RESEARCH ARTICLE

RAPID AND SENSITIVE LC-MS/MS METHOD FOR THE SIMULTANEOUS ESTIMATION OF AMOXICILLIN AND CLAVULANIC ACID IN HUMAN PLASMA

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Abstract: A simple, rapid, specific and sensitive liquid chromatography - tandem mass spectrometric method has been developed and validated for the simultaneous estimation of amoxicillin and clavulanic acid in human plasma. Both amoxicillin and clavulanic acid were extracted from human plasma by liquid-liquid extraction. Diclofenac was used as internal standard. Detection was performed using an applied Biosystems Sciex API 3200 mass spectrometer. Chromatographic separation of analytes and internal standard were carried out using a symmetry C_{18} (50 x 4.6 mm i.d, 5 µ) column. The assay of amoxicillin and clavulanic acid were linear over the range of 0.5 to 40 µg/mL and 0.1 to 6.0 µg/mL respectively with a precision of <15% and the limit of quantification in plasma for amoxicillin and clavulanic acid was 0.5 µg/mL and 0.1 µg/mL respectively.

Keywords: Amoxicillin, clavulanic acid, human plasma.

INTRODUCTION

Amoxicillin is an broad spectrum of semi - synthetic antibiotic derived from the basic penicillin nucleus, 6-amino penicillanic acid. Amoxicillin is chemically (2S, 5R, 6R)-6-[(R)-(-)-2-amino-2-(p-hydroxy phenyl) acetamido] -3,3-dimethyl -7-oxo-4 thia-1-aza bicycle[3.2.0] heptanes-2-carboxylic acid [1]. Clavulanic acid is chemically designed as (2R, 5R, z)-3-(2-hydroxy ethylidene)-7-oxo-4-oxo-1-aza-bicyclo [3.2.0] heptanes -2-carboxylic acid [2]. The present paper deals with the development of rapid, precise, selective and sensitive LC-MS/MS method for the estimation of amoxicillin and clavulanic acid in human plasma that have short and simple extraction procedure, consumes small amount of solvents and biological fluid for extraction and a short turn-around time.

MATERIALS AND METHODS

Materials

Working standards of amoxicillin and clavulanic acid were provided by Ranbaxy Laboratories, Delhi. Both were having a purity of >99%. Diclofenac was used as internal standard. HPLC grade acetonitrile and methanol were purchased from J T Baker, Phillipsburg, NJ, USA. AR grade dichloromethane and citric acid were purchased form Merck, India. MS grade formic acid was purchased from Sigma-Aldrich, Fluka.

Working standard preparation

The stock solutions of amoxicillin, clavulanic acid and diclofenac (IS) were prepared in water at free base concentration of 2000 µg/mL, 1000 µg/mL and 1000 µg/mL respectively. Working solutions in the required concentration range were prepared by appropriate dilution of their stock solutions in methanol:water (50:50 % v/v) as and when required. All the solutions were stored at 2-8 °C and were brought to room temperature before use.

Calibration curves and quality control samples [3]

The calibration standards and quality control (QC) samples were prepared by spiking blank plasma with working solutions (5 %) to preserve the integrity of the samples. Mixed calibration standards were prepared at concentrations of 0.50, 1.0, 4.0, 8.0, 12.0, 20.0, 30.0, and 40.0 µg/mL for amoxicillin and 0.10, 0.20, 0.60, 1.20, 3.0, 4.0, 5.0 and 6.0 µg/mL for clavulanic acid. Mixed QC samples were prepared at 0.500 µg/mL (LLQC), 1.5 µg/mL (LQC), 20 µg/mL (MQC), and 30 µg/mL (HQC) for amoxicillin and at 0.10 µg/mL (LLQC), 0.20 µg/mL (LQC), 3.0 µg/mL (MQC) and 4.5 µg/mL (HQC) for clavulanic acid. All aliquots of spiked plasma samples were taken in micro centrifuge tube and stored at -70±5 °C.

Sample preparation [4]

Plasma samples to be processed were thawed at room temperature. Protein precipitation combined with liquid-liquid method using dichloromethane was selected because of its greater recovery. 200 µL of plasma sample was transferred into a polypropylene ria vial. In this 50 µL of 300 ng/mL internal standard solution and 50 µL of 0.1M citric acid was added and vortex for 30 seconds. Then 400 µL of acetonitrile was added as precipitating agent and centrifuged for 3 minutes at 4000 rpm. The supernatant liquid was collected and extracted with dichloromethane.

Instrumentation and chromatographic conditions

The LC system (SHIMADZU) consists of an isocratic pump an auto-sampler and a symmetry C_{18} (50 x 4.6mm, i.d, 5 µ) column. The mobile phase consists of acetonitrile and 0.1% formic acid (90:10 % v/v). Chromatographic separation was performed at ambient temperature with flow rate of 0.5mL/minute. Detection was performed by an applied biosystems sciex API 3200 mass spectrometer using atmospheric turbo ion spray as ionization source. API was performed in the negative ion mode. The LC-MS/MS detector was operated at unit resolution in the multiple reaction monitoring mode (MRM). The transitions of the molecular ions of amoxicillin at m/z 364→223.5, clavulanic acid at m/z 198.2→136.2 and diclofenac at m/z 296.1→250.1. Parent ion mass spectra of amoxicillin and clavulanic acid are shown in Figure 1 and 2 respectively. Product ion mass spectra of amoxicillin and clavulanic acid are shown in Figure 3 and 4 respectively.

RESULTS AND DISCUSSION

Validation procedures [5, 6, 7]

Selectivity

The selectivity of the method towards endogenous plasma matrix was evaluated in six different batches of human plasma by analyzing blank and spiked samples at LLOQ levels. Endogenous peaks at the retention time of the analytes were not observed for any of the plasma batches. Figure 5 and 6 shows representative chromatograms of selectivity for amoxicillin and clavulanic acid.

Linearity

Linearity was studied over concentration of 0.5 µg/mL to 40 µg/mL for amoxicillin and 0.10 µg/mL to 6.0 µg/mL for clavulanic acid respectively. Calibration curves appear linear and were well described by least squares lines with correlation coefficient ≥0.9980 for amoxicillin and 0.9974 for clavulanic acid. A weighing factor of $1/conc^2$ was chosen to achieve homogeneity of variance.

Accuracy and precision

The precision and accuracy of the developed method was determined by analysis of four quality control levels including LLOQ level. The results obtained for within-run precision & accuracy (Table 1, 2) and between-run accuracy & precision of amoxicillin and clavulanic acid (Table 3, 4) respectively.

Recovery

For the recovery experiment, plasma extracted samples were prepared by spiking both amoxicillin and clavulanic acid at three different QC level concentrations. Extraction was done by liquid

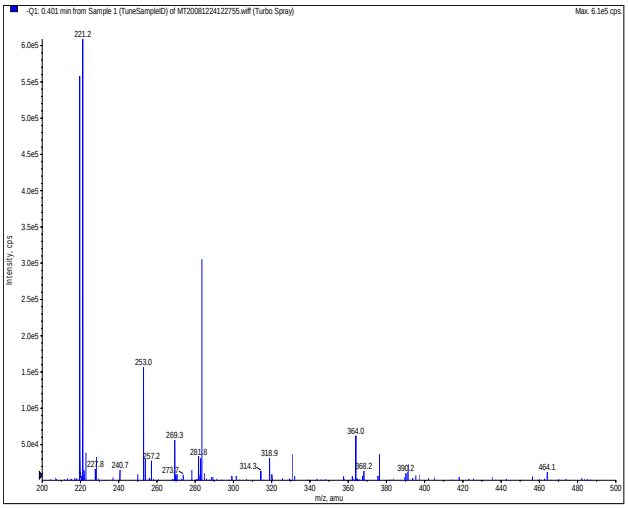

Figure 1. Mass spectrum of amoxicillin (parent ion scan)

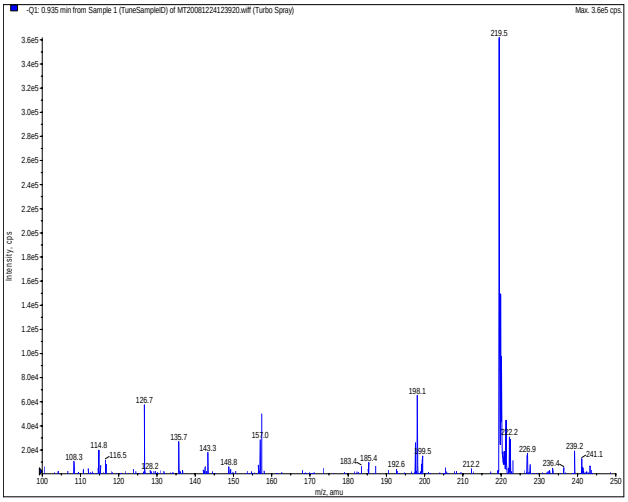

Figure 2. Mass spectrum of clavulanic acid (parent ion scan)

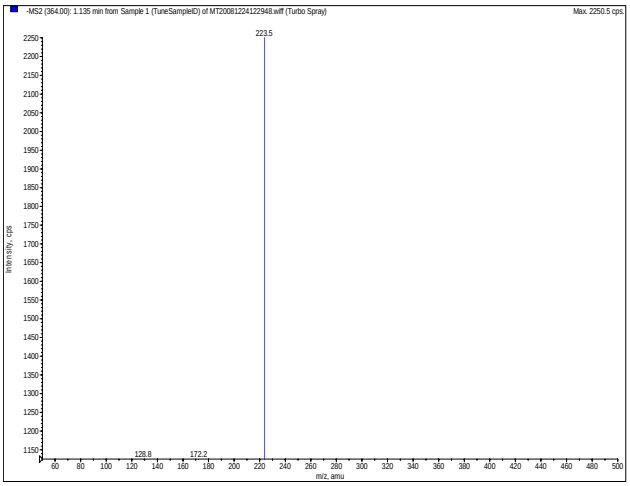

Figure 3. Mass spectrum of amoxicillin (product ion scan)

-liquid extraction procedure. Recovery was carried out by comparing the area obtained from and extracted sample with standard (unextracted) sample.

Stability

The stock solution stability at room temperature for 24.0 hours was compared with freshly prepared stock solution of amoxicillin and clavulanic acid. Freeze thaw stability was done for LQC and HQC after three cycles at -20°C. Room temperature stability in plasma was done for 24 hours at two concentrations LQC and HQC. Auto sampler stability was done for 24 hours at tow concentrations LQC and HQC (Table 5, 6).

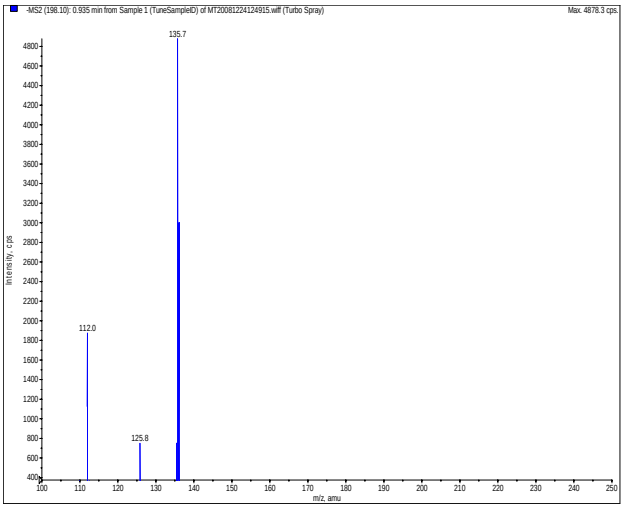

Figure 4. Mass spectrum of clavulanic acid (product ion scan)

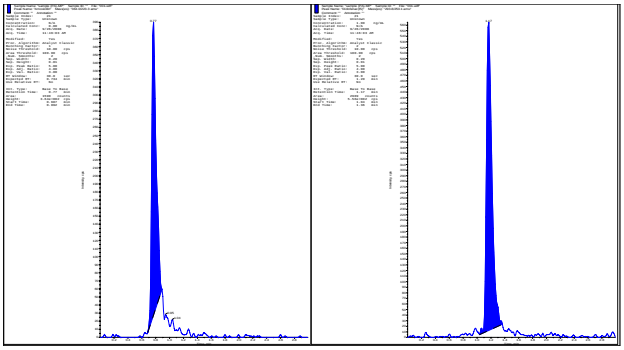

Figure 5. Chromatogram of amoxicillin

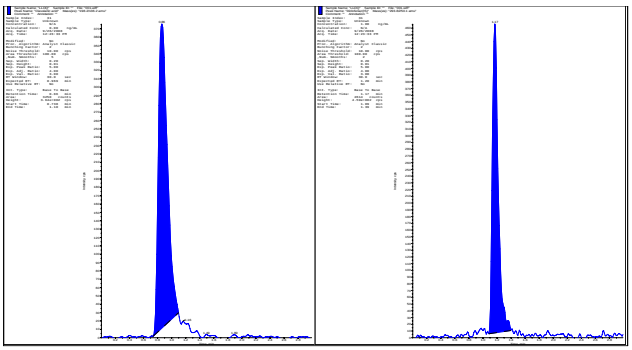

Figure 6. Chromatogram of clavulanic acid

CONCLUSION

The proposed method was found to be simple, precise, accurate and rapid for determination of amoxicillin and clavulanic acid in human plasma. Expected recoveries were observed in the present processing technique for LQC, MQC and HQC. The values obtained from system suitability studies demonstrated the suitability of the system for the analysis of the amoxicillin and clavulanic acid in plasma. The method can be applied for bioavailability studies and for analyzing patient samples in clinical trails.

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Table 1. Within - run accuracy and precision for amoxicillin

S. No	LLOQ (0.5µg)		LQC (1.5 µg)		MQC (20 µg)		HQC (30 µg)	
	Conc. found	Accuracy	Conc. found	Accuracy	Conc. found	Accuracy	Conc. found	Accuracy
1	0.478	95.60	1.451	96.73	20.04	100.20	32.54	108.47
2	0.485	97.00	1.455	97.00	22.44	112.20	28.25	94.17
3	0.457	91.40	1.478	98.53	19.56	97.80	32.56	108.53
4	0.456	91.20	1.501	100.07	19.55	97.75	30.14	100.47
5	0.488	97.60	1.481	98.73	21.54	107.70	30.87	102.90
6	0.479	95.80	1.488	99.20	20.69	103.45	33.91	113.03
Mean	**0.47**		**1.48**		**20.64**		**31.38**	
			1.31		**5.64**		**6.49**	
% CV	**2.94**		**98.38**		**103.18**		**104.59**	
% Nominal	**94.77**							

Table 2. Within - run accuracy and precision for clavulanic acid

S. No	LLOQ (0.1µg)		LQC (0.2 µg)		MQC (3.0 µg)		HQC (4.5 µg)	
	Conc. found	Accuracy	Conc. found	Accuracy	Conc. found	Accuracy	Conc. found	Accuracy
1	0.098	98.00	0.213	106.50	3.145	104.83	4.125	91.67
2	0.089	89.00	0.211	105.50	3.110	103.67	4.015	89.22
3	0.092	92.00	0.198	99.00	3.240	108.00	4.587	101.93
4	0.102	102.00	0.189	94.50	2.987	99.57	4.286	95.24
5	0.099	99.00	0.179	89.50	2.756	91.87	4.871	108.24
6	0.111	111.00	0.208	104.00	3.012	100.40	4.571	101.58
Mean	**0.100**		**0.20**		**3.04**		**4.41**	
			6.79		**5.50**		**7.33**	
% CV	**7.88**		**99.83**		**101.39**		**97.98**	
% Nominal	**98.50**							

Table 3. Between - run accuracy and precision for amoxicillin

Parameters	LLOQ (0.5µg)	LQC (1.5 µg)	MQC (20 µg)	HQC (30 µg)
	Conc. found	Conc. found	Conc. found	Conc. found
ACCURACY & PRECISION DAY 1 ANALYST 1	0.47	1.48	20.64	31.38
ACCURACY & PRECISION DAY 1 ANALYST 2	0.51	1.51	20.13	30.29
ACCURACY & PRECISION DAY 2 ANALYST 1	0.45	1.55	18.58	29.55
Mean	**0.48**	**1.51**	**19.78**	**30.41**
% CV	**6.41**	**2.32**	**5.42**	**3.03**
% Nominal	**95.33**	**100.89**	**98.92**	**101.36**

Table 4. Between - run accuracy and precision for clavulanic acid

S. No	LLOQ (0.1µg)	LQC (0.2 µg)	MQC (3.0 µg)	HQC (4.5 µg)
	Conc. found	Conc. found	Conc. found	Conc. found
ACCURACY & PRECISION DAY 1 ANALYST 1	0.100	0.2	3.04	4.41
ACCURACY & PRECISION DAY 1 ANALYST 2	0.098	0.211	3.24	4.36
ACCURACY & PRECISION DAY 2 ANALYST 1	0.110	0.201	2.89	4.89
Mean	**0.10**	**0.20**	**3.06**	**4.55**
% CV	**6.26**	**2.98**	**5.74**	**6.43**
% Nominal	**102.67**	**102.00**	**101.89**	**101.19**

Table 5. Recovery of amoxicillin

S. No	LQC		MQC		HQC	
	Extracted response	Un extracted response	Extracted response	Un extracted response	Extracted response	Un extracted response
1	2261	2582	4157	5078	8628	10451
2	1917	2647	4078	4983	8358	10781
3	2089	2584	4232	4925	8598	9741
4	2177	2303	4899	5043	8966	10886
5	1966	2468	4256	5178	8252	11024
6	2078	2541	3915	5069	8898	10781
Mean	**2081**	**2521**	**4256**	**5046**	**8617**	**10611**
SD	**127.91**	**121.90**	**338.17**	**86.71**	**283.39**	**466.15**
% CV	**6.15**	**4.84**	**7.95**	**1.72**	**3.29**	**4.39**
% Recovery	**82.57 %**		**84.35 %**		**81.21 %**	
Global mean Recovery	**82.71 %**					

Table 6. Recovery of clavulanic acid

S. No	LQC		MQC		HQC	
	Extracted response	Un extracted response	Extracted response	Un extracted response	Extracted response	Un extracted response
1	1021	1151	2914	3245	7025	7898
2	1124	1389	3448	3352	7843	7789
3	1054	1241	2878	3461	6989	8014
4	1287	1246	3165	3478	7785	7985
5	989	1187	2788	3396	7154	7896
6	1044	1047	3358	3258	6980	7963
Mean	**1087**	**1210**	**3092**	**3365**	**7296**	**7924**
SD	**107.95**	**113.91**	**273.03**	**98.96**	**406.46**	**81.27**
% CV	**9.94**	**9.41**	**8.83**	**2.94**	**5.57**	**1.03**
% Recovery	**89.78 %**		**91.88 %**		**92.07 %**	
Global Mean Recovery	**91.25 %**					

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