On-line robotic high-throughput liquid chromatography–tandem mass spectrometric method for simultaneous quantification of Hydroxyzine and Cetirizine in human plasma

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Abstract

A simple, sensitive and reliable method have been developed for simultaneous quantification of Hydroxyzine and Cetirizine in human plasma by using on-line robotic HTLC–MS/MS method. Quetiapine was used as an internal standard(IS). A typical two-column setup featuring two six-port switching valves was employed for method development and Turbo flow on-line technique was applied for separation of analyte from plasma sample. The analytical procedure involves on-line coupling of sample extraction with Cyclone P (50 mm × 0.5 mm 50 µm) HTLC column by injecting 15µL sample and chromatographic separation is performed with Inertsil ODS-3(5 µm ,4.6 x100 mm.). To achieve required chromatograms with consistency we have performed different combinations of the solvents and gradient system. Finally we succeeded with the solution combinations of 5mM Ammonium Acetate:Methanol: Acetonitrile (5:5:90) in pump A, Pure methanol in pump B, 0.1% formic acid in pump C and washing solution in the ratio of 70:25:5 (methanol:water:IPA) in pump D and analyzed more than 150 samples with out overloading of the chromatographic columns with improved real throughput efficiency. Detection was performed at transitions of m/z 375.300→201.100 for Hydroxyzine, m/z 389.200→201.100 for Cetirizine and m/z 384.200→253.100 for Quetiapine by positive electro-spray ionization (ESI+) in multiple reaction monitoring (MRM) mode using tandem mass spectrometry. The calibration curves were linear over a concentration range of 0.100 ng/mL to 150.000 ng/mL of Hydroxyzine and 0.500 ng/mL to 500.000 ng/mL of Cetirizine for quantification with the correlation coefficients demonstrating good linearity (0.994-0.999). The total run time of analysis was 5 min and the lower limits of Quantification were 0.100 ng/mL for Hydroxyzine and 0.500 ng/mL for Cetirizine, respectively. The method validation was carried out in terms of specificity, sensitivity, linearity, precision, accuracy and stability. The validated method was successfully applied in bioavailability and bioequivalence study.

Materials and Methods

Chemicals and reagents :

Hydroxyzine dihydrochloride, Citirizine dihydrochloride, Quetiapine fumarate and Ammonium Acetate were purchased from Sigma-Aldrich Chemicals. Acetonitrile and Methanol was obtained from JT Baker (LC-MS grade). Formic acid was obtained from Parchem chemicals. Isopropyl alcohol obtained from A.B.Enterprises. Water (LC-MS grade) was purchased from Fisher Chemicals.

Standard solutions preparation :

Stock solution preparation:

Approximately 5 mg of Hydroxyzine dihydrochloride/ 5mg of Citirizine dihydrochloride / 2 mg of Quetiapine fumarate (ISTD) working standard is weighed and transferred to 10.0 mL volumetric flask, to this 5.0 mL of methanol is added and sonicated to aid dissolution and the final volume is made up with methanol.

Preparation of internal standard dilution :

The Quetiapine internal standard (ISTD) dilution of about 100 ng/mL from the ISTD stock solution (ISTD stock) using (50% v/v Methanol in Water)as the diluent is prepared.

Preparation of calibration curve (CC) standards and quality control (QC) samples :

Appropriate dilutions of the stock solutions with diluent were made subsequently in order to prepare the working standard solutions for Hydroxyzine and Citirizine. All the solutions were stored in a refrigerator between 2°C and 8°C. Calibration standards and quality control samples, in the range of 0.100 ng/mL to 150.000 ng/mL of Hydroxyzine and 0.500 ng/mL to 500.000 ng/mL of Cetirizine were prepared for calibration. Accuracy and precision, quality control and stability assessment was done by spiking 0.5mL of drug free plasma with appropriate volume of working solution.

Solutions used for robotic on-line sample extraction system :

5mM Ammonium Acetate:Methanol: Acetonitrile (5:5:90) in pump A, Pure methanol in pump B, 0.1% formic acid in pump C and washing solution in the ratio of 70:25:5 (methanol:water:IPA) in pump D

Sample preparation:

Retrieved the frozen CC, QC and subject samples from the deep freezer and thaw in water bath maintained at room temperature, vortexed to mix. Removed the caps from the polypropylene tubes. Aliquoted 0.2 mL of CC, QC and subject samples into pre-labelled HPLC vials. Added 50.0 µL of ISTD dilution (100 ng/mL) followed by 50.0 µL of 10mM Ammonium Acetate buffer of pH 7.5 into vials, caped, vortexed to mix and transferred vials to auto sampler. Then, 15 µL aliquot was injected on to the LCMS/MS system. For optimal stability, the auto-sampler temperature was set at 5 ˚C.

Data processing:

Chromatograms were acquired on a TSQ tandem mass spectrometry (Thermo Finnigan, Sanjose, CA, USA) equipped with Electrospray ionization (ESI) and connected to a PC runs with the standard software Xcalibur 2.0.7 and LC Quan 2.5.6. Mass spectroscopic detection was performed on a Triple quadrapole instrument (Thermo, TSQ Quantum Discovery Max). Robotic liquid handling system is operated using the software package supplied from the cohesive technologies Aria^{TM}. The calibration curve is constructed by weighted 1/x^2 least-square linear regression analysis of the peak area ratio (drug/ISTD) vs. the concentration of drug.

Results

Chromatographic and mass spectrometric conditions :

The LC/MS/MS system consisted of four pumps for gradient solvent delivery, and a divert valve to direct LC effluent to the mass spectrometer in the analyte elution window. The analytical column effluent is directed through the divert valve to a thermo electron TSQ quantum discovery mass spectrometer. Source specific and Compound specific parameters are presented in Table 1. Detection was performed at transitions of m/z 375.300→201.100 for Hydroxyzine, m/z 389.200→201.100 for Cetirizine and m/z 384.200→253.100 for Quetiapine by positive electro-spray ionization (ESI+) in multiple reaction monitoring (MRM) mode using tandem mass spectrometry. A resolution of one unit (at half peak height) is used for both Q1 and Q3.

Steps involved in on-line robotic method development:

A typical two-column setup featuring two six-port switching valves is employed for method development. The procedure consisted of four steps:

(1) The eluent loop is filled with 50% acetonitrile in 10 mM ammonium formate.

(2) 15µL sample is loaded onto the Cyclone P (50 mm × 0.5 mm, 50 µm) HTLC column at a flow rate of 2 mL/min during 60 s.

(3) The eluent loop is discharged at 0.5 mL/min for 60 s to transfer the analytes from HTLC column onto the Cohesive Propel C18 (50 mm × 2.1 mm i.d., 5 µm) column and 0.5% aqueous formic acid at 0.2 mL/min in added post column.

(4) LC–MS/MS is performed using ballistic gradient at 2.0 mL/min (10–90% acetonitrile in 0.5% formic acid).

On-line sample extraction:

The gradient program accomplished a Cyclone HTLC column for sample extraction, elution with four pumps. TLX turbo flow on-line technique is employed for separation of analyte from sample molecules. The mechanism involved in sample preparation may be affinity. The small drug molecules bind to the HTLC column, and molecules that have lower binding affinity quickly diffuse into the column particles and large sample molecules are flushed to waste, then the mobile phase elutes the analyte molecules that are binded at HTLC column to analytical column, from this analytical column analytes are entered to mass detector. To achieve required chromatograms with consistency we have performed different combinations of the solvents and gradient system. Finally we succeeded with the solution combinations as mentioned in Table 2 and analyzed more than 150 samples with out overloading of the chromatographic columns with improved real throughput efficiency.

Method Validation:

The method validation was carried out in terms of specificity, sensitivity, linearity, precision, accuracy and stability. The mean accuracy and precisions for back calculated concentrations of each standard calculated from calibration curves are tabulated as Table 3. The accuracy and precision for inter day and intra day was tabulated for drug in Table 4. Representative chromatograms of extracted human blank plasma and extracted human blank plasma spiked with both Drugs are shown in Fig.1.

In our study quality control plasma samples were used subject to bench top (10h), Auto injector (48 h), freeze–thaw (-80 to +20 °C) cycles, wet extract (30 h) at room temperature, wet extract at 2-8°C (30h) and long term (90 days) at deep freezer (at -80 °C) tests are performed. The values obtained for present stability studies are tabulated (Table 5), which are within the acceptance criteria.

Application of the method:

The present method was applied for a randomized cross-over bioequivalence study of two different preparations in 12 healthy male volunteers. After single oral administration of the drug blood samples were collected at a suitable time intervals. This method was successfully used to measure the Plasma concentrations of Hydroxyzine and Citrizine.

Step	Start	S	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	0.00	30	2.00	Step	0.0	100.0	0.0	0.0	-	Out	0.80	Step	20.0	80.0
2	0.50	90	0.40	Step	50.0	0.0	50.0	0.0	T	In	0.80	Step	20.0	80.0
3	2.00	30	2.00	Step	0.0	0.0	0.0	100.0	-	In	0.80	Ramp	20.0	80.0
4	2.50	60	2.00	Step	0.0	0.0	0.0	100.0	-	In	0.80	Step	20.0	80.0
5	3.50	60	2.00	Step	50.0	50.0	0.0	0.0	-	In	0.80	Step	20.0	80.0
6	4.50	30	2.00	Step	50.0	50.0	0.0	0.0	-	In	0.80	Step	20.0	80.0
7	5.00	60	2.00	Step	0.0	100.0	0.0	0.0	-	Out	0.80	Step	20.0	80.0

Table-2. Steps nvolved in Online Robotic Method

Parameter	Hydroxyzine	Cetirizine	Quetiapine
Declustering potential	33.00	30.00	45.00
Entrance potential	10.00	10.00	10.00
CEP	3.70	21.55	21.39
Collision energy	24.00	55.00	40.00
CXP	3.70	5.00	5.00
Polarity	Positive	Positive	Positive
Curtain gas (CUR)	30.00		
CAD Gas	5.00		
Ion spray voltage	5000.00		
Heater temperature	500.00°C		
Nebulizer gas (GS1)	45.00		
Heater gas (GS2)	40.00		
Dwell time	300.00 msec		
Ihe	ON		

Table-1:Source specific and compound specific mass spectrometric parameters

Drug Name	Hydroxyzine		Citirizine	
Standards	% C.V	% Accuracy	% C.V.	% Accuracy
STD-1	3.3	100.8	3.9	100.5
STD-2	13.4	98.9	17.3	101.1
STD-3	4.2	92.5	4.8	94.8
STD-4	4	99.9	5.3	101
STD-5	7.2	102.9	4.1	97.3
STD-6	3.2	102.8	3.8	99.3
STD-7	0.7	93.6	2.9	93.1
STD-8	6	107.8	2.1	113.2

Table 2: Back calculated concentrations from calibration curves

Molecule Name	Experiment	HQC	MQC	LQC	LLOQ
Hydroxyzine	Intra-day accuracy (%) (day1)	95.6	98.4	98.8	111.3
	Intra-day precision (%) (day1)	7.2	9.2	8.8	14
	Intra-day accuracy (%) (day2)	104.2	102.3	103.1	103.6
	Intra-day precision (%) (day2)	7.6	6.5	12.1	13.6
	Intra-day accuracy (%) (day3)	85.1	91.2	95	99
	Intra-day precision (%) (day3)	10.8	14.9	6.2	8.7
	Overall accuracy (%)	95	97.3	99	104.6
	Overall Precision (%)	10.1	5.8	4.1	5.9
	Number of determinations	18	18	18	18
Citrizine	Intra-day accuracy (%) (day1)	92.6	93.3	86.7	106.1
	Intra-day precision (%) (day1)	9.3	10.8	7.1	11.6
	Intra-day accuracy (%) (day2)	108.3	103.6	96	102.5
	Intra-day precision (%) (day2)	13.3	8	7.3	6
	Intra-day accuracy (%) (day3)	95.6	103.6	100.4	102.5
	Intra-day precision (%) (day3)	6.2	9.1	7.5	5.2
	Overall accuracy (%)	98.8	102.1	94.3	103.7
	Overall Precision (%)	8.4	5.9	7.4	2
	Number of determinations	18	18	18	18

Table 3.Assessment of Accuracy and precision of the method

Experiment	Hydroxyzine				Citirizine			
	Accuracy		Precision		Accuracy		Precision	
	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC
Freeze-thaw stability	107.1	101.4	8.0	14.2	104.7	101.7	9.6	14.6
Bench top stability	100.7	96.7	4.6	6.6	105.9	99.8	4.9	6.7
Auto sampler stability	93.7	92.4	7.1	12.8	102.5	96.0	11.3	9.6
In injector stability	93.8	90.2	6.0	8.1	105.9	103.7	8.1	8.0

Table 4.Stability results

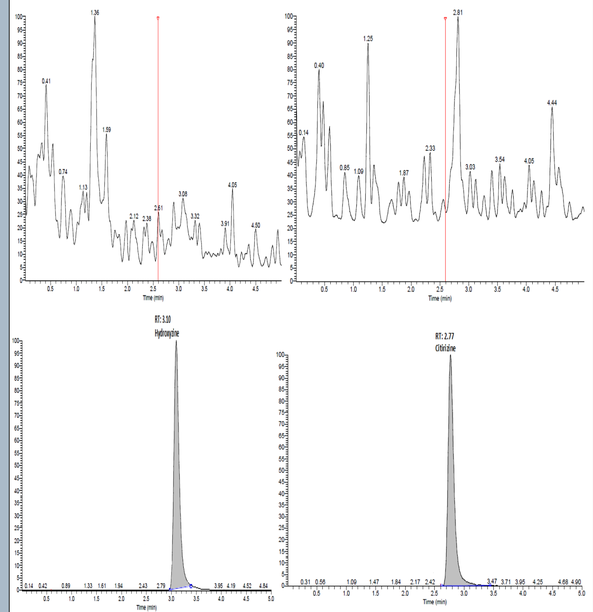

Fig.1. Representative chromatograms of extracted human blank plasma and extracted human blank plasma spiked with both Drugs

Conclusion

The method was applied successfully to the analysis of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence study after therapeutic doses of Hydroxyzine and Citrizine. . The established HTLC-MS/MS method is sensitive and suitable for the study of Hydroxyzine and Citrizine in human plasma. The method is easy to follow and can be adopted for clinical drug monitoring.

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