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Highly sensitive quantitative analysis of Amoxicillin and Clavulanic acid from plasma using LC/MS/MS

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Introduction

Amoxicillin and clavulanic acid is a combination drug product in which amoxicillin is a β-lactam antibiotic, and clavulanic acid is a β-lactamase inhibitor. In this combination, clavulanic acid acts by preventing the destruction of antibiotic amoxicillin which is used to treat various bacterial infections[1].
LC/MS/MS has been increasingly employed in pharmacokinetic studies due to its specificity and sensitivity. This also allows the development of assays with minimal sample preparation.

LC/MS/MS method has been developed for highly sensitive quantitation of these molecules from plasma using LCMS-8040, a triple quadrupole mass spectrometer from Shimadzu Corporation, Japan. Ultra fast polarity switching of LCMS-8040 enabled simultaneous analysis of amoxicillin (in positive mode) and clavulanic acid (in negative mode) even in absence of chromatographic separation. Both these molecules were quantitated at low levels with good repeatability even in presence of complex matrix like plasma.

Amoxicillin

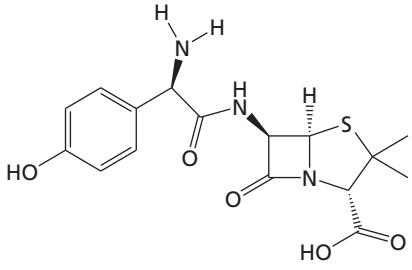

Figure 1. Structure of amoxicillin

Amoxicillin is a semi synthetic antibiotic. Its chemical name (2S,5R,6R)- 6-{[(2R)-2-amino- 2-(4-hydroxyphenyl)-acetyl]amino}- 3,3-dimethyl- 7-oxo- 4-thia-1-azabicyclo[3.2.0]heptane- 24-carboxylic acid and it has a molecular formula as $C_{16}H_{19}N_3O_5S$. It's structure is shown in Figure 1.

Clavulanic acid

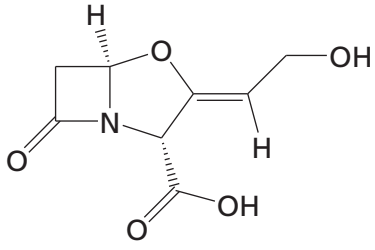

Figure 2. Structure of clavulanic acid

Clavulanic acid is a novel beta-lactam compound which was isolated from the culture fluid of *Streptomyces clavuligerus*. Its chemical name is (2R,3Z,5R)-3-(2-Hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylic acid and has a molecular formula as $C_8H_9NO_5$. It's structure is shown in Figure 2.

Method of analysis

Sample preparation

- **Preparation of aqueous calibration levels**

Amoxicillin and clavulanic acid mix standards at concentration levels of 1 ng/mL, 2 ng/mL, 3 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, 30 ng/mL, 50 ng/mL and 100 ng/mL were prepared in water : acetonitrile (1:1 v/v).

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• Preparation of matrix matched calibration levels

To 500 µL plasma, 100 µL sodium carbonate (1 mol/L) and 5 mL of diethyl ether : hexane (1:1 v/v) was added[2]. It was placed in rotary shaker at 20 rpm for 15 minutes for uniform mixing and centrifuged at 7000 rpm for 20 minutes. Supernatant was collected and evaporated to dryness at 60 °C. It was finally reconstituted in 1000 µL water : acetonitrile (1:1 v/v). This reconstituted solution was then used as a diluent to prepare matrix matched calibration levels from 1 ng/mL to 100 ng/mL.

Figure 3. Nexera with LCMS-8040 triple quadrupole system by Shimadzu

LCMS-8040 triple quadrupole mass spectrometer by Shimadzu (shown in Figure 3), sets a new benchmark in triple quadrupole technology with an unsurpassed sensitivity (UFsensitivity), ultra fast scanning speed of 15,000 u/sec (UFscanning) and polarity switching speed of 15 msec (UFswitching). This system ensures highest quality of data, with very high degree of reliability.

LC/MS/MS analysis

Amoxicillin and clavulanic acid were simultaneously analyzed using Ultra High Performance Liquid Chromatography (UHPLC) Nexera coupled with LCMS-8040 triple quadrupole system (Shimadzu Corporation, Japan). The details of analytical conditions are given in Table 1.

Table 1. LC/MS/MS conditions for amoxicillin and clavulanic acid

Column	: Shim-pack XR-ODS (50 mm L x 3 mm I.D.; 2.2 µm)
Guard column	: Phenomenex SecurityGuard ULTRA cartridge
Mobile phase	: A: 10 mM ammonium formate in water B: acetonitrile
Gradient program (B %)	: 0.0–2.0 min → 0-100 (%); 2.0–3.0 min → 100 (%); 3.0–3.5 min → 100-0 (%); 3.5–7.0 min → 0 (%)
Flow rate	: 0.3 mL/min
Oven temperature	: 40 °C
Injection volume	: 30 µL
MS interface	: Electro Spray Ionization (ESI)
Polarity	: Positive and negative
Nitrogen gas flow	: Nebulizing gas 2 L/min; Drying gas 10 L/min
MS temperature	: Desolvation line 275 °C; Heating block 500 °C

Results

LC/MS/MS analysis results of amoxicillin and clavulanic acid

LC/MS/MS method was developed for simultaneous quantitation of amoxicillin and clavulanic acid. Analysis was performed using aqueous as well as matrix matched standards. MRM transitions used for these compounds are given in Table 2. Linearity studies were carried out using external standard calibration method and results of linearity studies are tabulated in Table 2 for both aqueous and matrix matched standards. MRM chromatogram of simultaneous analysis of amoxicillin and clavulanic acid aqueous standard at 5 ng/mL is shown in Figure 4. Overlay of MRM chromatograms of diluent, 1 ng/mL and 100 ng/mL level for amoxicillin aqueous standards is shown in Figure 5. Overlay of MRM chromatograms of blank plasma, 1 ng/mL and 100 ng/mL level for amoxicillin matrix matched standards is shown in Figure 6. Similarly, overlay of MRM chromatograms of diluent, 3 ng/mL and 100 ng/mL for clavulanic acid aqueous standards is shown in Figure 7. Overlay of MRM chromatograms of blank plasma, 3 ng/mL and 100 ng/mL for clavulanic acid matrix matched standards is shown in Figure 8. No interfering peaks were seen in diluent or in blank plasma at the retention time of these compounds, confirms the absence of any interference.

LOQ was determined for these compounds based on the following criteria – **(1)** % RSD for area < 20 %, **(2)** % accuracy between 80-120 % and **(3)** Signal to noise ratio (S/N) > 10. LOQ of 1 ng/mL was achieved for both amoxicillin aqueous and matrix matched standards. Similarly, LOQ of 3 ng/mL was achieved for both clavulanic acid aqueous and matrix matched standards. Accuracy and repeatability results for amoxicillin and clavulanic acid are given in Tables 3 and 4 respectively.

Table 2. Details of MRM transitions and linearity results

Name of the compound	MRM transitions	Retention time (min)	Linearity (r^2)		Polarity
			Aqueous	Matrix matched	
Amoxicillin	365.90 > 114.10	1.06	0.9939	0.9967	ESI positive
Clavulanic acid	TIC (198.30 > 107.95 + 198.30 > 136.05)	0.99	0.9946	0.9958	ESI negative

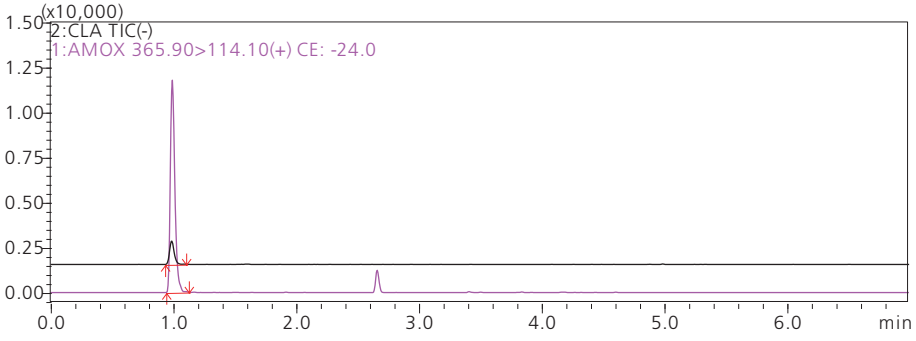

Figure 4. MRM chromatogram of simultaneous analysis of amoxicillin and clavulanic acid at 5 ng/mL level

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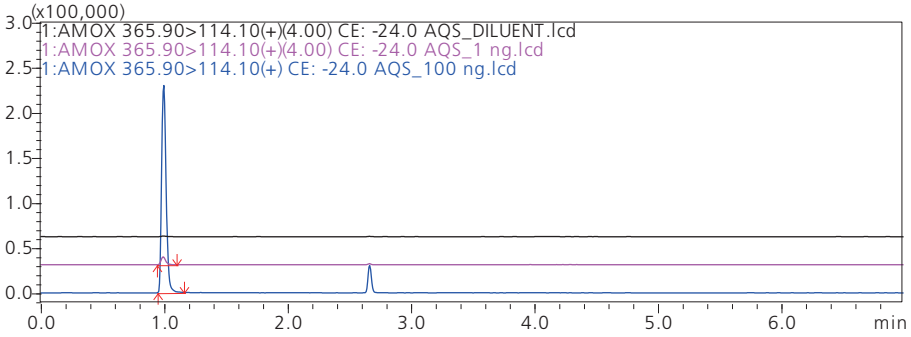

Figure 5. Overlay of MRM chromatograms of diluent, 1 ng/mL and 100 ng/mL for amoxicillin aqueous standard

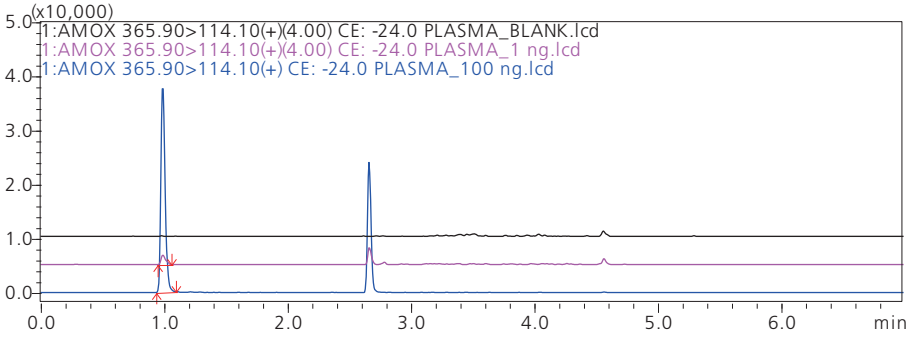

Figure 6. Overlay of MRM chromatograms of blank plasma, 1 ng/mL and 100 ng/mL for amoxicillin matrix matched standard

Table 3. Results of accuracy and repeatability for amoxicillin

Name of compound	Standard concentration (ng/mL)	Calculated average concentration from calibration graph (ng/mL) (n=3)		Average % accuracy (n=3)		Average % RSD for area counts (n=3)	
		Aqueous	Matrix matched	Aqueous	Matrix matched	Aqueous	Matrix matched
Amoxicillin	1	0.96	0.95	95.73	95.10	7.04	18.92
	2	2.01	2.17	100.30	108.63	5.97	3.39
	3	3.36	3.01	111.83	100.27	4.93	15.27
	5	5.36	4.91	107.17	98.30	1.05	5.29
	10	10.30	10.60	100.80	105.93	2.19	4.59
	20	18.12	20.76	90.57	103.80	0.50	6.03
	30	30.53	27.87	101.77	92.90	1.46	0.95
	50	47.04	49.87	94.07	99.73	2.83	4.07
	100	102.32	98.75	102.30	98.77	2.65	2.00

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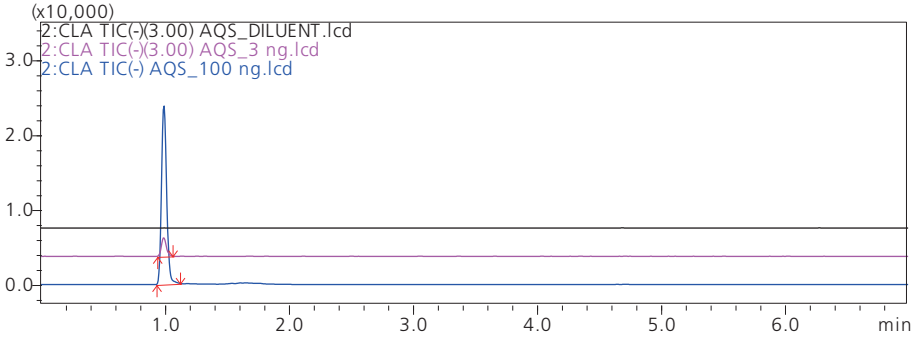

Figure 7. Overlay of MRM chromatograms of diluent, 3 ng/mL and 100 ng/mL for clavulanic acid aqueous standard

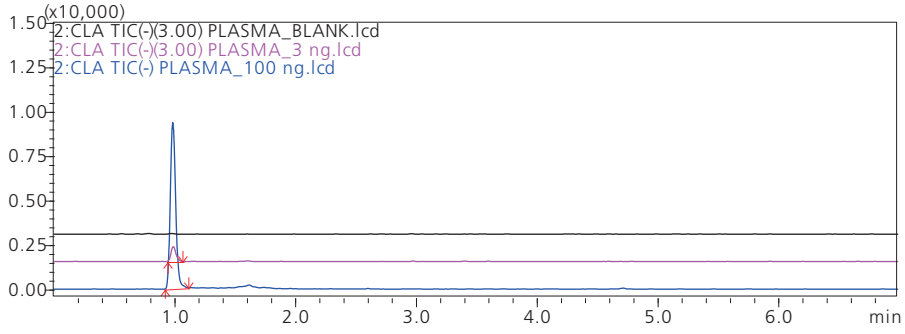

Figure 8. Overlay of MRM chromatograms of blank plasma, 3 ng/mL and 100 ng/mL for clavulanic acid matrix matched standard

Table 4. Results of accuracy and repeatability for clavulanic acid

Name of compound	Standard concentration (ng/mL)	Calculated average concentration from calibration graph (ng/mL) (n=3)		Average % accuracy (n=3)		Average % RSD for area counts (n=3)	
		Aqueous	Matrix matched	Aqueous	Matrix matched	Aqueous	Matrix matched
Clavulanic acid	3	2.95	2.99	98.37	99.57	5.20	4.93
	5	5.06	5.18	101.13	103.60	9.13	2.86
	10	10.80	9.38	108.03	93.77	0.47	9.63
	20	18.79	21.62	93.97	108.07	0.33	1.26
	30	29.32	28.52	97.77	95.07	4.93	5.12
	50	47.81	49.82	95.60	100.83	2.36	2.45
	100	108.96	103.29	108.93	103.27	0.49	1.10

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Conclusion

- Ultra-high sensitivity and ultra fast polarity switching (UFswitching) enabled simultaneous analysis of amoxicillin and clavulanic acid from plasma samples even in absence of chromatographic separation.
- LOQ of 1 ng/mL was achieved for amoxicillin aqueous and matrix matched standards whereas it was 3 ng/mL clavulanic acid aqueous and matrix matched standards.

References

[1] Avinash Gaikwad, Sumit Gavali et.al., Journal of Pharmacy Research, Volume 6, Issue 8, (2013), 804-812.
[2] Rajinder Singh Gujral, Sk Manirul Haque, Int J Biomed Sci, Volume 6(4), (2010), 335-343.

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