Development and validation of a LC-MS/MS method for the determination of multi-class antibiotics in human serum and its application to maternal women exposure

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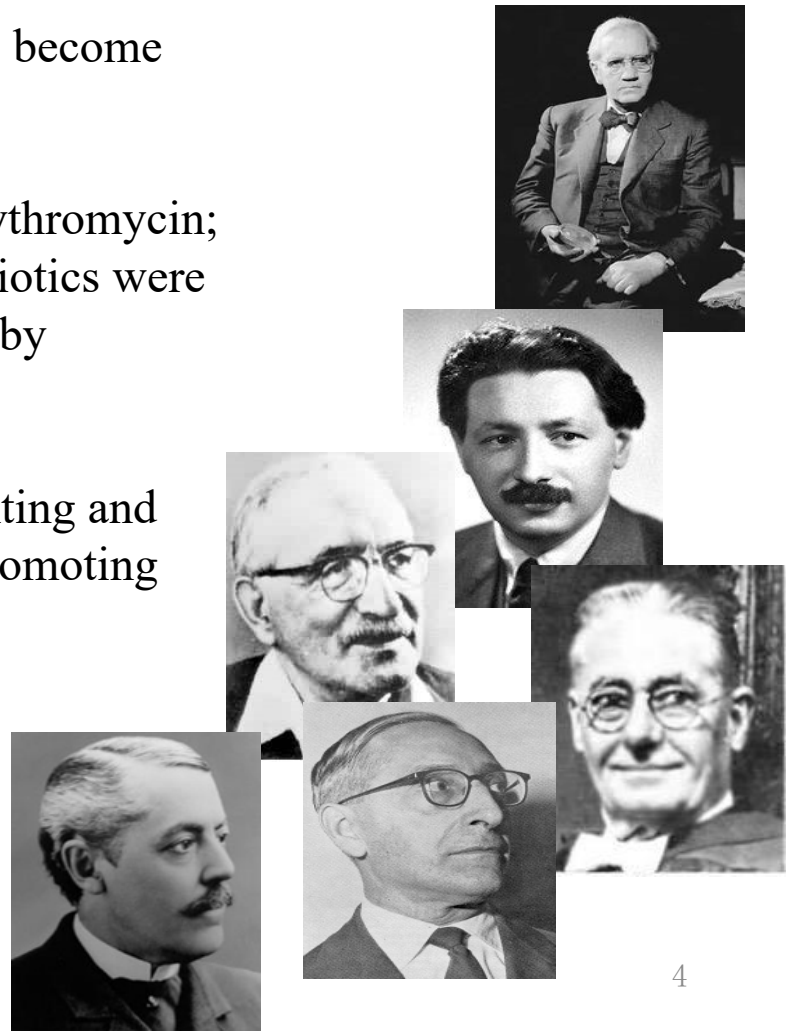
1959-2019

CONTENTS

CONTENTS

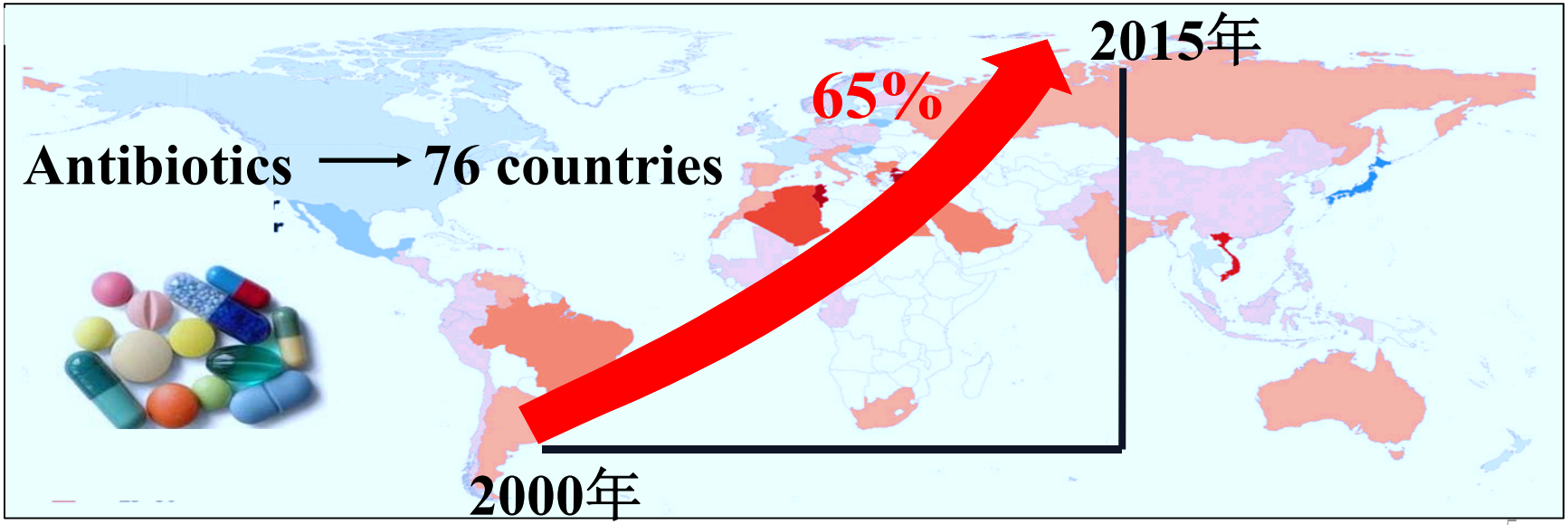
Antibiotics

- Antibiotics are a type of antimicrobial agent used specifically to treat infections.
- since the advent of penicillin in 1929, antibiotics have become the boon for improving human health.
- 1944, streptomycin; 1947, chloramphenicol; 1952, erythromycin; 1953,Tetracycline; 1964, Cefalotin. Most of the antibiotics were discovered in the ‘golden era’ in the 1950s and 1960s by screening soil-derived actinomycetes.
- In addition, antibiotics have also been used for preventing and treating animals and plants infections as well as for promoting growth in animal farming.



Antibiotics usage

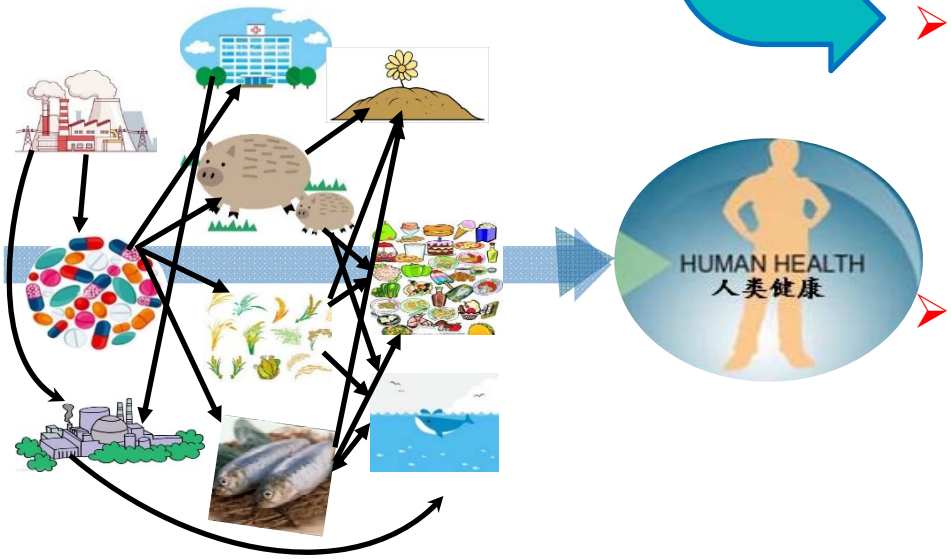
- Antibiotic use has increased markedly. Between 2000 and 2015, global antibiotic consumption increased by 65%, from 21.1 to 34.8 billion defined daily doses (DDS), while the antibiotic consumption rate increased 39% from 11.3 to 15.7 DDDs per 1,000 inhabitants per day over the study period.
- The increase was driven by low- and middle-income countries (LMICs), where rising consumption was correlated with gross domestic product per capita (GDPPC) growth.
- The rapid increase in the use of last-resort compounds,, such as glycylcyclines, oxazolidinones, car-bapenems, and polymyxins, both in HICs and LMICs.



-*Klein E Y et al.* *PNAS, 2018, 15(15):201717295*

Occurrence and risk of antibiotics

- Antibiotics can be more or less extensively metabolized by humans and animals.
- The intensive use of antibiotics for human, veterinary and agriculture purposes, would lead to residual antibiotics can be released into the environment with the air missions and the disposal of large volumes of animal waste.
- These pollutants not only negatively affect the quality of air, surface water, soil, and groundwater, but also pose risks to public health, such as adverse drug reaction (ADR) and antibiotic resistance



- Allergic reaction is one type of ADR and a large proportion of antibiotics have antigenicity and consuming tainted products may cause allergic symptoms.
- Another ADR called “chronic toxicity” is that antibiotics accumulate in the human body and then cause organ lesions through low dose consumption over a long term.

Maternal antibiotics exposure

- Pregnancy women
  - Placental carriers mediate the uptake of hormones and nutrients from maternal blood into the fetus and the removal of metabolites back to maternal blood, and take part in the homeostasis of placental tissue itself.
  - At least eleven antibiotics can enter fetal circulation by crossing placental carriers.
  - The consequences of fetal (and placental) exposure to antibiotics can be benign or involve structural or behavioral teratogenicity, or even termination of pregnancy.
- Lactation women
  - Breastfeeding is extremely important for both the mother and the infant, considering its nutritional, immunological and socioeconomic benefits.
  - Antibiotics with smaller molecular weight and higher pKa have a greater tendency to accumulate as an ionized form in breast milk.
  - The infant gut microbiome is influenced by several factors including genetics, gestational age, mode of delivery, feeding practices (breastfeeding vs formula feeding), and exposure to antibiotics.

Gut microbiome

REVIEW

Interactions Between the Microbiota and the Immune System

doi:10.1038/nature11400

Antibiotics in early life alter the murine colonic microbiome and adiposity

Cell Host & Microbe

Review

Control of Brain Development, Function, and Behavior by the Microbiome

Immune system

It is clear that resident microbes provide signals that foster normal immune system development and influence the ensuing immune responses.

Metabolic disorders

Administration of subtherapeutic antibiotic therapy increased adiposity in young mice and increased hormone levels related to metabolism

Nervous system

Microbiome extends its influence to the brain via various pathways connecting the gut to the central nervous system.

How to detect antibiotics

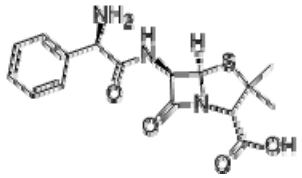
- The determination of antibiotic residue is generally performed by immunoassays, bioassay or HPLC suitable detectors, including UV, fluorescence diode-array.
- Liquid chromatography coupled with mass spectrometry (LC-MS/MS), has become the method of choice for many quantitative analysis applications due to its robustness, high sensitivity, selectivity, and structural elucidation capabilities.
- Most of the methods reported for multi-residue determination focus on closely related compounds usually those belonging to a single class of antibiotics. More recently, new methods have concentrated on multi-class analysis. However, few studies were reported about the multi-residue in human serum.
- Hence, in this study, we developed a more sensitive quantitative LC–MS/MS method that used a triple quadrupole mass spectrometer to evaluate antibiotics exposure level in maternal women.

CONTENTS

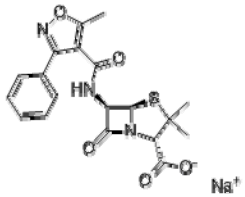
Targeted antibiotics

1959-2019

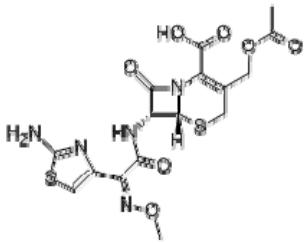
β-lactams



Ampicillin

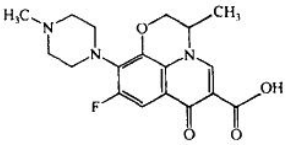


Oxacillin

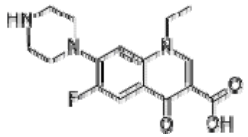


Cefotaxime

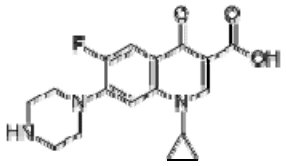
Quinolones



Ofloxacin

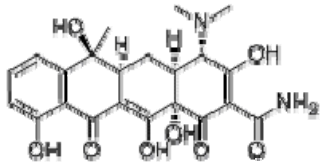


Norfloxacin



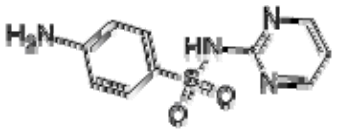
Ciprofloxacin

Tetracyclines

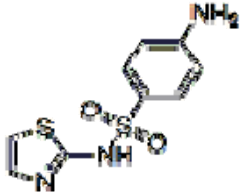


Tetracycline

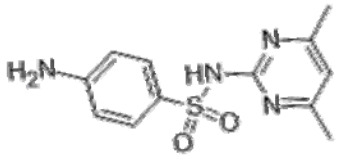
Sulfonamides



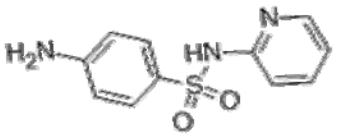
Sulfadiazine



Sulfathiazole

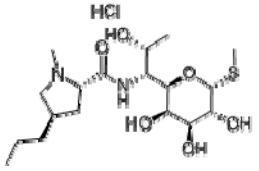


Sulfamethazine



Sulfapyridine

Lincomycin series



Lincomicin

Mass spectrometry

- A triple–quadrupole mass spectrometer (Triple QUAD™ 5500, Applied Biosystems, CA, USA) equipped with a TurboIonSpray™ interface was used.
- Ionization was achieved using electrospray ionization (ESI) source operating in the positive mode and the data were collected in the schedule-MRM mode.
- Source conditions were as follows: ion spray voltage 4.5 kV, source temperature 450◦C, collision gas 7 units, curtain gas 20 psi, nebulizer Gas 50 psi, source gas 50 psi.

Acquisition parameters					
Number	Antibiotic	Q1	Q3	DP	CE
1	Sulfapyridine	250.2	156.1	80	23
2	Sulfadiazine	251.2	156.1	70	22
3	Sulfathiazole	256.1	156.1	60	21
4	Sulfamethazine	279.2	186.1	80	24
5	Norfloxacin	320.4	233.2	110	35
6	Ciprofloxacin	332.4	245.3	110	34
7	Ofloxacin	362.4	318.4	100	28
8	Ampicillin	350.3	160.2	70	19
9	Oxacillin	402.2	160.2	90	20
10	Lincomicin	407.0	126.0	100	38
11	Cefotaxime	456.1	396.2	80	15
12	Tetracycline	445.2	410.1	80	27

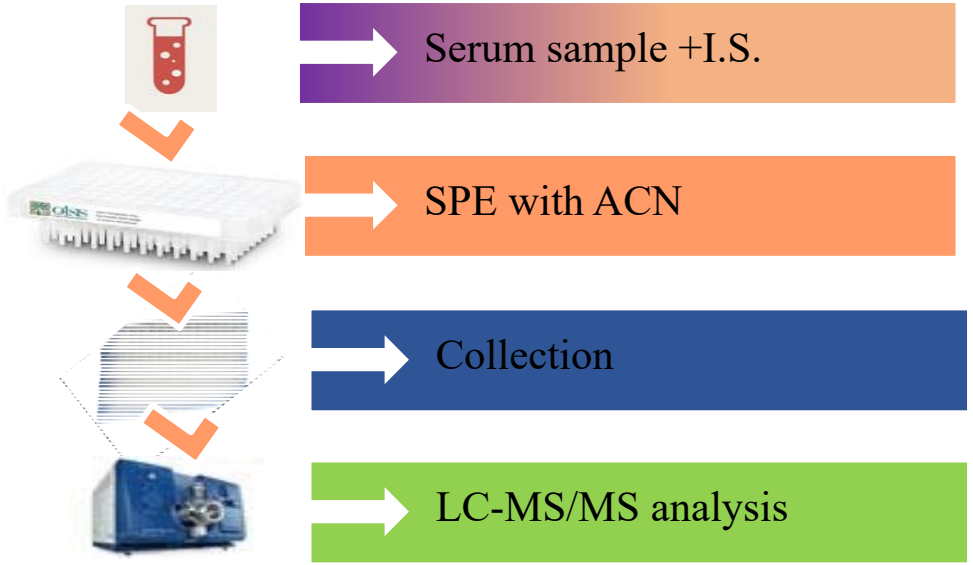
High performance liquid chromatography

- A Shimadzu Nexrea XR Prominence liquid chromatograph (Shimadzu, Columbia, MD, USA) equipped with a refrigerated autosampler and a binary pump system was used for chromatographic separation.
- The autosampler tray was set to 4◦C.
- A CORTECS T3 column 2.7 μm 100 mm×2.1 mm purchased from Waters was used at 35◦C.
- Mobile phases A and B were 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile, respectively, with a combined flow rate of 400 μL/min.
- The gradient started at 5% mobile phase B and ramped to 60% by 5 min, 90% by 5.5 min, and was held at 90% until 6.5 min. For re-equilibration, mobile phase B was decreased to 5% by 6.6 min and held until 8 min.

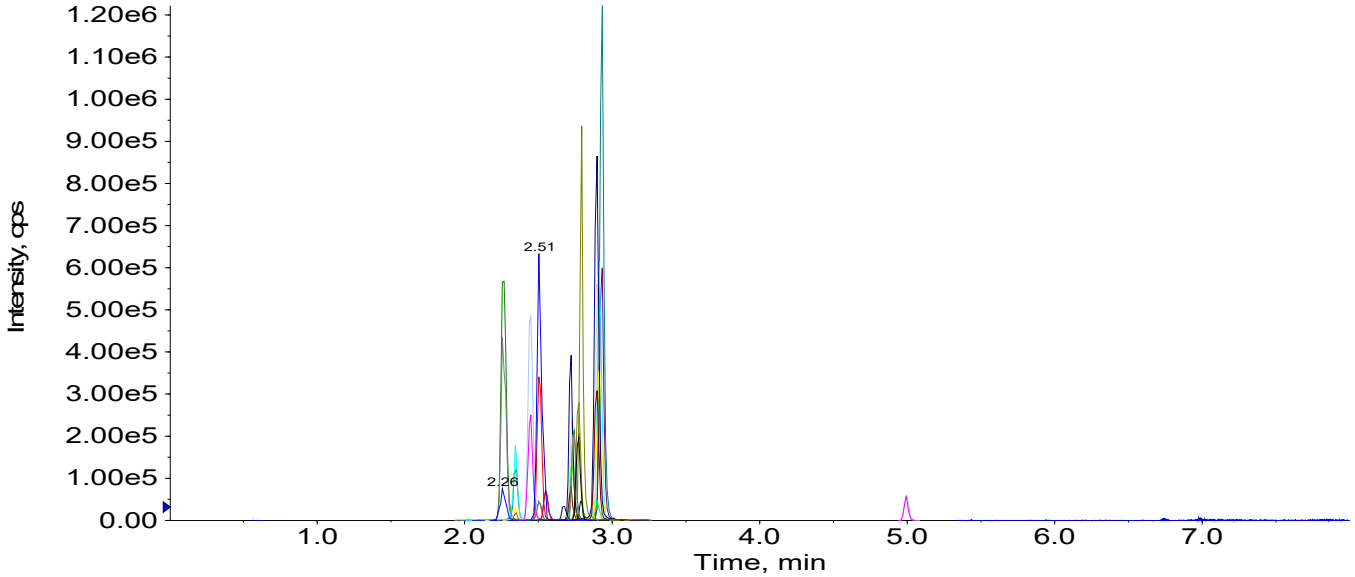
Time（min）	A （%）	B（%）	流速 (μl/mL)
0	95	5	400
5	40	60	400
5.5	10	90	400
6.5	10	90	400
6.6	95	5	400
8	95	5	400

Sample preparation

- The volume of 200 μL of serum mixed with 10 μL of IS and 190 μL 4% Phosphoric acid solution and 300 μL of supernatant were analyzed by solid phase extraction (Waters Oasis HLB μElution Plate).
- Prior to extraction, the cartridge was first washed with water and methanol, respectively.
- After percolation of the whole solution, the columns were washed with 100 μl of water twice to remove the matrix materials from the cartridge and dried for 2 min.
- Finally, the target analytes retained on the columns were eluted with 150μl of 60% acetonitrile, the eluates were collected into a test tube and 2 μL of eluates was injected to LC–MS/MS analysis.



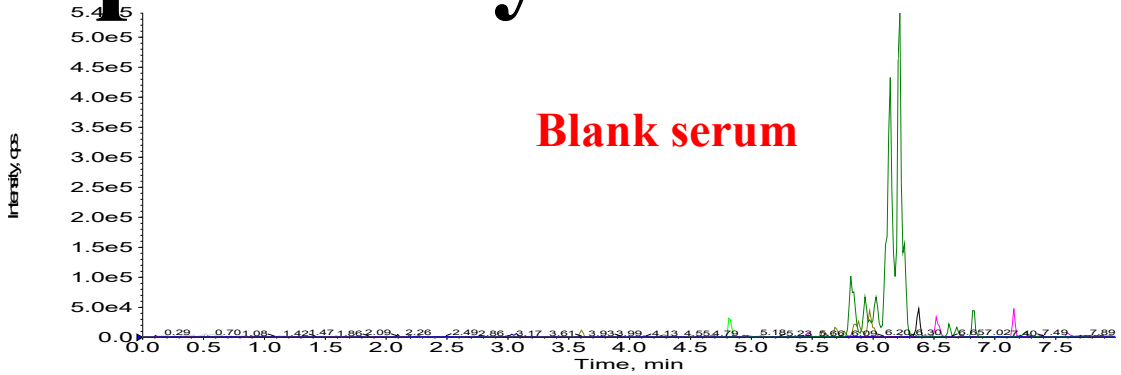
Chromatography

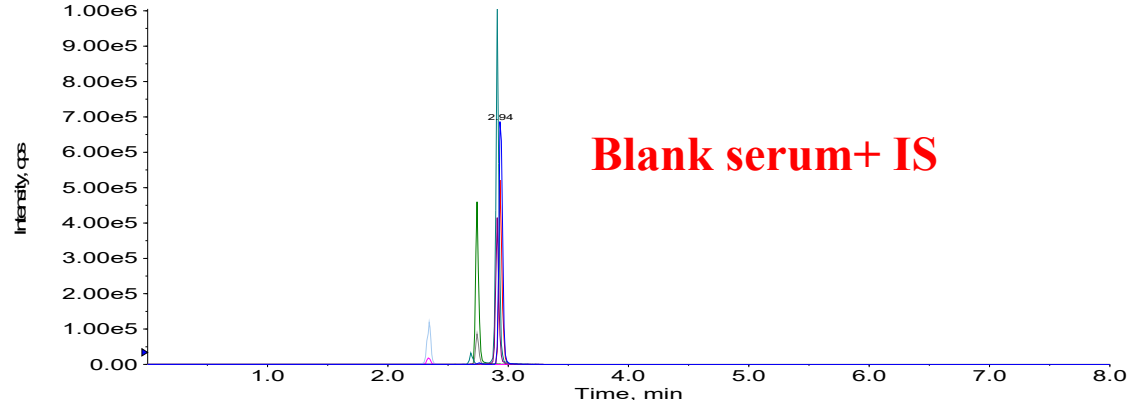


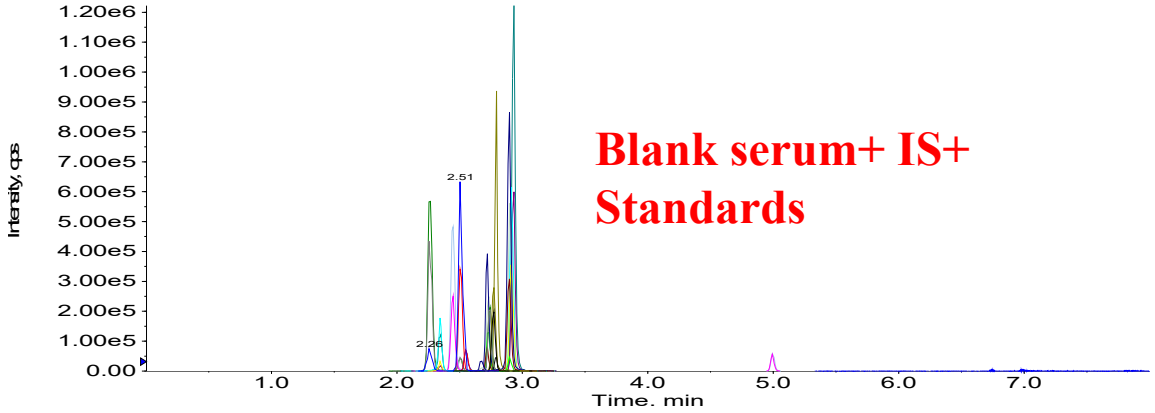
Antibiotic	RT (min)	Antibiotic	RT (min)
Sulfapyridine	2.52	ampicillin	2.58
Sulfadiazine	2.27	Ofloxacin	2.76
Sulfathiazole	2.45	Oxacillin	4.99
Sulfamethazine	2.93	Lincomicin	2.35
Norfloxacin	2.75	Tetracycline	2.92
Ciprofloxacin	2.81	Cefotaxime	2.79

Method validation

Specificity







- The absence of any interfering peaks around the analytes and IS retention times demonstrated the selectivity of the clean up procedure.
- The comparison between the chromatogram of the standard solution with the blank samples highlighted the specificity of the method.

Linearity and sensitivity

Antibiotic/ range (ng/mL)	Regression equation	r^2	LOQ (ng/mL)
Sulfapyridine (0.5 - 50)	y = 0.12047 x + 0.02745	0.995	0.02
Sulfadiazine (0.5 - 50)	y = 0.11701 x + -8.50245e-4	0.998	0.05
Sulfathiazole (0.5 - 50)	y = 0.09688 x + 0.00108	0.995	0.10
Sulfamethazine (0.5 - 50)	y = 0.21379 x + 0.00721	0.997	0.02
Norfloxacin (0.5 - 50)	y = 0.06404 x + -0.00502	0.997	0.05
Ciprofloxacin (0.5 - 50)	y = 0.18253 x + 0.00810	0.993	0.01
Ampicillin (1.0 - 100)	y = 0.02092 x + 0.00580	0.995	1.0
Ofloxacin (0.5 - 50)	y = 0.06899 x + 0.00257	0.991	0.10
Oxacillin (0.5 - 50)	y = 0.00653 x + 2.22603e-4	0.992	0.10
Lincomicin (0.5 - 50)	y = 0.08297 x + 0.00147	0.996	0.20
Tetracycline (0.5 - 50)	y = 0.05962 x + -0.00159	0.999	0.05
Cefotaxime (1.0 - 100)	y = 0.01034x + -8.23402e-4	0.997	0.20

Carryover

- All analytes showed minminimal carryover (< 0.5%)

Accuracy

- Accuracy was determined by fortifying serum matrix at three concentration levels (LQC, MQC and HQC) and analyzing and comparing the calculated and expected concentrations.
- The accuracy at three QC levels of serum ranged from 85.15% to 114.11%.

Precision

- Intra-day and inter-day precision and accuracy were evaluated by analyzing six replicate QC samples at three concentration levels (LQC, MQC and HQC) on the same day and on four consecutive days.
- Precision was determined by the coefficient of variation (%CV) of the analyzed QC's.
- The intra-day and inter-day precision for each analyte at three QC levels of serum ranged from 2.06% to 12.09% and 1.03% to 10.63%.

Matrix effect

- Matrix effect was assessed by calculating the matrix factor for three QC levels in 6 lots of matrix. Matrix factor is calculated as the peak area ratio of analyte in extracted serum (post spiked) to neat samples (mobile phase).
- Matrix factor was within the range of 0.80-1.05 with precisions < 15% for all analytes.

Extract recovery

- Recovery % was determined by comparing extracted samples to samples spiked post extraction at each of the QC concentration levels.
- the mean recovery across the different QCs was 60% and 114% for all analytes and consistent with %CV <15% across the 3 QC, except lincomicin.

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Method validation was demonstrated the reliability of this method for biological samples analysis

CONTENTS

Sample collect and analysis

- Serum samples from 1000 pregnancy women or lactation women were collected from several region of china.
- Before the experiment, the subjects have not been treated with any drugs.
- Serum samples were prepared according to the developed method.
- Data acquisition was carried out using Analyst 1.6 software..
- Raw data files from the LC-MS/MS analysis were processed using MultiQuant™ 3.0.2 software (version 3.0.2, AB SCIEX).
- The absolute concentration of each analyte was calculated according to the calibration curve.

Sample collect and analysis

Compound	Positive samples	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14
Sulfapyridine	5	8.19	-	-	-	2.57	-	>50	-	0.65	-	-	0.91	-	-
Sulfadiazine	7	8.89	8.19	-	-	>50	>50	-	1.34	4.39	-	-	-	2.38	-
Sulfathiazole	2	10.9	-	-	-	-	0.76	-	-	-	-	-	-	-	-
Sulfamethazine	4	6.65	1.56	-	-	-	1.65	-	37.3	-	-	-	-	-	-
Norfloxacin	5	3.37	16.4	2.76	0.95	-	-	-	-	-	-	-	-	-	>50
Ciprofloxacin	2	4.48	-	-	-	-	-	-	-	-	-	>50	-	-	-
Ofloxacin	9	>50	10.4	>50	>50	-	12.6	>50	7.19	-	>50	-	4.05	-	-
ampicillin	1	-	-	-	-	-	-	-	-	-	-	-	-	81.8	-
oxacillin	—	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lincomicin	5	6.36	4.55	3.21	1.51	0.67	3.91	-	-	-	-	-	-	-	-
Tetracycline	6	3.81	-	0.56	0.71	-	-	-	-	>50	-	-	1.8	-	-
Cefotaxime	1	2.58	-	-		-	-	-	-	-	-	-	-	-	-

S means “Sample”, ng/mL.

Sample collect and analysis

- Of all the 1 000 samples analyzed, 14 samples were positive.
- It can be seen that most of antibiotics are quinolones and sulfonamides
- Quinolones are broad-spectrum synthetic antimicrobial agents used in the treatment of livestock and in aquaculture.
- Sulfonamides are bacteriostatics. Sulfonamides are used as veterinary drugs for prophylactic and therapeutic purposes; they also act as growth-promoting substances.
- The presence of quinolones, sulfonamides, tetracyclines, and the antibiotics that are used exclusively for veterinary applications in the serum samples of wmen, could be mainly due to the contaminated environment or food.
- The unintended consumption of antibiotics present in food or in water could lead to allergy and toxicity problems, even transported to fetus or infants.

Comparison

Antibiotic	LC-MS/MS		TRFIA	
	Sample number	Positive ratio	Sample number	Positive ratio
Sulfapyridine	S1, S5, S7, S9, S12	5/14	S2, S7	2/14
Sulfadiazine	S1, S2, S5, S6, S8, S9, S13	7/14	S2, S5, S6	3/14
Sulfathiazole	S1, S6	2/14	S2	1/14
Sulfamethazine	S1, S2, S6, S8	4/14	S2, S8	2/14
Norfloxacin	S1, S2, S3, S4, S14	5/14	S1, S2, S3, S4, S14	5/14
Ciprofloxacin	S1, S11	2/14	S2, S11	2/14
Ofloxacin	S1, S2, S3, S4, S6, S7,S8,S10,S12	9/14	S1,S2,S3,S4,S10	5/14
Ampicillin	S13	1/14	S1，S2，S13	3/14
Oxacillin	-	-	S2	1/14
Lincomicin	S1，S2，S3，S4，S5，S6	6/14	S2，S3	2/14
Tetracycline	S1，S2，S3，S4，S9，S12	6/14	S1，S2，S3，S9	4/14
Cefotaxime	S1	1/14	S2	1/14

- The data obtained from LC-MS/MS didn't completely match with that from TRFIA.
- The positive samples detected by LC-MS/MS were more than that by TRFIA.

CONTENTS

Conclusions

- we developed and validated a sensitive, simple liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) method to determine 12 antibiotics belonging to 5 different groups in human serum.
- The proposed method has been applied to the determination of drug residues for maternal women at low-dose exposure.
- Out of 1 000 samples, 14 samples were positive; Out of 12 selected pharmaceuticals, 11 were detected.
- The unintended consumption of antibiotics present in food or in water could bring negative impacts on health of maternal women, fetus and infants. We should pay attention to the antibiotics in the environment and food.

Acknowledgement

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