Rapid and Sensitive Analysis of Antibiotics in Children's Urine Using the X500R QTOF System

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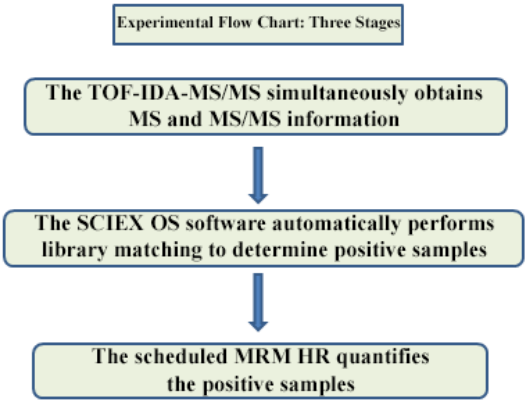
Introduction

Antibiotics are a class of secondary metabolites produced by microorganisms (including bacteria, fungi, and actinomycetes) which restrain the growth and survival of other microorganisms, as well as analogous compounds that are chemically synthesized or semi-synthesized. In 1928, Alexander Fleming discovered penicillin and officially began using it in clinical settings in 1941, marking the arrival of antibiotic era. In recent decades, antibiotics have evolved by leaps and bounds, with more than 200 varieties on the market. The annual use of antibiotics is alarming and amounts to tens of tons, including not only human antibiotics, but also large amounts used in animal breeding. The abuse of antibiotics has caused serious pollution to food and the environment. It is widely known that many antibiotics can cause liver and kidney damage, allergies, local irritation, and other side effects to the human body. Because the physiological functions of childrens' organs are still immature, direct and indirect antibiotic exposure may cause negative effects on the development of their bodies. Aside from clinical and prescribed medication, children also are more likely to ingest antibiotics from meat, eggs, milk, other food, and water pollution. Therefore, the monitoring of level of antibiotics in biological samples has become a major research area.

In this study, we use the SCIEX X500R **Quadrupole-Time of Flight Mass Spectrometer** to build screening and quantitative methods. Urine samples containing more than 200 types of antibiotics from hundreds of children were screened, and the positive samples were quantitatively analyzed.

The X500R **Quadrupole-Time of Flight Mass Spectrometer system** features the world's fastest sampling rate, the intelligent TOF MS-ID / MS / MS sampling mode, and a stable and durable ion source, making it ideal for the analysis of a large number of complex matrix samples (Figure 1). This system truly achieves the goal of instant, comprehensive collection of high-quality primary and secondary mass spectrometry data by needle injection. It allows the target compound to pass the screening of "Four Critical Points": precursor mass accuracy, compound isotope pattern, retention time, and high-precision mass accuracy, providing the most accurate qualitative screening in a timely manner. SCIEX has a professional spectral library with hundreds of antibiotics, which delivers added confirmatory proof of the analyte detected.



In addition, the excellent sensitivity of the SCIEX X500R and unique *Scheduled* MRM^{HR} function is comparable to the quantitative function of triple quadrupole (see Figure 2). The experiment procedures are as follows:

Sample Collection

A total of 114 urine samples were collected. 55 boys and 59 girls from Beijing participated, 12 of whom were 2-5 years old and 110 of were 8-11 years old. Samples were from from morning urine. Samples were immediately stored in a -80 refrigerator after collection.

Pretreatment Method

1. Sample hydrolysis

Urine hydrolysis: 1000 µL juvenile urine, +200 µL ammonium acetate buffer solution (pH 5.0) +15 µL glucuronidase. Allow the mixture to undergo hydrolysis reaction at 37°C overnight. Add 100 µL of McIlvaine-Na2 EDTA buffer to mixture.

2. SPE extraction

1) **Activate:** Water-activate the Cleanert MAS-MIX SPE cartridge (60 mg / 3 mL) using 1 mL of methanol and 1 mL of water.

2) **Sample loading:** Load 1300 µL of urine hydrolysate sample.

3) **Leaching:** Leach with 1 mL of 5% aqueous methanol

4) **Elution:** 2 mL (ammonia: methanol: water) = 5: 85: 10

5) **Dissolution after nitrogen blowdown:** 35 nitrogen blow to dry, 20% methanol dissolved in water to 200 µL, mix with sample.

Aqueous phase conditions

Chromatographic column: Phenomenex Analytical Column, Kinetex 2.6 µm F5 100 Å, 100 X 3.0 mm

Mobile phase Phase A consists of 0.1% formic acid; Phase B consists of 0.1% acetonitrile

Flow rate: 0.4 mL / min

Column temperature: 40ºC

Mass Spectrometry Method

Scanning method: TOF-IDA-MS/MS qualitative screening

Scanning method: *Scheduled* MRM^{HR} positive sample accurate quantification

ESI ion source parameters:

Air curtain air CUR: 30 PSI;
Collision gas CAD: 7
IS voltage: 5500V / -4500V;
Source temperature: 600;
Atomizing gas GAS1: 60 PSI;
Auxiliary gas GAS2: 60 PSI

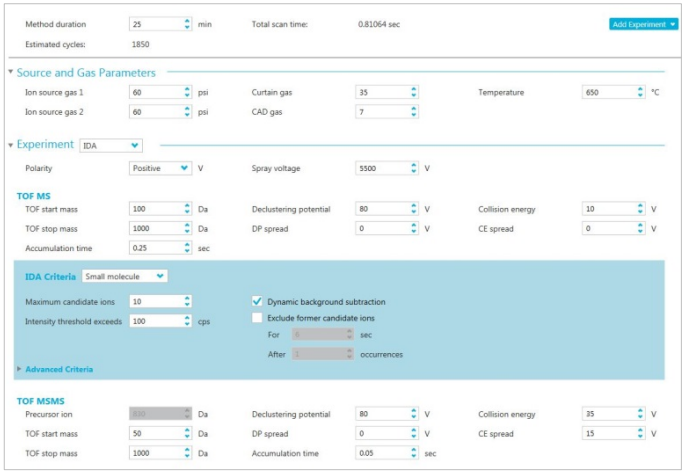


Figure 1 Screening method TOF MS-IDA-MS / MS method editing interface

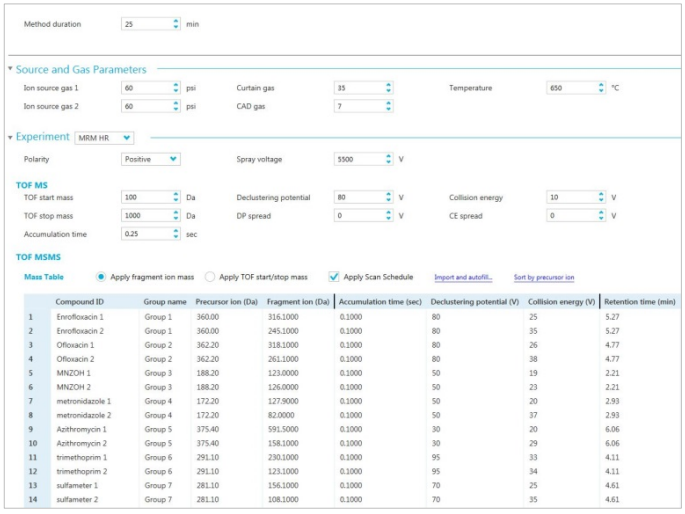


Figure 2 MRM^{HR} quantitative editing interface

Screening of target antibiotics

A total of more than 200 varieties of antibiotics in eight categories were screened in this study including: 8 varieites of penicillins, 38 cephalosporins, 18 macrolide compounds, 10 lincomycin compounds, 18 Tetracycline compounds, 39 quinolones, 41 imidazolium compounds, and 50 sulfonamides and sulfonamide compounds.

Screening test results

The extracted ion chromatogram for more than 200 antibiotic compounds under the above LC-MS conditions (see Fig. 3).

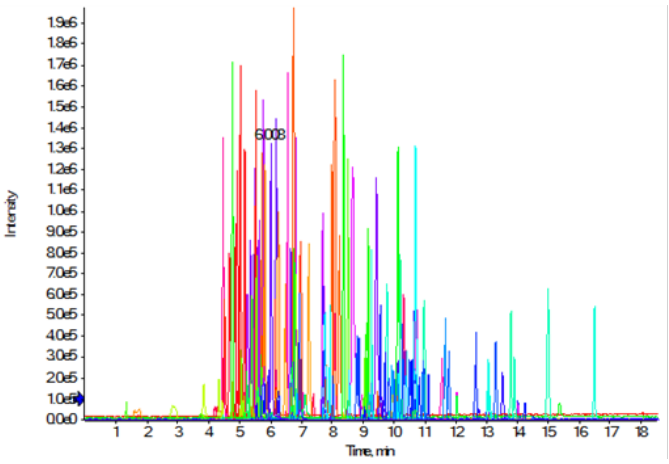


Figure 3 The extracted ion chromatogram for more than 200 antibiotic compounds

SCIEX OS software's integrated interface is highly automated, easy to use and self-explanatory. The software automatically screens target compounds on "Four Critical Points": compound mass error, isotope distribution, retention time and secondary fragments spectral configuration to ensure the accuracy of the results.

As shown in Fig. 4, according to the results shown by the software, azithromycin was detected in several samples. The green check mark on mass error, isotopic distribution, retention time and secondary fragments spectral configuration means they matched well. The mass deviation of azithromycin in several samples was less than 1 ppm, which indicated the instrument had mass accuracy and stability. The match scores between measured second-order spectra and the database were all over 90 points, which indicated that the instrument retained excellent secondary fragmentation performance even with complex matrix samples.

According to the experimental results, eight compounds in four categories were detected in 104 samples (see Table 1). This includes enrofloxacin and ofloxacin in the quinolone category, which were detected, respectively, in 1 and 13 of the samples, accounting for 0.88% and 11.40% of the total sample size,

respectively. Three examples of the sulfonamides category, namely Sulfamonomethoxine, trimethoprim, sulfamethoxazole pyrimidine were found in 5, 11, and 4 of the samples, accounting for 4.39%, 9.65% and 3.51% of the total sample size, respectively. The macrocyclic lactone azithromycin was detected in 11 samples, accounting for 9.65% of the total sample. Metronidazole and metronidazole of imidazole were detected in 12 and 13 samples respectively, accounting for 10.53% and 11.40% of the total sample size respectively.

The detection rate is shown in Fig 6. A total of 39 samples were found to be positive for antibiotics, accounting for 34.2% of the total samples. More than one kind of antibiotic (as many as three) were found in some samples. In some samples, hydroxylmetronidazole was detected. This may be caused by the internal biotransformation of metronidazole.

Table 1 Compounds tested in urine samples and urine sample numbers

Compound Name	Molecular formula	Child urine sample number (#)
Enrofloxacin	*C19H22FN3O3*	*90*
Ofloxacin	*C18H20FN3O4*	*37、38、56、58、63、64、65、66、68、69、85、90、92*
Hydroxymetronidazole	*C6H9N3O4*	*3、4、7、13、16、18、19、31、63、80、81、95、112*
metronidazole	*C6H9N3O3*	*3、4、7、13、16、18、19、31、63、80、95、112*
Sulfamonomethoxine	*C11H12N4O3S*	*19、63、66、68、69*
Trimethoprim (TMP)	*C14H18N4O3*	*55、63、66、68、69、72、80、86、87、90、91*
Sulfameter (SMD)	*C11H12N4O3S*	*63、66、68、69*
Azithromycin	*C38H72N2O12*	*7、8、21、23、41、47、50、57、67、72、74*

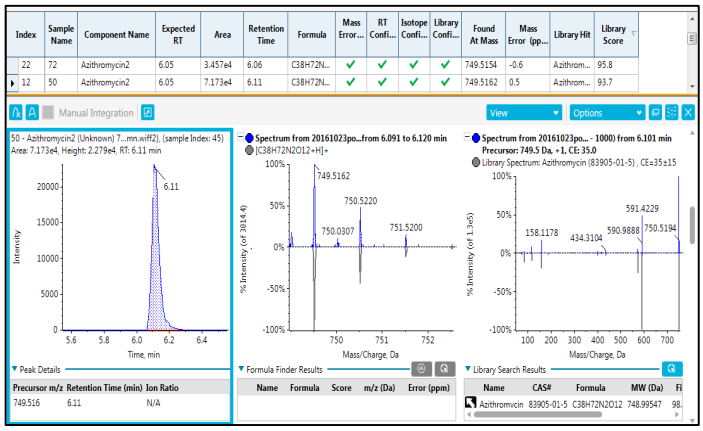


Figure 4 Azithromycin spectra from screening results in some samples

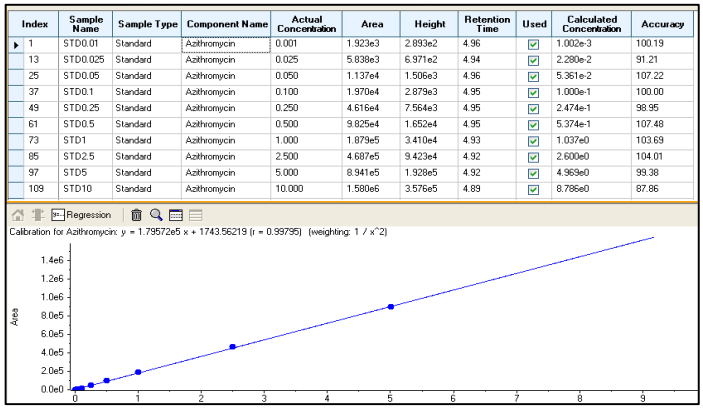


Figure 5 Trimethoprim standard curve and accuracy

Quantitative experimental results

This experiment used *Scheduled* MRM^{HR} scan mode to detect the varieties of compounds to determine the exact quantity. In the case of azithromycin, it was linear from 0.01 ppb to 5 ppb, good in both linearity and accuracy (see Figure 5). Table 2 shows the linear range of other compounds, the range of contents in the sample, and the average content. The distribution of the content of each compound is shown in Figure 7. Some samples contained very high levels of antibiotics. From the results of the survey, of the 39 samples that had positive results, only 5 instances of the samples with azithromycin were derived from prescribed medication. The remaining 34 children (87% of the total samples) had not taken the detected antibiotics within the week prior to sampling, so therefore the antibiotics in these samples likely came from food and water, etc.

Table 2 The linear range of each compound, the range of content in each sample, and the average content

Compound Name	Linear Range (ng/mL)	Content Range (ng/mL)	Average Content (ng/mL)
Enrofloxacin	*0.05-50 ppb*	*0.58*	*0.58*
Ofloxacin	*0.01-50 ppb*	*0.49-56.2*	*10.18*
Hydroxylmetronidazole	*0.5-50 ppb*	*0.73-37.3*	*6.02*
Metronidazole	*0.01-50 ppb*	*0.42-25.5*	*4.87*
Sulfamonomethoxine	*0.01-50 ppb*	*1.33-9.57*	*5.60*
Trimethoprim	*0.01-50 ppb*	*3.45-7.33*	*0.59*
Sulfameter (SMD)	*0.01-50 ppb*	*0.02-4.73*	*1.73*
Azithromycin	*0.01-50 ppb*	*0.02-106.3*	*21.46*

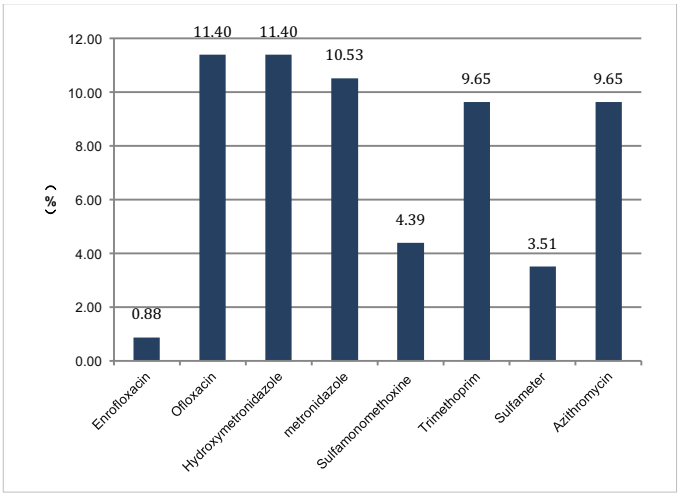


Figure 6 The detection rate of each antibiotic component (%)

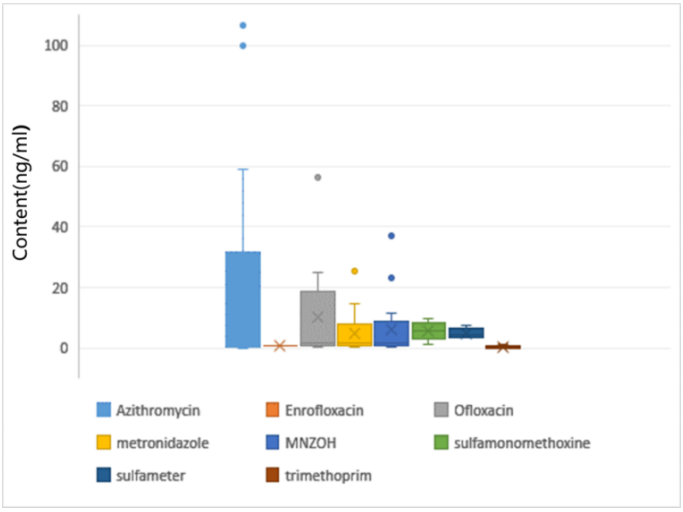


Figure 7 Box plot of each compound content

Summary

1. The combination of the X500R's ultra-fast scanning speed and SCIEX's scanning method with unique dynamic background subtraction and a complete secondary library of antibiotics to ensures that the most comprehensive and high-quality first and second degree mass spectrometry data on complex matrix samples can be obtained with a single injection. This is the foundation and prerequisite of any successful screening experiment.

2. High-quality first and second degree mass spectrometry data on the analyte in complex matrix samples can be obtained with a single injection by passing "Four Critical Points": high precision mass accuracy of less than 1 ppm, isotope pattern, retention time and secondary mass spectra in order to quickly and accurately provide screening results.

3. SCIEX OS Software is highly automated and easy to use, combined with a professional secondary library of antibiotics to ensure efficient and accurate screening.

4. The X500R's mass spectrometer system can provide a ppt level of sensitivity, combined with the unique MRM quantitative mode, making it comparable to the quantitative function of quadrupole MRM.

5. The results of this experiment were from blind screening. The final survey found that some of the samples were from the same household and the same screening results were obtained. Although the surname is different, sample 85 and 92 turn out to be from twins. Likewise, for sample 64 and sample 65, and sample 56 and sample 58, who are siblings. The results of this experiment are exactly the same as the feedback from the parents, which supports the accuracy and reliability of this screening method.

References

1. Hexing Wang, Bin Wang, Ying Zhou, Qingwu Jiang, Rapid and sensitive screening and selective quantification of antibiotics in human urine by two-dimensional ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. Anal Bioanal Chem (2014) 406:8049–8058.

2. Hexing Wang, Bin Wang, Qi Zhao.etc. Antibiotic Body Burden of Chinese School Children: A Mu-ltisite Biomonitoring-based Study. Environmental Science & Technology.

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