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# A Qualitative LC-MS/MS Method for Simultaneous Screening of 15 commonly used Antibiotics from dried Blood and Urine spots

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**Abstract :** A simple, fast, sensitive and selective qualitative liquid chromatography with tandem mass spectrometry (LC-MS/MS) method for identification of fifteen (15) antibiotics in dried blood spot (DBS) and dried urine spot (DUS) was developed and validated. Amoxicillin, ampicillin, penicillin G, penicillin V, cloxacillin, cephalexin, sulfamethoxazole, trimethoprim, erythromycin, ciprofloxacin, tetracycline, clarithromycin, metronidazole, chloramphenical, and azithromycin were identified in DBS and DUS samples. DBS and DUS samples were prepared by applying blood or urine (50 µL) to filter papers and dried at room temperature for 3 hours in the dark. The whole diameter disk (containing 50 µL of blood or urine) was cut out from each DBS and DUS and extracted using methanol and acetonitrile (20:80). The extracted sample was chromatographed without further treatment using an LC-MS/MS instrument equipped with C18 column, (Agilent ZORBAX C-18 Eclipse Plus 2.1 × 150 mm, 1.8 µm (p/n 959759-902)). The mobile phase was A) Water + 0.1 % formic acid + 0.1% ammonium formate, B) 80% acetonitrile + 20% methanol + 0.1% formic acid. The run time was 25 minutes and post run time was 2 minutes. Two multiple reaction monitoring (MRM) transitions were selected for all target compounds to ensure selectivity and robustness. The following method parameters were validated; limit of detection (LOD), selectivity (SLR), sensitivity (SNR), reliability (RLR), false positive rate (FPR) and false negative rate (FNR). The method has been applied for the detection of the 15 antibiotics in DBS and DUS from blood and urine of volunteers who were administered the antibiotics.
**Keywords :** Qualitative; antibiotics; LC-MS/MS; dry blood spot (DBS); dry urine spot (DUS).

## 1. Introduction

The WHO global report on antimicrobial resistance (AMR) indicates that resistance of common bacteria has reached alarming levels in many parts of the world [1]. Antibiotic consumption is a primary driver of antibiotic resistance [2]. The association between antibiotic consumption and resistance is well documented across individual hospitals [3], communities [4], and countries [5]. The Global Action Plan on AMR recommends that all countries collect and report antibiotic consumption data. This is to monitor national and global consumption trends over time; compare antibiotic use among countries; provide a baseline for the evaluation of future efforts to reduce antibiotic use; enable epidemiological analysis of the association between antibiotic use and resistance over time; and support policies that aim to reduce antibiotic resistance[6].

Although such surveillance reports are being collected and documented, the method of self-reported medicine use that is often used to collect this information in most low and middle income countries (LMICs) have been shown to have a low validity and reliability [7]. Much as self-reports are relatively simple to use and cheap, the concerns about their validity arise from the fact that they are prone to recall bias and usually respondents provide information that conform to their perceived expectations of their interviewer [8]; therefore chances are high that the burden of antibiotic consumption and misuse is higher than reported.

Given the threat posed by rising AMR level and the unreliability of self-reported medicines use; there is need for alternative and reliable methods of data collection on antibiotic consumption patterns across countries. Studies have reported the simultaneous identification of multiple antibiotics in biological fluids, such as milk, dried plasma spots and dried urine spot [9,10], however, none of these studies used LC-MS/MS method for the simultaneous screening of fifteen commonly used antibiotics in LMICs from DBS and DUS. We therefore developed and validated a qualitative LC-MS/MS method for the simultaneous screening of fifteen commonly used antibiotics in LMICs from DBS and DUS. . This will help in monitoring of antibiotics commonly used in the population. This paper describes the method development, validation and it's application to detect the 15 antibiotics from DBS and DUS collected from healthy volunteers.

## 2. Materials and Methods

### 2.1 Equipment, chemicals and materials

Instrumentation separation of analytes for this method was performed using an Agilent 1290 infinity II LC with a 20 µL injection port and multiwash capability. An Agilent 6420 Triple Quadrupole LC/MS was used as the detector. The column used was Agilent ZORBAX C-18 Eclipse Plus 2.1 × 150 mm, 1.8 µm (p/n 959759-902). The optimized conditions included; column temperature (45 $^{o}$C), injection volumn (10 µL), mobile phase (A: Water + 0.1 % formic acid + 0.1% ammonium formate, B: 80% acetonitrile + 20% methanol + 0.1% formic acid), run time (25 minutes), post run time (2 minutes), flow rate (0.25), gas temperature (330 $^{o}$C), gas flow rate (11 L/min), nebulizer pressure (30 psi), capillary voltage (4000 V (3000V)) and delta EMV (200V).

Electrospray ionization was performed in simultaneous positive and negative mode. All data acquisition and processing were performed using Agilent MassHunter software (version B 07.00). Antibiotic reference standards of amoxicillin, ampicillin, penicillin G, penicillin V, cloxacillin, cephalexin, sulfamethoxazole, trimethoprim, erythromycin, ciprofloxacin, tetracycline, clarithromycin, metronidazole, chloramphenicol and azithromycin were kindly donated by Department of Laboratory Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge (Sweden) as pure solid materials. LC/MS grade acetonitrile, methanol, water, formic acid and ammonium formate were obtained from EMD Millipore (Darmstadt, Germany). Whatman Filter Paper Grade 591 was donated by the Laboratory of Clinical Pharmacology, Huddinge, Sweden. Blank whole blood and urine were obtained from volunteers who had not taken any antibiotics for at least 1 month. The scissors used to cut out the DBS and DUS were procured from a local retail shop in Kampala, Uganda.

### 2.2 Preparation of stock and standard solutions

Mixed standard stock solution (40 mg/L) was prepared by weighing 2 ± 0.1 mg of each standard substance, followed by quantitative transfer to a 50 mL volumetric flask and filling to volume with methanol. The working standard solution was prepared by diluting the mixed standard stock solution with methanol to a series of the required concentrations. The standard stock antibiotic mix was protected from sun light and stored at -20 ˚C until analysis.

### 2.3 Sample preparation and sample extraction procedure

2 mls of blank whole blood and urine were obtained from volunteers who had not taken any antibiotics for at least 1 month. The spiking of the blood and urine was done by adding antibiotic standard mix (0.1ng/mL, 1ng/mL, 10ng/mL, 20ng/mL, 30ng/mL, 40ng/mL, 50ng/mL) to blood/urine in a test tube. To prepare the DBS and DUS, 50 µL of the spiked blood or urine standards were spotted onto filter paper and left to dry in the dark for at least 3 h at room temperature before processing. The whole diameter disk (containing 50 µL of blood or urine) was cut out from each DBS and DUS. The cut disc was placed in an Eppendorf tube (1.5 mL capacity) and mixed with 1000 µL of methanol (20%) and acetonitrile (80%).The sample was vortex-mixed twice for 20 s at 10-min intervals and then centrifuged at 3500 revolutions per minute (RPM) for 5 minutes. After the extraction period, the filter paper was removed, and 500 µL of the extract was transferred into an auto-sampler vial to be injected onto the LC-MS/MS system for analysis.

### 2.4 Method validation

The output of qualitative methods, such as the one described here are typically categorical, reporting binary results such as “presence”/ “absence” or “above cut-off”/ “below cut-off” of the analyte. We used guidelines which evaluate performance by relying on the discrete results instead of continuous measurements for validation [11-13]. EURACHEM specifies that the qualitative performance parameters that should be evaluated are: confirmation of identity; sensitivity; selectivity/specificity; and, precision. Precision may be expressed as true and false positive (and negative) rates and it has to be taken into account that these rates are related to sensitivity and specificity [14]. Association of Official Analytical Chemists (AOAC International) proposes four performance indicators which include; sensitivity, specificity, false negative and false positive rates [15].

#### 2.4.1 Determination of the limit of detection (LOD)

The limits of detection (LODs) 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. 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 [16].

### 2.4.2 Confirmation of identity

The different antibiotics were considered present by the presence of both the major and minor transitions in table 1.

**Table 1. MRM transitions used for the detection of the different antibiotics**

| Analyte | Major | CE (V) | Minor | CE (V) | Polarity |
|---|---|---|---|---|---|
| Azithromycin | 749.5/591.4 | 20 | 749.5/158.1 | 40 | Positive |
| Clarithromycin | 748.5/590.4 | 20 | 748.5/158.1 | 20 | Positive |
| Erythromycin | 734.5/576.4 | 20 | 734.5/158.1 | 40 | Positive |
| Tetracycline | 445.2/427.1 | 10 | 445.2/410.1 | 20 | Positive |
| Cloxacillin | 436/277 | 15 | 436/160 | 15 | Positive |
| Amoxicillin | 366.1/349.1 | 10 | 366.1/114 | 20 | Positive |
| Penicillin V | 351/192 | 5 | 351/160 | 10 | Positive |
| Cephalexin | 348.24/158.1 | 15 | 348.24/106.2 | 25 | Positive |
| Penicillin G | 335.1/176.1 | 10 | 335.1/160 | 10 | Positive |
| Ciprofloxacin | 332.1/314.1 | 20 | 332.1/231.1 | 40 | Positive |
| Trimethoprim | 291.1/230.1 | 20 | 291.1/123.1 | 40 | Positive |
| Sulfamethoxazole | 254.1/156 | 10 | 254.1/92 | 20 | Positive |
| Metronidazole | 172.1/128 | 10 | 172.1/82.1 | 20 | Positive |
| Ampicillin | 348.1/207.1 | 10 | 348.1/74 | 40 | Negative |
| Chloramphenicol | 321/257 | 10 | 321/152 | 20 | Negative |

***CE: Collision Energies; V: Volts***

### 2.4.3 False negative (FNR), false positive (FPR), selectivity (SLR), sensitivity (SNR) and reliability (RLR) rates

Method performance parameters were calculated on DBS/DUS prepared from blood/urine samples spiked with mixed standard antibacterials at 50% and 150% of the detection limit concentration [12]. Results for the 15 samples at 50% of the detection limit were classified as true negative (TN) or false positive (FP), whereas results for the 15 samples at 150% of detection limit were classified as true positive (TP) or false negative (FN) [12].

$$\mathrm{FNR} = \frac{\mathrm{FN}}{\mathrm{FN} + \mathrm{TP}} \times 100$$

$$\mathrm{FPR} = \frac{\mathrm{FP}}{\mathrm{FP} + \mathrm{TN}} \times 100$$

$$\mathrm{RLR} = \frac{\mathrm{TP} + \mathrm{TN}}{\mathrm{n}} \times 100 = 100 - \mathrm{FPR} - \mathrm{FNR}$$

$$\mathrm{SLR} = \frac{\mathrm{TN}}{\mathrm{TN} + \mathrm{FP}} \times 100$$

$$\mathrm{SNR} = \frac{\mathrm{TP}}{\mathrm{TP} + \mathrm{FN}} \times 100$$

The acceptance criteria for the rates were as follows: FNR ≤ 7%, FPR =0%, RLR ≥ 93%, SLR = 100%, SNR ≥ 93% [11].

### 2.4.4 Volunteer Sampling

For further validation of the method, six volunteers were recruited in the study after getting informed consent. Only those who had reported not taking any antibiotics for atleast a month were included in the study. Each volunteer was given a single dose of a combination of three antibiotics to swallow, one volunteer was given penicillin G by intramuscular injection. These combinations were chosen after considering that there was no drug-drug interactions among them, which could otherwise affect their presence or absence in the samples. The combination of antibiotics given to each volunteer included; A (Azithromycin, penicillin V, amoxicillin), B (Ampicillin, tetracycline, metronidazole), C (Clarithromycin, cephalexin, cotrimoxazole), D (Chloramphenicol, ciprofloxacin, cloxacillin), E (Amoxicillin, ampicillin, erythromycin) and F (Chloramphenicol, penicillin V, penicillin G). Blood and urine samples were collected from each volunteer by a finger prick before administration of the antibiotics to ensure that they didn't have any antibiotics in their system and 3 hours after administration because that is the average time most of the antibiotics take to reach peak plasma levels [17,18]. To prepare DBS approximately 50 µL of blood was collected from the volunteers before antibiotics intake, another 50 µL of blood was collected 1 and 3 hours respectively after antibiotics intake via a finger prick then spotted onto the filter paper and allowed to dry for 3 hours in the dark at ambient temperature and then stored at -20°C until analysis.

To prepare DUS approximately 50 µL of urine was sampled from the volunteers before antibiotics intake, another 50 µL of urine was collected 1 and 3 hours respectively after antibiotics intake, it was spotted on filter paper and allowed to dry for 3 hours in the dark at ambient temperature and then stored at −80°C until analysis.

### 2.5 Ethical approval

The study was approved by Makerere University School of Biomedical Sciences Research and Ethics Committee (reference SBS-570) and the Uganda National Council of Science and Technology (reference HS235ES). Written informed consent was obtained from the volunteers.

### 2.6 Data analysis

All data were analysed using Microsoft® Excel 2010 (Microsoft Corporation, USA).

## 3. Results and discussions

### 3.1 Chromatography

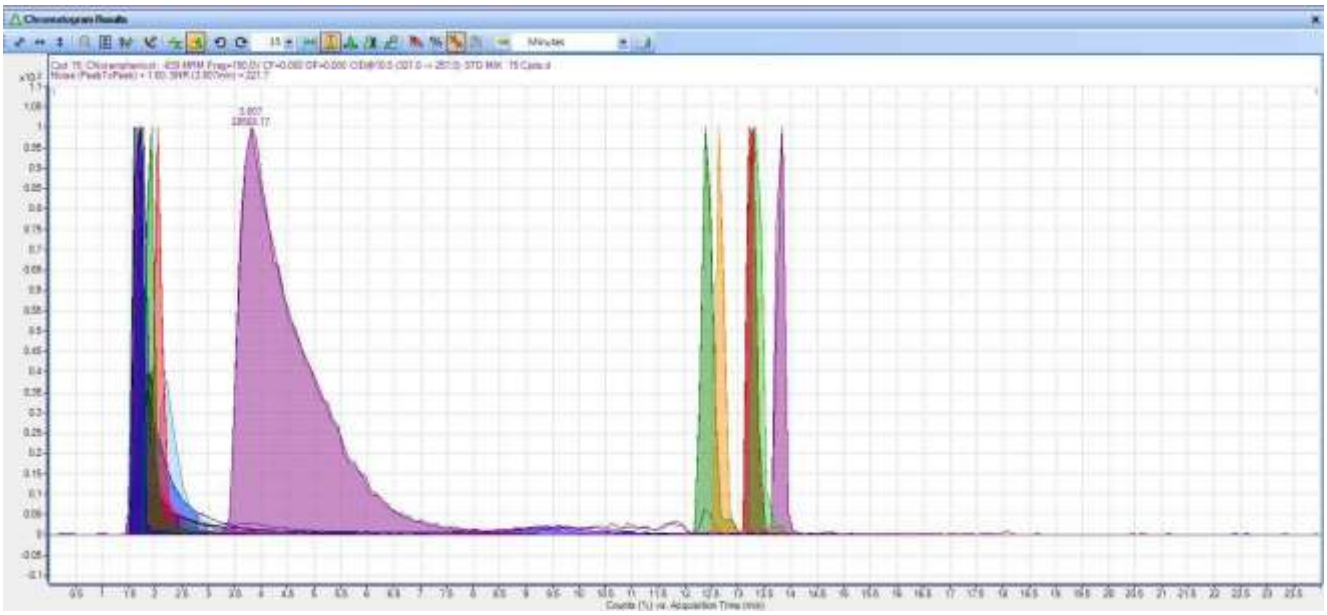
We optimized LC-MS/MS chromatographic conditions to simultaneously detect 15 antibiotics in blood and urine. The optimized conditions included, gradient mobile phase (A, Water + 0.1 % formic acid + 0.1% ammonium formate, and B, 80% acetonitrile + 20% methanol + 0.1% formic acid. The gradient elution program was carefully adjusted until it permitted the best separation ability. The run time was 25 minutes and post run time was 2 minutes. Previous studies have developed methods for simultaneous detection of multiple antibiotics in blood/urine [9,10]. Our study has optimized conditions for simultaneous detection of upto 15 different antibiotics both in blood and urine collected urine filter papers. This will help in monitoring of antibiotics commonly used in the population. We selected two multiple reaction monitoring (MRM) transitions (Table 1) for all target antibiotics to ensure selectivity and robustness of the chromatographic conditions for accurate measurement of the antibiotics.. For the purpose of correct identification the LC-MS/MS analysis was performed on standard solutions under the LC-MS/MS optimized. The chromatogram of all the 15 antibiotics in the standard antibiotics mix solution is shown in figure 1.

### 3.2 Extraction

Processing of samples to ensure efficient and maximum extraction of antibiotics in the matrix is key in accurate identification of the antibiotics. In our study we optimized simultaneous extraction of antibiotics in DBS and DUS using 20% and 80% repectively. Maximum extraction of antibiotics in DBS and DUS is key for accurate identification of antibiotics. The drying of blood spots and urine spots applied to filter paper for 3 hours in the dark resulted in high and reproducible recoveries. Drying of blood spots applied to filter paper in the dark for 3

hours has been used in a previous study where a HPLC method was developed and validated for the determination of metronidazole in DBS in neonatal whole blood samples [19].

**Figure 1: Chromatogram of all the 15 antibiotics in standard antibiotics mix solution**



### 3.3 Method validation

Our method was validated for false negative (FNR), false positive (FPR), selectivity (SLR), sensitivity (SNR) and reliability (RLR) rates, table 3. The acceptance criteria for the rates were as follows: FNR ≤ 7%, FPR =0%, RLR ≥ 93%, SLR = 100%, SNR ≥ 93% [11]. The FNR, SNR and RLR of ampicillin in DUS is better than that in DBS. This could be because urine is cleaner than blood therefore less matrix effect. This could also be due to excretion of ampicillin in urine, hence, making the concentration of ampicillin to be higher in urine than blood. T (Table 2).

**Table 2. False negative (FNR), false positive (FPR), selectivity (SLR), sensitivity (SNR), reliability (RLR) rates and limit of detection (LOD) for DBS and DUS**

| Antibiotics | LOD (ng/mL) | FNR | | FPR | | SLR | | SNR | | RLR | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DBS | DUS | DBS | DUS | DBS | DUS | DBS | DUS | DBS | DUS |
| Amoxicillin | 1.3402 | 7 | 7 | 0 | 0 | 100 | 100 | 93 | 93 | 97 | 97 |
| Ampicillin | 0.0011 | 7 | 0 | 0 | 0 | 100 | 100 | 93 | 100 | 97 | 100 |
| Penicillin G | 0.0051 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| Penicillin V | 0.0283 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| Cloxacillin | 0.2057 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| Cephalexin | 0.2178 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| Sulfamethoxazole | 0.2057 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| Trimethoprim | 0.5173 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| Erythromycin | 1.0510 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| Ciprofloxacin | 0.1000 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| Tetracycline | 0.1363 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| Clarithromycin | 1.3993 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| Metronidazole | 1.0000 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| Chloramphenical | 0.0001 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| Azithromycin | 0.2155 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |

### 3.4 Volunteer sampling

Our validated method was applied in the detection of 15 commony used antibiotics. The method simultaneously detected 15 antibiotics in the DBS and DUS (Table 3). The observed results from the healthy volunteers can be explained by the pharmacokinetics of the different antibiotics (Table 4).

**Table 3. Qualitative results of antibiotics taken by each volunteer in DBS and DUS**

| Volunteer | Antibiotics | Before intake | | After 1 hour of intake | | After 3 hours of intake | |
|---|---|---|---|---|---|---|---|
| | | DBS | DUS | DBS | DUS | DBS | DUS |
| A | Azithromycin (oral) | X | X | X | X | √ | √ |
| | Penicillin V (oral) | X | X | √ | √ | X | X |
| | Amoxicillin (oral) | X | X | √ | √ | √ | √ |
| B | Ampicillin (oral) | X | X | √ | √ | √ | √ |
| | Tetracycline (oral) | X | X | √ | √ | √ | √ |
| | Metronidazole (0ral) | X | X | √ | √ | √ | √ |
| C | Clarithromycin (oral) | X | X | X | X | √ | √ |
| | Cephalexin (oral) | X | X | √ | √ | √ | √ |
| | Sulfamethoxazole (oral) | X | X | √ | √ | √ | √ |
| | Trimethoprim | X | X | √ | √ | √ | √ |
| D | Chloramphenicol (oral) | X | X | √ | √ | √ | √ |
| | Ciprofloxacin (oral) | X | X | √ | √ | √ | √ |
| | Cloxacillin (oral) | X | X | √ | √ | √ | √ |
| E | Amoxicillin (oral) | X | X | √ | √ | √ | √ |
| | Ampicillin (oral) | X | X | √ | √ | √ | √ |
| | Erythromycin (oral) | X | X | √ | √ | √ | √ |
| F | Chloramphenicol (oral) | X | X | √ | √ | √ | √ |
| | Penicillin V (oral) | X | X | √ | √ | X | X |
| | Penicillin G (IM) | X | X | √ | √ | √ | √ |

X = Absent; √ = present

**Table 4. Summary of key pharmacokinetic properties of the antibiotics that could explain their presence or absence in DBS and DUS**

| Antibiotics | Time to peak plasma concentrations (hours) | Time it takes to be cleared from the system | Terminal Half-life (hours) | Reference |
|---|---|---|---|---|
| Amoxicillin | 1-2 | 6-8 hours | 1.7 ± 0.3 | [20,21] |
| Ampicillin | 1-2 | 5.5 hours | 1-1.8 | [22-24] |
| Penicillin G | 0.25-0.5 | 3 hours | 0.5 ± 0.1 | [20] |
| Penicillin V | 0.5-1 | 2 hours 45 minutes | 0.5-0.6 | [22,24] |
| Cloxacillin | 0.58-1 | 3-5.5 hours | 0.5 | [25,26] |
| Cephalexin | 1.4 ± 0.8 | 8 hours | 0.90 ± 0.18 | [27] |
| Sulfamethoxazole | 4 | 33-55 hours | 10.1 ± 2.6 | [28,29] |
| Trimethoprim | 2 | 24-48 hours | 10 ± 2 | [28,29] |
| Erythromycin | 1 | 11 hours | 1.6 ± 0.7 | [30] |
| Ciprofloxacin | 1-2 | 22 hours | 3.3 ± 0.4 | [31,32] |
| Tetracycline | 2-4 | 2 days | 10.6 ± 1.5 | [33,34] |
| Clarithromycin | 2.8 | 22-38 hours | 3.3 ± 0.5 | [35,36] |
| Metronidazole | 0.3-3 | 2 days | 8.5 ± 2.9 | [37] |
| Chloramphenical | 1-2 | 22 hours | 1.5-4.6 | [24,38] |
| Azithromycin | 2-3 | 15.5 days | 40 | [39] |

## 4. Conclusions

A qualitative LC-MS/MS method has been developed for the simultaneous screening of 15 commonly used antibiotics from blood and urine samples dried on filter paper. The method was validated for limit of detection (LOD), selectivity (SLR), sensitivity (SNR), reliability (RLR), false positive rate (FPR) and false negative rate (FNR). It has been applied for the detection of the 15 antibiotics in DBS and DUS from volunteers who were administered the antibiotics. A consideration of all of the chromatograms obtained from this study indicates that the method can be used to detect the 15 antibiotics in DBS and DUS in patients.

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