Journal of Cardiovascular Disease Research

ISSN:0975-3583,0976-2833 VOL12,ISSUE04,2021

METHOD DEVELOPMENT AND VALDATION OF CEPHAPIRIN IN HUMAN PLASMA BY LC-MS/MS

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Abstract

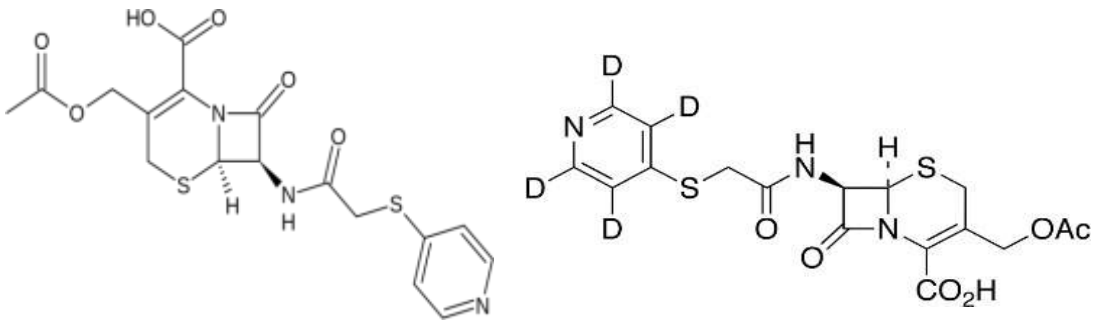
A simple, sensitive and accurate liquid chromatography tandem mass spectrometric (LC-MS/MS) method has been developed and validated for determination of Cephapirin in human plasma using Cephapirin-D4 as internal standard. The analytes were separated by Zorbax SB-C18, 4.6 x 75 mm, 3.5 µm, 80 Å column, and 5mM ammonium formate: acetonitrile (10:90 v/v) mobile phase was used for Chromatographic separation. The Cephapirin and Cephapirin-d4 were monitored by electrospray ionization in positive ion multiple reaction monitoring (MRM) mode was used to detect the Cephapirin at m/z 423.11/292.24 and Cephapirin-d4 (IS) at 427.47/292.24. Liquid-liquid extraction was employed in the extraction of analytes from human plasma. This method is validated over a linear concentration range of 50.0–10000.0 pg/mL with a correlation coefficient (r) of ≥ 0.9997. Both drug and internal standards (IS) were stable in plasma samples.

Keywords: LC-ESI-MS/MS; Cephapirin; Cephapirin-d4; Human plasma;

Introduction

Cefapirin is a first generation cephalosporin indicated in the treatment of susceptible bacterial infections. Cefapirin (INN, also spelled cephapirin), commonly marketed under the trade name Cefadyl, is a first-generation cephalosporin antibiotic that is available in injectable formulations [1-5]. Production for use in humans has been discontinued in the United States. Cefapirin is partly plasma-bound and is effective against gram-negative and gram-positive organisms. The compound molecular weight 423.463 and $C_{17}H_{17}N_3O_6S_2$. The bactericidal activity of cephapirin results from the inhibition of cell wall synthesis via affinity for penicillin-binding proteins (PBPs). IUPAC name was (6R,7R)-3-[(acetyloxy)methyl]-8-oxo-7-[2-(pyridin-4-ylsulfanyl)acetamido]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid [6-12].

ISSN:0975-3583,0976-2833 VOL12,ISSUE04,2021



A **B**

Fig.1. Chemical Structures of A) Cephapirin B) Cephapirin-d4

Based on the literature survey as per best of our knowledge very few methods were reported on pharmacokinetics of Cephapirin which includes HPLC [13, 17, 18], radio immune assay [14, 15], LC-MS/MS [16, 19, 2-25]. None of the method was reported on estimation of Cephapirin using Cephapirin-d4 as internal standard in spiked human plasma samples by LC-MS/MS. Hence, present study describes the development and validation of an isocratic LC-MS/MS with highly efficient, more specific and highly sensitive, simple extraction, good linear method for quantitative determination of Cephapirin in human plasma with the small amount of plasma usage [26].

Materials and Methods

Chemicals and reagents

Cephapirin reference standard was purchased from Symed labs, Cephapirin-d4 was obtained from Torrent research chemicals Canada (fig.1). Tertiary butyl methyl ether (TBME), HPLC grade methanol and acetonitrile were purchased from J.T. Baker USA. Potassium dihydrogen phosphate (KH_2PO_4, reagent grade), Ammonium formate (reagent grade) was purchased from Merck Limited, Worli, Mumbai. Human plasma was obtained from Navajeevan blood bank, Hyderabad, India. Ultra pure water from MilliQ-system (Millipore) was used through the study.

Instrumentation

The 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany). Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using MRM. A turbo electrospray interface in positive ionization mode was used. Data processing was performed on Analyst 1.4.1 software package (SCIEX).

Detection

The mass transitions were selected as *m*/*z* 423.11/292.24 and 427.47/292.24 for quantification of Cephapirin and Cephapirin-D4 respectively. (Figure-3).

ISSN:0975-3583,0976-2833 VOL12,ISSUE04,2021

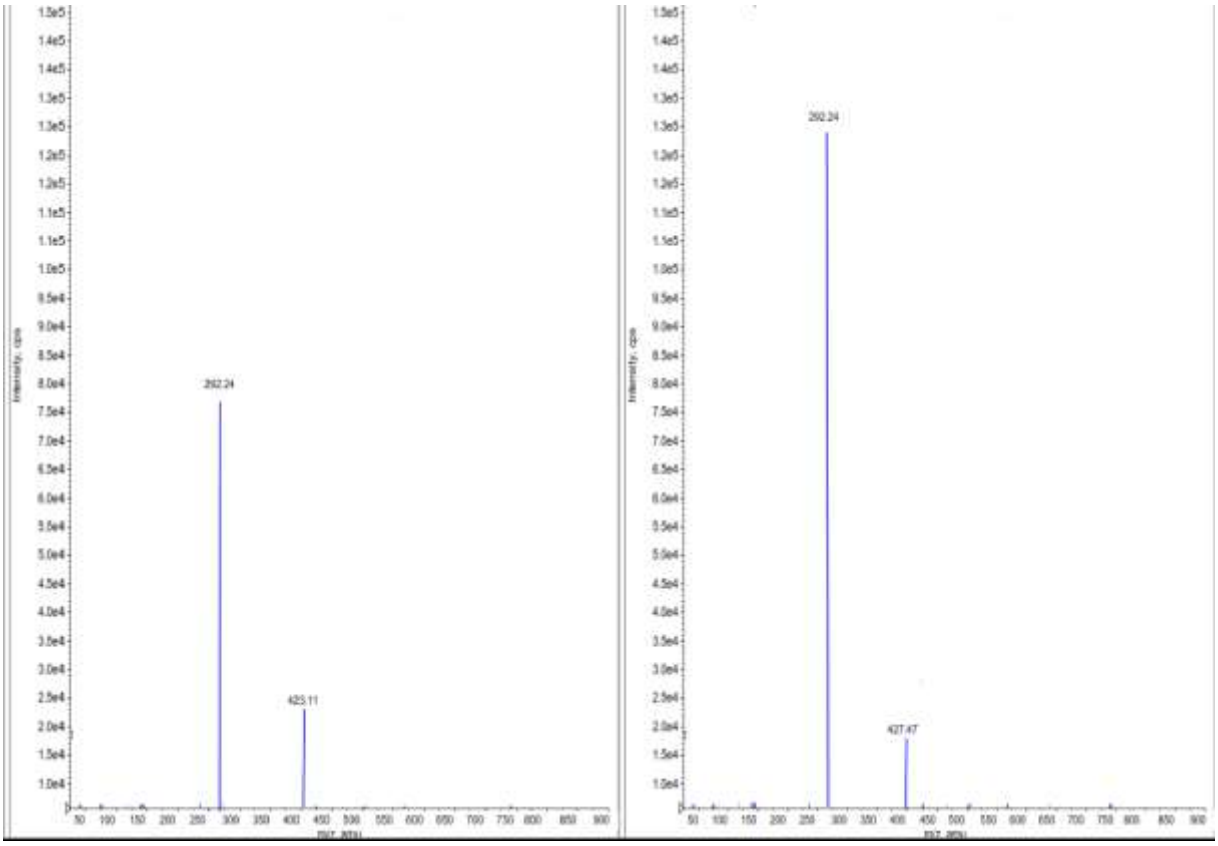


Figure.3.0.Mass spectrum Q1 and Q3 ions of Cephapirin and Cephapirin-D4

Chromatographic conditions

Chromatographic separation was achieved with 5 mM ammonium formate: acetonitrile (10:90v/v), gave the best peak shape and low baseline noise was observed using the Zorbax SB-C18, 4.6 x 75 mm, 3.5 µm 80 Å. The total analysis time was 13 min and flow rate was set to 0.6 mL/min. The temperature was set to 40°C for the column oven. The sample volume for the injection into mass spectrometry was adjusted to 20 µL for better ionization and chromatography.

Cephapirin-d4 was selected as IS (internal standard) due to its compatibility with analyte chromatographic conditions. The analyte and internal standard were eluted at 9.2, 8.2 Cephapirin-d4 minutes with total runtime of 13 minutes for each injection.

Calibration standards and quality control Samples

Standard Stock solutions of Cephapirin (100.0 µg/mL) were prepared in methanol. From each stock solution 500.0 ng/mL, 25.0 ng/mL, 2.5 ng/mL intermediate dilutions were prepared in plasma. Aliquots of 500.0 ng/mL, 25.0 ng/mL and 2.5 ng/mL were used to spike blank human plasma in order to obtain calibration curve standards of 50.0, 100.0, 500.0, 1000.0, 2000.0, 4000.0, 6000.0, 8000.0 and 10000.0 pg/mL. Four levels of QC concentrations at 50.0, 150.0, 3000.0 and 8000.0 pg/mL (LLOQ, LQC, MQC and HQC) were prepared by using the different

ISSN:0975-3583,0976-2833 VOL12,ISSUE04,2021

plasma. Spiked calibration curve standards and Quality control standards were stored at -30 ºC. Standard stock solutions of Cephapirin-D4 (100.0 µg/mL) were prepared in methanol. Cephapirin-D4 was further diluted to 30.0 ng/mL (Spiked concentration of internal standard) using 50% methanol and stored in the refrigerator 2-8 ^{0}C until analysis.

Sample preparation

Liquid-liquid extraction was carried out to extract the drug and IS for this purpose 100 µL of respective concentration of plasma sample was taken into polypropylene tubes and mixed with 50µL of internal standard (30.0 ng/mL). This was followed by addition of 100 ⌈L of 10mM KH_2PO4 solution and 2.5 mL of methyl tertiary butyl ether and vortexed for approximately 5 minutes. Then the Samples were centrifuged at 4000 rpm for 10 minutes at 20°C. Further, the supernatant was transferred into labeled polypropylene tubes and evaporated with nitrogen gas at 40°C. Then the samples were reconstituted with the reconstitution solution (Acetonitrile: 5mM ammonium formate (90:10)) and vortexed for 2 minutes. Finally, Sample was transferred into auto sampler vials to inject into the LC-MS/MS.

Selectivity and specificity

Selectivitywas performed by analyzed the human blank plasma samples from six different sources (donors) with an additional hemolysed group and lipedimic group to test for interference at the retention times of analytes. The peak area of Cephapirin in blank samples should not be more than 20% of mean peak area of LOQ of Cephapirin. Similarly, peak area of Cephapirin-D4 in a blank sample should not be more than 5% of mean peak area of LOQ of Cephapirin-D4.

Linearity

Linearity of the instrument was checked by analyzing nine cephapirin standards (50-10000 pg/mL) with each concentration injected three times. A calibration curve was constructed by plotting peak areas for the standards against its concentrations. The calibration equation and correlation coefficient from the regression analysis were used to validate linearity (Table-1).

Table.1.0: Calibration curve details of Cephapirin

Spiked plasma concentration (pg/mL)	Concentration measured(mean) (pg/mL) (*n* = 5)	Precision RSD. (*n* = 5)	Accuracy %
50.0	51.0 ± 1.3	2.5	102.00
100.0	96.6 ± 4.7	4.9	96.60
500.0	498.4 ± 24.7	5.0	99.68
1000.0	1000.0 ± 17.1	1.7	100.00
2000.0	2013.0 ± 74.6	3.7	100.65
4000.0	4008.4 ± 206.6	5.2	100.21
6000.0	5956.5 ± 190.7	3.2	99.28
8000.0	7952.2 ± 165.6	2.1	99.40
10000.0	10317.1 ± 487.6	4.7	103.17

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Precision and accuracy

Precision and accuracy was determined by replicate analysis of quality control samples (n = 6) at LQC (low quality control), MQC (medium quality control) and HQC (high quality control) levels. The % CV should be less than 15%, and accuracy should be within 15% except LLOQ where it should be within 20% (Table-2).

Table.2.0: Precision and accuracy (analysis with spiked plasma samples at three different concentrations)

Spiked plasma concentration (pg/mL)	Within-run (n=6)			Between-run (n=6)		
	Concentration measured (pg/mL) (mean±S.D.)	Precision RSD	Accuracy %	Concentration measured (pg/mL) (mean±S.D.)	Precision RSD	Accuracy %
50.00	51.4±2.3	4.5	102.7	55.5±4.1	7.4	110.6
150.00	154.9±3.4	2.2	105.5	157.9±5.7	3.6	105.2
3000.00	3103.8±102.0	3.3	103.2	3133.0±108.2	3.5	104.3
8000.00	7297.1±89.9	1.2	91.7	7278.7±275.5	3.8	103.9

Matrix effect

The matrix effect due to plasma was used to evaluate the ion suppression/enhancement in a signal when comparing the absolute response of QC samples after pretreatment (Liquid-liquid extraction with MTBE) with that of the reconstituted samples. Experiments were performed at MQC levels in triplicate with six different plasma lots. The acceptable precision (%CV) of ≤ 15% was maintained.

Recovery

The extraction efficiencies of Cephapirin and Cephapirin-D4 were determined by analysis of six replicates at each quality control concentration level and at one concentration for the internal standard Cephapirin-D4.

The percent recovery was evaluated by comparing the peak areas of extracted standards to the peak areas of non extracted standards (spiked into mobile phase).

Limit of detection and quantification (LOD and LOQ)

The limit of detection (LOD) is a parameter that provides the lowest concentration in a sample that can be detected from background noise but not quantitated. LOD was determined using the signal-to-noise ratio (s/n) of 3:1 by comparing test results from samples with known concentrations of analytes with blank samples. The limit of quantitation (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy.

The LOQ was found by analyzing a set of mobile phase and plasma standards with a known concentration of Cephapirin.

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Stability (Freeze- thaw, Auto sampler, Room temperature, Long term)

Stock solution stability was performed by comparing the area response of analyte and internal standard in the stability sample, with the area response of sample prepared from fresh stock solution.

Stability studies in plasma were performed at the LQC and HQC concentration level using six replicates at each level. Analyte was considered stable if the % Change is less than 15% as per US FDA guidelines [18-20]. The stability of spiked human plasma samples stored at room temperature (bench top stability) was evaluated for 48 h. The stability of spiked human plasma samples stored at -30 °C in autosampler (autosampler stability) was evaluated for 55.5 h. The autosampler sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were reinjected after storing in the autosampler at 20°C for 55.5 h. The reinjection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were re-injected after storing in the autosampler at 20°C for 27 h. The freeze–thaw stability was conducted by comparing the stability samples that had been frozen at –30 °C and thawed three times, with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration levels were used for the freeze–thaw stability evaluation. For long term stability evaluation the concentrations obtained after 71days were compared with initial concentrations (Table-3).

Table.3.0: Stability of Cephapirin in human plasma samples

Stability experiments	Storage condition	Spiked plasma concentration (pg/ml)	Concentration measured (*n*=6) Mean ± SD	RSD (*n*=6) (%)	Accuracy (%)
Bench top in plasma	**RT 48 hr**	150.0	148.3 ± 8.1	5.5	98.9
		8000.0	6728.3±206.3	3.1	81.5
Processed (extracted sample)	**Autosampler 55.5 hr**	150.0	162.3 ± 2.4	1.5	108.2
		8000.0	7536.7±294.5	3.9	90.4
Freeze/Thaw stability	**-30^{0}C Cycle-3**	150.0	156.5 ± 4.0	2.5	104.3
		8000.0	7381.7±173.4	2.3	90.4
Long-term stability in human plasma	**- 30^{0}C 71 days**	50.0	160.3±13.2	8.2	106.9
		8000.0	7450.0±229.1	3.1	90.5

Results and Discussion

Method development and validation

The goal of this work is to develop and validate a simple, rapid and sensitive assay method for the quantitative determination of Cephapirin from plasma samples. LC-MS/MS has been used as one of the most powerful analytical tool in clinical pharmacokinetics for its selectivity, sensitivity and reproducibility. The MS optimization was performed by direct

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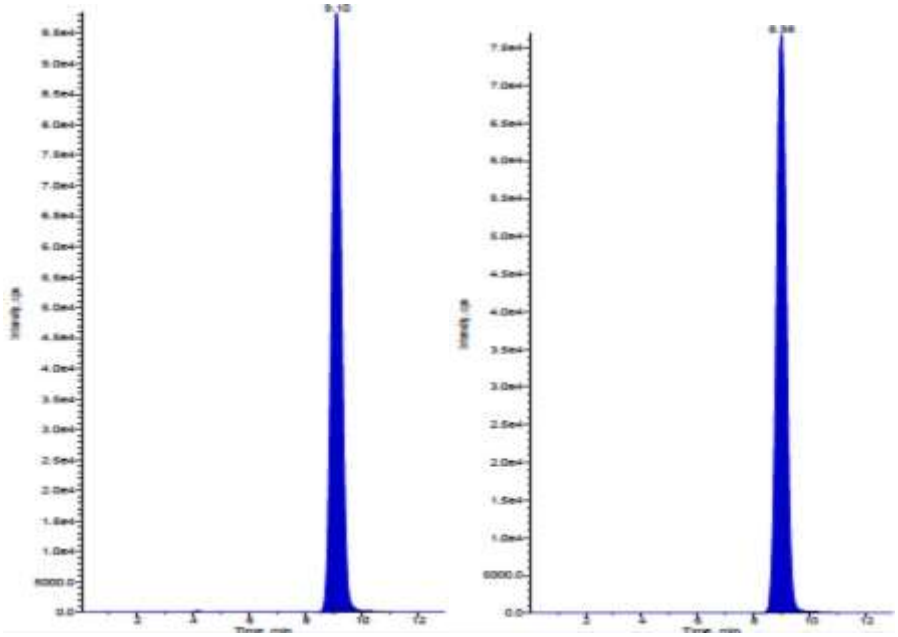
infusion of solutions of Cephapirin and Cephapirin-D4 into the ESI source of the mass spectrometer. The vital parameters like ionization type, temperature, and voltage, gas parameters such as nebulizer and heater gases, compound parameters like DP, EP, FP, CE and CXP were optimized to obtain a better spray shape and ionization to form the respective productions from the protonated Cephapirin and Cephapirin-D4 molecules.Chromatographic conditions, especially, composition of the mobile phase, selection of suitable column was optimized through several trials to achieve the best resolution and increase the signal of analyte and internal standard. Different extraction methods like solid phase extraction, Liquid-liquid extraction, precipitation methods were optimized for extraction of Cephapirin and Cephapirin-D4 from the plasma sample. A good separation and elution were achieved using 5 mM ammonium formate: acetonitrile (10:90v/v) as the mobile phase, at a flow-rate of 0.6 mL/minutes and injection volume of 20 µL. Liquid-liquid extraction was chosen to optimize the drug and internal standard. The retention time was optimized 9.2 minutes for Cephapirin and 8.2 minutes for Cephapirin-D4 (Figure-4).



A **B**

Figure.4.0. Chromatograms of A) Cephapirin B) Cephapirin-D4

Linearity

Calibration curve was plotted as the peak area ratio (Cephapirin/Cephapirin-D4) versus (Cephapirin) concentration. Calibration was found to be linear over the concentration range of 50.0 – 10000.0 pg/mL. The correlation coefficient (r^2) was greater than 0.9997 for all curves (Table.1.0).

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Selectivity

The selectivity of the method assessed by comparing chromatograms of blank plasma. There were no significant endogenous peaks were observed at respective retention time of Cephapirin and Cephapirin-D4. The results indicate that the method exhibited both good specificity and selectivity.

Precision and Accuracy

Precision and accuracy for this method were controlled by calculating the Within-run and Between-run variations at three concentrations (150.0, 3000.0 and 8000.0 pg/mL) of QC samples in six replicates. As shown in Table.2 the Within-run Precision and Accuracy were between 1.2 to 4.5 and 91.7 to 105.5 % for Cephapirin. Similarly, the Between-run Precision and Accuracy were between 3.5 to 7.4 and 103.9 to 110.6 % for Cephapirin. These results indicate the adequate reliability and reproducibility of this method within the analytical range.

Matrix effect

The ion suppression/enhancement in the signal at MQC level was found % CV 1.27. These results indicating that there is no effect on ion suppression and ion enhancement.

Recovery

The %recoveries of Cephapirin were determined at three different concentrations 150.0, 3000.0 and 8000.0 pg/mL, were found to be 99.6 ±3.53, 88.2 ±2.7 and 97.60 ± 4.7 %. The overall average recoveries of Cephapirin and Cephapirin-D4 was found to be 95.1 ± 6.1 and 98.1 ± 4.47%. Recoveries of the analyte and IS were consistent, precise and reproducible.

Limits of Detection and Quantification (LOD&LOQ)

The LOQ& LOD signal-to-noise (S/N) values found for six injections of Cephapirin at LOQ & LOD concentrations were 31.95pg/ml and 10.5pg/ml.

Stability (Freeze - thaw, Auto sampler, Room temperature, Long term)

Stock solution stability was performed to check stability of Cephapirin and Cephapirin-D4 in stock solutions prepared in methanol and stored at 2-8 °C in a refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 26 days. The % change for Cephapirin and Cephapirin-D4 were -0.02% and 0.03% respectively indicate that stock solutions were stable at least for 26 days. Room temperature and auto sampler stability for Cephapirin was investigated at LQC and HQC levels. The results revealed that Cephapirin was stable in plasma for at least 72 h at room temperature, and 78 h in an auto sampler. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with Cephapirin at LQC and HQC levels did not affect their stability. The long-term stability results also indicated that Cephapirin was stable in a matrix up to 71days at a storage temperature of -30 °C. The results obtained from all these stability studies are tabulated in Table 3. Precision (%CV) is less than 5 for Room temperature, long-term, Freeze thaw, auto sampler stability.

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Conclusion

The proposed research work is highly specific due to the inherent selectivity of tandem mass spectrometry and has significant advantages over other described methods in previously. Quantification of Cephapirin was compared with respective isotope labeled internal standard (Cephapirin-D4). Extraction of analyte and IS were achieved by using LLE. Linearity range, column, mobile phase, flow rate, injection volume, plasma usage volume for analysis was improved. Hence this method has significant advantages over previously reported methods in-terms of Selectivity, sensitivity, Linearity, Reproducibility.

Acknowledgements

Authors wish to thank the support received from Ray analytical labs PVT Ltd, Hyderabad for providing Literature survey and carry out this Research work.

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