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Liquid Chromatography-Mass Spectrometry Determination of Cetrizine Hydrochloride in Rabbit Plasma

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A sensitive and highly selective liquid chromatography-tandem mass spectrometry (LC-MS) method was developed to determine cetrizine chloride in rabbit plasma. The analyte and internal standard levocetrizine were extracted from plasma samples and liquid-liquid extraction with ethyl acetate and chromatographed on a C_{18} column. The mobile phase consisted of acetonitrile:water (70:30 v/v). Detection was performed on a single quadrupole mass spectrometer by selected ion monitoring (SIM) mode *via* atmospheric pressure chemical ionization (APCI) source. The method has a limit of quantification of 10 ng/mL. The linear calibration curves were obtained in the concentration range of 10-500 ng/mL. The intra- and inter-day precisions were lower than 4.4 % in terms of relative standard deviation (RSD). This validated method was successfully applied for the evaluation of pharmacokinetic profiles of cetrizine chloride tablets.

Key Words: Cetrizine hydrochloride, LC-MS.

INTRODUCTION

Cetrizine hydrochloride (RS)-2-[2-[4-[(4-chlorophenyl)phenylmethyl]-piperazin-1-yl]ethoxy], a human metabolite of hydroxyzine[1] is an antihistamine and its principal effects are mediated *via* selective inhibition of peripheral H_1 receptors. The antihistaminic activity of cetirizine has been clearly documented in a variety of animal and human models. *In vivo* and *ex vivo* animal models have shown negligible anticholinergic and antiserotonergic activity. In clinical studies, however, dry mouth was more common with cetirizine than with placebo. *In vitro* receptor binding studies have shown no measurable affinity for other than H_1 receptors. Autoradiographic studies with radiolabeled cetirizine in the rat have shown negligible penetration into the brain. *Ex vivo* experiments in the mouse have shown that systemically administered cetirizine does not significantly occupy cerebral H_1 receptors.

A number of analytical methods have been developed for analyzing cetrizine hydrochloride drugs in both biological fluids and pharmaceutical formulations[1-4]. This paper describes the development and validation of a simple, specific, rapid

and sensitive liquid chromatography-mass spectrometry method for the determination of cetrizine hydrochloride in rabbit plasma with a limit of quantification (LOQ) of 10.0 ng/mL for cetrizine hydrochloride with a run time of 5.0 min using levocetrizine as an internal standard.

EXPERIMENTAL

The reference standards of cetrizine hydrochloride (purity 99.67 %) and levocetrizine (purity 98.44 %) were obtained from M/s. Orchid Pharmaceuticals (Chennai, India) and Cadila Pharma (Ahmedabad, India) respectively. High purity water was prepared in-house using a Milli-Q water purification system obtained from M/s Millipore (India) Pvt. Ltd. (Bangalore, India). HPLC grade acetonitrile were purchased from E. Merck Ltd. (Mumbai, India).

A Shimadzu 2010 A LC-MS (including two LC-10ADvp pumps) an online vacuum deaerator, a constant temperature automatic sampler, a quadrupole mass spectrometer equipped with an electrospray ionization interface (ESI) source and LC-MS solution (Version 2.04) was used for data processing.

Calibration curves: The stock solutions of cetrizine hydrochloride and internal standard (levocetrizine) were prepared in acetonitrile at free base concentration of 1000 µg/mL. Secondary and working standard solutions were prepared from stock solutions by dilution with water. These diluted working standard solutions were used to prepare the calibration curve and quality control samples. Blank rabbit plasma was screened prior to spiking to ensure it was free of endogenous interference at retention times of cetrizine hydrochloride and internal standard. An eight points standard curve of cetrizine hydrochloride was prepared by spiking the blank plasma with appropriate amount of cetrizine hydrochloride with calibration curve range from 10.0 to 500.0 ng/mL. Quality control samples, prepared at three concentration levels of 25, 200 and 450 ng/mL for cetrizine hydrochloride were made with blank plasma. The samples were vortexed and stored at -70 ± 2 ºC until further processing.

Sample preparation: A 0.25 mL aliquot of rabbit plasma sample was mixed with 100 µL of internal standard working solution (2500 ng/mL of levo cetrizine) and 1.0 mL of borate buffer of pH 9.00 were added and mixed. The resulting solution was vortexed and extracted with ethyl acetate (3 × 2 mL). The upper organic layer was separated, evaporated and the residue was reconstituted using 0.25 mL of the mobile phase and analyzed.

Validation: The method was validated as per FDA guidelines[5]. The method was validated for selectivity, sensitivity, linearity, precision, accuracy and stability. The selectivity of the method was evaluated by comparing the chromatograms obtained from the samples containing cetrizine hydrochloride and internal standard levocetrizine with those obtained from blank samples. Sensitivity was determined in terms of LLOQ (lower limit of quantification) where the response of LLOQ should be at least five times greater than the response of interference in blank matrix at the

retention time or mass transitions of the analyte. The linearity of different concentrations of standard solutions was prepared to contain 10 to 500 ng/mL of cetrizine hydrochloride containing 2500 ng/mL of levocetrizine. These solutions were analyzed and the peak areas and response factors were calculated. The calibration curve was plotted using response factor against concentration of the standard solutions. The standard curve fitting was determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighing and statistical tests for goodness of fit. The precision of the method was determined by intraday and interday precision. The intraday precision was evaluated by analysis of blank plasma sample containing cetrizine hydrochloride at three different concentrations namely low, medium and high quality control concentrations using nine replicate determinations for three occasions. The interday precision was similarly evaluated over 2 weeks period.

Accuracy of the developed method was determined by relative and absolute recovery experiments. The relative recovery of the drug was calculated by comparing the concentration obtained from the drug supplemented plasma to that of the actually added concentration. Recovery studies were carried out for three levels at six times and the percentage recovery, mean, standard deviation and coefficients of variation were calculated.

As a part of the method validation, stability and partial volume analysis was evaluated. Room temperature stock solution stability, refrigerated stock solution stability, freeze thaw stability, short term stability and long term stability were determined. Room temperature stock solution stability was carried out at 0, 3 and 8 h by injecting four replicates of prepared stock dilutions of cetrizine hydrochloride equivalent to middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration. Comparison of the mean area response of cetrizine hydrochloride and internal standard at 3 and 8 h was carried out against the 0 h value. Refrigerated stock solution stability was determined at 7, 14 and 27 days by injecting four replicates of prepared stock dilutions of the analyte equivalent to the middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration. The stability studies of plasma samples spiked with cetrizine hydrochloride were subjected to three freeze-thaw cycles, short term stability at room temperature for 3 h and long term stability at -70 ºC over 4 weeks. In addition, stability of standard solutions were performed at room temperature for 6 h and freeze condition for 4 weeks. The stability of triplicate spiked rabbit plasma samples following three freeze thaw cycles were analyzed. The mean concentrations of the stability samples were compared to the theoretical concentrations. The stability of triplicate short term samples spiked with cetrizine hydrochloride were kept at room temperature for 1 to 3 h before extraction. The plasma samples of the long term stability were stored in the freezer at -70 ºC until the time of analysis.

RESULTS AND DISCUSSION

Method development: The aim of this work is to develop and validate a simple, rapid and sensitive assay method for the quantification of cetrizine hydrochloride, suitable to determine the pharmacokinetics of this compound in clinical studies. To achieve this goal, during the method development of different options were evaluated to optimize sample extraction, detection of parameters and chromatography. The standard solutions of cetrizine hydrochloride were analyzed by LC-MS system using direct injection probe with APCI interfaces. From the mass spectrum recorded, the detection molecular ion selected was 389 for cetrizine hydrochloride.

Optimization of the chromatographic conditions: The LC-MS method for the determination of cetrizine chloride in human plasma was investigated on the basis of literature. Initially cetrizine chloride and levocetrizine were introduced directly to mass spectrometer using APCI ionization. Parameters such as corona discharge, orifice voltage, ring voltage, flow of sheath and auxiliary gas (N_2) were optimized in order to obtain more abundant protonated molecular ions of analytes. It shows product ion spectra of $[M+H]^+$ ions of cetrizine chloride and levocetrizine. By positive APCI mode, the analyte and internal standard formed predominately protonated molecules $[M + H]^+$ in full scan mass spectra. The major fragment ions at m/z 389 and 388 were chosen in the SIM acquisition for cetrizine chloride and levocetrizine, respectively.The most suitable collision energy was determined by observing the maximum response obtained for the ion peak m/z.The possibility of using electrospray ionization (ESI) or atmospheric pressure chemical ionization sources under positive ion detection mode was evaluated during the early stage of method development. It was found that APCI could offer higher sensitivity and better linearity for the analyte than ESI. Higher flow rate of 0.5 mL/min could be used in APCI mode than that in ESI mode (usually less than 0.5 mL/min) that reduced the chromatographic separating time. The stable response of APCI source provided reproducibility of the measurement. The chromatographic conditions were investigated to optimize for sensitivity, speed and peak shape. The compositions of mobile phase were optimized with varying percentages of organic solvent. It was found that high organic solvent contents (*ca*. 80 %) in HPLC system decreased the background noise and provided rapid separation and stable MS signal throughout an analytical run, allowing the enhancement of sensitivity. Methanol was chosen as the organic solvent because it was less toxic and cheaper than acetonitrile while providing same sensitivity as acetonitrile. The mobile phase could improve the sensitivity by promoting the ionization of the analytes, but the contents of formic acid in mobile phase had no significant effect on the sensitivity of the analytes under APCI conditions. To achieve symmetrical peak shapes, a short chromatographic run time and to eliminate the matrix effect, a mobile phase consisting of acetonitrile:water (70:30 v/v) was used in the experiment. Some kinds of protein precipitation and solid phase extraction methods had been published for the extraction

of cetrizine chloride from plasma samples. In the present experiment, different liquid-liquid extraction conditions were evaluated including different extraction solvents and aqueous pH buffers.

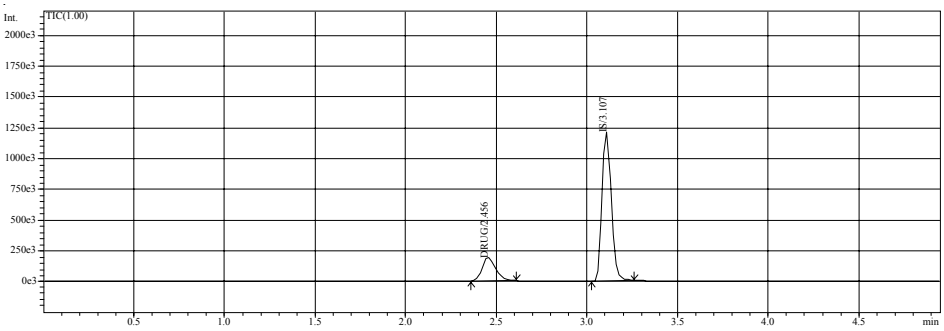

Fig. 1. Sample chromatogram of cetrizine hydrochloride and internal standard

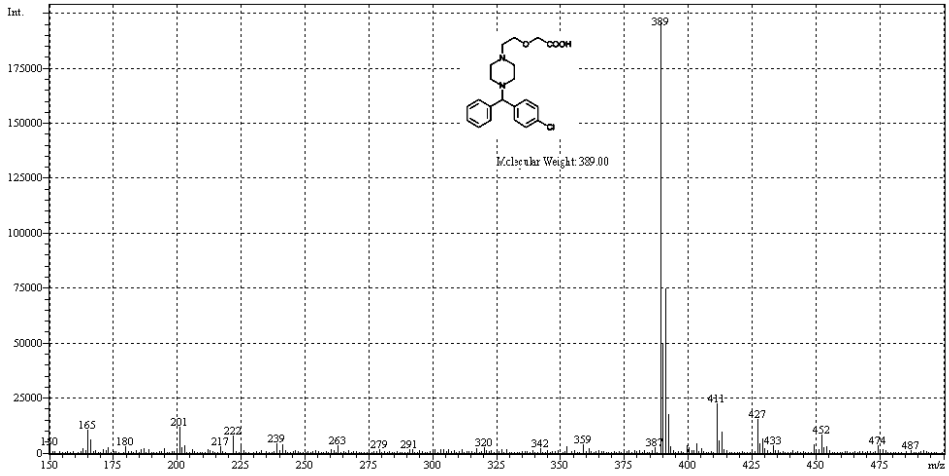

Fig. 2. Mass spectrum of cetrizine hydrochloride at positive mode scan

Method validation: Plasma samples were quantified using the ratio of the peak area of cetrizine hydrochloride to that of levocetrizine as the assay response. Linear calibration curves were obtained with correlation coefficients of greater than 0.999 using a $1/x^2$ weighted linear regression model. To evaluate linearity, plasma calibration curves were prepared and assayed in triplicate on three consecutive days. Accuracy and precision were also assessed by determining QC samples at three concentration levels on the three different validation days. The extraction recoveries of cetrizine hydrochloride at three QC levels were determined by comparing the peak area ratios of analyte to internal standard in sample that had been spiked with analyte prior to extraction with samples to which the analyte had been added

post-extraction. The internal standard was added to both sets of samples post-extraction. The stability of cetrizine hydrochloride in the reconstituted solution under feeble yellow light was assessed by placing QC samples under ambient conditions for 24 h. The freeze-thaw stability of cetrizine hydrochloride was also assessed by analyzing QC samples undergoing three freeze (-20 ºC)-thaw (room temperature) cycles.

Precision and accuracy: The intra- and inter-day precision and accuracy were calculated by analysis of variances based on replicate analyses (3 days, 3 concentrations, each n = 6) of QC samples. The results are summarized in Table-1. In this study, the intra- and inter-day precisions were less than 7.0 % for each QC level. The relative error was within ± 2.5 %. These data indicated reproducible LC-MS results and that the assay was accurate and reliable.

TABLE-1
PRECISION STUDIES OF CETRIZINE HYDROCHLORIDE

Quality control sample	Nominal concentration	Intra-assay (n = 5)	SD	CV (%)	Accuracy (%)	Inter-assay (n = 5)	SD	CV (%)	Accuracy (%)
		Mean concentration found (ng/mL)				Mean concentration found (ng/mL)			
LLOQ	10.00	9.880	0.060	0.60	98.80	9.840	0.095	0.96	98.40
LOC	25.00	24.430	0.794	3.25	97.72	24.486	0.690	2.81	97.92
MQC	200.00	198.930	1.111	0.55	99.46	198.896	1.307	0.65	99.44
HQC	450.00	449.120	0.807	0.17	99.80	449.200	0.838	0.18	99.82

SD= Standard deviation, CV = Coefficient of variance, LLOQ = Lower limit of quantification, LQC = Lower quality control, MQC = Middle quality control, HQC = High quality control.

Specificity: Specificity of the method was carried out by analyzing six blank plasma samples and the chromatograms were recorded. These chromatograms were compared with the chromatograms obtained from standard solutions. Each chromatogram was tested for interference. The combination of the sample preparation procedure and chromatography provided an assay which is free from significant interfering endogenous plasma components at the retention times of the cetrizine hydrochloride and the internal standard. These observations show that the developed assay method is specific and selective.

Linearity: It was observed that the optimized methods were linear within a specific concentration range for cetrizine hydrochloride. The linearity range for were found to be 10 to 500 ng/mL (Fig. 3). The calibration curves were constructed on 11 different days over a period of 4 weeks to determine the variability of the slopes and intercepts. The results indicate that no significant interday variability of slopes and intercepts over the optimized concentration range.

Stability studies: The stability studies of plasma samples spiked with selected drugs were subjected to three freeze-thaw cycles, short term stability at room temperature for 3 h and long term stability at -70 ºC over four weeks. In addition, stability of standard solutions was performed at room temperature for 6 h and freeze condition

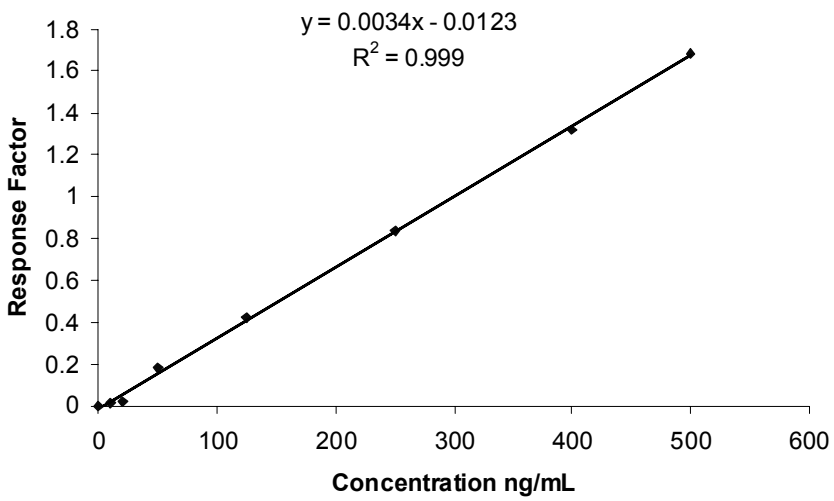

Fig. 3. Calibration curve of cetrizine hydrochloride

for four weeks. The mean concentrations of the stability samples were compared to the theoretical concentrations. The results (Table-2) indicate that the selected drugs in plasma samples can be stored for a month without degradation in frozen state. The results of short term storage at room temperature stability and freeze-thaw cycles indicate no degradation of cetrizine hydrochloride in plasma as well as in sample solution and hence plasma samples could be handled without special precautions.

TABLE-2
STABILITY OF HUMAN PLASMA SAMPLES OF CETRIZINE HYDROCHLORIDE

Sample concentration (ng/mL) (n = 6)	Concentration found (mean ± SD) (ng/mL)	Coefficient of variance (%)
Short-term stability (1, 2, 3 h)		
25	24.190 ± 0.487	2.19
200	195.810 ± 1.323	0.72
450	448.800 ± 9.895	2.30
Long-term stability (4 weeks)		
25	23.647 ± 1.659	7.66
200	192.760 ± 6.750	3.65
450	439.534 ± 8.328	1.96
Stock solution stability (7, 14, 21 days)		
25	23.628 ± 0.656	2.77
200	194.806 ± 2.037	1.04
450	440.470 ± 2.588	0.58
Freeze thaw stability (3 cycle)		
25	24.356 ± 0.735	3.28
200	194.985 ± 3.586	1.96
450	447.803 ± 6.705	1.57

Conclusion

An LC-MS method was developed and validated for the determination of cetrizine hydrochloride in human plasma. The method is rapid, sensitive and highly selective with an LLOQ of 10.0 ng/mL using 0.25 mL rabbit plasma. The determination of one plasma sample only needed 4 min and more than 120 samples could be assayed daily, including sample preparation, data acquisition and processing. It was proved superior in sensitivity and speed than the reported methods.The method was successfully applied to evaluate the pharmacokinetics of cetrizine hydrochloride after an oral dose healthy volunteer. The present method provides an example for biological samples analysis of cetrizine hydrochloride drugs.

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