Original Paper

Simultaneous Separation and Identification of Antibiotic Drugs in Complex Mixtures by using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS-MS)

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ABSTRACT

Antibiotics are generally considered to be potential life-saving drugs. In recent times it has been observed that these drugs are being used indiscriminately by many individuals, including youngsters and the elderly. It is well known that most drugs including antibiotics, can cause anaphylactic shock in certain individuals, and this can lead to forensic investigation because of the unexpected nature and abruptness of the death.

It is important therefore to have an effective method for the identification of these antibiotic compounds in forensic samples. Earlier workers had performed the separation and identification of fluoroquinolone antibiotics by using various analytical techniques such as High Performance Thin Layer Chromatography (HPTLC), High Performance Liquid Chromatography (HPLC), and Gas Chromatography-Mass Spectrometry (GC-MS).

In this study, a simple and selective qualitative method was developed for the separation and identification of four fluoroquinolone antibiotic drugs (Ciprofloxacin, Norfloxacin, Ofloxacin and Gatifloxacin) in forensic samples by using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS-MS) by using Multiple Reaction Monitoring (MRM) functions. The limit of detection (LOD) was observed to be 10 ng/mL for Norfloxacin, Ciprofloxacin and Gatifloxacin, while it was 0.5 ng/mL for Ofloxacin.

Key Words: Antibiotic, Fluoroquinolone, Qualitative analysis, Liquid chromatography-tandem mass spectrometry, LC-MS-MS

Introduction

Fluoroquinolone antibiotics are commercially used in case of urinary tract infections by prescription only.[1] But people often procure these antibiotics without prescription to prevent the time and the cost of consultation with the doctor. If they do not get enough relief from one fluroquinolone antibiotic, they may even switch over to another. In this way, antibiotics are increasingly being abused, and in some cases, it proves fatal in the form of a strong immunity reaction (anaphylactic shock).[2-4] In some such cases, forensic intervention becomes necessary to finalize the cause of death, owing to the unexpected nature and abruptness of death. There is a need therefore to develop new and simple detection methods for the identification of these antibiotics in forensic samples.

Ines et al[5] and Neckle et al[6] have developed methods for simultaneous determination of antibiotics by using HPLC, and many others have done the same with regard to biological samples for the detection of various prescription drugs such as analgesics, antidepressants, etc. Unfortunately, enough studies do not exist for forensic samples. In the present study, an identification and separation method is proposed for the identification of antibiotic drugs in the context of forensic analysis.

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Materials and Methods

1. *Standards*
   Ciprofloxacin, Norfloxacin, Gatifloxacin and Ofloxacin (supplied by Blue Star Pharmaceuticals, India), Methanol (HPLC grade), Chloroform (AR grade), Ammonium Acetate (AR Grade), Water (HPLC grade), Glassware (Borosil, India) were used.
2. *Equipment*
   The LC system used was a Perkin Elmer 200 liquid chromatograph equipped with a gradient pump (200 series), an autosampler, and a degausser. Mass spectrometric analysis was performed by using a Triple quadrupole with ion trap capability from Applied Biosystems, USA. The data acquisition and system controller software was Analyst 1.4.1. Nitrogen was produced by an on-site nitrogen generator from peak scientific instrument.

   The stationary phase for the analytical run was C-18 packed in a 150 x 2.1 mm and 5 micrometre particle size column from Restek. All analytical runs were preceded by a guard column packed with C18 from Restek.
3. *Preparation of Standards*
   All four fluoroquinolone standards were weighted and a stock solution of 1 mg/mL concentration was prepared. Methanol and ammonium acetate solution in water (10 mM conc and pH 4.7) in the ratio 4:1 v/v was used to make the final solution of each fluoroquinolone antibiotic to 500 ng/mL conc in 50mL volume each.
4. *Preparation of Mixed Solution*
   A mixture of these four standard samples was prepared by pippeting 10 mL from each individual standard sample, making a final volume of 40 mL. The standards and the mixture having the same volume were used, i.e., 40 mL each.
5. *Preparation of Urine Sample and Extraction*
   10 mL of mixture solution was pippeted and spiked in 20 mL of urine (blank) sample. The prepared urine sample was extracted with choloroform three times. The remaining urine sample was discarded, and the three extracts were mixed and air dried. The dried sample was reconstituted by using methanol and ammonium acetate solution in water (10 mM conc and pH 4.7) in the ratio 4:1 v/v.
6. *Optimization of the MS-MS Conditions*
   Mass spectrometric conditions were optimized by using flow injection method for each standard sample, whereby the Q1 scan mode was used to identify the parent ion (protonated molecule), and product ion scan was done to select the most abundant fragmented ion. It was optimized as selected identifier ion for the parent molecular ion. The respective product ion masses for the four different fluoroquinolone antibiotics are displayed in **Table 1**.
7. *Optimization of LC Conditions*
   The liquid chromatographic conditions were optimized by using different proportions of isocratic mobile phase of methanol and water, and then gradient method was used to develop the selective separation in the mixture. Repeated trial and error method finally yielded a stable gradient mobile phase consisting of ammonium acetate (10 mM conc and pH 4.7), methanol and water. The mobile phase gradient used is shown in **Table 2**.

Results & Discussion

Data acquisition and processing were done by Analyst 1.4.1 software, and the MRM (Multiple Reaction Monitoring) data obtained from the standard antibiotic mixture, and the extracted urine sample were compared to establish the presence of fluoroquinolone antibiotics in the sample. The LOD was calculated by the use of stan-

Table 1

S.No.	Compound Name	Parent ion (*m/z*)	Product ion (*m/z*)	Dwell Time (*msec*)	DP (*V*)	EP (*V*)	Collision Energy (*eV*)
1.	Ciprofloxacin	332.14	314.13	200	47	3	29
2.	Norfloxacin	320.14	302.13	200	47	3	29
3.	Ofloxacin	362.15	318.16	200	59	5	25
4.	Gatifloxacin	376.17	332.18	200	59	4	28

Table 2

S.No.	Time (*min*)	Flow Rate (*microlitre/min*)	Ammonium Acetate Buffer (*10 mM*)	Methanol	Water	Gradient
1.	0.0	200	10	13	77	-1
2.	10.0	200	10	43	47	-1
3.	30.0	200	10	43	47	-1

dard in diluted concentrations of standard fluoroquinolone antibiotics, which was found to be as follows: Norfloxacin (10 ng/mL), Ciprofloxacin (10 ng/mL), Ofloxacin (0.5 ng/mL) and Gatifloxacin (10 ng/mL).

The observed chromatograms were used to quantify the approximate concentration in the spiked urine sample by using the following formula (the observed quantitation results are shown in **Table 3**):

$$\frac{I\,(s)}{I\,(u)} = \frac{C\,(s)}{C\,(u)}$$

Where,
I (s): Observed intensity of known standard (*mean value*)
I (u): Observed intensity in unknown sample (*mean value*)
C (s): Known concentration of the standard (*mean value*)
C (u): Concentration of the compound in unknown sample (*mean value*).

The standard fluoroquinolones Ciprofloxacin, Norfloxacin, Gatifloxacin and Ofloxacin show the presence of the protonated molecules (MH^+) of *m/z* 332.14, 320.14, 376.17 and 362.15 respectively. The product qualifier ion selected for these parent ions were of *m/z* 314.13, 302.13, 332.18 and 318.16 respectively. The intensity of these product ions was found to be maximum and stable among all abundant ions formed after the cleavage of the parent ions.

The Multiple Reaction Monitoring (MRM) function was used to identify the main parent ions in the urine sample, and the matrix effect could be observed in the resulting chromatogram. The observed intensities were used as a reflection value towards the concentration of standard and the extracted sample of urine, which was used further to calculate the recovery of sample.

Thus the proposed method for the qualitative separation and quantitation rule could be used effectively to calculate the concentration of drugs in unknown samples, and qualitative identification of these fluoroquinolone drug samples in biological matrices. Although the recovery by liquid-liquid extraction process was low, the sensitivity and selectivity of the proposed method were found to be accurate enough for the needs of forensic scientists.

Table 3

S.No.	Compound Name	Observed Intensity (*Standard*)* in cps**	Observed Intensity (*Unknown*)* in cps**	Concentration (*Standard*) ng/mL	Extraction Method$	Calculated Concentration (*Spiked Sample*) ng/mL
1.	Ciprofloxacin	3491	1690	500	Liquid-Liquid	242
2.	Norfloxacin	3336	1452	500	Liquid-Liquid	217
3	Ofloxacin	3901	1435	500	Liquid-Liquid	183
4.	Gatifloxacin	3215	1060	500	Liquid-Liquid	165

*Mean values of five consecutive samples run in MRM mode
**Count per second
$Extraction by chloroform (AR)

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REFERENCES

1. Bertino J, Fish D. The safety profile of the fluoroquinolones. Clin Ther 2000; 22 (7): 798.
2. Williams LV. Antibiotics in Laboratory Medicine. 4th edn, 1996. p. 591.
3. Hardman JG, Limbird LE, Gilman AG (eds). Goodman & Gilman's The Pharmacological Basis of Therapeutics. 10th edn, 2001. McGraw-Hill, USA.
4. Belal F, Al-Majed A, Al-Obaid AM. Methods of analysis of 4-quinolone antibacterial. Talanta 1999; 50(4): 765.
5. Ines M, Santoro RM, Singh AK, Erika RM, Hackmam K. Quantitative determination of gatifloxacin, levofloxacin, lomefloxacin and pefloxacin fluoroquinolone antibiotics in pharmaceutical preparations by high-performance liquid chromatography. J Pharm Biomed Anal 2006; 40(1): 179.
6. Neckle U, Joukhadar C, Mayer BX. Simultaneous determination of levofloxacin and ciprofloxacin in microdialysates and plasma by high-performance liquid chromatography. Anal Chim Acta 2002; 463(2): 199.