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Development and validation of a stability-indicating HPLC method for the determination of related Substances of cephradine drug substance

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ABSTRACT

A simple and sensitive reverse phase high-performance liquid chromatographic method, for the determination of related substances of Cephradine drug substance has been developed and validated. The degraded products and the isolated impurities were analysed by RP-HPLC utilizing a Octadecylsilane Column (YMC Pack ODS-AM 150 × 4.6 mm, 5µ), followed by ultraviolet detection at 220 nm and a mixture of Acetonitrile, Phosphate buffer 0.02M used as a mobile phase in a gradient elution. This method was validated in terms of Selectivity, Linearity, Precision, Accuracy, Robustness, Limit of detection (LOD), Limit of quantitation (LOQ). This method has been succefully applied for drug substance of cephradine.

KEYWORDS

Cephradine;
Cephalosporins;
Validation;
HPLC;
Impurities.

1. INTRODUCTION

Cephradine, 7-[α-D- (cyclohexa-1, 4-dienyl)-glycyl-amino]-3-methyl-3-cephem-4-carboxylic acid, is a first generation cephalosphorin. It has broad-spectrum antibacterial activity against gram-positive and gram negative microorganisms, through inhibition of bacterial cell wall synthesis[1,2]. Cephradine is useful for treatment of infections of the urinary and respiratory tract, skin and soft tissues. Several analytical methods are available in the literature for the determination of cephradine and cefadroxil in their pharmaceutical preparations including HPLC[3-5], capillary electrophoresis[6,7], polorography[8,9], chemiluminescence[10,11] and spectroscopic methods[12-16]. Our strategy was to develop a valid high-performance liquid chromatography using UV detection method to analyse the process related impurities as well as the degraded products of cephradine. This paper explicates the method development and validation to determine the related substances of cephradine drug substance and formulated products.

2. EXPERIMENTAL

2.1. Samples and reagents

The well-examined samples of cephradine bulk material in powdered form (B.No –CDNP/115/2001), compacted form (B.No- CDNC/114/2001) and the Cephalexin (B.No-WS-14), were obtained from Orchid Chemicals & Pharmaceuticals Ltd., Chennai, India.

Sodium dihydrogen orthophosphate dihydrate and Orthophosphoric acid (85% v/v), all AR grade were obtained from SD fine-chem Ltd., India.Acetonitrile HPLC grade was obtained from Merck, India. High pure Milli-Q water was used with the help of Millipore Milli-Q plus purification system (MILLIPORE SA,

67120 MOL SHEM, France).

2.2. Apparatus

A Waters Model Alliance 2690 separation model equipped with a Waters 996 photo diode array UV detector and 2690 separation model equipped with a Waters 2487 UV-VIS detector were used. Waters Millennium ^{32}Chromatography software was used for calculation of results. Samples were weighed in Mettler Toledo Model AT 261

2.3. HPLC conditions

An in-house liquid chromatography method was developed for the analysis of Cephradine, its impurities and the intermediates, where a C-18 Column (YMC Pack ODS-AM 150 × 4.6 mm, 5µ) with a mobile phase consisting of a mixture of 0.02M $NaH_2PO_4.2H_2O$ (pH 4.5; 0.02 M) and acetonitrile in the gradient elution was used with UV detection 220 nm at a constant flow rate of 1.5 ml/min for the resolution of all impurities. The gradient condition started with 99.5% of phosphate buffer and 0.5 % of acetonitrile up to 5 minutes and then the acetonitrile was raised to 10 % at 20 minutes, which was maintained up to 26 minutes, then slowly raised to 20% at 38 minutes. In every injection the delay time of 10 minutes was maintained at the initial gradient condition. The overall analysis was performed at ambient temperature and the injection volume 20µL.

2.4. Preparation of resolution solution and evaluation of system suitability

Equal quantity of Cephradine and cephalexin working standards were accurately weighed and dissolved in phosphate buffer solution, diluted to the final concentration of each 0.1 mg ml^{-1}, and 20µL was injected to check the system is suitable for analysis, the resolution between the peaks corresponding to cephalexin (retention time around 21 minutes) and cephradine (retention time around 23 minutes) was not less than 5.0.The tailing factor for cephradine peak was not more than 2.0.

2.5.Preparation of standard, sample solutions and quantitative determination for related substances

Around 50 mg of Cephradine working standard was weighed and dissolved in phosphate buffer solution in 50 ml volumetric flask, diluted to the final concentration of 0.01 mg ml^{-1}, and 20µL was injected.

The Cephradine sample to be examined was weighed about 50 mg, diluted with the same phosphate buffer solution to the final concentration of 1.0 mg ml^{-1}, and 20µL was injected. The following formula was used to calculate the content of related substance.

% of each related substance = $A_T \times DS \times P/A_S \times DT$

Where A_T and A_S represent the individual impurity peak area of sample and the peak area of standard respectively, DT and DS represent the dilution factor of sample solution and standard solution respectively and the P represents the purity (%w/w) of Cephradine working standard.

2.6. Stability of cephradine under stressed conditions

Stability of the solid state of both cephradine powdered and compacted forms were demonstrated by storing for 5 weeks at 90°C in a Petri dish. Cephradine drug substance was separately treated with 1N hydrochloric acid, 0.1N sodium hydroxide and 30 % w/w solution of hydrogen peroxide. The sample was exposed to Sunlight for 35Hrs, also the sample was subjected to humidity degradation by keeping at 25^0C and 97% relative humidity.

3. RESULTS AND DISCUSSION

3.1.Optimization of HPLC conditions

In order to obtain a precise and rugged method, several trials with low pH, higher pH and the different buffers like acetate and citrate were tried in the mobile phase. Finally the complete resolution among all the related substances of cephradine and the degraded products was achieved by using phosphate buffer and acetonitrile mixture with ODS column in a selective gradient condition. Instead of acetonitrile, methanol was tried in the gradient elution, some of the related substances that is highly non-polar when compared to cephradine was not eluted out. Since the cephalexin was the process related impurity and also good resolution between cephradine, it was preferred to check the suitability test. (Figure 1).

3.2. Validation of determination of related substance

After optimization of analytical conditions, the evalu-

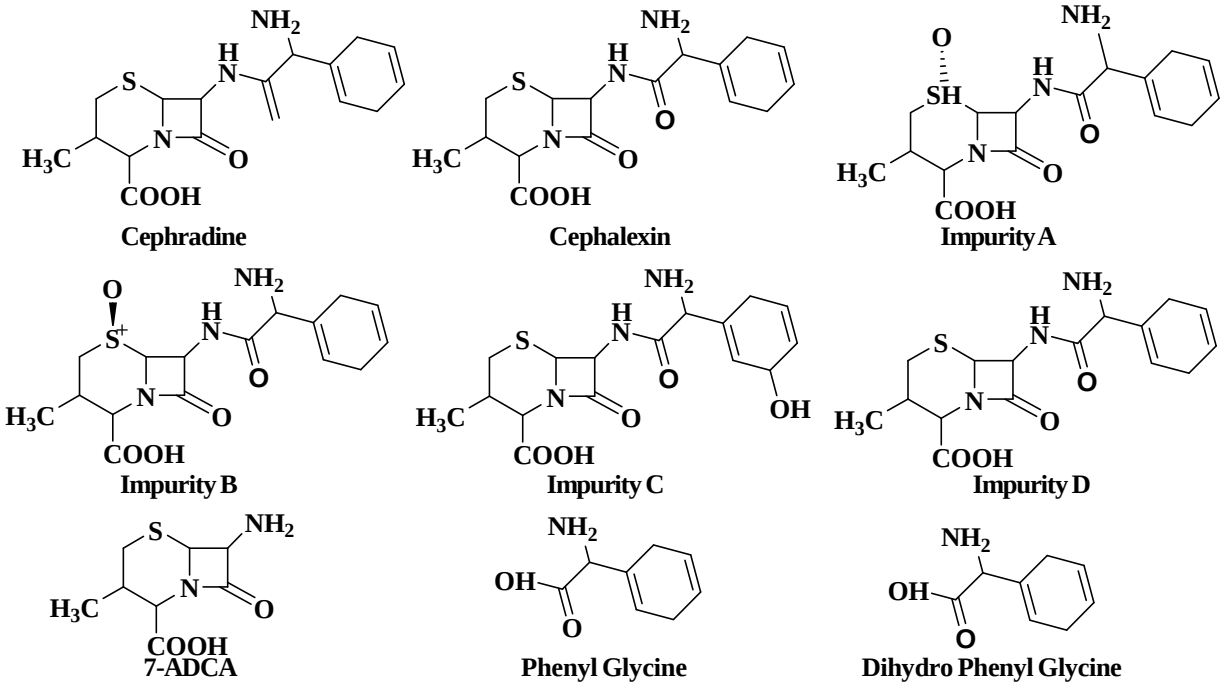

Figure 1

TABLE 1: The relative retention time (RRT) of known peaks with respect to Cephradine

S.no.	Name	RRT
1	Phenyl Glycine*	0.091
2	7 ADCA*	0.106
3	Dihydro Phenyl Glycine*	0.130
4	Dimethy formamide*	0.171
5	Impurity A	0.385
6	Impurity B	0.545
7	Impurity C	0.742
8	Cephalexin*	0.916
9	Cephradine	1.000
10	Impurity D	1.193

***Intermediates, which are not present in the final substance**

TABLE 2: Degradation of cephradine

Mode of degradation	Conditions		% of degradation
Acid	1N HCl	(1.5Hrs/95°C)	20
Alkali	0.1N NaOH	(1.0Hr/RT)	9
Oxidative	H_2O_2 30%w/w	(Initial /RT)	9
Thermal	95°C	(1Hr)	17
Photolytic	Sunlight	(35Hrs)	12
Humidity	25°C 97%RH	(30.8Hrs)	0

ation of parameters such as specificity, linearity, LOD, LOQ, precision, accuracy, ruggedness and robustness were completed for the validation of the method.

Specificity

In order to show this method is highly specific, each related substances and the intermediates of cephradine were injected individually in the concentration about 0.5 mg ml^{-1}. Further to confirm the specificity, the known related substances and the intermediates of cephradine were spiked with the sample in 1% level to the cephradine concentration 1.0 mg ml^{-1}. It was observed that the related substances are well separated from each other and also from the cephradine peak (Figure 2). The relative retention time (RRT) of all well resolved peaks were tabulated in TABLE 1.

This method is not only specific in the normal analysis, but also in the analysis of cephradine samples, which endure in stressed conditions. The percentage of degradation and the peak purity values of cephradine were given in TABLE 2.

Linearity

The solutions of cephradine and its known related impurities were prepared at low concentrations from 0.12 μg ml^{-1} and at higher concentrations 12 μg ml^{-1}, and the relationship between peak area (Y) and concentration (X) was observed. An excellent linearity[for cephradine Y = 14565 X + 498 (r = 0.99999)] was obtained within the above concentration range for all related substances. Microsoft Excel software used to plot the peak areas versus micrograms injected. (TABLE 3).

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Limit of quantitation and detection

The limit of quantitation (LOQ) of known related substances of cephradine were determined by using the residual standard deviation[STEYX, that is the standard error of the predicted Y value for each X in the regression. The standard error is a measure of the amount of error in the prediction of Y for an individual X] and the slope values from the linearity data of respective related substances using the following formula[LOQ = (STEYX / slope) × 10]. The each related substance solutions were prepared at about the predicted LOQ concentration level and its precision was verified (TABLE 4).

Similarly the limits of detection (LOD) of known related substances of cephradine were determined by using the following formula[LOD = (STEYX / slope) ×3.3]. The each related substance solutions were prepared at about the predicted LOD concentration level and its precision was verified. (TABLE 5).

TABLE 3: Linearity

Name	Conc. (µg ml-1) Lowest-Highest	Slope	Intercept	Correlation coefficient
Phenyl Glycine	0.126 - 12.072	17781	912	0.99991
7 ADCA	0.126 - 12.131	17693	841	0.99991
Dihydro phenyl glycine	0.122 - 11.749	4883	67	0.99997
Impurity A	0.126 - 12.136	14448	211	0.99997
Impurity B	0.127 - 12.174	13424	377	0.99999
Impurity C	0.126 - 12.111	13002	289	0.99998
Cephradine	0.126 - 12.090	14565	498	0.99999
Impurity D	0.126 - 12.092	10961	-704	0.99993

Precision or reproducibility and ruggedness

The precision of the method was determined by preparing a sample solution of single batch of cephradine drug substance (in the concentration of 1.0 mg ml^{-1}) six

TABLE 4: Limit of quantitation

Name	LOQ (%w/w)	Area of LOQ preparation						%RSD*
		1	2	3	4	5	6	
Phenyl Glycine	0.011	2367	2337	2287	2196	2520	2354	4.57
7 ADCA	0.009	2111	2252	2133	2196	2119	2146	2.50
DHPG*	0.010	531	554	511	570	516	523	4.31
Impurity A	0.009	1572	1718	1622	1763	1753	1713	4.50
Impurity B	0.009	1767	1786	1825	1741	1781	1689	2.61
Impurity C	0.011	1871	1719	1734	1754	1787	1819	3.20
Cephradine	0.009	2219	1754	2134	2145	2064	2257	8.59
Impurity D	0.018	1634	1942	1931	1983	1981	1632	9.19

***Dihydro Phenyl Glycine, *The acceptance criteria are the %RSD should not more than 10% for LOQ**

TABLE 5: Limit of detection

Name	LOD (%w/w)	Area of LOD Preparation						%RSD*
		1	2	3	4	5	6	
Phenyl Glycine	0.003	572	614	615	680	750	740	11.03
7 ADCA	0.003	581	593	574	589	446	525	10.34
DHPG	0.003	188	178	190	194	225	165	10.53
Impurity A	0.003	441	414	441	419	280	408	15.21
Impurity B	0.003	428	434	427	431	315	396	11.36
Impurity C	0.003	379	397	421	443	289	407	13.88
Cephradine	0.003	731	769	809	926	996	819	11.88
Impurity D	0.005	491	439	377	530	450	409	12.25

***The acceptance criteria are the %RSD should not more than 33% and not less the 10% for LOD**

TABLE 6 : Precision (Cephradine compacted)

Name	%w/w of Impurities						%RSD*
	1	2	3	4	5	6	
Impurity A	0.060	0.059	0.061	0.059	0.061	0.060	1.50
Impurity B	0.106	0.108	0.106	0.108	0.108	0.106	1.03
Impurity C	0.073	0.073	0.076	0.075	0.076	0.077	2.27
Impurity D	0.617	0.619	0.614	0.613	0.621	0.605	0.93
Highest Unknown	0.034	0.035	0.034	0.034	0.033	0.035	2.35
Total Unknown	0.143	0.148	0.146	0.147	0.141	0.144	1.79
Total Related substances	0.999	1.077	1.003	1.002	1.007	0.992	0.56

***The acceptance criteria are the %RSD should not more than 10%**

times and analyzed as per the proposed method. The related substances of cephradine were calculated against the cephradine standard (TABLE 6).

Two different analysts conducted the six replicate determination of cephradine drug substance in the same concentration on different days using different instruments in two different columns of same brand. The comparative results are summarized in TABLE 7. There is no significant deviation between the results of two different values, it has clearly indicates that this method is precise and rugged.

Accuracy

Method accuracy was demonstrated by spiking a known amount of related substances of cephradine in the sample preparation (1.0 mg ml^{-1}) in three different levels, like 20%, 60% and 120% in the concentration of 2.0μg ml^{-1}, 6.0μg ml^{-1} and 12.0μg ml^{-1} respectively in triplicate. There is no significant change in the values between the amount added and the amount recovered after the corrections of the known sample, which is already present. The percentage recoveries of all substances were in between 96 to 102. (The acceptance criteria is 80 % to 120 %) The % RSD of recovery of three levels were < 3.0. (TABLE 8).

TABLE 7: Ruggedness

Analysis	(Total related substances in %w/w)	
	Analyst-1	Analyst-2
1	0.999	0.989
2	1.007	1.001
3	1.003	0.998
4	1.002	1.015
5	1.007	1.014
6	0.992	1.007
%RSD	0.560	1.000
Over all % RSD*	0.780	

***% RSD of over all values of both analysts**

Stability of analytical solution

The solution (1.0 mg ml^{-1}) of cephradine with the known impurities (spiked in 1% level) was studied at room temperature at different time intervals. The cumulative %RSD of each related substances were calculated and concluded that the cephradine and its related substances were stable for about 10 hrs at room temperature (≈ 25^{0}C).

Robustness

The chromatographic conditions were deliberately changed to demonstrate the robustness. The flow rate (± 10 %), detection wavelength (± 5 nm), the composition of acetonitrile (± 2 % absolute) and the column oven temperature (at 35^{0}C) were changed to check the difference in the resolution between the all related

TABLE 8 : Accuracy

Name	20%			60%			120%		
	AA*	AR*	Rec*	AA	AR	Rec	AA	AR	Rec
	0.101	0.102	100.99	0.303	0.305	100.66	0.605	0.597	98.68
Phenyl Glycine	0.101	0.102	100.99	0.303	0.307	101.32	0.605	0.599	99.01
	0.101	0.102	100.99	0.303	0.305	100.66	0.605	0.599	99.01
	0.101	0.101	100.99	0.302	0.306	101.32	0.605	0.586	96.86
7 ADCA	0.101	0.101	100.99	0.302	0.307	101.66	0.605	0.585	96.69
	0.101	0.101	100.99	0.302	0.307	101.66	0.605	0.586	96.86
	0.098	0.102	104.08	0.293	0.299	102.05	0.586	0.592	101.02
DHPG	0.098	0.103	105.10	0.293	0.299	102.05	0.586	0.590	100.68
	0.098	0.102	104.08	0.293	0.299	102.39	0.586	0.594	101.37
	0.101	0.100	99.01	0.302	0.301	99.67	0.604	0.602	99.67
Impurity A	0.101	0.100	99.01	0.302	0.301	99.67	0.604	0.600	99.34
	0.101	0.101	100.00	0.302	0.300	99.34	0.604	0.602	99.67
	0.102	0.103	100.98	0.306	0.304	99.35	0.611	0.610	99.84
Impurity B	0.102	0.103	100.98	0.306	0.306	100.00	0.611	0.609	99.67
	0.102	0.103	100.98	0.306	0.305	99.67	0.611	0.613	100.33
	0.101	0.100	99.01	0.304	0.311	102.30	0.607	0.607	100.00
Impurity C	0.101	0.100	99.01	0.304	0.310	101.97	0.607	0.608	100.16
	0.101	0.100	99.01	0.304	0.310	100.00	0.607	0.607	100.00
	0.101	0.102	100.99	0.303	0.313	103.30	0.607	0.610	100.49
Impurity D	0.101	0.102	100.99	0.303	0.311	102.64	0.607	0.609	100.33
	0.101	0.103	101.98	0.303	0.312	102.97	0.607	0.607	100.00

The % RSD of recovery of all substances in three levels are <3 %, *AA-Amount added in mg, AR-Amount recovered in mg and Rec-recovery in percentage

substances of cephradine. There is no noteworthy variation in results were clearly indicates that this method is robust.

System suitability

The system suitability testing, which is part of an integral part of chromatographic methods, and used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed.

4.CONCLUTIONS

According to complete validation studies, the cephradine peak of both powdered and compacted were free of interference from the related substances and its degradation products, point out that the proposed RP-HPLC method is simple, precise, accurate, rugged and robust in all situation.

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