Antibiotics in a general population: Relations with gender, body mass index (BMI) and age and their human health risks

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HIGHLIGHTS
• Serum levels of 40 antibiotics were determined in 107 normal adults.
• 28 antibiotics were detected and 5 were above 1000 ng/mL in 3.7% of the samples.
• Males had higher levels of the veterinary antibiotics than females (p < 0.05).
• There were significant BMI-related and age-related differences for sulfonamides.
• No health risks occurred except for azithromycin in one sample.

GRAPHICAL ABSTRACT

ABSTRACT

Recently, increasing regulatory and public attention has been paid to the exposure risks of antibiotics due to their occurrence and antibiotic resistance worldwide. However, limited information on antibiotic levels in general populations is available. Forty antibiotics, including 9 sulfonamides, 5 fluoroquinolones, 4 macrolides, 4 tetracyclines, 3 chloramphenicolcs, 12 β-lactams and 3 others, were analyzed in 107 serum samples of normal adults collected from a hospital in Dalian, North China, between 2015 and 2016 using solid-phase extraction (SPE) coupled with HPLC-MS/MS. The results clearly showed that antibiotics were present in the serum of these adults. Specifically, 28 antibiotics were detected in the samples, with detection frequencies ranging from 0.9% to 17.8%. The total antibiotic concentrations in 26.2% of the serum samples were between the LOD and 20.0 ng/mL. Importantly, the maximum concentrations of 5 antibiotics (trimethoprim, ciprofloxacin, cefaclor, lincomycin and erythromycin) were above 1000 ng/mL in 3.7% of the samples. Furthermore, the detection frequencies of 5 veterinary antibiotics, 7 human antibiotics and 16 human/veterinary antibiotics in the serum samples were 23.4%, 17.8% and 29.0%, respectively. Significant differences of the veterinary antibiotics between female and male adults and of the sulfonamides between different BMI (body mass index) groups were observed (p < 0.05). The concentrations of sulfonamides in elderly individuals were significantly higher (p < 0.05) than those in young people. Finally, our results showed that almost all of the adults had no health risks related to exposure to antibiotics at such levels despite the high effect ratio (ER = 1.74) for azithromycin in one sample. This study is the first to report the current status of antibiotics in human blood, which can help in better understanding the long-term effects of antibiotics on general populations and in identifying susceptible populations that are at high risk to antibiotic exposure.

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1. Introduction

Antibiotics, as a class of pharmaceuticals, are extensively used in humans and animals for disease prevention, treatment and growth promotion (Kemper, 2008; Nathan and Cars, 2014). A high percentage of antibiotics or their metabolites are excreted from the bodies of humans and animals (Bound and Voulvoulis, 2004). These antibiotics are released into the environment via wastewater treatment plants (WWTPs) (Kim et al., 2005; Li and Zhang, 2013) and are thus frequently detected in the surrounding such as in waters, soils and sediments (Gao et al., 2012; Topp et al., 2016). Owing to their high use among human populations, their continuous introduction makes antibiotics “pseudo-persistent” in the natural environment (Gavalchin and Katz, 1994; Hektoen et al., 1995; Kemper, 2008).

Antibiotics are often abused and overused in animal husbandry and aquaculture (Lam et al., 2013; Liu and Wong, 2013), which leads to antibiotic residues in human foods. To date, over 70 antibiotics have been identified in various foods of animal origin, including chicken, pork, beef, and fish products (Chen et al., 2015; He et al., 2012; Yamaguchi et al., 2015). Thus, humans can suffer from antibiotic exposure by dietary intake of these contaminated foods. Furthermore, antibiotics can enter into human bodies via drug abuse (Li et al., 2012), drinking water (Leung et al., 2013; Wang et al., 2016b), and the inhalation of contaminated dust (Hamscher et al., 2003). The issue of antibiotics has repeatedly been reported after the administration of sulfonamides (Wawruch et al., 2002). In addition, evidence has demonstrated that some antibiotics can cause adverse effects on human bodies in the long term, such as childhood obesity (Riley et al., 2013), tooth discoloration (Sanchez et al., 2004), and photobiological damage (Habif, 2006; Wang and Lin, 2012). In addition, antibiotics can lead to the formation of resistant genes of bacteria strains (Zhu et al., 2013), which may ultimately transfer to humans via food chains (Zhi et al., 2016). According to a report by the World Health Organization (WHO), there will be ~1,000,000 deaths annually due to antibiotic resistance by 2050 (WHO, 2014). Therefore, the existence of antibiotics in humans is of great concern to assess the human health risks.

Several studies have demonstrated that antibiotics are present in the urine of Chinese children (Wang et al., 2015; Wang et al., 2016a; Wang et al., 2016b). For example, 18 antibiotics were detected in the urine of Chinese healthy children, and the concentrations of ampicillin reached >40,000 ng/mL in a recent study (Wang et al., 2015). Furthermore, a number of clinical studies have shown that the uptake, volume of distribution, elimination, and associated half-lives of antibiotics in human bodies can be affected by age and gender (Cox and Blaser, 2015; Mueller et al., 2006). In addition, human gut microbiota can be altered after antibiotic treatment, which may lead to a change of human metabolism and body weight (Mikkelsen et al., 2016). Thus, it is plausible that the antibiotic body burden is related to age, gender, and body weight. However, as important biomarkers of many contaminations in human bodies (Bosca, 2012), no information on antibiotic levels in human blood is currently available.

The main objective of the present study is to extensively examine the human body burden of antibiotics in serum from a population of 107 normal adults (55 females, ages: 25–85 years old) in a local hospital in Dalian from November 2015 to February 2016. All subjects participating in this study were volunteers who provided their informed written consent. These blood samples were collected at the same time of one day, place, and preservation conditions. Fasting blood in the morning was required. The biological indexes of the subjects (Table S2), including their age, gender, weight and height, were recorded in the hospital. Five milliliters of blood from a cubital vein were collected in a sterile serum tube (Sani, China) without anticoagulant. The serum was separated by centrifugation at 4000 rpm for 5 min using a high speed centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) and then transferred to another tube. All serum samples were kept at −20 °C until further analyzed.

2. Materials and methods

2.1. Sampling

All blood samples were collected from 107 normal adults (55 females, ages: 25–85 years old) in a local hospital in Dalian from November 2015 to February 2016. All subjects participating in this study were volunteers who provided their informed written consent. These blood samples were collected at the same time of one day, place, and preservation conditions. Fasting blood in the morning was required. The biological indexes of the subjects (Table S2), including their age, gender, weight and height, were recorded in the hospital. Five milliliters of blood from a cubital vein were collected in a sterile serum tube (Sani, China) without anticoagulant. The serum was separated by centrifugation at 4000 rpm for 5 min using a high speed centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) and then transferred to another tube. All serum samples were kept at −20 °C until further analyzed.

2.2. Standards and reagents

Forty antibiotics, including 9 sulfonamides (sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfadimidine, sulfamethoxazole, sulfadimethoxine, sulfamonomethoxine and sulfachlorpyridazine), 5 fluoroquinolones (enoxacin, ofloxacin, ciprofloxacin, norfloxacin and enrofloxacin), 4 macrolides (roxithromycin, erythromycin, azithromycin and clarithromycin), 12 β-lactams (cefotiofur, cephalonium, cefuroxime, cefapirin, cefalexin, cefuqniome, cepfime, cepoferase, cefazoline, cefaclor, penicillin and amoxicillin), 4 tetracyclines (tetracycline, oxytetracycline, chlorotetracycline and doxycycline), 3 chloramphenicol (chloramphenicol, thiamphephiloc and floxenilicin) and 3 others (trimethoprim, rifampin and lincomycin), were purchased from the Dr. Ehrenstorfer Co., Ltd. (Germany). The purity of all of these standards was >98%. The labeled compounds, including sulfamethoxazole-D4, atrazine-D6, trimethoprim-D3, ciprofloxacin-D6, chloramphenicol-D6, and caffeine-15N2, were also purchased from the Dr. Ehrenstorfer Co., Ltd. The stock solutions of 1.00 mg/mL for these standards were prepared in methanol and stored at −4 °C for 6 months.

Methanol and acetonitrile were of HPLC grade from the Tedla Inc. (Ohio, USA). Ammonium formate (analytical purity) was obtained from the Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Formic acid (analytical purity) was purchased from the Kermel Chemical Reagent Co., Ltd. (Tianjin, China). Ammonium hydroxide (guaranteed purity) was purchased from the Guangfu Fine Chemical Research Institute (Tianjin, China). Ultrapure water (18.2 Mt·cm, 25 °C) was obtained with an OkPure water system (Lakie Instrument Co., Ltd., Shanghai, China).
2.3. Sample extraction

The sample extraction was performed by following the same procedure described by Kwok et al. (2010), with minor modifications. Briefly, 1.0 mL of plasma specimens were spiked with 10 ng of surrogate standards (sulfamethoxazole-D₃, atrazine-D₃, trimethoprim-D₃, chloramphenicol-D₃, and ciprofloxacin-D₃), diluted with 1 mL of 1.0 M ammonium acetate (pH = 5) to denature the serum albumin, vortexed thoroughly and kept overnight. The mixture was transferred onto an Oasis HLB cartridge (60 mg, 3 mL; 30 μm) pre-conditioned with 2 mL of methanol and equilibrated with 2 mL of ultrapure water. The cartridge was sequentially washed with 4 mL of ultrapure water to remove matrix interferences and the ammonium acetate buffer. Afterwards, the cartridge was dried under vacuum for 30 min. The retained analytes and surrogates were eluted with 2 mL of methanol containing 5% ammonium hydroxide. The eluent was completely evaporated at 40 °C under a gentle stream of nitrogen. The dry residues were reconstituted with 200 μL of initial mobile phase (95% A: 5% B). Ten nanograms of caffeine-¹⁵N₂ were added. Finally, the solution was filtered by 0.22 μm film and 10 μL of extracts were injected for LC/MS/MS analysis.

2.4. Instrument analysis

Chemical analysis was performed using an Agilent 1100 high performance liquid chromatography-tandem 6410B quadrupole mass spectrometer (LC-MS/MS) equipped with an electrospray ionization (ESI) source using the multiple reaction monitoring (MRM) mode. An XTerra® MS C18 column (2.1 mm × 100 mm, 3.5 μm; Waters, Milford, MA, USA) was used to separate the analytes, and the column temperature was 40 °C. The gradient elution program was initiated with 95% A (0.1% v/v formic acid with 15.9 mmol/L ammonium formate in water, pH = 2.4) and 5% B (methanol/acetonitrile, 1/1 in v/v) and followed by a linear segment with 5–88% A (0–30 min) at a flow rate of 0.3 mL/min. The MRM transitions and fragment voltages, collision energies, and retention times are shown in Table S3. Other MS conditions are: drying gas flow, 8 L/min; drying gas temperature, 350 °C; nebulizer pressure, 38 psi; capillary voltage, 4000 V; delta electron multiplier voltage, 400 V; and collision gas, N₂ at 1.75 × 10⁻² Torr.

2.5. Quality assurance and quality control

An eight-point matrix-matched calibration curve from 0.5 to 2000 ng/mL was prepared for all target analytes. The regression coefficients (r²) ranged from 0.979 to 0.999 (Table S4). The limits of detection (LODs) and limits of quantitation (LOQs) were defined as three times and ten times the standard deviation (SD) of the mean procedural blanks (n = 6), respectively. The LODs of the target antibiotics were 0.0163–1.81 ng/mL (Table S4). To prevent the carryover of analytes from sample to sample, a solvent blank was analyzed before each batch of 12 samples to check all appliances, equipment, and solvents. In addition, one procedural blank was analyzed within each batch to monitor the contaminants. All target antibiotics in blanks were below the LODs. As a check for instrumentation drift in the response factors, a midpoint calibration standard was injected after each batch. The accuracy and repeatability of the overall method were determined by the analysis of six replicates of blank matrices (i.e., 5% fetal calf serum) spiked at 10 and 100 ng/mL. The spiked recoveries of the target antibiotics ranged from 41.3% to 119% (Table S4). The relative standard deviations (RSDs) of the recoveries were 2–24.6%. All samples were spiked with the surrogate standards prior to extraction to assess the recoveries of the target compounds in the serum samples. The surrogate recoveries varied from 69.4% to 119%. To account for the potential loss of analytes during sample preparation, the matrix-induced signal suppression or enhancement in ionization, and the variation in the instrumental response, the concentrations of analytes were quantified with respect to the mass of the respective surrogate standards using the isotope dilution method. The instrumental standard (IS), caffeine-¹⁵N₂, was used to compensate for the variation in the volumes of the final extracts and to check the instrument performance during the whole measurement series. That is, the peak area of the IS was monitored to detect problems in regard to instrumental sensitivity or the injection volume. If the IS area decreased significantly (i.e., a signal reduction of >20% within the same matrix), then the series was stopped, and the instrument was cleaned.

2.6. Health risk assessment of human antibiotic exposure

To assess the human health risks of these adults related to antibiotics, a risk model was improved based on the “read-across” hypothesis, which can be used to assess the potential impacts of human pharmaceuticals on fish in environmental toxicology (Huggett et al., 2003). In this improved model, an effect ratio (ER) value of an antibiotic can be derived from dividing its measured concentration in the blood of one person (C_measured) by its human therapeutic plasma concentration (HTPC, Table S1) and applying an uncertainty factor (UF) of 10, which accounts for the inter-individual variability (e.g., gender, age, lifestyle, nutritional status, pharmacokinetics, and pharmacodynamics) in the general population with antibiotic exposure (Ginsberg et al., 2004; Renwick, 1998). It is stipulated that a drug will result in a pharmacological effect only if its plasma concentration reaches or exceeds the HTPC value (Rand-Weaver et al., 2013). Thus, in the present study, we assumed that a high human health risk would occur when the ER value of an antibiotic for an adult was not < 1.

\[ ER = \frac{C_{\text{measured}}}{HTPC \times UF} \]

2.7. Data analysis

Values below the LODs were treated as zero for the calculation of total concentrations, means, and medians (Thomas et al., 2006). However, when performing statistical analyses, those below the LODs were taken as 1/2 LODs. The data were log-transformed prior to conducting statistical tests. Student’s t-test was used to compare the antibiotic concentrations in different groups (gender, body mass index (BMI), or age) if the data passed the normality test and presented equal variance. If the data were not normally distributed, the nonparametric Mann-Whitney U test was employed for intergroup comparisons. A multiple linear regression model was performed to adjust for potential effects on the relations of the serum concentrations with gender, age, and BMI. All these relations were analyzed based on a small population (n = 44) that excluded the undetected samples. The statistical analyses were conducted using SPSS 19.0 software (IBM Co, Armonk, NY, USA). The level of significance was set to p < 0.05.

3. Results and discussion

3.1. Frequencies and concentrations in serum

Twenty-eight antibiotics from eight categories were detected in the serum samples (Table 1, Fig. S1). The overall detection frequency of these antibiotics was 41.1%, and the detection frequencies of individual antibiotics ranged from 0.9% to 17.8%. Specifically, the dominant categories of detected antibiotics were SAs and FQs, with the detection frequencies of each antibiotic categories being 30.8% (SAs + trimethoprim), 25.2% (FQs), 7.5% (Cfs), 6.5% (Mls), 2.8% (Tcs), 3.7% (lincomycin), and 1.9% (penicillin). Five antibiotics, including sulfamethazine (14.0%), sulfadimethoxine (13.1%), trimethoprim (10.3%), enrofloxacin (17.8%) and ofloxacin (13.1%), were detected in >10% of the serum samples. Among these detected antibiotics, five
antibiotics are used only for animals, seven only for humans, and 16 for both animals and humans in China (Table S1); their detection frequencies in the serum were 17.8%, 23.4%, and 29.0%, respectively (Fig. S1). As a result, we speculated that the study participants were exposed to antibiotics via multiple sources such as dietary intake of contaminated foods and the common use and prescription of antibiotics. For example, the maximum concentration of lincomycin (log_{10}C_{max} = 0.6), a veterinary antibiotic (Kemper, 2008), reached 3.82 × 10^3 ng/mL in the serum of these adults, which indicated that it was mainly from daily dietary intake.

3.2. Relations of antibiotic concentrations with gender, BMI, or age

Fig. 2 shows the geometric means of the concentrations of different antibiotic categories, classified by their antibacterial mechanisms and usages in the detected serum samples in relation to (a) gender, (b) BMI or (c) age group. The concentrations of the veterinary antibiotics in males were significantly higher (p < 0.05) than those in females (Fig 2a). This significant sex-related difference could be affected by age and BMI. To exclude their possible effects, a multiple linear regression model was thus implemented. The result shows that the relation was not significant after adjusting for age and BMI (Table S5). The total antibiotic concentrations in males were also higher than those in females but not significant (p > 0.05). A significant sex-related difference of antibiotics in the urine of children has been previously reported (Wang et al., 2015; Wang et al., 2016a). For example, boys had a significantly higher exposure risk to sulfonamides than girls (Wang et al., 2015). One possible explanation is the different food habits, lifestyles, and morbidities of various diseases between males and females, which will affect the intake of antibiotics. The sex differences in dieting behavior indicate that men consume more calories than women, whereas eating disorders are much more prevalent in females (Rolls et al., 1991), which may unintentionally lower the antibiotic exposure risk of females via intake of various contaminated foods relative to males.

A significant difference (p < 0.05) in the sulfonamides between different BMI groups (BMI < 24 and BMI ≥ 24) was observed, regardless of whether adjusting for gender and age (Fig. 2b and Table S5). Higher levels of antibiotics in the normal weight group (BMI < 24) were also found for the other antibiotic categories, except for the fluoroquinolones, but the differences were not significant (p > 0.05). These differences suggest that antibiotic exposure can affect body weight, which may be due to the influence of different bioaccumulation profiles, exposure pathways or exposure doses. However, we failed to conduct a questionnaire survey on diet and the usage of antibiotics due to the improper or incomplete information. Additionally, more anthropometric data (e.g., percent body fat) is necessary to adequately elucidate the potential association between body weight and antibiotic use in these adults.
The concentrations of different antibiotic categories among different age groups were compared. An age categorization that defined young adults as persons 20 to 59 years of age and older adults as persons 60 years of age and older was used in the present study according to the study by Kuczynska et al. (2001). To explore the age-related change in the antibiotic body burden, the young adults were further divided into three groups by an age range of 10, the standard deviation of the ages.

Fig. 2c shows that different antibiotic categories demonstrated different concentration profiles related to age. There was a significant age-related difference ($p < 0.05$) for the sulfonamides in the serum samples (Fig. 2c and Table S5) even after adjusting for gender and BMI. Moreover, except for the fluoroquinolones, the concentration trends for the other antibiotic categories in the samples first decreased and, then, increased in the 25–60 age range. The levels of the fluoroquinolones in the serum had an increasing trend with increasing ages. Overall, the concentrations for all antibiotic categories, except for the fluoroquinolones and the tetracyclines, were the lowest in the 40 to 50 age group. The antibiotic levels were significantly higher ($p < 0.05$) in the adults in the ≥50 age group than those in the 25–50 age group. A recent finding in the urine of children has also reported that children in the age 10–11 group had a lower antibiotic exposure risk than those in the 8–10 age group (Wang et al., 2015). There may be two reasons for our interesting observation: 1) advanced age accompanied by many physiologic alterations (e.g., lower numbers of gut microbiota and reduced species diversity in the bodies of elderly individuals) may have negative effects on the metabolism and excretion process of antibiotics (Ljungberg and Nilsonnebole, 1987; O’Toole and Claesson, 2010; Woodmansey et al., 2004); and ii) for adults, elderly individuals are more susceptible to respiratory and skin infections due to their declining immune response (Gardner, 1980), which may lead to the consequence that antibiotics are more frequently prescribed to elderly individuals than to the young people. Unfortunately, the history of recent prescribed antibiotic use by this population was not clearly understood.

The detection frequencies of antibiotics in the positive detected samples ($n = 44$) exhibited the same trends as the observed concentration profiles in relation to gender, age or BMI (Fig. S2), and no significant differences were found ($p > 0.05$). The similar sex-related difference in the frequencies of all detected antibiotics in the urine of children was reported by Wang et al. (2015). In addition, we did not analyze the relations of individual antibiotics between different groups because the detection frequencies of individual antibiotics were so low (Table 1) that the statistical results would not be persuasive despite the substitution of non-detectable values with 1/2 LODs and the exclusion of the undetected samples.

3.3. Human risks of antibiotics

Human health risks of antibiotic blood exposure for adults were assessed using the ER values of these antibiotics in the serum. The ER values of twenty detected antibiotics were calculated (Fig. 3) even though 28 antibiotics were detected because the HPC values of the remaining antibiotics were not available. The ERs for these adults ranged from $1.12 \times 10^{-5}$ for cefoperazone to 1.74 for azithromycin. More than 80% of the adults were exposed to these antibiotics at the level of <0.9 μg/kg/day. More than half of the adults were exposed to these antibiotics at the level of approximately 0.5 (geometric mean: 0.6) μg/kg/day, which was much lower than that of children from Shanghai, China (Wang et al., 2016b).

For almost all of the adults investigated, no human health risks to exposure to these antibiotics were identified, even at such exposure levels in their serum. Only azithromycin had a relative high ER value (1.74) in one serum sample, indicating a high risk of pharmacological effects occurring. This finding might be related to its low human therapeutic plasma concentration ($HPC: 0.04 \mu g/mL$) and relatively high half-life ($t_{1/2}: 60$ h) in human blood (Table S1). Another explanation may be the common use and prescription of this drug.

Two limitations of this improved model based on the “read-across” hypothesis should be borne in mind. First, it might not always be the case that a chemical will exert a toxicological response only when above its pharmacological effect concentration, even though the model accounts for inter-individual variability by applying a 10-fold uncertainty factor. Second, the model only assesses human health risks in terms of causing pharmacological effects, without considering microbiome disruption, bacterial resistance or any other adverse effects. Thus, although there are no risks in terms of a therapeutically active dose, there may still be risks related to antibiotic resistance. According to pharmacodynamic models, resistant bacteria can be selected only at concentrations between the minimal inhibitory concentration (MIC) of the susceptible wild-type population (MIC$_{susc}$) and that of the resistant bacteria (MIC$_{res}$) (Dricola, 2003; Dricola and Zhao, 2007). For example, the MICs of tetracycline for the susceptible Salmonella enterica (Var. Typhimurium LT2) wild-type and resistant mutants (resistance marker: Tn10dTeT strains) are 1.5 and 128 μg/mL; respectively, the MICs of ciprofloxacin for Escherichia coli wild-type and one of its resistant markers (gyrA 837L) are 0.023 and 0.38 μg/mL, respectively (Gullberg et al., 2011). Thus, exposure to such antibiotic concentrations may result in enrichment for the resistant mutants of E. coli in 3% of these adults but not for S. typhimurium.
4. Conclusions

Our work is the first to report the human serum concentrations of 40 antibiotics in a general population. These pilot data are very helpful for improving the understanding of the long-term effects of antibiotics on human health and for identifying susceptible populations that are at high risk to antibiotic exposure for regulators. First, the adults in the study population suffered from a multi-source and combined antibiotic exposure, and the maximum concentrations were as high as $4.74 \times 10^3$ ng/ml for erythromycin. Moreover, concentrations of the veterinary antibiotics in males were significantly higher than those in females. Sulfonamides exhibited significant differences between the different BMI groups and age groups. However, more studies are warranted to further clarify the reasons behind the relations of antibiotics with gender, BMI, or age. Finally, there were no health risks for almost all of the adults exposed to these antibiotics although pharmacological effects may occur occasionally. Given that the risks associated with antibiotic resistance are not considered in conventional assessment models, developing new risk assessment methods for antibiotic resistance in human bodies in the future is urgently needed. In addition, some under-predicted risks such as synergistic effects will occur while humans suffer from the combined exposure to these antibiotics. However, the combined exposure risks of these antibiotics in humans also remain to be further elucidated.

Conflicts of interest

The authors have declared that no conflicts of interest exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.04.216.

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