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LC-MS-MS Method for Determination of Metolazone in Human Plasma

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Abstract: A rapid, sensitive and specific method for quantification of metolazone in human plasma using metaxalone as internal standard is described. Sample preparation involved a simple liquid-liquid extraction procedure. The extract was analyzed by high performance liquid chromatography coupled to electrospray tandem mass spectrometry (LC-MS-MS). Chromatography was performed isocratically on a 5 μm C₁₈ analytical column (50 mm \times 4.6 mm *i.d.*) with buffer-acetonitrile 20:80 (v/v) as mobile phase. The response to metolazone was a linear function of concentration over the range 1.00 to 2000.00 ng mL⁻¹. The lower limit of quantification in plasma was 1.0 ng mL⁻¹. The method was successfully applied in a bioequivalence study of a metolazone formulation after administration as a single oral dose.

Keywords: Metolazone, Human plasma, LC-MS-MS.

Introduction

Metolazone has the molecular formula C₁₆H₁₆ClN₃O₃S and the chemical name 7-chloro-1,2,3,4-tetrahydro-2-methyl-3-(2-methylphenyl)-4-oxo-6-quinazolinesulfonamide with molecular weight⁷ of 365.83. Metolazone is a quinazoline diuretic with properties generally similar to the thiazide diuretics. The actions of metolazone result from interference with the renal tubular mechanism of electrolyte reabsorption. Metolazone acts primarily to inhibit sodium potassium excretion. Metolazone does not inhibit carbonic anhydrase. A proximal action of metolazone has been shown in humans by increased excretion of phosphate and magnesium ions and by a markedly increased fractional excretion of sodium in patients with severely compromised glomerular filtration⁶⁻¹⁶. Previous work shows metaxalone has been determined in biological fluids such as human plasma, blood and urine with high pressure liquid chromatography with fluorescence detector and coupled mass detector¹⁻⁵. The objective of this study was to develop a simple, inexpensive, sensitive, rapid, and accurate method for analysis of metolazone in human plasma with reliable reproducibility suitable for pharmacokinetic studies.

Experimental

Chemicals and reagents

Working standard of metolazone was obtained from Centaur Chemical Pvt. Ltd, Mumbai-400055, India. Working standard of metaxalone was obtained from Lannett company, Inc.9000 state Rd., Philadelphia. Methanol and acetonitrile of HPLC grade were from J.T. Baker. Ammonium acetate and formic acid of HPLC grade were from BDH Laboratory Reagents, England. HPLC-grade water was from Merck.

Instrumentation and chromatographic conditions

The HPLC system (Shimadzu LC-20AD) consisted of a binary pump and an autosampler (SIL-HTc). Detection was performed with an Applied Biosystems Sciex (API 2000) mass spectrometer with atmospheric ion spray for ion production, which was controlled by Analyst 1.4 software. Chromatography was performed isocratically on a 50 mm × 4.6 mm *i.d.*, 5 μm particle, thermo hypurity C₁₈ analytical column. The mobile phase was buffer-acetonitrile 20:80 (v/v) at a flow rate of 0.4 mL min⁻¹. Buffer was 2 mM ammonium acetate, pH adjusted to 3.0 with formic acid. Chromatography was performed at ambient temperature. The ion-spray potential was set at 5.5 kV and the source temperature was 400°C. The collision activation dissociation (CAD) gas setting at 4.0; nitrogen was used as collision gas. The instrument was set up in multiple reaction monitoring (MRM) mode; the transition m/z 366.1 → 259.0 was monitored for metolazone and the transition m/z 222.2 → 161.1 for metaxalone. Figure 1 and Figure 2 shows MS-MS scan for metolazone and metaxalone.

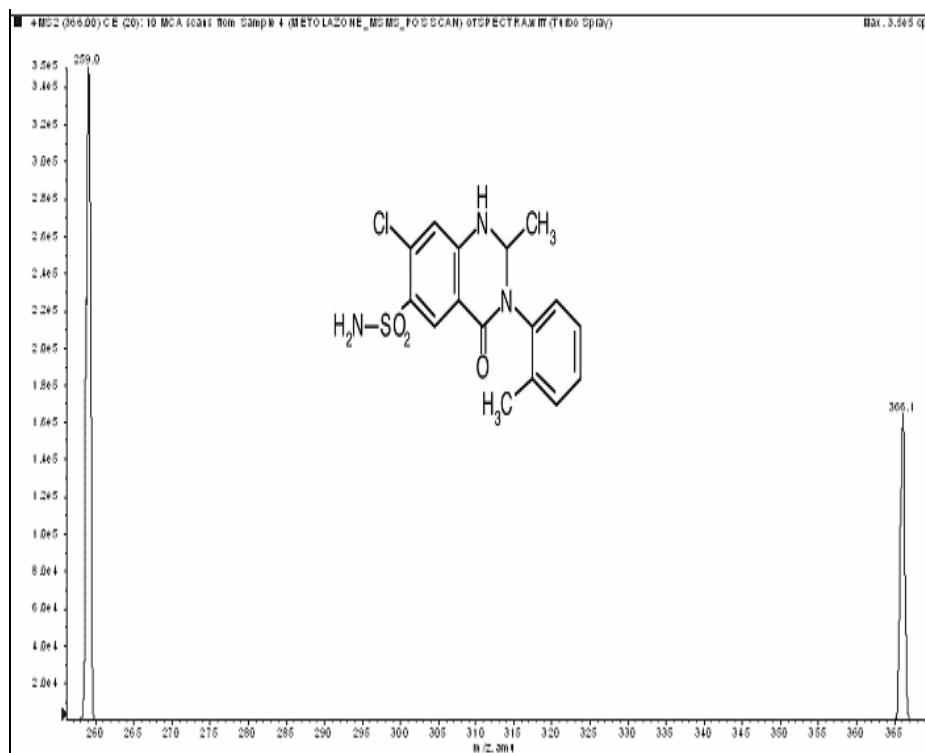


Figure 1. Metolazone MS-MS scan (366.1 > 259.0).

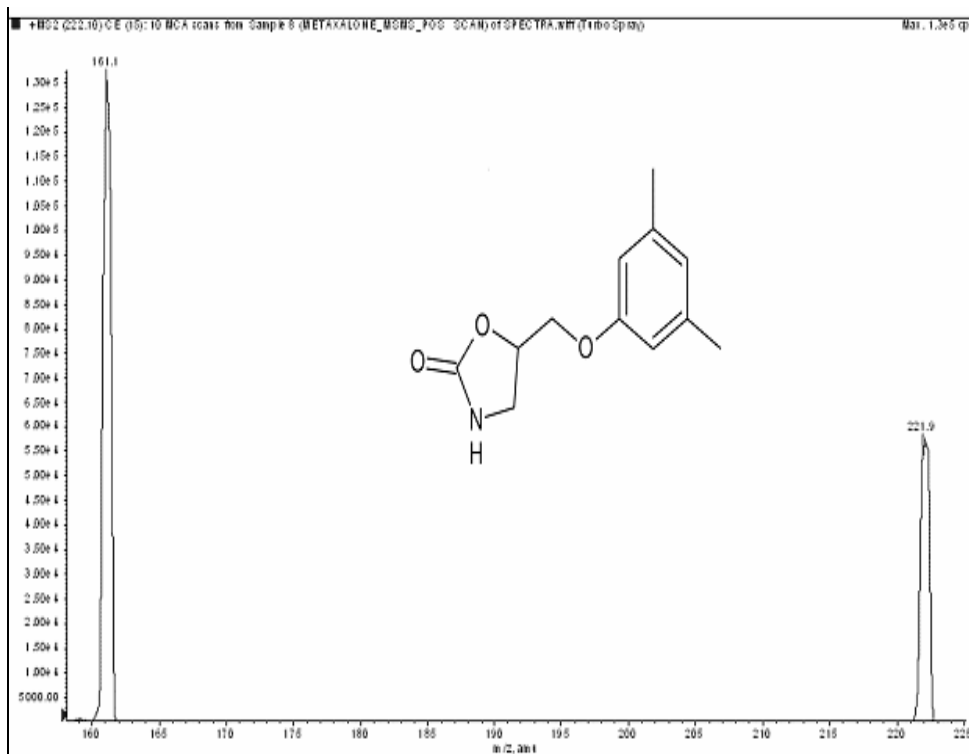


Figure 2. Metaxalone msms scan (222.2 > 161.1)

Preparation of stock solution and calibration and quality control samples

A stock solution of metolazone ($1000.00 \mu\text{g mL}^{-1}$) and metaxalone ($1000.00 \mu\text{g mL}^{-1}$) were prepared in methanol. A series of working standard of metolazone containing 20, 50, 500, 1000, 10000, 20000, 30000 and 40000 ng mL^{-1} was prepared by diluting the stock solution with diluent methanol–water 60:40 (v/v). Working internal standard solution ($10.00 \mu\text{g mL}^{-1}$) was prepared in methanol and water in the ratio 60:40 (v/v). Low, medium and high concentration quality control solutions (60.00 , 4000.00 and $35000.00 \text{ ng mL}^{-1}$, respectively) were prepared in diluents methanol and water in the ratio 60:40 (v/v). Calibration plot standards were prepared by spiking blank plasma with metolazone at concentration of 1.00, 2.50, 25.00, 50.00, 500.00, 1000.00, 1500.00 and $2000.00 \text{ ng mL}^{-1}$. Quality control samples were prepared by spiking blank plasma with 3.00, 200.00 and $1750.00 \text{ ng mL}^{-1}$ metolazone. Stock solutions were stored at $4\text{--}8^\circ\text{C}$ and used within 33 days of preparation

Sample preparation

To 2.0 mL polypropylene centrifuge tube, 500 μL of plasma sample was spiked with 50.00 μL of internal standard ($10 \mu\text{g mL}^{-1}$) solution, 25 μL diluent (or Metolazone working calibration standard solution or quality-control solution) and 1.5 mL methyl *tert*-butyl ether were added. Samples were vortexed for 10.0 minutes followed by centrifugation at $15000 \times g$ for 10 minutes. The supernatant layer was separated and evaporated to dryness under a stream of nitrogen at 50°C . The dry residue was dissolved with 250 μL of mobile phase, vortexes for 1.0 minute and 10 μL of the sample was injected

Validation procedures

The method was validated in accordance with current acceptance criteria. The specificity of the analytical method was investigated by extraction and analysis of blank plasma samples from six different sources to assess potential interference from endogenous substances. The apparent response at the retention times of metolazone and metaxalone was compared with that at the lower limit of quantification (1.0 ng mL^{-1}). Representative chromatograms illustrating the specificity of the method are shown in Figure 3 and 4. The acceptance criterion for metolazone was that the mean interference from the six individual sources should be $\leq 20\%$ of the signal at the LLOQ and that the mean interference for metaxalone (internal standard) from the six individual sources should be $\leq 5\%$ of the signal at working concentration

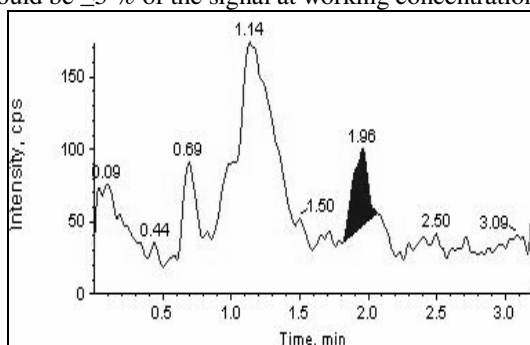


Figure 3. Representative chromatogram plasma blank for Metolazone

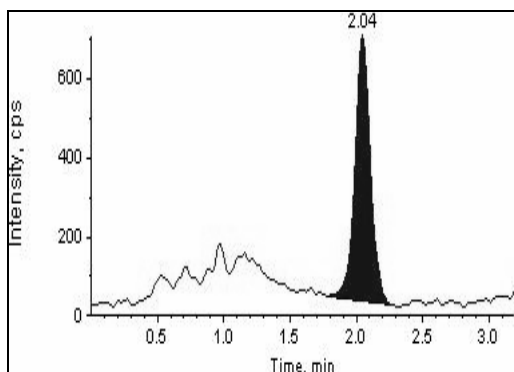


Figure 4. Representative chromatogram plasma spiked with metolazone at the lower limit of quantification

The calibration equation was determined by least-squares linear regression (weighting $1/X^2$) over the range 1.0 to $2000.0 \text{ ng mL}^{-1}$ in plasma. The precision and accuracy of the methods were determined at the three QC sample levels for six replicates together with calibration samples from different validation batches. The basic fundamental properties studied during method validation were the stability of stock solution stored at $4-8^\circ\text{C}$ for one month, freeze thaw stability through three cycles, short term stability, an autosampler stability and long term stability. For freeze thaw stability, QC plasma samples were subjected to three cycles from -20°C to room temperature. Short term bench top stability was determined by placing samples on the bench top at ambient temperature for 24 h. An autosampler stability was assessed by placing processed QC samples in an autosampler at

10°C for 24 h and long term stability was evaluated by freezing QC samples at -20°C for a month. Recovery was assessed by comparing the peak areas of neat analyte standards with those for spiked standards at three concentrations before and after extraction¹⁷.

Pharmacokinetic application

The method was used to determine pharmacokinetic data for metolazone in human volunteers.

Results and Discussion

The assay was found to be linear for metolazone concentrations in the range 1.0 to 2000.00 ng mL⁻¹, ($r = 0.9998$).

Table 1. correlation coefficient (r)

Calibration curve	Slope	Intercept	Regression
1	0.007220	0.000789	0.9973
2	0.007310	0.00284	0.9990
3	0.007570	0.00384	0.9985
4	0.007110	0.00138	0.9970
Mean	0.007303	0.002212	0.9980

$n = 4$

Precision and accuracy were studied satisfactory at three QC concentrations. The intra-day precision and accuracy of the method at QC levels (3.0 ng mL⁻¹, 200.0 ng mL⁻¹, and 1750.0 ng mL⁻¹, $n = 6$) were 5.43, 5.63 and 4.27% and 106.10, 104.59 and 102.07%, respectively. The inter-day precision and accuracy of the method at QC levels ($n = 6$) were 5.82, 5.27 and 5.13% and 106.58, 103.97 and 100.55% respectively. The results obtained from measurement of linearity, precision and accuracy are listed in Tables 2 and 3.

Table 2. Concentrations of metolazone in calibration standards prepared in human plasma.

	Nominal*	Mean*	CV, %
Level 1	1.000	0.972	0.81
Level 2	2.500	2.667	1.78
Level 3	25.000	26.366	6.10
Level 4	50.000	47.465	1.09
Level 5	500.000	482.972	1.00
Level 6	1000.000	981.670	8.19
Level 7	1500.000	1491.657	4.08
Level 8	2000.000	2030.249	2.37

*ng mL⁻¹; $n = 4$

Table 3. Data of inter day precision and accuracy.

Drug	Nominal*	Mean*	Inter Day		Intra Day		
			Accuracy, %	CV, %	Mean*	Accuracy, %	CV, %
LQC, $n=6$	3.000	3.197	106.58	5.82	3.183	106.10	5.43
MQC, $n=6$	200.000	207.933	103.97	5.27	209.176	104.59	5.63
HQC, $n=6$	1750.000	1759.618	100.55	5.13	1786.211	102.07	4.27

*ng mL⁻¹; $n = 3$

The extraction recovery of the method at QC levels ($n = 6$) was 62.23, 67.40 and 78.58%, respectively. Absolute mean recovery of metolazone and the internal standard (metaxalone) were 69.41 & 79.46% respectively. Stock solution stored at 4–8°C was found to be stable for 33 days. When drug stability at the LQC and HQC concentrations was measured after three freeze thaw cycles, the differences from freshly prepared samples 1.28

and 3.54% respectively were low. When bench top stability at the LQC and HQC concentrations for 24 h was measured the differences from freshly prepared QC samples were -4.33 and -1.07% respectively. When an autosampler stability at the LQC and HQC concentrations for 24 h was measured, the differences from freshly prepared samples were approximately 1.52 and 0.77% respectively. When drug stability in the matrix at -20°C for 31 days was measured at the LQC and HQC concentrations difference from freshly prepared samples were 4.78 and -2.57% respectively.

Table 4. Concentrations of metolazone in stability sample prepared in human plasma

Stability	Nominal*	% Difference	CV, %
Bench top	3.000	-4.33	9.64
	1750.000	-1.07	5.32
Freeze thaw	3.000	1.28	3.54
	1750.000	3.54	5.32
Auto sampler	3.000	1.52	2.32
	1750.000	0.77	5.32
Drug stability in matrix	3.000	4.78	12.23
	1750.000	-2.57	9.99

*ng mL⁻¹; n = 6

Pharmacokinetic application

After oral administration to volunteers, the observed peak plasma concentration (C_{\max}) was 49.00 ng mL⁻¹ for test and 49.66 ng mL⁻¹ for reference. The time (T_{\max}) taken to achieve peak plasma concentration were 2.83 hours for test and 2.75 hours for reference. In addition, the calculated 90% confidence interval (CI) for mean C_{\max} , AUC_{last} , and $AUC_{0-\infty}$ individual ratios were within the 80–125% interval stipulated by the US Food and Drug Administration.¹⁸

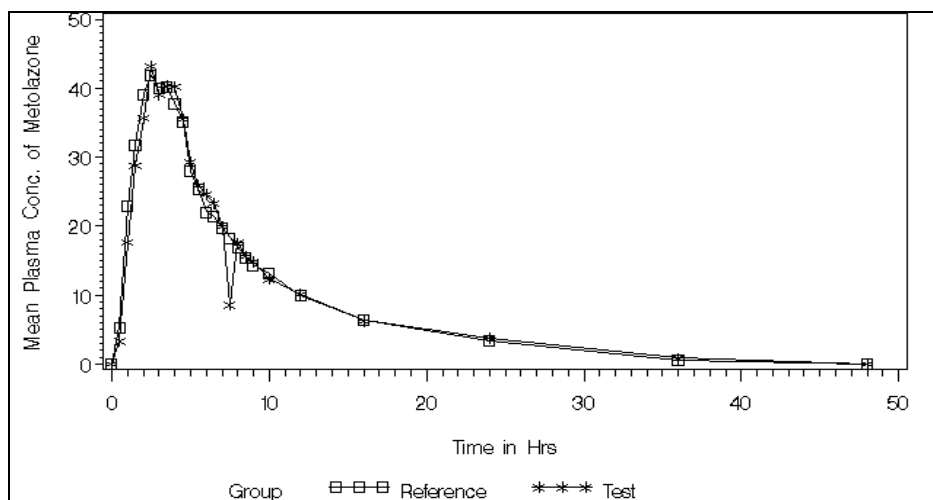


Figure 5. Mean plasma concentration –time profile of metolazone after a single oral dose of metolazone (USP 5mg) tablet to healthy male volunteers.

LC–MS–MS analysis with a reversed-phase column and low aqueous high organic mobile phases was found to be ideal for the analysis. Increasing the organic content of the mobile phase resulted in improved sensitivity by enhancement of the ionization yield.^{19–21} Use of methanol as

the organic component of the mobile phase did not result in adequate sensitivity and selectivity, owing to bad peak shape and increased interference from the plasma. Acetonitrile as organic component and pH of buffer, resulted in better sensitivity, but variation of the amount of acetonitrile in the mobile phase effected the run time. The mobile phase was optimized to provide sufficient selectivity in a short separation time. Analyte and internal standard responded best to positive ionization using atmospheric turbo ion spray for ion production. In order to get higher response hypersil hypurity (4.6 x 50 mm), 5 μ , Column was used. The pH of mobile phase adjusted to 3.0 with formic acid. The assay was found to be linear in the conc. range 1.000 to 2000.000 ng mL⁻¹ for metolazone. Precision and accuracy were satisfactory at the three conc. studied. Absolute mean % recovery of the metolazone and metaxalone is 69.41 & 79.46% respectively. Recovery of an analyte and internal standard is consistent, precise and reproducible Stability of analyte and internal standard in methanol stock sol. was verified on storage for 33 days at 2-8°C. The proposed method proved accurate and selective and met the standards for bioanalytical Method Validation accepted by the FDA¹⁷

Conclusion

Rapid and sensitive LC-MS-MS method is reported for the determination of metolazone in human plasma. The Assay was successfully applied to determine concentration of the drug in a bioequivalence study of metolazone. The method developed allows high samples through put due short run time and relatively simple sample preparation procedure. Advantages of wide Linearity range 1.0 ng mL⁻¹ to 2000 ng mL⁻¹ of the method is applicable for various dose of metolazone.

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