Pharmaceutica Analytica Acta
ISSN: 2153-2435

Elhag et al., Pharm Anal Acta 2018, 9:2
DOI: 10.4172/2153-2435.1000578

Research Article Open Access

ESI-LC/MS Method Development and Validation for the Determination of Some Selected Antibiotics in Hospital Wastewater

Elhag DE[1*], Abdallah BS[1], Hassan M[2,3] and Suliman A[1]

[1]*Analytical Research Centre, UMST University, Khartoum, Sudan*
[2]*Swiss Tropical & Public Health Institute, Socinstrasse 57, 4051 Basel, Switzerland*
[3]*Basel University, Peterplatz 1, 4001, Basel, Switzerland*

Abstract

For the last decade, significant attention has been paid to the occurrence, bioaccumaltion and fate of drugs in effluent hospital water. Therefore, the aim of this study was to develop and validate analytical method to identify and quantify the antibiotics tetracycline HCl, (Tetra) doxycycline, (Doxy), ampicillin trihydrate (Ampi), amoxicillin trihydrate (Amoxi) and cephalexin monohydrate (Cefalex). The LCMS instrument used was equipped with with C18 column, (150 mm length x 4.6 mm inner diameter x^5 um particle size). The mobile phase was acetonitrile/formic acid (1%) under gradient elution mode. The MS employs ESI unit and quadrupole mass analyzer. The analysis time was less than 15 minutes. The method was validated in terms of linearity, precision, accuracy, robustness, limit of detection and limit of quantitation, specificity, stability and excellent results were obtained.

Keywords: Development; Optimization; LCMS; Antibiotics; Aquatic environment

Introduction

Antibiotics are natural, semisynthetic or synthetic drugs used as antibacterial, antifungal or antiparasitic. Antibiotics can be grouped by either their chemical structure or mechanism of action [1-3]. Antibiotics represent a major source of micro pollutants as they may as chemical mixtures that exhibit a wide range of mechanisms of action [4-9,1]. Moreover, they can undergo chemical and/or physical reactions leading different metabolites by the action of microorganisms, as well as by other physical or chemical means, resulting in mixtures with higher toxicities and risks to human health than those of the individual compounds [4,1,10-16]. In contrast to their therapeutic outcome, these antibiotics often disadvantageous for those target and non-target organisms. In addition to this, improperly disposal of unused antibiotics and non-metabolized antibiotics excreted by humans can all enter the sewer system in low concentrations. However, the use of antibiotics is growing and their input to the aquatic environment is increasing making them of increasing environmental relevance. The increased awareness that synthetic drugs can lead to serious side effects in the environment has prompted researchers to launch several monitoring studies into the most commonly administered compounds in urban wastewater [17-24]. In this work, we developed and validated ESI. LC-MS method for the determination of some antibiotics in hospital waste water.

Materials and Methods

Antibiotics Standards

Antibiotics reference standards tetracycline HCl, (Tetra) doxycycline, (Doxy), ampicillin trihydrate (Ampi), amoxicillin trihydrate (Amoxi) and cephalexin monohydrate (Cefalex) were kindly donated by Azal Pharmaceutical, Company, Khartoum Sudan.

Chemicals and reagents

Acetonitrile, formic acid (HPLC grade), methanol 99% (analytical grade and HPLC grade), phosphoric acid (98%), acetone 99% triethylamine (analytical reagent grade) were purchased from Scharlu, Spain.

Samples

Three samples were collected from wastewater (sewerage system) and from different locations in Khartoum North Hospital. The samples were preserved and stored in 500 ml amber borosilicate glass bottles to prevent photo degradation. The samples collected were mixed before cleanup.

Samples pretreatment and clean up

The samples were filtered through 0.45 um filter paper, acidified to pH 3.0 by adding phosphoric (0.1 M) and then were passed through activated C18 cartridge which was activated with 5 ml methanol/water 50:50 (v/v). The cartridge was washed further with 5 ml of acidified water (pH 3.) and then was eluted with 5 ml of triethylamine (5% v/v) in methanol. The eluted solution was evaporated at normal room temperature (28°C). Finally, sample was made to 1 ml by adding water/acetonitrile 95:5 (v/v) and introduced to the LC-MS instrument where 10 uL were injected.

Instrument

LC-MS 2020 (Shimadzu Corporation, Kyoto, Japan) equipped with C18 column, (150 mm length x 4.6 mm inner diameter x^5 um particle size). Pump mode binary gradient (LC-20AD), flow rate 0.5000 ml/min (Tables 1 and 2).

The LCMS experimental parameters are shown in Table 3.

Preparation of standard solution stock

A weight of exactly 0.05 g of each antibiotic tetracycline HCl, (Tetra) doxycycline, (Doxy), ampicillin trihydrate (Ampi), amoxicillin trihydrate (Amoxi) and cephalexin monohydrate (Cefalex) was transferred into a 50 ml volumetric flask and the volume was completed

***Corresponding author:** Dhia Eldin Elhag, Analytical Research Centre, UMST University, Khartoum, Sudan, Tel: +249 183 228614; E-mail: magied20@gmail.com

Received January 16, 2018; **Accepted** January 25, 2018; **Published** January 31, 2018

Citation: Elhag DE, Abdallah BS, Hassan M, Suliman A (2018) ESI-LC/MS Method Development and Validation for the Determination of Some Selected Antibiotics in Hospital Wastewater. Pharm Anal Acta 9: 578. doi: 10.4172/2153-2435.1000578

by the diluents which is acetonitrile and formic acid (1%), (1:1 v/v) and then ultrasonicated. 1 ml of this solution was transferred into a 10 ml flask and was completed to volume by using the same diluent. From this solution, 1 ml was pipettted into a 10 ml flask and was completed to volume by addition of the same diluent as above to obtain a solution of a concentration of 1 ppm.

Time In min	Module	command	Component A value (formic acid1%) Pump A	Component A value (acetonitrile) Pump B
0.01	pump	Pump B conc.	0.00	100
20.00	pump	Pump B conc.	70.00	30
22.00	pump	Pump B conc.	0	100
25.00	pump	Pump B conc.	0	100
25.10	pump	stop	0	

Table 1: Gradient elution programme.

Auto sampler model	SIL-20AC
Enable auto sampler	Use
Sample rack	Rack 1.5 ml 105 vials
Rinsing volume	500 ul
Needle stroke	52 mm
Control vial needle stroke	52 mm
Rinsing speed	35 ul/sec
Sampling speed	15 ul/sec
Purge time	25.0 min
Rinse mode	Before/After
Rinse Dip time	0 sec

Table 2: Auto sampler settings.

Start time	0.00 min
End time	25.10 min
Acquisition mode	Scan & SIM
Polarity	Positive
Event time	1.00 sec
Detector voltage	+1.20 kV
Start m/z	100.00
End m/z	1000.00
Scan speed	938 u/sec
Interface	ESI
DL temperature	250°C
Nebulizing gas flow	1.5 L/min

Table 3: Common MS settings.

Results and Discussion

During the last two decades LCMS has been extensively used in the environmental research for identification and quantification of pollutants and this due to its performance characteristics such as accuracy reproducibility, low detection limit and sensitivity. The current study reports a novel and validated method for quantitative analysis of nine antibiotics commonly found in hospital effluents using LC-MS. Sample preparation and clean up was achieved by using solid-phase technique as it is a powerful sample clean up method in various antibiotic matrices [25-27].

LC-MS soptimization

Several gradient programs were tried to achieve the optimum separation of the entire antibiotics standard. Gradient elution was necessary to avoid excessive retention. Well resolved peaks were obtained within short analysis time.

The positive and the negative electrospray ionization (ESI) scan modes were investigated for attaining the highest sensitivity during the method development process. The full scan of the antibiotics mixture in positive mode showed that the signal-to- noise ratios obtained in this mode were higher than those of the in negative mode. Hence, positive mode was used to obtain the precursor ion [M+H] for the qualitative and quantitative analysis [9,17,28-31]. During the method development, the quadrupole mass analyzers operated in selected ion monitoring (SIM) mode where it monitors only a few mass-to-charge ratios. By using electrospray ionization and subsequent analysis produced the chromatogram shown in Figure 1.

Although all peaks were well resolved in this study, LC MS capability allows analysis of co-eluted analytes. This allows fast analysis time and minimal sample preparation. Table 4 shows the precursor ion and the retention time.

Method performances and validation

Developing and validation of a method for LC-MS involves demonstrating all the performance characteristics such as linearity, precision, accuracy, limits of detection and quantitation, solution stability and robustness [32]. The linearity of a test procedure is its ability (within a given range) to produce results that are directly proportional to the concentration of analyte in the sample. Acceptability of linearity data is often judged by examining the correlation coefficient (r^2) and y-intercept of the linear regression line for the response versus concentration plot. Regression line equations are shown in Table 5. Excellent correlation between the instrumental response and the concentration were obtained.

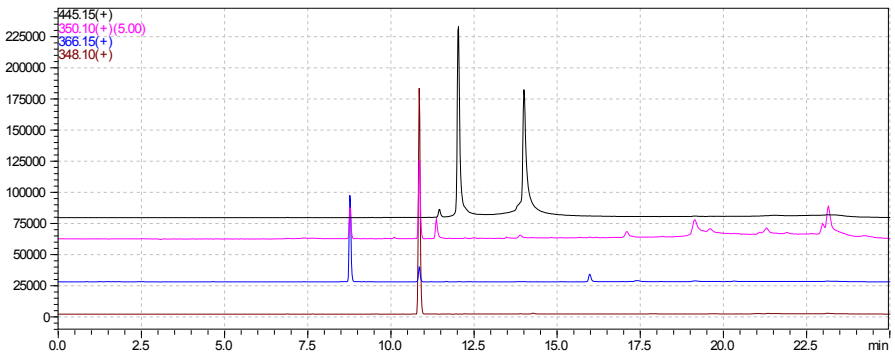

Figure 1: Chromatogram of the antibiotics standard.

Drug	Retention time	M/z
Tetra	12.034	445.15
Doxy	14.014	445.15
Ampi	10.862	350.1
Amoxi	8.773	366.15
Cefalex	10.858	348.1

Table 4: The ion peaks (M/z) and the retention times of the antibiotic standards.

Drug	Regression line equation	R^2
tetra	Y=86865x-14669	0.999
Doxy	Y=1E06x+11923	0.999
Ampi	Y=37168x-2454	0.998
Amoxi	Y=41663x-1983	0.998
Cefalex	51884x+22985	0.999

Table 5: The linearity testing results.

Drug	1	2	3	4	5	6	AVG	SD	RSD
tetra	1756479	1759324	1781687	1759548	1793867	1753518	1767404	16391.91	0.93
Doxy	1685461	1650101	1679713	1621835	1652958	1639810	1654980	24072.5	1.45
Ampi	102656	103065	103160	103330	104035	103103	103224.8	455.1032	0.44
Amoxi	552041	551651	554843	561591	556520	571101	557957.8	7382.376	1.32
Cefalex	1429264	1428162	1431949	1434767	1442267	1425304	1431952	6001.813	0.42

Table 6: The results of repeatability testing.

Day 1			Day 2		Day 3	
Drug	SD	RSD	SD	RSD	SD	RSD
tetra	34824.82	1.93428	20111.91	1.083573	14413.97	0.78
Doxy	33701.61	1.917657	32774.64	1.942115	15980.39	0.95
Ampi	1556.515	1.542105	918.1362	0.81053	964.5576	0.855
Amoxi	3936.037	0.642846	10674.47	1.702397	7980.729	1.27
Cefalex	10462.19	0.702811	15534.94	0.994861	12659.74	0.81

Table 7: The results of reproducibility testing.

		80%	100%	130%
tetra	std con	101.9607	99.99998	101.8249
Doxy	1	101.6675	100	101.0471
Ampi	1	99.5806	99.87969	103.0607
Amoxi	1	99.17957	99.71749	104.4347
Cefalex	1	100.0237	99.87456	103.6223

Table 8: The accuracy test results.

Drug	LOD	LOQ
tetra	0.02	0.12
Doxy	0.02	0.18
Ampi	0.02	0.22
Amoxi	0.08	0.8
Cefalex	0.02	0.05

Table 9: The results of Limit of detection and quantitation testing.

The precision of the method (intraday) was examined by repeatedly injecting the antibiotic solutions. Precision criteria for an assay method are that the instrument precision and the intra-assay precision (RSD) will be ≤ 2%.0.28% RSD. The intraday precision was in the range of 1.450-44% (Table 6).

Excellent values were obtained for interday precision and the values range was 0.78-1.39 (Table 7).

The accuracy of the method was evaluated by determination of the recovery of the antibiotics at four concentration levels (80,100 and 130%). The accuracy of the method was determined by calculating recoveries of each standard. The results showed good recoveries (Table 8).

The limit of detection (LOD) and the limit of quantitation (LOQ) and for the analyzed samples were calculated using the standard deviation of the response (σ) and the slopes (s) i.e. LOD=3.3σ/s and LOQ=10 σ/s. Low detection and quantitation limits were obtained (Table 9).

Drug	injection1	injection2	AVG area	std area	std con	found con	AVG RE %
tetra	1813011	1856073	1834542	1856073	1	0.9884	98.83997
Doxy	1788795	1687575	1738185	1687575	1	1.02999	102.999
Ampi	108434	113276	110855	113276	1	0.978627	97.86274
Amoxi	572373	627026	599699.5	627026	1	0.956419	95.64189
Cefalex	1502397	1561519	1531958	1561519	1	0.981069	98.10691

Table 10: The solution stability test results.

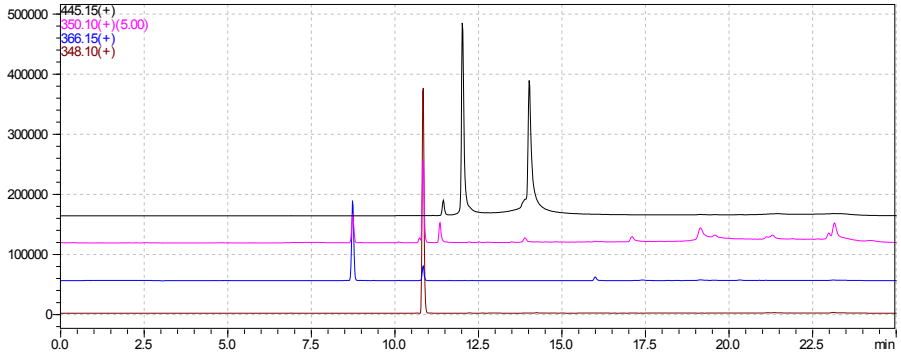

Figure 2(a): Antibiotics standard chromatogram.

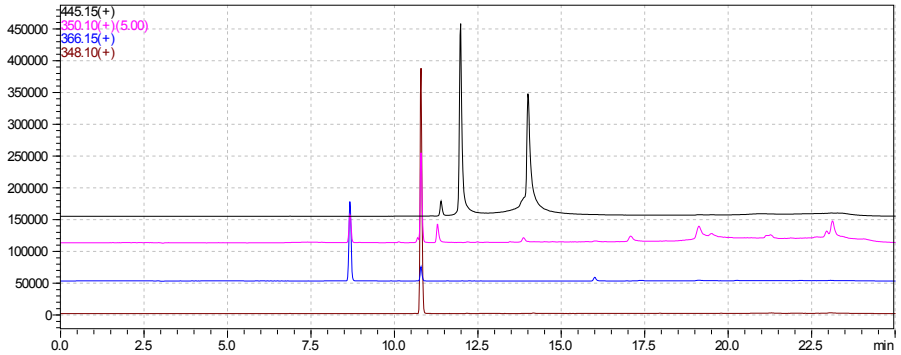

Figure 2(b): Antibiotics standard chromatogram after six hours from the first injection.

Specificity which is the ability of the method to accurately measure the analyte response in the presence of all potential sample components. The obtained results blank injection showed absence of any interferents. Solution stability of the antibiotics standards solution was also assessed after 6 h room temperature storage. For solutions to be considered stable, the results of the percentage difference between the mean response for the fresh and stored solutions should be ≤ 5.0% (Figure 2a, 2b and Table 10).

Robustness which is the reliability of an analysis with respect to deliberate variations in method parameters of an analytical procedure should show the reliability of an analysis with respect to deliberate variations in method parameters. The evaluation of robustness should be considered during the development phase. The standard pH was 3.0 and the room temperature was 28˚C. These two parameters were varied in order to evaluate the robustness of the methods and excellent results are shown in Table 11.

Sample Analysis

The developed method was applied for the determination of antibiotics in hospital wastewater samples. The results of the sample analyses are tetracycline HCl, doxycycline, and cephalexin monohydrate with concentrations of 0.124, 0.134 and 0.084 ppm, respectively. Ampicillin trihydrate and amoxicillin trihydrate were not detected (Figure 3).

Such findings necessitate the need for more efficient wastewater treatment plants and stricter quality control measures. However, there numerous routes by which the disposed of antibiotics and other drugs can reach aquatic environment. However, antibiotics persist and degrade slowly, pass through water treatment plants and thereafter transported to sediment or aquatic environment.

Chemical degradation includes hydrolysis oxidation, decarboxylation, isomerization and elimination. Hydrolysis spitting by water, is a potential degradation pathway for organic pollutants in the aquatic environment and it is probably the most commonly encountered mode of drug degradation. Examples of antibiotics that undergo hydrolysis include lactones, amide sand macrolides. The pH has a profound effect in hydrolysis reaction. For instance, at neutral pH, hydrolysis of sulphonamides is very slow whereas lactams hydrolyse under acidic conditions [33-37]. It is noteworthy that the environmental occurrence, persistence, fate and bioaccumulation ability of antibiotics differ depending on their chemical properties and on the environmental conditions [38,39].

Parameter	Drug	Run1	Run2	Run3	AVG	SD	RSD
29°C	tetra	1761821	1759775	1768001	1763199	4282.631	0.24289
	Doxy	1560235	1558049	1532373	1550219	15493.69	0.999452
	Ampi	99096	99071	101316	99827.67	1288.995	1.29122
	Amoxi	548559	537823	546558	544313.3	5709.144	1.048871
	Cefalex	1382887	1401628	1399124	1394546	10174.6	0.729599
27°C	tetra	1787640	1768801	1789080	1781840	11315.32	0.635036
	Doxy	1677628	1649295	1647241	1658055	16982.09	1.024218
	Ampi	98033	98845	98586	98488	414.7758	0.421144
	Amoxi	541732	546526	546459	544905.7	2748.68	0.504432
	Cefalex	1371988	1383966	1367474	1374476	8522.859	0.620081
PH3.1	tetra	1713358	1704663	1714488	1710836	5376.036	0.314234
	Doxy	1571982	1554607	1544523	1557037	13889.89	0.892072
	Ampi	92592	92732	94447	93257	1032.945	1.107632
	Amoxi	491749	497037	503680	497488.7	5978.31	1.201698
	Cefalex	1296192	1316877	1319020	1310696	12606.74	0.961835
PH2.9	tetra	1601163	1615916	1621137	1612739	10359.14	0.642332
	Doxy	1576493	1554135	1556553	1562394	12270.09	0.785339
	Ampi	95074	94744	95123	94980.33	206.1318	0.217026
	Amoxi	501861	511828	513200	508963	6188.651	1.215933
	Cefalex	1312012	1326165	1341340	1326506	14666.97	1.105684

Table 11: The results of robustness testing.

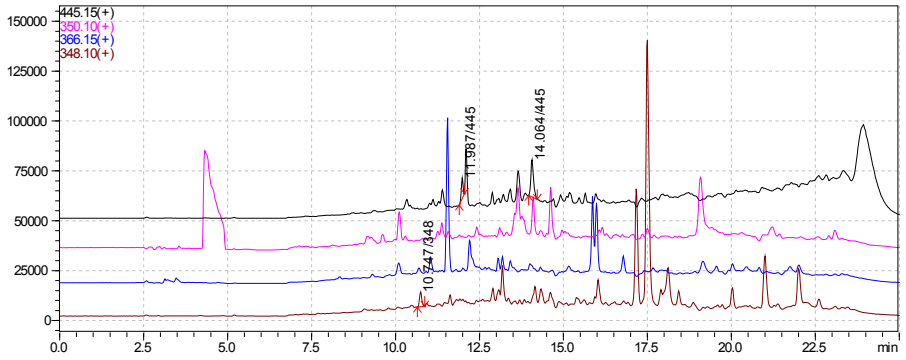

Figure 3: chromatograms of the antibiotic found in hospital effluent sample.

Conclusion

A novel LC-MS method was developed for analysis of amoxicillin trihydrate, ampicillin trihydrate, cephalexin monohydrate, norfloxacin HCl, ciprofloxacin, tetracycline HCl, azithromycin, doxycycline and clarythromycin. The method proved to be accurate, precise linear, reproducible and robust. The method can be used conveniently for identification and quantification of these antibiotics in aqueous samples.

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