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Qualitative Analysis of Amoxicillin, Ampicillin, Cephalexin by Quadrupole –Time of Flight (LCMS) Using Electrospray Ionization.

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Abstract: A simple, fast, novel method with dual Electrospray ionization (dual ESI), multistage tandem mass spectrometry (LC-MS) were used to identify of Amoxicillin, Ampicillin and Cephalexin. The established method with excellent separation and good capacity factorwas successfully applied. Ion detection was performed usingQuadrupole –Time of Flight coupled with dual ESI ion sourceand identified corresponding ions as m/z 365, 349, 347[M+H] with respective of Cephalexin, Amoxicillin, Ampicillin. The results of the study showed that the proposed LCMS method is simple, rapid, precise and accurate, which is useful for the routine determination of Cephalexin, Amoxicillin, and Ampicillinin bulk drug and in its pharmaceutical dosage forms.
Key words: Amoxicillin; Ampicillin; Cephalexin;LCMS; dual ESI; Quadrupole Time of Flight; Antibiotics.

INTRODUCTION

Production of combination method development always creates a challenge for the pharmaceutical analyst. The modern analytical investigation of antibiotic drugs, content and purity estimations of active compounds, very often involve Liquid chromatography–mass spectrometry (LCMS).
Amoxicilin[1] and ampicillin[2] is b-lactam antibiotic that belongs to the group of penicillins. Amoxicilin and Ampicillin are extremely active against both Gram-positive and Gram-negative organisms, including several pathogenic enteric organisms.
Amoxicillin(AMOX)[[2S-[2a,5a,6a(S*)]]-6-[Amino (4- hydroxyphenyl) acetyl]amino]-3,3-dimethyl-7-oxo-4-thia- 1-azabicyclo[3.2.0]heptane-2-carboxilic acid] is widely used in veterinary practice for the treatment of gastro-intestinal and systemic infections. Ampicillin the more frequent occurrence of β-lactamase producing clinically important bacterial strains has limited the usage of these antibiotics. Co-administration of the labile β-lactam together with another antibacterial capable of

inhibiting the β-lactamase was developed to improve the activity and overcome bacterial resistance. It is generally indicated for a number of bacterial infections including shigellosis (dysentery), gonorrhoea, meningitis, Escherichia coli, Streptococcal and Staphylococcal infections.

Ampicillin (AMP)[(2S,5R,6R)-6-([(2R)-2-amino-2-phenylacetyl]amino)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2- carboxylic acid]can inhibit the third and final stage of bacterial cell wall synthesis, which ultimately leads to cell lysis[3]. Ampicillin is one of the most widely prescribed antibiotics. It is considered a penicillin and is a close relative of another penicillin, amoxicillin. Unlike penicillin, ampicillin and amoxicillin can penetrate and prevent the growth of certain types of bacteria, called gram-negative bacteria. Ampicillin is used mainly to treat infections of the middle ear, sinuses, bladder, kidney, and uncomplicated gonorrhea. It is also used intravenously to treat meningitis and other serious infections[4]. A semisynthetic penicillin having a broader antibacterial spectrum of action than that of penicillin G. It is effective against gram-negative and gram-positive bacteria and used to treat gonorrhea and infections of the intestinal, urinary, and respiratory tracts [5].

Cephalexin (CPL)[6R, 7R)-7-{[(2R)-2-amino-2-phenylacetyl] amino}-3-methyl-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene- 2-carboxylic acid] is used to treat urinary tract infections, respiratory tract infections (including sinusitis, otitis media, pharyngitis, tonsillitis, pneumonia, and bronchitis), and skin and soft tissue infections. In addition to being a rational first line treatment for cellulitis, it is a useful alternative to penicillin's in patients with penicillin hypersensitivity.[6]

The proposed LCMS (liquid chromatography mass spectrometry) method for determination of amoxicillin, ampicillin and cephalexin are a direct, sensitive and robust involving no laborious sample preparation steps. We report the development and validation of a new rapid and sensitive quantitative chromatographic method for determination of amoxicillin, ampicillin and cephalexinin premixes.The proposed method involves dual ESI mode for quantification of amoxicillin, ampicillin and cephalexin with electrospray ionization to achieve very low LOD and LOQ, the method validation study is carried out as per ICH guidelines.[7,8].

1. MATERIALS AND METHODS

1.1. Chemicals, reagents and materials

Reference standards of amoxicillin, ampicillin and cephalexinwere obtained from the Sigma-Aldrich, India. Organic solvents for chromatography, LCMS grade, were purchased from ACS grade Acetonitrile, water, were purchased from Honeywell-Burdick & Jackson (USA), All the chemicals used were of Analytical Reagent grade, and the solvents were of ACS.The purity of each reference wasdetermined to be over 98% by normalization of the peak area detected by HPLC PDA detector and LCMS. All solvents and samples were filtered through MILLEX FG (Millipore), 13mm, 0.2µM, FLUROPORE, NON-STERILE membrane sample filter paper before injecting into HPLC.

1.2. Apparatus and chromatographic conditions

1.2.1. HPLC analysis

The analyses were performed using an Agilent 1200 Series HPLC system, equipped with a binary pump, an auto-sampler, a column oven, and a mass hunter software version B.02.01 (B2116.20) (Agilent Technologies, USA), was connected to the liquid chromatography for detection of beta-lactam antibiotics and cephalosporin antibiotics. The separation was carried out on a reverse phase RESTEK Pinnacle® DB Cyano C_{18} column (5.0mmx150mm, 5u) at a column temperature of 25°C. the isocratic elution was employed using water (solvent A) and Acetonitrile (solvent B), and eluted by the following program at the flow rate of 0.2ml/min; 0 min 20% B, 10-min 40% B, 12 min 20%.

1.2.2. Quadrupole Time Of Flight LCMS-analysis

LCMS was used to confirm the identification of chromatographic peaks of interest. The LCMS–multimode analyses were conducted on an Agilent 1200 series HPLC system (Agilent Technologies, USA) is equipped with Binary gradient pump, Auto Sampler, thermostatted column compartment, variable wavelength detector, Auto sampler thermostatted (G 1330B), coupled with a Q-TOF LCMS 6500 series multimode source (Agilent Technologies, USA). Mass spectra were acquired in positive mode using with scan range from m/z 100 to 500. The conditions of multimode source were as followed: drying gas (N2) flow rate, 8.0 l/min; gas temperature, 325°C; pressure of nebulizer, 30 psi; skimmer, 65, and fragmentor voltage, 175 V. Data were acquired and analyzed by Agilent mass hunter software version B.02.01 (B2116.20) (Agilent

Technologies, USA). The output signal is monitored and processed using mass hunter software on Intel ® Core (TM) 2 Duo computer (HP xw 4600 Workstation).

1.3. Preparation of standard solutions

Mixed standard stock solution was prepared by accuratelyweighing 3antibiotics, i.e., Amoxicillin, Ampicillin and Cephalexin were weighed 1mg each and dissolved them in 1mLAcetonitrile. The working standard solution was prepared by diluting the mixed standard solution with Acetonitrile to a series of proper concentrations. The standard stock and working solutions were all stored at4 ◦C until use.

1.4. Calibration Curves

The working standard solutions were brought to room temperature and an aliquot of 5µl was injected into LCMSfor the construction of calibration curves. At least six concentrations in triplicate were analyzed, and the calibration curves were calculated by linear regression of the double logarithmic plots of the peak area versus the amount of antibioticsinjected.

1.5. Limits of Detection and Quantitation

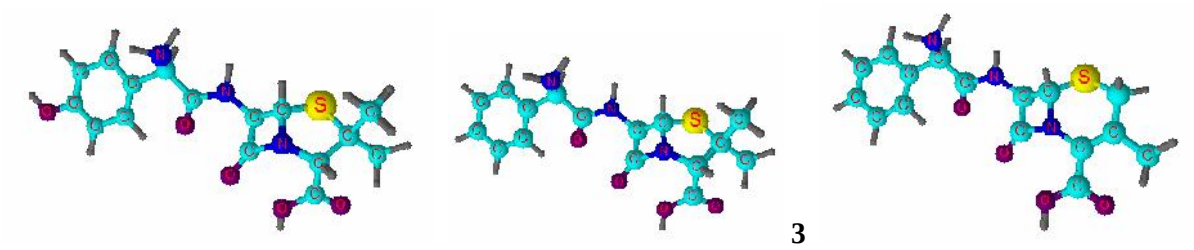
The limits of detection (LODs) and quantification (LOQs) under the present chromatographic conditions were determined by diluting the standard solution when the signal-to-noise ratios(S/N) of analytes were almost 3 and 10, respectively. The S/N was calculated as the peak height divided by thebackground noise value. The background noise was measured from the background start to background end time.

2. RESULTS AND DISCUSSION

2.1. Qualitative analysis of three antibiotics by LCMS-multimode

The previous chromatographic conditions for determination of three antibiotics by HPLC were used as the basis for mobile phase selection and optimization. Unfortunately, the reported gradient elution of methanol–water could not be applied to the separation of antibiotics, the gradient elution program was carefully adjusted and after several trials the new gradient program was selected until it permitted the best separation ability for all the analytes investigated.

For the purpose of correct identification, a HPLC–ESI-MS analysis was performed on standard solutions under the HPLC–ESI-MS conditions described in Section 1.2.2. The mass spectra data of three antibiotics in positive ion modes are listed in Table 1. In positive ion mode, the compounds of interest exhibited mainly protonated ions. Finally, three antibiotics AMP,AMOX and CPL were identified by comparing their retention times and MS data with those of reference compounds (Fig. 3).



A **B** **3** **C**

Figure 1: 3-Dimentional molecular structure of the A. Amoxicillin, B. Ampicillin, C. Cephalexin.

Table 1: Retention times, mass spectra for each analytes by using TIC and DAD.

	Retention time					Characteristic ions (m/z)
S.No.	**TIC**	**DAD**	**Analytes**	**Molecular Formula**	**Molecular weight**	**Positive ions**
1	3.61	3.37	Amoxicillin	C16H19N3O5S	365.40	366.10992
2	4.70	4.49	Ampicillin	C16H19N3O4S	349.41	350.11534
3	2.44	2.54	Cephalexin	C16H17N3O4S	347.40	348.09860

2.2. Validation of the method

2.2.1. Linearity, LOD and LOQ

As shown in Table 2, acceptable results of the regression analysis, the correlation coefficients (r^2), LODs and LOQs were obtained for all the analytes: the LODs and LOQs of the three antibiotics were in the range of 2.97 -6.33 ppm,89-190 ppm respectively. A linearity curve has been obtained by injecting various concentrations of 8, 12, 16, 20, 24, 28 ppmare showed in table no 4 and the pictorial diagram has been showed in Fig.5.

2.3. Qualitative analysis of antibiotics by LCMS

The proposed LCMS method was successfully applied to simultaneous determination of threeantibiotics of AMP, AMOX and CPL commercially purchased from sigma. The qualitative analyses were performed and the analytical results are summarized in Table 2 and Table 3, the overlaid HPLC and TIC chromatograms of all samples are presented in Fig. 2 and Fig.3 and the typical Mass spectra ESI–MS of three antibiotics positive ion mass spectrum of cephalexin, Amoxicillin and Ampicillin acquired analysis and its chemical structure in Fig.4.

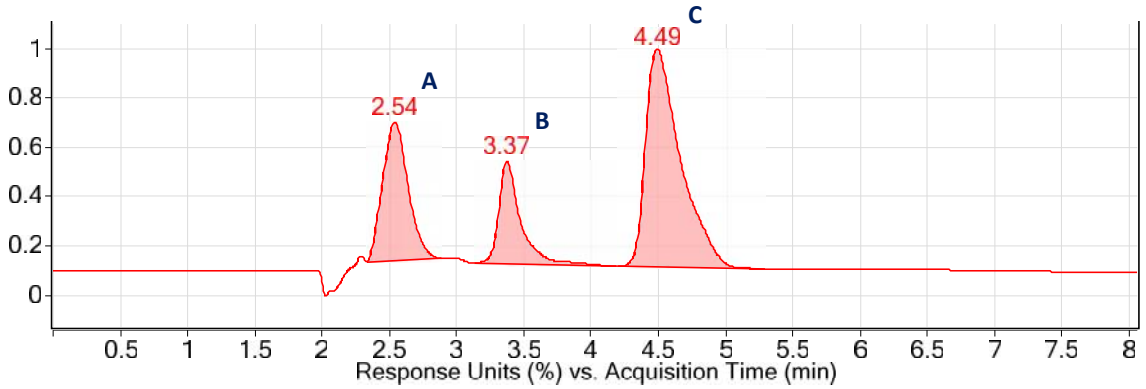


Figure 2: DAD Representative HPLC elution profile of(A) Cephalexin, (B) Amoxicillin and (C) Ampicillin standard recorded at 257 nm is shown.

Table 2: System Suitability parameters for Amoxicillin, Ampicillin and Cephalexin with DAD

Peak Number	1	2	3
Compound	**Cephalexin**	**Amoxicillin**	**Ampicillin**
Retention time (rt)	2.54	3.37	4.49
Area	1238	787.71	2670.3
Area %	46.35	29.5	100
Height	94.08	69.70	148.07
Max Y	100.65	74.10	150.36
Width	0.55	1.05	1.09
Capacity factor (k')	-0.7	-0.6	-0.4
Theoretical plates(*N*)	844	2933	1621
Resolution	1	2.8	3.2
Symmetry	0.78	0.45	0.38
Tailing factor	1.2	1.7	1.8
LOD [a] (ppm)	4.03	2.97	6.33
LOQ [a] (ppm)	121.00	89.00	190.00

[a] LOQ and LOD were determined practically

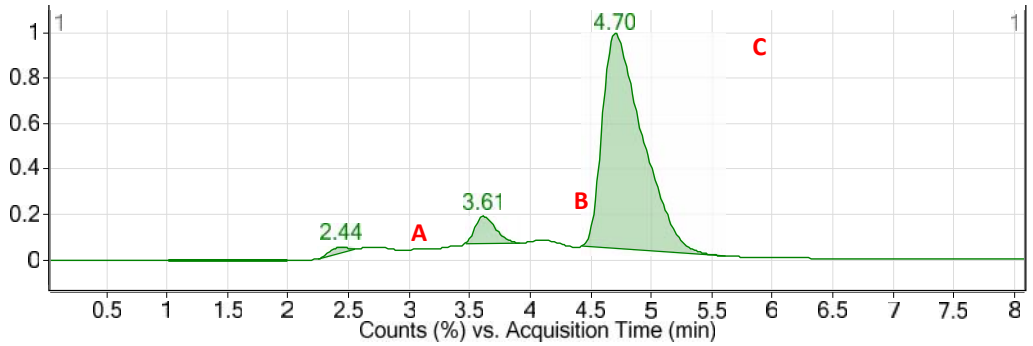


Figure 3: EIC Representative HPLC elution profile of (A) Cephalexin, (B) Amoxicillin and (C) Ampicillin standard

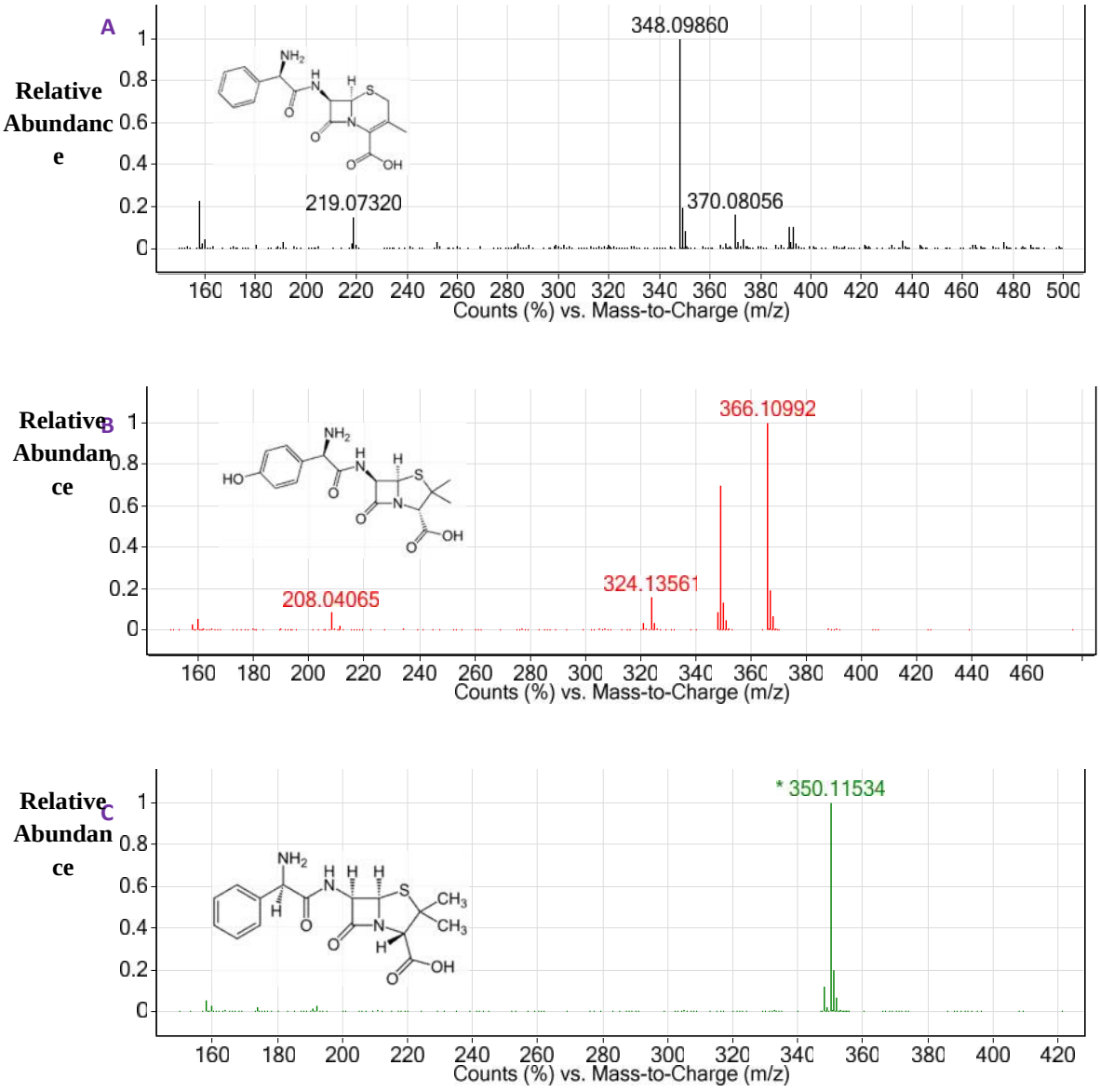


Fig 4: The typical Mass spectra of antibiotics (A) Positive ion mass spectrum of Cephalexin acquired during the HPLC–ESI–MS analysis and its chemical structure. (B) Positive ion mass spectrum of Amoxicillin acquired during the HPLC–ESI–MS analysis and its chemical structure. (C) Positive ion mass spectrum of Ampicillin acquired during the HPLC–ESI–MS analysis and its chemical structure.

Table 3: System Suitability parameters for Amoxicillin, Ampicillin and Cephalexin with EIC

Peak Number	1	2	3
Compound	**Cephalexin**	**Amoxicillin**	**Ampicillin**
Retention time (rt)	2.44	3.63	4.7
Area	478237	2423419	33474884
Area Sum %	1.21	6.01	92.78
Height	40453	180924	1445801
Base Peak	348.09845	366.10992	350.11534
Width	0.3	0.45	1.19
Tailing factor	0.8	1.4	1.8
Theoretical plates	1628	2193	983
Symmetry	1.67	0.57	0.39

Table 4: linearity data for Amoxicillin, Ampicillin and Cephalexin

S.no.	Concentrations (ppm)	Cephalexin	Mean* peak area	Amoxicillin	Mean* peak area	Ampicillin	Mean* peak area
1	8	198.08	501.54	126.03	429.77	427.25	1902.11
2	12	297.12	760.63	189.05	651.66	640.87	2885.21
3	16	396.16	1014.17	252.07	868.88	854.50	3846.94
4	20	495.20	1260.28	315.08	1080.11	1068.12	4779.84
5	24	594.24	1516.50	378.10	1299.91	1281.74	5751.19
6	28	693.28	1763.01	441.12	1511.71	1495.37	6687.29

*average of six determinations

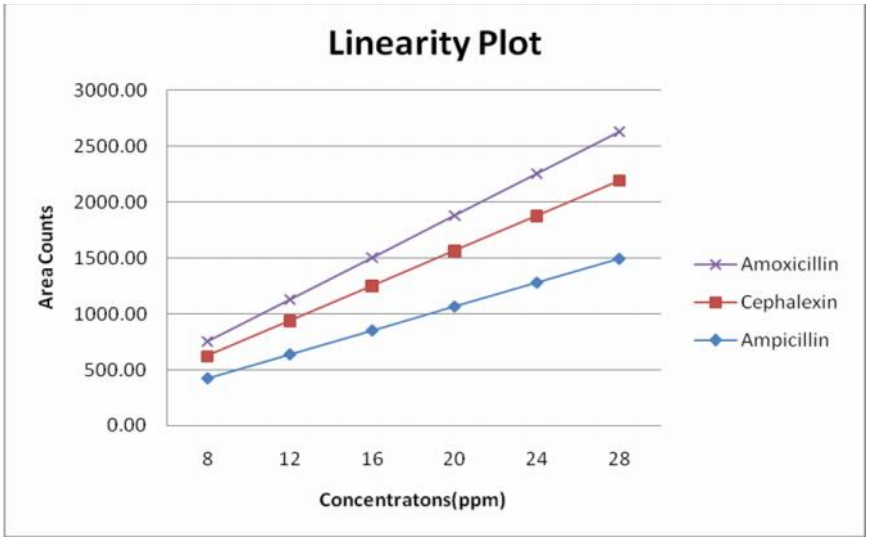


Fig 5: linearity Plot for Amoxicillin, Ampicillin and Cephalexin

3. CONCLUSION

This study developed simple and reliable HPLC-LCMSmethods for the estimation of amoxicillin, ampicillin and cephalexin in combined dosage form. A direct tandem mass spectrometric method was described for screening and qualitative analysis of AMP, AMOX and CPL in the dual ESI mode. The LCMS profile was more sensitive, the method was accurate and reproducible for measurement. The method was validated and found to be simple, sensitive, accurate and precise. Therefore the proposed method can be used for quantification of amoxicillin, ampicillin and cephalexin in combined dosage form as well as for routine analysis in quality control.

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DISCLOSURE

The authors report no conflicts of interest.

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