengal, India).

Chromatographic condition and optimization

The chromatographic separation was optimized with variations in the column, mobile phase composition, and flow rate. The optimal condition was achieved using Agilent Zorbax XDB C18 column (150mm x 4.6mm, 5µm; Agilent technologies, Hyderabad, India). The temperature of the column was regulated at 30°C. The components were eluted using a mobile phase composed of solvent A (0.1% formic acid) & (Acetonitrile) solvent B in the ratio of 20:80 v/v isocratic elution was used with the mobile phase flow of 0.4 mL/min of a total run time of 3.5 minutes. Aliquots of 15µL were injected for the analysis.

Mass spectrometric conditions

Quantification was achieved by using MS/MS detection in an ESI positive ion mode. The Mass spectrometer settings for Cyclophosphamide (262.90/141.60) and Cyclophosphamide

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D8 (IS) was (270.90/148.60). Scheduled MRM was employed for the quantification of Cyclophosphamide and IS. The optimized selective MRM transitions and their respective energies were listed in Table 1. MassLynx software (Version 1.4, Waters Corporation, India) was used for data acquisition & processing.

Table 1: Optimized LC retention time & MRM transitions for Cyclophosphamide and Cyclophosphamide D8 (IS)

Analyte	Q1 m/z	Q2 m/z	Dwell time (secs)	CE (V)	Cone Volt (V)
Cyclophosphamide	262.90	141.60	0.200	20.0	30.0
Cyclophosphamide D8	270.90	148.60	0.200	20.0	30.0

Abbreviation: Q1, Precursor ion; Q2, Product ion; CE, Collisional energy (V); Cone Volt, Cone Voltage (V)

Working standard, Calibration, and Quality control samples:

Stock solutions at 1 mg/mL were prepared for Cyclophosphamide & IS. The working standard solutions were prepared from the serial dilution method. A Calibration curve was constructed in the range of 20-15000 ng/mL. Eight point calibration was made at concentrations of 20, 40, 200, 1000, 5000, 8000, 12000, 15000 ng/mL. The lower limit of quantification (LLOQ), low-quality control (LQC), Geometric mean quality control (GMQC), medium quality control (QMC), high-quality control (HQC) were prepared at Cyclophosphamide concentrations of 20, 60, 670, 7500 & 13000 ng/mL respectively.

A solution of IS (100 ng/mL) was prepared from a stock solution with methanol. All the prepared stocks, calibration standards were stored at 2-8°C. The CC, QC and plasma samples were stored at -20°C & brought to room temperature before analysis.

Sample extraction

The liquid-liquid extraction (LLE) method was employed to extract drug & IS from human plasma. An aliquot of 50 µl of was transferred into Eppendorf tube, followed by the addition of 20 µl of IS and 1ml of MTBE. This mixture was vortexed & subjected to centrifugation (4000 rpm) for 10 min at 20^0 C. 0.800 ml of the supernatant organic layer was collected, transferred to another tube, and evaporated to dryness at 40^0 C in nitrogen evaporator.

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Reconstituted in 100 µl aliquots of mobile phase, and 15 µl were injected into the UPLC-MS/MS system for detection.

Method validation parameters

Method validation was carried out according to the US FDA guidelines [26] guidelines. This included evaluation of selectivity, sensitivity, linearity, precision and accuracy, recovery, matrix effects and stability.

3. Results and discussion

Mass spectrometry

Method development starts with the setting of the mass spectrometric conditions. The ESI was operated in the positive ion modes for both Cyclophosphamide and the IS by preparing the sample in diluent. Multiple reaction monitoring (MRM) mode was used for data acquisition. The source dependent parameters (nebulizer gas, collision gas, curtain gas, and auxillary gas) and compound dependent parameters (collision energy, declustering potential and collision cell exit potential) were suitably altered to get satisfactory and reproducible response by infusing the sample into ESI source of the mass spectrometer. The most sensitive mass transition observed were Cyclophosphamide (262.90/141.60) (Figure 3) and IS was (270.90/148.60) (Figure 4). A dwell time of 200 ms was set for each transition.

Figure 3: [M+ H]$^+$ ion spectra of Cyclophosphamide in positive ESI modes

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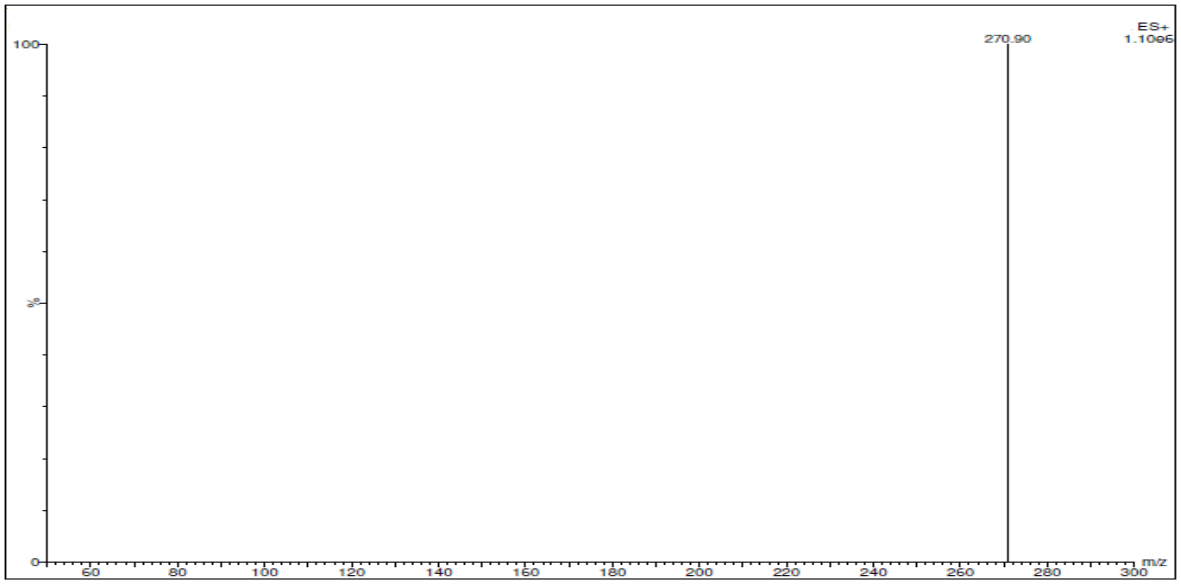

Figure 4: $[M+ H]^+$ ion spectra of Cyclophosphamide D8 in positive ESI mode

Optimization of chromatographic conditions

Here, we investigated different mobile phase compositions and its pH to obtain adequate retention, sharp peak shape and short run time for Cyclophosphamide and the IS, with minimum matrix interference and solvent consumption. Additionally, a variety of analytical columns (Zodiac C18, Zorbax SB C18, Zorbax SB C8, Hypersil BDS, Inertsil ODS, Ace 5 C18, Kromasi etc) were tested for better selectivity and sensitivity of an assay. Initially, volatile acids and buffers in aqueous phase in combination with methanol & acetonitrile were checked by altering the flow rate from 0.3 to 1.0 mL/min. Nevertheless, an isocratic mobile phase composed of An isocratic mobile phase composed of 0.1% formic acid/acetonitrile in the ratio of 20:80 v/v and Zorbax XDB C18 (150mm x 4.6mm, 5µm) column gave good peak shape and response even at lowest concentration level for the analyte and the IS.

The retention time obtained for the analyte and the IS was 2.53 min with the flow rate of 0.4 mL/min. The total chromatographic time was set at 3.5 min. Further, use of stable labeled isotopes as internal standard which had identical chromatographic behavior helped to achieve better method performance. Also, these standards will increase the bioanalytical assay precision and accuracy. Hence, Cyclophosphamide d8 was employed as an internal standard.

Calibration model

Four non-zero calibration standards were prepared and analyzed in three separate runs. Linearity of the calibration model was determined by plotting the peak area ratio of the analyte/IS against the corresponding concentration (x) of the calibration standard. The

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reciprocal of the squared concentrations (1/x2) was used as a weighting factor for all analytes. For each calibration curve the calibration concentrations were back-calculated from the response ratios. The deviations of the nominal concentrations should be within ±15%. At the LLOQ level a deviation of • ±20% was permitted. All calibration curves (n=3) of all analytes met these criteria. The assay was linear for the validated concentration ranges of 20–15000 ng/mL.

Accuracy and precision

Intra- and inter-assay bias and precisions of the method were determined by analyzing five replicate QC samples in three consecutive runs at LLOQ, mid and upper limit of quantification (ULOQ) concentration levels. The intra- and inter-assay biases and precisions should be within • ±20% and ≤20%, respectively, for the LLOQ concentration and within • ±15% and ≤15%, respectively, for other concentrations. Table 2&3 summarizes the intra- and inter-assay biases and precisions of the assay. All values were within the acceptance criteria.

The accuracies were observed between 88.6-110.8% with the coefficient of variation between 2.65-10.84%. All the results of the tested samples were meeting the acceptable criterion (RSD < 15 %), indicated that the developed method was accurate and reliable. The representative chromatograms were given in Figure 5-10.

Table 2: Intra-day precision & accuracy:

Statistical parameter	LLOQ QC	LQC	GMQC	MQC	HQC
Nominal QC concentration (ng/ml)	20	60	670	7500	13000
Mean QC concentration	17.7	52.2	631.5	7124.8	12711.1
SD (±)	1.97	1.74	17.59	363.92	657.78
CV (%)	11.13	3.34	2.78	5.10	5.17
% Accuracy	88.6±9.75	87.1±2.90	94.2±2.63	95±4.87	97.8±5.03
n	6	6	6	6	6

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Abbreviation: QC, Quality Control; LQC, low quality control; MQC, medium quality control; HQC, high quality control; RSD, relative standard deviation

Table 3: Inter-day precision & accuracy:

Statistical parameter	LLOQ QC	LQC	GMQC	MQC	HQC
Nominal QC concentration (ng/ml)	20	60	670	7500	13000
Mean QC concentration	18.28	53.28	640.53	7683.43	13458.85
SD (±)	1.95	2.13	18.10	353.34	628.22
CV (%)	10.70	4.00	2.82	4.59	4.66
% Accuracy	90±10.84	87.1±2.65	100.25±3.24	99.8±3.70	110.8±4.29
n	18	18	18	18	18

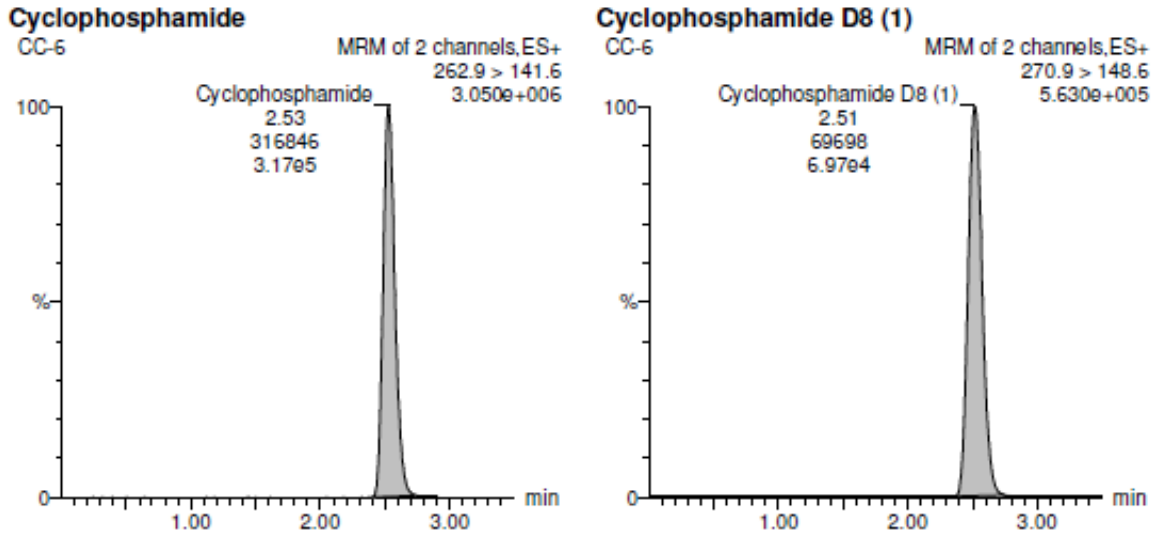

Figure 5: Representative chromatogram of ULOQ sample

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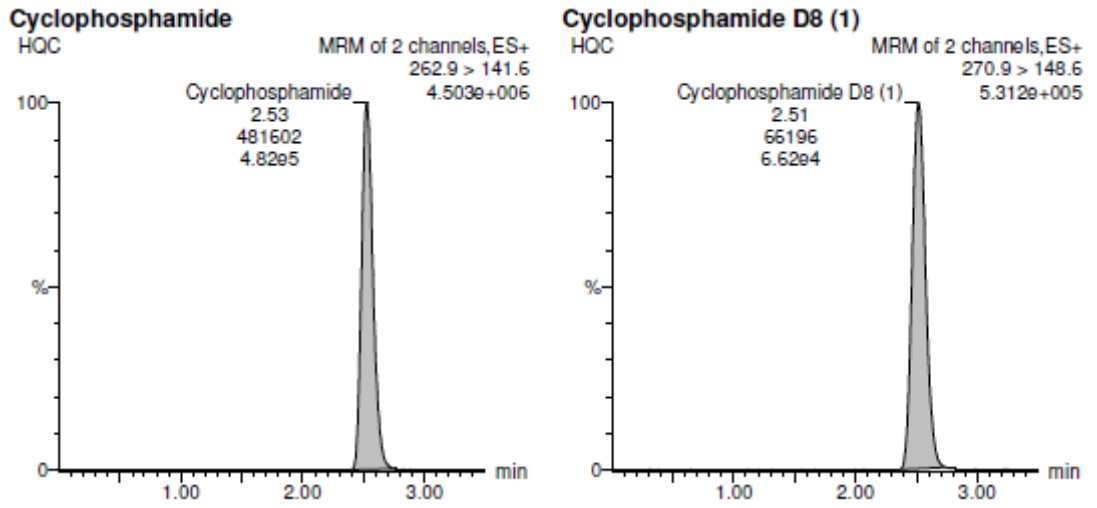

Figure 6: Representative chromatogram of HQC sample

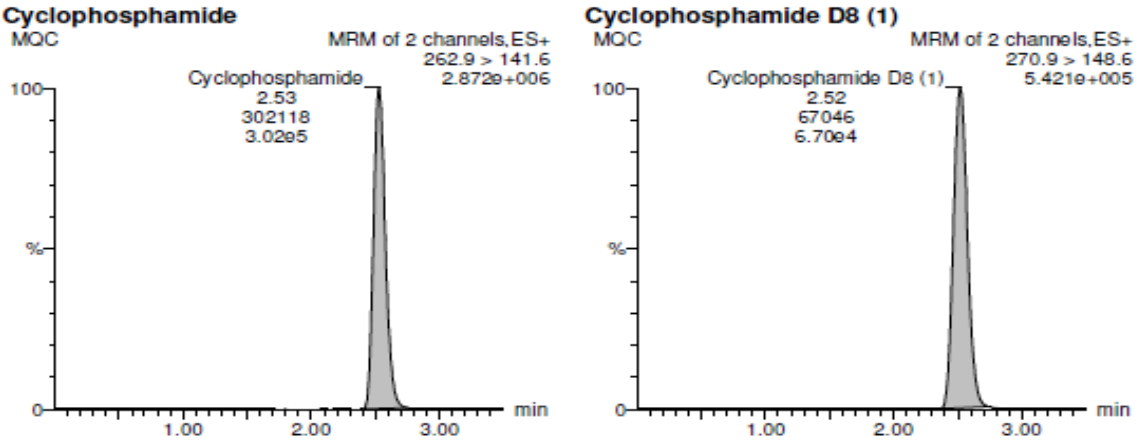

Figure 7: Representative chromatogram of MQC sample

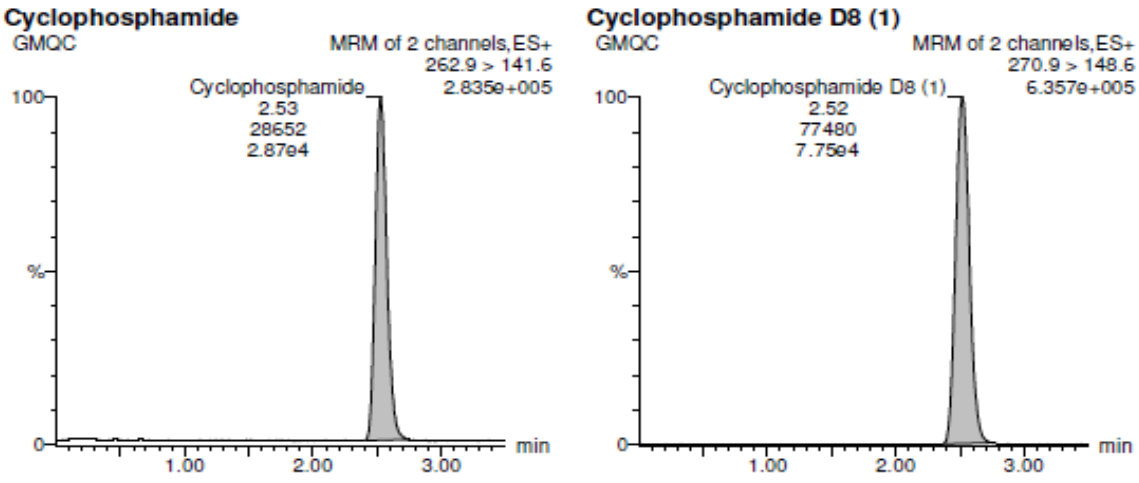

Figure 8: Representative chromatogram of GMQC sample

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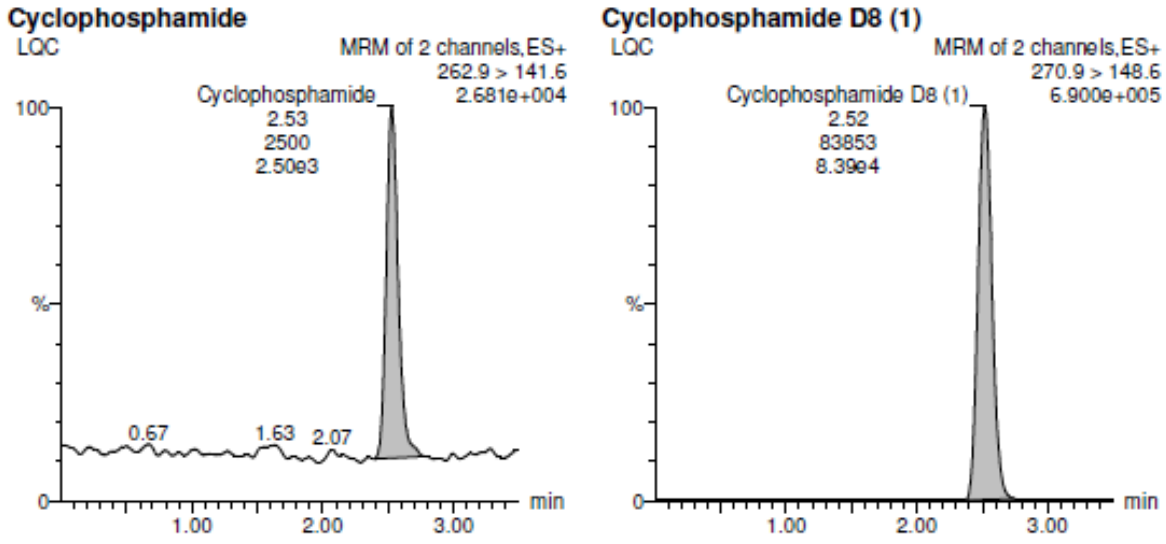

Figure 9: Representative chromatogram of LQC sample

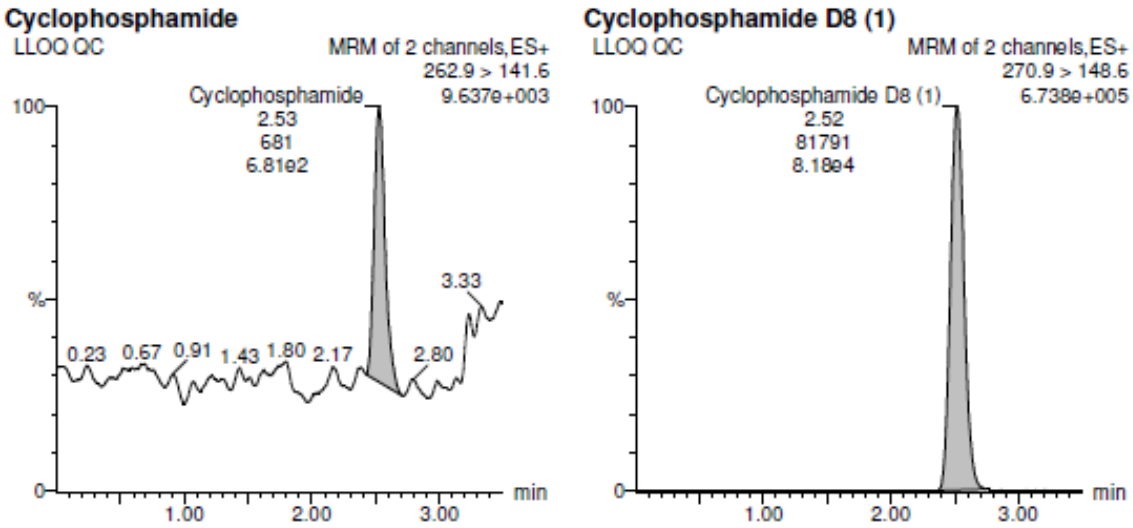

Figure 10: Representative chromatogram of LLOQ QC sample

Selectivity and specificity

Method selectivity was determined with 8 different sources of human plasma (6 were normal and one lipemic and one haemolyzed). These were analyzed as blanks and spiked LLOQ samples. The area response obtained in blank samples was compared with mean LLOQ samples area response. As displayed in Figure 11&12, no significant interference in the blank plasma samples including haemolyzed plasma as well as hyperlipidemic plasma samples was observed from endogenous substances in drug free human plasma at the retention time of Cyclophosphamide and the IS.

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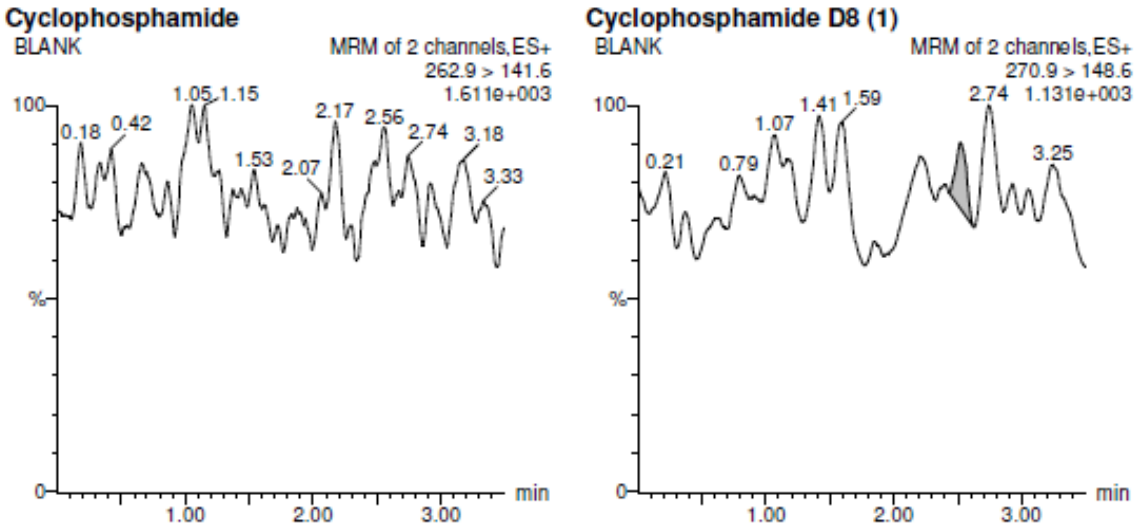

Figure 11: Blank chromatogram

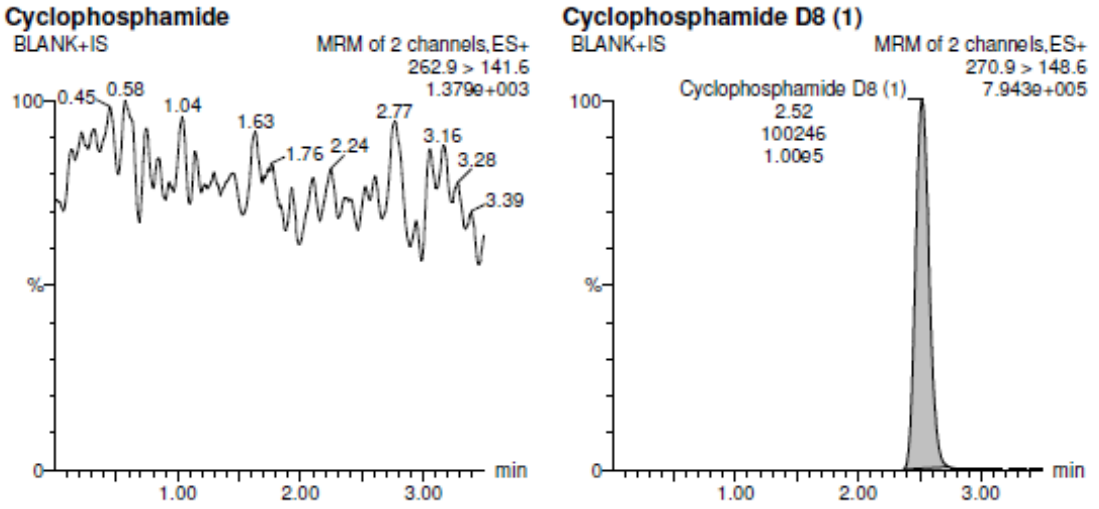

Figure 12: Representative chromatogram of blank + IS

Matrix effect & Recovery

The matrix effect test was performed to evaluate the suppression or enhancement of the ionization of analytes and the internal standard by the presence of matrix components in biological samples. In the present study, the matrix effect experiment was carried out using the ratio between spiked mobile phase solutions and unextracted samples. The extraction recoveries were evaluated by dividing the extracted sample mean response by the unextracted (spiked blank plasma extract) sample mean of the corresponding concentration. Three replicates of respective LQC, MQC and HQC were prepared according to the established extraction procedure. The reproducible recoveries were obtained for Cyclophosphamide and

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the IS. The mean recovery of Cyclophosphamide was 101, 100.1 & 100.25 with %RSD <2.78% at the level of LQC, MQC & HQC respectively.

The results show that no significant ionization suppression or maximization resulting from sample matrices. Moreover, the method resulted in a high recovery value at all QCs showing good efficiency. A variety of extraction procedures was tested, as described, and the best recovery was achieved with liquid-liquid extraction. The results are presented in Table 4.

Table 4: Recovery & Matrix effect of Cyclophosphamide in Human plasma

QC Level	Matrix effect				Recovery	
	Conc. (ng/mL)	N	Mean matrix effect (%)	%RSD	Mean Recovery (%)	%RSD
LQC	60	3	98.06	7.55	101	2.78
MQC	7500	3	92.81	7.50	100.1	2.44
HQC	13000	3	97.70	6.69	100.25	1.84

Stability study

Stability studies in plasma samples were assessed at three QC levels under four different storage conditions: at room temperature for 12 h, frozen at -20^0C for 30 days, three freeze-thaw cycles and samples after prepared at 4^0C for 12 h. The acceptable criteria of accuracy, extraction recovery, matrix effect and stability were all within ±15 % and the precision less than 15 %.

LQC and HQC samples were used to evaluate the stability of Cyclophosphamide under a variety of conditions and the results are all shown in Table 5. The stock solution of Cyclophosphamide was stable over 24 hrs at room temperature. The bench top stability, long term stability (at -20^0C) was 04 hrs & 02 days respectively. Frozen samples were observed to be stable even after subjecting three freeze-thaw cycles.

Table 5: Stability study

Stability type	Nominal Concentration	Calculated concentration Mean ± SD (n=6)	%RSD

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Freeze-thaw stability	55.2	55.2±5.81	10.53
(three cycles)	13097.8	12749.63±63	2.93
Long term stability	55.2	53.86±4.34	4.62
(-20^{0}C for two days)	13097.8	12605.8±737.35	5.84
Stock solution stability	55.2	51.86±5.38	10.38
(room temperature for 24 hrs)	13097.8	12809.47±311.43	2.43
Bench top stability (for four	55.2	52.2±4.93	9.44
hrs)	13097.8	12559±372.67	2.96

Clinical application

This assay was developed to support therapeutic drug monitoring of Cyclophosphamide. As part of routine clinical care, K2EDTA blood samples (4 mL) were collected from a total of 25 patients by their consent that were treated with Cyclophosphamide at the Ajara Hospital (Ajara Health Care and Research Center Pvt. Ltd.) Warangal, Telangana, India. Approval was taken from Kakatiya institutional ethical committee (KIEC/KMC/NCT/NIS/2020/P10) Plasma samples were collected, processed and analyzed as described in this method and the Cyclophosphamide levels were found to be in the range 8523-8954 ng/mL after patient receiving a 1-h infusion of 1500 mg Cyclophosphamide the representative chromatogram was given in figure 13.

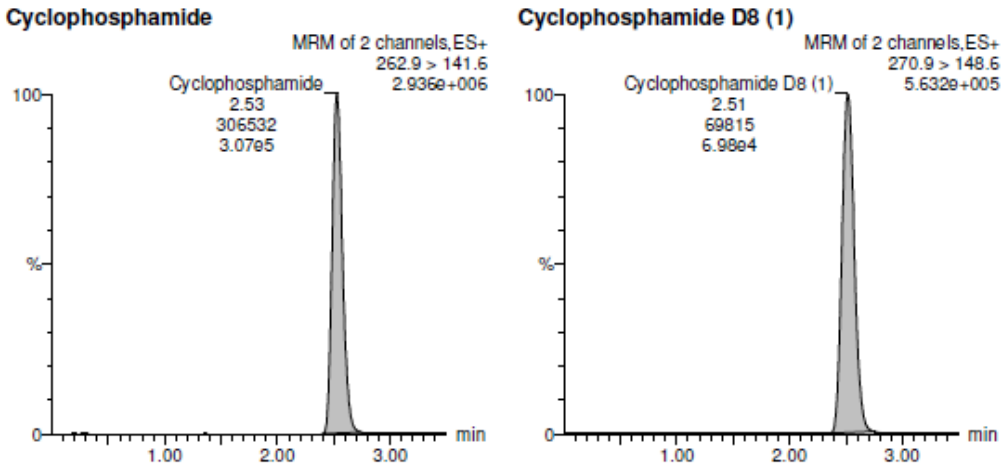

Figure 13: Representative chromatogram of patient sample receiving a 1-h infusion of 1500 mg Cyclophosphamide.

4. Conclusion

In ending, the projected UPLC-MS/MS method is novel, sensitive and rapid for the quantitative determination of Cyclophosphamide in human plasma. The method describes the complete method development and validation process as per US FDA guidelines and is well suitable for pharmacokinetic or bioavailability/bioequivalence application. The extraction procedure with LLE without drying and reconstitution steps helped in achieving consistent and reproducible recovery for analyte and the IS. Also, chromatography was shortened with the run time of 3.5 min, which allows analysis of many samples in a single day with low cost. The analyte stability in neat samples and in plasma samples under different conditions has been extensively evaluated. The method was found to applicable to clinical studies in humans.

Conflicts of interests

The authors declare no conflict of interest.

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