Development and Validation of Reversed-Phase High Performance Liquid Chromatographic Method for Analysis of Cephradine in Human Plasma Samples

[1]MAHMOOD AHMAD*, [1]MUHAMMAD USMAN, [1]ASADULLAH MADNI, [1]NAVEED AKHTAR, [1]NAYAB KHALID, [1]WAHEED ASGHAR AND [2]SATTAR BAKHSH
[1]*Department of Pharmacy, Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur, Pakistan.*
[2]*Faculty of Pharmacy, Gomal University, D.I. Khan, Pakistan.*

(Received on 26th January 2009, accepted in revised form 3rd November 2009)

Summary: An HPLC method with high precision, accuracy and selectivity was developed and validated for the assessment of cephradine in human plasma samples. The extraction procedure was simple and accurate with single step followed by direct injection of sample into HPLC system. The extracted cephradine in spiked human plasma was separated and quantitated using reversed phase C_{18} column and UV detection wavelength of 254 nm. The optimized mobile phase of new composition of 0.05 M potassium dihydrogen phosphate (pH 3.4)-acetonitrile (88:12) was pumped at an optimum flow rate of 1 $mL.min^{-1}$. The method resulted linearity in the concentration range 0.15-20 $\mu g.mL^{-1}$. The limit of detection (LOD) and limit of quantification (LOQ) were 0.05 and 0.150 $\mu g.mL^{-1}$, respectively. The accuracy of method was 98.68 %. This method can be applied for bioequivalence studies and therapeutic drug monitoring as well as for the routine analysis of cephradine.

Introduction

Cephradine, (Fig. 1) a broad spectrum antibiotic was developed in 1969 at the Squibb Institute for Medical Research, Princeton, N.J. is a semisynthetic derivative of cephalosporin C, differs in side-chain substitution but has the same cephalosporanic acid nucleus as other cephalosporins [1].

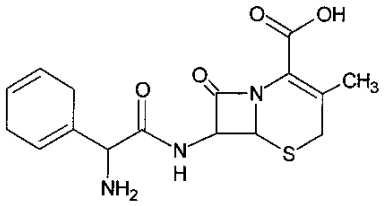

Fig. 1: Chemical structure of cephradine.

Cephradine has broad spectrum antibacterial activity and is effective against Gram positive and negative bacteria through inhibition of cell wall synthesis and highly resistant to beta-lactamase degradation [2, 3]. Cephradine is a drug of choice in eradicating penicillinase-producing organisms known to be resistant to penicillin G, penicillin V and ampicillin. Clinical trials have shown cephradine is effective in treating a wide variety of infections caused by susceptible organisms both in adults and children with low incidence of adverse reactions [4-7].

Cephradine is useful for the treatment of urinary and respiratory tract, skin and soft tissue and gastrointestinal infections [8, 9]. Average serum protein binding capacity of cephradine is 10 % [10].

Limited methods are available for the estimation of cephradine using HPLC with UV, UV-Vis and UV-DAD (Diode Array Detector) detection in pharmaceutical formulations as well as in human serum and plasma matrix [11, 12, 14]. A spectroscopic method for the determination of cephradine was developed in human urine. The method was based on batch and flow injection procedures. The method used only spectrometric technique which is not considered sensitive for the estimation of drugs in biological fluids [13]. Another method found in review employing cephradine as internal standard for the determination of cephalexin in plasma and urine [15]. The previously reported methods were found complex in terms of extraction procedures, greater retention time, low resolutions and separation of peaks. While, the present work described a modified and simple method with

*To whom all correspondence should be addressed.

improved sensitivity and resolution. We have developed simple liquid-liquid extraction procedure which resulted in high extraction yields and hence increased precision, accuracy and selectivity of the cephradine.

Results and Discussion

Method Development

Different proportions of methanol, acetonitrile and buffer were used to choose best mobile phase with optimum ratio to achieve maximum results at different pH values. A mobile phase consisting of 0.05 M potassium dihydrogen phosphate (pH 3.4)-Acetonitrile (88:12) was preferred to attain highest resolution, sensitivity and reproducibility.

Flow rates between 0.5 and 1.8 $mL.min^{-1}$ were examined. A flow rate of 1.0 $mL.min^{-1}$ gave an optimal signal to noise ratio with a reasonable separation time. Using a reversed-phase C_{18} column, the retention time was observed at 9.0 min. Total time of analysis was less than 12 min which showed an economic method in comparison to previously reported methods for the determination of cephradine required more time for the analysis as described by Wu *et al.* [11] and Virginia et al.[12]. The maximum absorption of cephradine as detected at 254 nm and this wavelength was preferred for the analysis same as previously reported methods.

Different procedures were checked for the extraction of drug from the spiked plasma to obtain optimum recovery. Various agents for the precipitation of plasma proteins, acetonitrile, diethyl-ether and perchloric acid with different percentages were tested at number of agitation speed of vortexing. Other parameters of extraction were also optimized for maximum recovery of drug from plasma.

A very short and reproducible extraction method was selected after various trials. 200 µl of 4 % (v/v) perchloric acid was added to 200 µl of drug-free plasma. All samples were mixed by vortex agitation for 30 sec and centrifuged for 5 min at 4000 rpm. Portions of the supernatant were transferred to small glass centrifuge tubes and 20 µl was injected into the HPLC system. The representative chromatogram is presented in Fig 3.

Method Validation

Linearity

The equation of the regression line, correlation coefficient (r^2), relative standard deviation (RSD) values of the slope and intercept for cephradine were determined. Excellent linearity was obtained between the peak areas and concentrations 0.150-20 $\mu g.mL^{-1}$ with r^2= 0.9993. Standard curve parameters for different cephradine batches in human plasma are also shown in Table-1. Standard curve is shown in Fig. 2.

Table-1: Standard curve parameters for cephradine in human plasma.

Batch Code	Slope	Intercept	r^2
CPD01	9701.75	579.74	0.9994
CPD02	9702.81	581.59	0.9991
CPD03	9699.65	580.61	0.9995
Mean	9701.40	580.65	0.9993
S.D.	1.6	0.9	0.0002
%RSD	0.0166	0.1594	0.0208

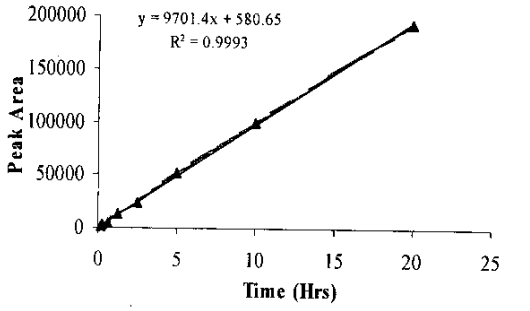

Fig. 2: Standard curve for known concentration of cephradine ($\mu g.mL^{-1}$) spiked in human plasma.

Limits of Detection and Quantitation

Limit of detection (LOD) and Limit of quantitation (LOQ) were established at a signal-to-noise ratio (S/N) of 3 and 9, respectively. Values of LOD and LOQ for cephradine were calculated as 0.050 and 0.150 $\mu g.mL^{-1}$, respectively. Limits of detection (LOD) and Limits of quantitation (LOQ) were experimentally verified by nine injections of cephradine spiked in plasma at the LOD and LOQ concentrations.

Suitability of the Method

The chromatographic parameters such as capacity factor (k′), separation factor (α) and

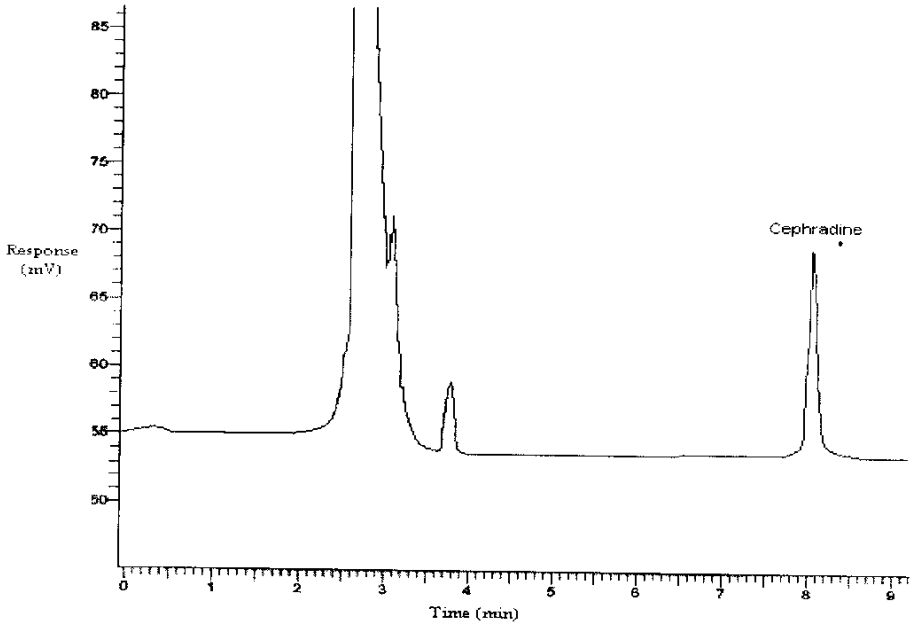

Fig. 3: Representative chromatogram of cephradine in human plasma.

Table-2: Separation and resolution parameters.

Drug	Capacity Factor (k')	RSD %	Separation factor (α)	RSD %	Resolution (R)	RSD %
Cephradine	2.476	0.25	1.18	0.32	1.87	0.38

resolution were determined and shown in Table-2. Value of resolution between peak pair was not less than 1.8.

Capacity factor (k) is a dimensionless measure of retention in liquid chromatography which describe the number of solute molecules in stationary phase to number of molecules in mobile phase.

Precision and Accuracy

The precision of method (Intra-day variations) was checked at the LOQ level. The precision of the method, expressed as the RSD % at the LOQ level was 1.217 %.

Accuracy in terms of percent deviation of the calculated concentrations from the actual concentrations was determined as 1.319 % with RSD % as 1.5 %. The values of Intra-day and Inter-day precision and accuracy of cephradine are shown in Tables-3 and 4, respectively.

Table-3: Intra-day precision and accuracy of cephradine in human plasma.

Batch Code	LQC	MQC	HQC
CPD01	0.150	2.50	20.00
	0.148	2.47	19.95
	0.149	2.48	19.85
	0.144	2.46	19.88
	0.146	2.48	19.78
	0.148	2.49	19.85
	0.147	2.47	19.79
Mean	0.147	2.475	19.850
S.D.	0.002	0.010	0.062
Nominal	0.150	2.50	20.00
%RSD	1.217	0.424	0.314
%Accuracy	98.000	99.000	99.250
%Deviation	2.000	1.000	0.750

Ruggedness

The ruggedness of the HPLC method was evaluated by carrying out the analysis using standard solution, same chromatographic system and column on different days. Small differences in areas and excellent consistency in retention times were observed with RSD % as 0.34. Similarly, ruggedness was also checked by injecting the standard solution in

Table-4: Inter-day precision and accuracy of cephradine in human plasma.

Batch Code	Nominal Concentrations		
	LQC	MQC	HQC
	0.150	2.50	20.00
CPD01	0.148	2.47	19.89
	0.147	2.48	19.75
	0.148	2.45	19.98
	0.149	2.47	19.85
	0.144	2.48	19.88
	0.146	2.46	19.78
CPD02	0.148	2.47	19.77
	0.147	2.48	19.84
	0.149	2.49	19.96
	0.145	2.45	19.74
	0.148	2.48	19.68
	0.146	2.47	19.86
CPD03	0.147	2.46	19.71
	0.149	2.48	19.87
	0.148	2.47	19.93
	0.144	2.49	19.93
	0.146	2.48	19.73
	0.148	2.45	19.83
Mean	0.147	2.471	19.832
S.D.	0.0016	0.0128	0.0894
Nominal	0.150	2.50	20.00
%RSD	1.0808	0.5173	0.4508
%Accuracy	98.04	98.84	99.16
%Deviation	1.963	1.156	0.839

different HPLC systems (Perkin Elmer 200 series, Agilent technologies 1200 series and Agilent technologies 1100 series). The developed method of cephradine is found satisfactorily rugged which is indicated by the high degree of reproducibility of detector responses and retention times.

Extraction Efficiency of the Method

A very simple method was adopted for the extraction of drug from the spiked plasma. Standard concentration of drug ranges from 0.16-20 $\mu g.mL^{-1}$ were spiked in plasma and extraction efficiency was determined by injecting the each concentration five times and percentage of recovery was determined as presented in the Table-5 and graphically shown in Fig. 4.

The previous study by Wu *et al.* [11] described the simultaneous determination of cephalosporins including the cephradine which reported the extraction recovery 98.7 % without spiking the plasma while in present study extraction recovery is 99.161 % with the spiking of drug into human plasma.

Application of Current Method in Pharmacokinetic Studies

The present method was successfully applied in pharmacokinetic evaluation of cephradine in 18 healthy volunteers. No significant difference at 95 % confidence interval was observed for C_{max} (P>0.05, 0.6372), T_{max} (P>0.05, 0.2355) and $AUC_{0-\infty}$ (P>0.05, 0.2684). The values of these parameters are

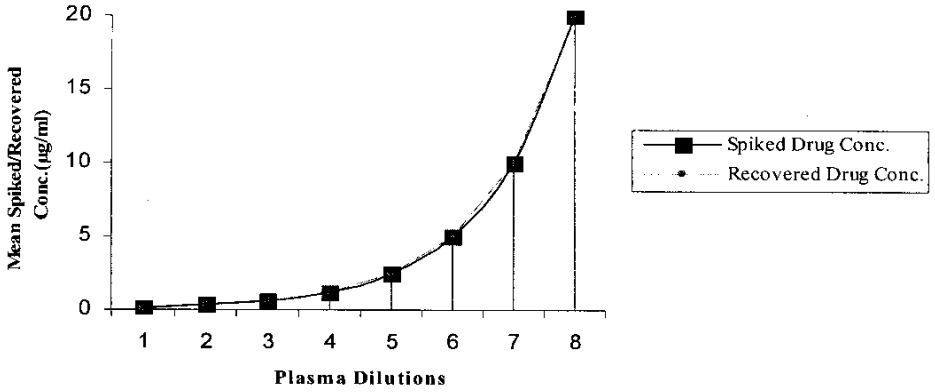

Fig. 4: Plot between spiked and recovered drug concentration.

Table-5: Percentage recovery of cephradine from spiked plasma.

Dilution No.	Spiked Quantity of cephradine ($\mu g.mL^{-1}$)	Cephradine Conc. Found after Extraction($\mu g.mL^{-1}$) Mean	Percent Recovery
1.	0.15	0.147	98.125
2.	0.31	0.299	96.452
3.	0.63	0.628	99.683
4.	1.25	1.249	99.920
5.	2.50	2.498	99.920
6.	5.00	4.989	99.780
7.	10.00	9.982	99.820
8.	20.00	19.918	99.590
Mean	4.981	4.965	99.161 %
			RSD %=1.174 %

given in Table-6 and were found in accordance with reported studies available in literature [2, 3, 8, 16]. The plasma levels of both brands are illustrated in Fig. 5.

Table-6: Comparative (Mean ± SEM) pharmacokinetic parameters of Velosef®-Squibb and Valodin®-Hilton in healthy male subjects.

S. No.	Parameters	Velosef®-Squibb	Valodin®-Hilton
1	AUC_{0-t} ($\mu g.h.mL^{-1}$)	23.07 ± 0.8151	22.01 ± 0.7041^{ns}
3	C_{max} ($\mu g.mL^{-1}$)	14.25 ± 0.5669	13.93 ± 0.4838^{ns}
4	T_{max} (h)	0.90 ± 0.0501	0.83 ± 0.0495^{ns}

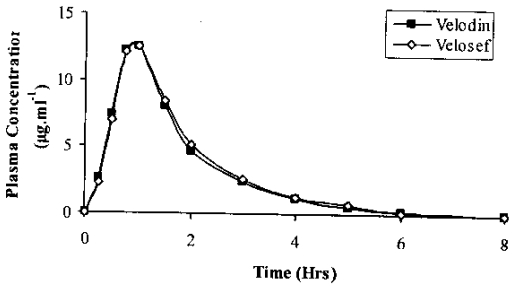

Fig. 5: Comparison of (mean ±SEM, n=18) plasma concentration vs time profile of Velosef®-Squibb and Valodin®-Hilton in healthy male subjects.

Experimental

Materials

Cephradine was gifted by Consolidated Chemical Laboratories (C. C. L) Pharmaceutical (Pvt.) Limited, Pakistan. Acetonitrile, phosphoric acid, perchloric acid and potassium dihydrogen phosphate were obtained from Merck-Germany. HPLC-grade water (double distilled deionised) was used during the development of method.

Method

Preparation of Mobile Phase

The mobile phase consisted of 0.05 M potassium dihydrogen phosphate (pH 3.4)-Acetonitrile (88:12) was prepared and filtered before use. The pH of buffer was adjusted with 5 % phosphoric acid. The flow rate of mobile phase was 1 $mL.min^{-1}$. Effluent was monitored at a wavelength of 254 nm with run time of 11min.

Preparation of Stock Solution

Stock solution of cephradine was prepared by dissolving the drug in filtered deionised water to give a final concentration of 1000 $\mu g.mL^{-1}$. Standard solutions were obtained by diluting this solution with filtered deionised water to give concentrations over the range of 0.156-20$\mu g.mL^{-1}$.

Preparation of Spiked Plasma Samples and Extraction Procedure

For plasma blank samples, 200 µl of 10 % (v/v) perchloric acid was added to 200 µl of drug-free plasma. All samples were mixed by vortex agitation for 30 sec and centrifuged for 5 min at 3500 rpm. Portions of the supernatant were transferred to small glass centrifuge tubes and 20 µl was injected into the HPLC system.

Preparation of Standard Curve

Standard curve was constructed to encompass anticipated range of plasma cephradine concentration found in human plasma after usual adult dose. Blank plasma was spiked with cephradine drug solutions to give concentrations of 0.156, 0.312, 0.625, 1.25, 2.5, 5,10 and 20 $\mu g.mL^{-1}$. Extraction

procedure was same as described above. Triplicate 20µl Injections were made for standard solution to see the reproducibility of the detector response at each concentration level. The peak area of drug was plotted against the known concentrations to obtain calibration curve. The seven concentrations were subjected to regression analysis to calculate the calibration equation and correlation coefficients.

High Performance Liquid Chromatography

Analyses were performed by using Perkin Elmer Liquid Chromatograph with Total Chrom software, pump 200 series, Perkin Elmer U.V. Visible Detector set at 254 nm. A reverse phase system was used consisting of C18 column (Hypersil ODS C18 250mm × 4.6mm I.D. 5 µ). The mobile phase was pumped at a rate of 1 $mL.min^{-1}$, injections of 20 µl were injected, with a run time of 11 minutes.

Pharmacokinetics Study

The protocols of the study were approved by the ethics committee of pharmacy. FDA (Food and Drug Administration) and ICH (International Conference for Harmonization) guidelines were followed to select eighteen human volunteers and written informed consent was taken from each. Two way crossover design was adopted to administer single oral dose of two brands of cephradine (Valodin®-Hilton as a Test and Velosef®-Squibb as a reference) on an empty stomach. The blood sample was collected before drug was given (zero time) and then at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0 hours. Blood Samples were centrifuged at 3500 rpm for 10 minutes and plasma was harvested and frozen at-20°C until assay. Pharmacokinetic analysis was performed as per non-compartmental method of analysis using MS Excel® (Microsoft Corporation 2003), Kinetica® 4.4 (Thermo Electron Corporation) and Windows professional XP® (Microsoft Corporation 2003). Paired t-test was used to calculate the difference between values of the bioparameters of two brands using SPSS-12.

Conclusion

It has been concluded that the method developed in the present study appeared as more simple, precise, linear and reproducible in comparison to methods published in past. This method has been successfully used in a pharmacokinetic study.

References

1. H. H. Weliky, K. Gadebusch, P. Kripalani and E. C. Schreiber, *Antimicrobial Agents and Chemotherapy*, **5**, 49 (1974).
2. Z. Atef, C. S. Eric, W. Irving, R. K. James and A. H. Jerome, *Journal of Clinical Pharmacology*, **14**, 118 (1974).
3. J. S. Choi, J. H. Lee, Y. J. Park and J. P. Burm, *Pharmacy Yakhak Hoeji*, **46**, 290 (2002).
4. B. Y. Marshall, *Current Medical Research Opinion*, **2**, 72 (1974).
5. R. R. Martin, *Journal of Iranian Medical Association*, **24**, 25 (1973).
6. W. J. Mogabgab, *Current Therapeutic Research, Clinical and Experimental*, **19**, 520 (1976).
7. E. S. Neiss, *Journal of Iranian Medical Association*, **24**, 1 (1973).
8. H. Y. Cho, S. Lee, H. A. Kang, I. J. Oh, D. K. Lim and J. D. Moon, *Journal of Korean Pharmaceutical Sciences*, **32**, 215 (2002).
9. L. C. Dionisio, W. S. Richard and E. B. Gerald, *Antimicrobial Agents and Chemotherapy*, **15**, 119 (1979).
10. B. Hoffstedt and M. Walder, *Antimicrobial Agents and Chemotherapy*, **20**, 783 (1981).
11. Z. J. Wu, W. B. Guo, Q. G. Zhang, K. Y. Ni and Y. S. Lin, *Chinese Journal of Chromatography*, **17**, 518 (1999).
12. M. J. Virginia, P. A. John, and C. C. Roger, *Journal of Chromatogr B.*, **740**, 71 (2000).
13. V. Rodenas, M. S. Gracia, C. Sanchez-Pedrino and M. I. Albero, *Journal of Pharmaceutical and Biomedical Analysis*, **15**, 1687 (1997).
14. V. F. Samanidou, E. A. Hapeshi and I. N. Papadoyannis, *Journal of Chromatography B.*, **788**, 147 (2003).
15. N. M. Najib, M. S. Suleiman, Y. M. El-Sayed and M. E. Abdulhameed, *Journal of Clinical Pharmacy and Therapeutics*, **12**, 419 (2008)
16. Y. H. Yana, K. W. Zhangb, G. Z. Hana, Z. S. Wangc, C. Y. Sua, H. X. Chun-Mei, Y. S. Zhaoa and X. W. Xiea, *Asian Journal of Drug Metabolism and Pharmacokinetics*, **5**, 139 (2005).