Rapid and Simultaneous Analysis of Multiple Classes of Antimicrobial Drugs by Liquid Chromatography-Tandem Mass Spectrometry and Its Application to Routine Biomedical, Food, and Soil Analyses

Anjali Mishra, Yashpal Singh Chhonker, Amol Chhatrapati Bisen, Yarra Durga Prasad, Sachin Laxman Tulsankar, Hardik Chandasana, Tushar Dey, Sarvesh Kumar Verma, Veenu Bala, Sanjeev Kanjoiya, Sandeep Ghatak, and Rabi Sankar Bhatta*

ABSTRACT: Antimicrobial agents (AMAs) are widely exploited nowadays to meet the high demand for animal-derived food. It has a significant impact on the food chain whose end consumers are human beings. The burden of AMAs on humans comes from either meat or crops cultivated on soil containing high residual antibiotics, which are responsible for the global crisis of antibiotic resistance. Thus, the objective of this study was to design a selective and sensitive liquid chromatography—mass spectrometry (LC-MS)/MS-based simultaneous bioanalytical method for estimation of twenty AMAs in human plasma, raw meat, and soil samples. The selective extraction of all analytes from the above matrices was performed by the solid-phase extraction clean-up method to overcome the interferences. Analytes were separated on a Waters Symmetry Shield C18 (150 × 4.6 mm, 5 μm) column, using an isocratic solvent system of methanol–0.5% formic acid (80:20, v/v) with 0.75 mL/min flow rate. The average extraction recoveries for all analytes in plasma were ranged from 42.0 to 94.0% with relative standard deviations (RSDs) below ±15%. All of the validation parameters are in accordance with the United State Food and Drug Administration (USFDA) guidelines. Moreover, the method was also valid for a broad plasma concentration range and can be proposed as an excellent method for routine pharmacokinetic studies, therapeutic drug monitoring, clinical analysis, and detection and quantitation of AMA remnants in raw meat as a standard quality control test for human consumption.

1. INTRODUCTION
Antimicrobials are commonly prescribed to treat infectious diseases in humans and animals. These are also primarily used as growth promoters in livestock to raise weight to satisfy the increasing demand for animal-derived food as well as for metaphylaxis and prophylaxis of diseases. Antimicrobial use in food animals is expected to rise to 105 596 tonnes worldwide by 2030, whereas it will rise to 82% in India, which was previously 3% in 2010. Among high-income nations, the USA, France, and Italy were the leading consumers of antibiotics in 2015. India, China, and Pakistan have been the main clients of antibiotics among low- and middle-income countries. Despite their advantages, excessive use of AMAs in animal feed and livestock production to meet worldwide requirement in minimal time affects both human and environment. AMA residues spill into the soil not only from farm manure but also from the pharmaceutical industries and research, hospital, and...
surface wastewater. High antibiotic concentrations in soil result in favorable outgrowth of antibiotic-resistant bacteria, leading to change in the sensitivity of whole microbial populations to AMAs. Even very low soil AMA concentrations [below the minimum inhibitory concentration (MIC)] create conditions for genetic modification in bacterial genomes. In soil, autochthonous bacteria may also be a source of environmental resistance genes that can be transmitted to the bacteria that colonize the human body. It leads to the increase of antimicrobial-resistant genes in pathogens having significant public health implications as end users are humans. This is a very serious concern to be addressed soon, as it acts to the emergence of antimicrobial resistance among humans.

AMAs in human food can affect public health including hypersensitivity, gastrointestinal disturbance, tissue damage, neurological disorders, and the development of antibiotic-resistant infections. In the case of critical illness, there is an alteration in the apparent volume of distribution and clearance of antibiotics, which explains the need for therapeutic drug monitoring (TDM).

A range of liquid chromatography–mass spectrometry (LC-MS)/MS methods are available for simultaneous estimation of various antibiotics in surface water, groundwater, and plasma; β-lactam antibiotics, and sulfonamides in environmental water and meat; macrolides in food, biological, and environmental matrices; cephalexins in pork and beef muscle; and tetracycline and sulfonamides in domestic wastewater. However, there were no reports in the literature on simultaneous estimation of multiple classes of antibiotics in the human food chain and environment.

The complex and diversified nature of antimicrobials is a major challenge in the development of a combined analytical method in different matrices like plasma, tissue, and soil. The major challenges are separation modes, detection strategies, various mobile phases, and sample preparation methods, which are not only time-consuming but also filled with multiple sources of errors.

Therefore, our aim is to develop a robust bioanalytical method using liquid chromatography-tandem mass spectrometry to establish a relationship between transfer of antimicrobial residues in food chain from meat and soil to human (Figure 1).

The method was validated for rapid, specific, and sensitive simultaneous determination of 20 marketed antimicrobial drugs in human plasma represented in Figure 2, animal tissue, and soil samples. The developed method had a shorter run time with the isocratic flow for rapid analysis. The analytes are representative of different classes of antimicrobials such as penicillin; cephalosporin; tetracycline; fluoroquinolone; nitroimidazole; sulfonamide; and antimalarial, antiviral, and antifungal agents.

This bioanalytical method can be used in pharmacokinetic trials, TDM, and routine clinical blood analysis, suitable for patients receiving combination of these medications for routine implementation. Furthermore, it can also be used to detect and estimate the concentration of AMAs in food products derived from animals as a quality control measure for human consumption. It is also possible to estimate the continuous exposure of AMAs to soil microbes that may be responsible for the development of resistant strains, which could be pathogenic to human as well as farm animals.

2. RESULTS AND DISCUSSION

2.1. Optimization of LC-MS/MS Parameters

2.1.1. Mass Spectrometric Variables. Several chromatographic and mass spectrometric parameters were optimized to achieve excellent selectivity and sensitivity for analytes. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources were comparatively studied during the method development process, and their selection was done based on their respective sensitivities. It was observed that ESI offers optimum analyte intensity as compared to APCI. The continuous infusion method was adopted for optimizing uniform ionization and fragmentation of analytes and IS using 400 ng/mL mixed stock solution at 10 μL/min rate into the ionization source of a mass spectrometer. The positive (ESI+) mode was found to be more sensitive than the negative ionization mode.

The most abundant precursor/product ions were chosen in multiple reaction monitoring (MRM) acquisition for better sensitivity and less interference. The optimization of compound-dependent parameters such as declustering potential (DP), collision energy (CE), collision cell exit potential (CXP), and entrance potential (EP), was performed. DP was tested between 0 and 400 V and was chosen according to precursor ion sensitivities; CE was tested between 0 and 130 eV and was selected when there was intensified ion production. Analyte response was unaffected by changes in EP and CXP. The compounds’ MRM transitions are listed in Table 1. Full scan spectra of analytes and their proposed fragmentation patterns are shown in the Supporting Information, Figures S1–S20. The ion-source-reliant constraints like source temperature 500 °C, ionization voltage 5500 V, curtain gas (CUR) 20 psi, ion source gas 1 (GS1) 55 psi, and ion source gas 2 (GS2) 50 psi were optimized to get maximum signal intensity for all compounds and IS.

2.1.2. Chromatographic Separation. Liquid chromatographic conditions, especially the mobile phase composition and selection of a suitable analytical column, were optimized to get symmetrical peaks, better resolution, and shorter run time. Various C8, C18, and cyano-bonded (CN) columns were cross-checked for their suitability, versatility, and robustness. The quick and complete chromatographic resolution was achieved on a Waters Symmetry Shield C18 (150 × 4.6 mm, 5 μm) column. Methanol (MeOH) was selected as the mobile phase as it was cheaper than acetonitrile (ACN), and different buffers such as ammonium acetate and ammonium formate with pH range of 3–6 were evaluated for ideal chromatographic resolution. The addition of 0.5% formic acid (FA) in ultrapure water decreases the pH of the mobile phase, resulting in

Antibiotics in livestock production → Animal meat

Farmaceutical → Urinary/Local excreta → Food

Soil → Food → Human infection & health issues → Drug susceptibility

Figure 1. A representative concatenation of antimicrobial residues in food chain.
Figure 2. continued
improved ionization efficiency and intensity of the signal response in ESI+ mode. The motive behind the selection of isocratic mode of elution is to avoid the need of column re-equilibration required in the case of gradient elution. A better chromatogram with a symmetrical peak shape was obtained using methanol and 0.5% FA in ultrapure water (80:20 v/v) at a flow rate of 0.75 mL/min, with a column temperature of 30 °C.

The analytes were not new chemical entities (NCEs); they already exist in the market, so it is better to use phenacetin (PHC) as an internal standard (IS) because it had similar chromatographic behavior and ionization response in ESI to analytes. Moreover, the extraction recovery and stability of PHC were acceptable during the entire analytical process.

Various extraction methods such as liquid–liquid extraction, protein precipitation, and solid-phase extraction (SPE) were evaluated for recovery efficiency. The optimization of the SPE procedure was based on the nature of sorbent, the buffer composition for cartridge conditioning, solvent composition for elution, and washing steps. The analytes were extracted from plasma samples using three materials, namely, silica-based C₈ and C₁₈ phase cartridges. First, the performance of the following three commercial cartridges was compared: C-18, 1 cc (Discovery DSC-18, Supelco); Phenomenex Strata-X
(polymeric-phase sorbent), and Oasis HLB (hydrophilic–lipophilic balanced reversed-phase sorbent). The C-18, 1 cc (Discovery DSC-18, Supelco) and Waters Oasis HLB (1 mL tubes, 30 mg) cartridges were adopted for human plasma and meat samples, respectively. Extraction efficiencies of analytes were found to be very high when methanol was used as an elution solvent. The effect of sample volume (50, 100, 150, 200, and 250 μL) on the extraction recoveries was also examined. In the case of 200 μL sample volume of plasma, the extraction recovery was found to be optimum. Before extraction, FA or ammonium hydroxide was used to test the effect of sample pH on the C18 sorbent partition system at pH values 2.5, 5.0, and 8.0. The effect of pH was evaluated, and analytes were found to be stable under acidic conditions. The effective elution of analytes from the sorbent was ensured by optimizing the desorption conditions like desorbing solvent and its volume required. Initially, different solvent systems [MeOH, ACN, or a mixture of MeOH and ACN (50:50, v/v)], 0.1% FA and the mobile phase [a mixture of MeOH and 0.5% FA (80:20, v/v)] were evaluated to optimize the elution efficiency of the desired analytes using the C18 cartridge.

2.2. Method Validation. 2.2.1. Specificity and Selectivity. The specificity of the method was established by analyzing the blank plasma, meat, and soil samples from six independent sources. Chromatograms of six batches of control drug-free plasma and meat were obtained without co-eluting peaks at the retention time of analytes and IS (>20% and >5% of analytes at ‘LLOQ - lower limit of quantification’). The representative LC-MS chromatograms of plasma spiked with the analytes are shown in Figure 2.

2.2.2. Recovery and Matrix Effect. For all 20 AMAs, the recovery of analytes measured from the low quality control (LQC), mid quality control (MQC), and high quality control (HQC) concentration spiked plasma and meat samples ranged from 42 to 94%. The overall recovery values of IS from spiked plasma and meat (at 400 ng/mL) were 86.23 ± 3.7 and 81.36 ± 4.3%, respectively (data is not disclosed). The mean matrix effect for analytes at LQC and HQC concentrations was < ±15%. This indicates that there was almost no endogenous interference at the retention time of analytes from the matrix (Table 2).

2.2.3. Calibration Curve and Carry-Over. The calibration curve shows reliable linearity and reproducibility over the entire concentration range given in Table 2. The average

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Table 1. Summary of MS/MS Parameters: Precursor Ion and Fragment Ion Retention Time (RT), Declustering Potential (DP), Collision Energy (CE), and Collision Cell Exit Potential (CXP) for Antimicrobial Drugs

| entry | drug | RT (min) | MRM transitions m/z (Q1/Q3) | DP (V) | CE (eV) | CXP (V) |
|-------|------|---------|-----------------------------|--------|---------|---------|
| 1     | ACY  | 1.88    | 226.1/152.1                 | 64     | 17      | 28      |
| 2     | AMB  | 1.37    | 924.6/743.5                 | 86     | 127     | 10      |
| 3     | AMP  | 1.39    | 350.1/106.1                 | 99     | 37      | 10      |
| 4     | ART  | 3.33    | 283.1/209.2                 | 35     | 16      | 10      |
| 5     | CDR  | 1.28    | 365.2/157.9                 | 81     | 19      | 10      |
| 6     | CFT  | 2.49    | 456.1/396.0                 | 85     | 15      | 10      |
| 7     | CEP  | 2.14    | 348.1/158.0                 | 49     | 15      | 10      |
| 8     | CPD  | 1.95    | 558.2/410.1                 | 70     | 37      | 10      |
| 9     | CLO  | 1.52    | 345.1/277.1                 | 26     | 11      | 10      |
| 10    | FLU  | 2.04    | 307.1/238.0                 | 67     | 23      | 22      |
| 11    | FLX  | 1.39    | 310.2/148.0                 | 45     | 13      | 10      |
| 12    | GRI  | 2.32    | 353.1/165.1                 | 85     | 27      | 10      |
| 13    | KET  | 1.30    | 531.2/120.1                 | 126    | 89      | 10      |
| 14    | NAT  | 1.39    | 666.4/503.3                 | 76     | 57      | 26      |
| 15    | NFLX | 1.39    | 296.2/134.1                 | 44     | 7       | 10      |
| 16    | OFL  | 1.29    | 362.1/318.1                 | 86     | 27      | 10      |
| 17    | ORN  | 2.09    | 220.1/128.1                 | 55     | 23      | 10      |
| 18    | PYR  | 1.38    | 249.2/198.2                 | 95     | 15      | 10      |
| 19    | SDX  | 1.99    | 311.1/156.0                 | 95     | 24      | 10      |
| 20    | TET  | 1.37    | 445.1/410.1                 | 51     | 25      | 15      |

Table 2. Calibrations, QC Range, and Mean Extraction Recoveries and Matrix Effect (P, Plasma; M, Meat)

| entry | drug    | calibration range (ng/mL) | LLOQ (ng/mL) | LQC (ng/mL) | MQC (ng/mL) | HQC (ng/mL) | mean extraction recovery (%) | mean matrix effect (%) |
|-------|---------|---------------------------|--------------|-------------|-------------|-------------|-----------------------------|-----------------------|
| 1     | ACY     | 1.56–500                  | 1.56         | 3.12        | 100         | 400         | 79                         | 12                     |
| 2     | AMB     | 3.12–500                  | 3.12         | 6.25        | 100         | 400         | 65                         | 6                     |
| 3     | AMP     | 12.5–500                  | 12.5         | 20.0        | 100         | 400         | 42                         | 10                    |
| 4     | ART     | 0.78–500                  | 0.78         | 1.56        | 100         | 400         | 82                         | 9                     |
| 5     | CDR     | 0.78–500                  | 0.78         | 1.56        | 100         | 400         | 76                         | 3                     |
| 6     | CFT     | 1.56–500                  | 1.56         | 3.12        | 100         | 400         | 69                         | 9                     |
| 7     | CEP     | 3.12–500                  | 3.12         | 6.25        | 100         | 400         | 76                         | 8                     |
| 8     | CPD     | 3.12–500                  | 3.12         | 6.25        | 100         | 400         | 91                         | 7                     |
| 9     | CLO     | 0.78–500                  | 0.78         | 1.56        | 100         | 400         | 94                         | 8                     |
| 10    | FLU     | 0.78–500                  | 0.78         | 1.56        | 100         | 400         | 85                         | 9                     |
| 11    | FLX     | 0.78–500                  | 0.78         | 1.56        | 100         | 400         | 87                         | 6                     |
| 12    | GRI     | 0.78–500                  | 0.78         | 1.56        | 100         | 400         | 73                         | 13                    |
| 13    | KET     | 0.78–500                  | 0.78         | 1.56        | 100         | 400         | 89                         | 11                    |
| 14    | NAT     | 1.56–500                  | 1.56         | 3.12        | 100         | 400         | 69                         | 9                     |
| 15    | NFLX    | 0.78–500                  | 0.78         | 1.56        | 100         | 400         | 85                         | 4                     |
| 16    | OFL     | 3.12–500                  | 3.12         | 6.25        | 100         | 400         | 79                         | 6                     |
| 17    | ORN     | 0.78–500                  | 0.78         | 1.56        | 100         | 400         | 94                         | 4                     |
| 18    | PYR     | 0.78–500                  | 0.78         | 1.56        | 100         | 400         | 87                         | 6                     |
| 19    | SDX     | 0.78–500                  | 0.78         | 1.56        | 100         | 400         | 85                         | 8                     |
| 20    | TET     | 12.5–500                  | 12.5         | 20.0        | 100         | 400         | 63                         | 2                     |
determination coefficient was found to be >0.997. The lowest concentration with relative standard deviation (RSD) < 20% was considered as LLOQ. The analytes did not show any considerable peaks (<20% of the area of LLOQ) in blank samples injected after the upper limit of quantification (ULOQ) samples, indicating no carry-over in the developed method.

### Table 3. Intraday (a) and Interday (b) Precision and Accuracy Data for Plasma

| entry | drug | LQC | MQC | HQC | LQC | MQC | HQC | LQC | MQC | HQC |
|-------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1     | ACY  | 4.6 | 5.9 | 7.8 | 4.5 | 9.2 | 2.6 | 6.9 | 7.5 | 4.7 |
| 2     | AMB  | 8.4 | 7.9 | 3.7 | 4.1 | 6.2 | 3.3 | -7.6 | 2.9 | 4.2 |
| 3     | AMP  | 2.6 | 3.9 | 7.2 | 9.5 | 11.3 | 3.5 | 3.7 | 7.8 | 2.7 |
| 4     | ART  | 5.7 | 3.6 | 5.6 | 11.6 | 8.7 | 9.4 | -4.6 | 6.7 | -7.8 |
| 5     | CDR  | 11.4 | 8.9 | 4.6 | 6.9 | 5.7 | 4.9 | 10.5 | 6.8 | 9.6 |
| 6     | CFT  | 2.7 | 5.8 | 10.2 | 7.9 | 11.2 | 5.6 | 7.8 | -6.8 | 3.6 |
| 7     | CEP  | 7.8 | 6.9 | 8.9 | 13.5 | 5.8 | 7.9 | 4.8 | 12.8 | 5.9 |
| 8     | CPD  | 10.4 | 5.7 | 7.9 | 6.8 | 5.9 | 11.8 | 5.9 | 9.9 | 10.7 |
| 9     | CLO  | 8.6 | 5.6 | 4.7 | 5.4 | 11.4 | 7.8 | 10.4 | 12.6 | 5.7 |
| 10    | FLU  | 5.9 | 10.4 | 14.6 | 4.7 | 5.8 | 6.3 | -12.6 | 5.7 | -6.8 |
| 11    | FLX  | 10.2 | 5.0 | 4.3 | 12.4 | 5.6 | 8.6 | -4.8 | 7.9 | 8.1 |
| 12    | GRI  | 5.4 | 3.9 | 11.8 | 7.3 | 10.1 | 4.7 | 8.9 | 3.5 | 6.7 |
| 13    | KET  | 4.3 | 12.3 | 4.6 | -8.7 | 12.4 | 10.4 | 5.8 | 8.7 | 9.0 |
| 14    | NAT  | 3.8 | 6.9 | 12.5 | 8.9 | 5.8 | 10.4 | 5.9 | -6.5 | 12.5 |
| 15    | NFLX | 8.6 | 7.9 | 8.9 | 10.4 | 5.7 | 10.5 | -9.5 | 5.9 | -10.3 |
| 16    | OFL  | 10.9 | 11.4 | 4.6 | 7.8 | 9.8 | 5.6 | 4.3 | -4.6 | 5.6 |
| 17    | ORN  | 12.3 | 8.6 | -5.6 | -7.1 | -8.3 | -5.6 | -3.4 | 5.6 | 7.8 |
| 18    | PYR  | 10.6 | 5.7 | 14.5 | 8.4 | 10.5 | 6.8 | 10.5 | 9.6 | -8.2 |
| 19    | SDX  | 9.1 | 5.8 | 12.5 | 7.3 | 10.8 | 9.3 | -7.6 | 8.7 | 6.8 |
| 20    | TET  | 9.2 | 7.4 | 13.3 | 10.4 | 8.6 | 7.6 | 9.8 | 7.9 | 8.1 |

Data are shown as the mean values (n = 5).

### Table 4. Intraday (a) and Interday (b) Precision and Accuracy Data in Meat Matrix

| entry | drug | LQC | MQC | HQC | LQC | MQC | HQC | LQC | MQC | HQC |
|-------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1     | ACY  | 0.6 | 2.7 | 11.7 | 4.9 | 10.1 | 12.9 | 6.1 | 3.9 | 2.1 |
| 2     | AMB  | 7.2 | 10.2 | 4.56 | 10.5 | 2.13 | 11.8 | 1.81 | -9.1 | -0.4 |
| 3     | AMP  | 11.9 | 3.3 | 1.9 | 6.53 | 10.5 | 12.8 | 8.7 | 0.96 | 6.1 |
| 4     | ART  | 10.9 | 4.6 | 14.3 | 5.32 | 12.8 | 9.8 | 12.3 | 3.8 | 10.6 |
| 5     | CDR  | 3.8 | 2.5 | 6.8 | 12.9 | 7.9 | 10.8 | 10.6 | 9.6 | -0.9 |
| 6     | CFT  | 10.8 | 4.56 | 13.6 | 3.52 | 8.3 | 2.89 | -7.8 | -1.1 | 8.7 |
| 7     | CEP  | 1.76 | 0.83 | 8.72 | 8.73 | 8.71 | 14.45 | 4.8 | 2.8 | -2.6 |
| 8     | CPD  | 13.4 | 7.91 | 3.42 | 4.11 | 8.85 | 941 | 9.05 | 8.6 | -5.3 |
| 9     | CLO  | 0.92 | 0.75 | 13.2 | 13.3 | 2.98 | 2.58 | -4.4 | -2.4 | -3.8 |
| 10    | FLU  | 12.4 | 14.6 | 5.8 | 6.2 | 6.6 | 14.16 | 8.9 | 12.5 | 6.8 |
| 11    | FLX  | 10.4 | 10.9 | 8.1 | 9.3 | 8.8 | 10.5 | 12.1 | 11.8 | -0.9 |
| 12    | GRI  | 13.3 | 14.6 | 12.9 | 3.5 | 5.04 | 3.8 | 1.52 | 8.4 | 6.8 |
| 13    | KET  | 13.3 | 13.6 | 14.8 | 9.76 | 10.26 | 6.45 | 8.7 | -0.9 | 6.1 |
| 14    | NAT  | 10.4 | 14.9 | 13.9 | 3.5 | 5.8 | 6.94 | 8.9 | 5.76 | 9.8 |
| 15    | NFLX | 12.1 | 11.6 | 12.8 | 7.7 | 9.26 | 5.45 | 4.3 | 14.6 | -4.4 |
| 16    | OFL  | 11.8 | 3.36 | 1.90 | 6.53 | 10.58 | 12.73 | 3.4 | 0.85 | 13.8 |
| 17    | ORN  | 13.5 | 13.1 | 4.56 | 5.16 | 1.18 | 2.71 | 3.8 | -0.4 | -5.9 |
| 18    | PYR  | 13.5 | 14.1 | 13.9 | 13.5 | 7.9 | 9.26 | 10.2 | 9.2 | 9.6 |
| 19    | SDX  | 8.9 | 11.8 | 3.21 | 10.9 | 9.05 | 2.91 | 14.4 | 8.0 | -8.08 |
| 20    | TET  | 10.7 | 11.5 | 6.56 | 11.2 | 1.5 | 2.88 | 10.8 | -0.9 | 0.80 |

Data are shown as the mean values (n = 5).

### 2.2.4. Accuracy and Precision

Tables 3 and 4 summarize the intra- and interday reliability and consistency values for the QC specimens of plasma and meat, respectively. The results showed that the bioanalytical approach produced was accurate because intra- and interday accuracy and percent bias were within acceptable limits of ±20% of the theoretical value at LLOQ and ±15% at remaining concentration levels.

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2.2.5. Stability. Stability studies were performed under different laboratory conditions, and the results are listed in Table 5. Various stability experiments were performed, viz., long-term storage, benchtop, autosampler, dry residue, and freeze/thaw stability, and the mean percentage nominal values of all analytes were calculated to be within ±15% of the estimated concentrations for the analytes at given LQC, MQC, and HQC levels. Thus, all of the analytes were found to be stable throughout the run as the results were within the acceptable limits during the entire validation.

### Table 5. Mean Stability Recoveries of the Antimicrobial Drugs at Different Storage Conditions (P, Plasma; M, Meat)

| entry | drug | mean stability recoveries (%) |
|-------|------|-------------------------------|
|       |      | freeze–thaw (−70 ± 10°C) | long-term storage (−70 ± 10°C, 15 days) | autosampler (4°C, 24 h) | benchtop (20 ± 5°C, 24 h) | dry extract (−20°C, 24 h) |
|       | P    | M    | P    | M    | P    | M    | P    |
| 1     | ACY  | 98   | 93   | 87   | 88   | 87   | 101  | 98   | 96   | 94   | 99   |
| 2     | AMB  | 92   | 91   | 86   | 87   | 94   | 95   | 96   | 94   | 89   | 89   |
| 3     | AMP  | 96   | 95   | 90   | 85   | 92   | 98   | 87   | 82   | 91   | 95   |
| 4     | ART  | 92   | 96   | 88   | 86   | 102  | 105  | 99   | 90   | 89   | 92   |
| 5     | CDR  | 97   | 98   | 89   | 86   | 95   | 98   | 104  | 102  | 93   | 94   |
| 6     | CFT  | 91   | 87   | 94   | 93   | 96   | 98   | 97   | 106  | 108  | 110  |
| 7     | CEP  | 89   | 88   | 97   | 91   | 86   | 92   | 103  | 110  | 95   | 98   |
| 8     | CPD  | 87   | 88   | 88   | 86   | 104  | 95   | 110  | 90   | 93   | 95   |
| 9     | CLO  | 89   | 86   | 92   | 90   | 94   | 96   | 86   | 89   | 86   | 86   |
| 10    | FLU  | 95   | 90   | 89   | 88   | 95   | 94   | 98   | 84   | 93   | 91   |
| 11    | FLX  | 94   | 93   | 94   | 87   | 94   | 90   | 94   | 91   | 94   | 92   |
| 12    | GRI  | 87   | 88   | 87   | 88   | 87   | 88   | 87   | 86   | 87   | 88   |
| 13    | KET  | 93   | 96   | 98   | 90   | 89   | 89   | 89   | 110  | 88   | 91   |
| 14    | NAT  | 86   | 87   | 92   | 94   | 86   | 87   | 95   | 95   | 89   | 91   |
| 15    | NFLX | 96   | 92   | 89   | 88   | 105  | 102  | 95   | 95   | 107  | 100  |
| 16    | OFL  | 98   | 92   | 87   | 86   | 94   | 95   | 105  | 102  | 93   | 99   |
| 17    | ORN  | 91   | 95   | 95   | 94   | 87   | 98   | 96   | 95   | 87   | 88   |
| 18    | PYR  | 95   | 92   | 87   | 88   | 89   | 92   | 91   | 92   | 92   | 95   |
| 19    | SDX  | 94   | 91   | 91   | 92   | 86   | 91   | 92   | 93   | 88   | 94   |
| 20    | TET  | 94   | 91   | 104  | 95   | 96   | 88   | 108  | 99   | 95   | 95   |

### Table 6. Comparative Evaluation of Clinical Dose, C⁰max, LLOQs, and ULOQs of literature data versus experimental data

| entry | drug | reported literature information | experimental data |
|-------|------|--------------------------------|--------------------|
|       |      | clinical oral dose | C⁰max (ng/mL) | LLOQs–ULOQ (ng/mL) | LLOQs–ULOQ (ng/mL) |
| 1     | acyclovir⁵⁹ | 1000 mg/tablet | 4216.5 | 47.6–10 255 | 1.56–400 |
| 2     | amphotericin B³⁰ | 5 mg/kg formulation | 83 000 | 1–2000 | 3.12–400 |
| 3     | ampicillin⁵⁹ | 500 mg/capsule | 5850 | 100–15 000 | 12.5–400 |
| 4     | artemisinin³⁰² | 500 mg/tablet | 4800 | 1.03–762 | 0.78–400 |
| 5     | cefadroxil⁵⁹ | 500 mg/tablet | 15 460 | 5–30 000 | 0.78–400 |
| 6     | cefotaxime³⁴ | 1 g/tablet | NA | 100–50 000 | 1.56–400 |
| 7     | cephalexin³⁵ | 250 mg/tablet | 40 000 | 280–16 000 | 3.12–400 |
| 8     | cefpodoxime proxetil³⁶ | 200 mg/tablet | 2130 | 40–4400 | 3.12–400 |
| 9     | clotrimazole³⁷ | 150 mg/suppository | 0.89 | 0.01563–1.0 | 0.78–400 |
| 10    | fluconazole³⁵ | 12 mg/kg formulation | 15 500 | 10–10 000 | 0.78–400 |
| 11    | fluoxetine³⁶ | 20 mg/tablet | 27 | 0.15–50 | 0.78–400 |
| 12    | griseofulvin⁴⁰ | 500 mg/ tablet | NA | 20–3000 | 0.78–400 |
| 13    | ketoconazole⁴¹ | 200 mg/tablet | 4220 | 2.0–500 | 0.78–400 |
| 14    | natamycin⁵⁶ | 200 mg/kg formulation | NA | 6.5–400 | 1.56–400 |
| 15    | norfloxetine³⁹ | 20 mg/tablet | 8.4 | 0.5–50 | 0.78–400 |
| 16    | ofloxacinn⁵⁵ | 200 mg/tablet | NA | 13–2000 | 3.12–400 |
| 17    | ornidazole³⁹ | 1 g/tablet | 16 185 | 100–24 000 | 0.78–400 |
| 18    | pyrimethamine⁴⁵ | 25 mg/tablet | 598 | 3.12–800 | 0.78–400 |
| 19    | sulfadoxine⁴⁵ | 500 mg/tablet | 106 000 | 3120–140 000 | 0.78–400 |
| 20    | tetracycline⁴⁶ | 300 mg/tablet | NA | 50–6000 | 12.5–400 |

2.3. Occurrence of Antibiotic Residues in Animal Tissue and Soil Samples. LC-MS/MS is widely used for the analysis of biological tissue samples due to its excellent sensitivity and selectivity for determining the residual levels of AMAs. The chromatograms of the reference standards and the calibration range for each of the antibiotics are shown in Figure 2 and Table 2, respectively. The calibration curve of antibiotics showed good linearity ranging from 0.78 to 500 ng/mL. The developed method was found to be more sensitive, accurate, and efficient over previously reported methods for bioanalysis of clinical samples shown in Table 6.
Table 7. Drug Concentration Present per Gram of Tissue (μg/kg)

| sl. no. | sample ID | sample type | acyclovir | amphotericin B | cefpodoxime | clotrimazole | ketoconazole | pyrimethamine |
|---------|-----------|-------------|-----------|----------------|-------------|--------------|--------------|---------------|
| 1       | M77/15    | pig muscle  |           |                |             |              |              | 168.5         |
| 2       | M78/15    | pig muscle  | 4.945     |                |             |              |              | 3.96          |
| 3       | M79/15    | beef muscle |           |                |             |              |              |               |
| 4       | M80/15    | pig intestine |          |                |             |              |              |               |
| 5       | M81/15    | pig kidney  |           |                |             | 2.27         |              |               |
| 6       | M83/15    | chicken muscle |         | 4.915          |             |              |              | 2.185         |
| 7       | M88/15    | pig kidney  |           |                | 196.5       |              |              | 38.15         |
| 8       | M89/15    | pig heart   |           |                |             |              |              | 35            |
| 9       | M91/15    | chicken muscle |         |                |             |              |              |               |
| 10      | M92/15    | chicken muscle |         |                |             |              |              |               |
| 11      | M94/15    | pig muscle  |           |                | 3.85        |              |              |               |
| 12      | M96/15    | chicken muscle |         | 9.7            |             |              |              | 3.36          |
| 13      | M97/15    | pig muscle  |           |                |             |              |              |               |
| 14      | M101/15   | beef muscle |           |                |             |              |              | 3.185         |
| 15      | M102/15   | pig lung    |           |                |             |              |              |               |
| 16      | M107/15   | chicken muscle |         |                |             |              |              | 184           |
| 17      | M111/15   | mutton intestine |      |                |             |              |              |               |
| 18      | M117/15   | chicken intestine |     | 3.645          |             |              |              |               |
| 19      | M131/15   | chicken muscle |         |                |             |              |              |               |
| 20      | M133/15   | pig muscle  |           | 4.765          |             |              |              | 2.1           |
| 21      | M144/15   | pig muscle  |           |                | 182.5       | 80.5         |              |               |
| 22      | M149/15   | pig muscle  |           |                |             |              |              |               |
| 23      | M159/15   | pig muscle  |           |                |             |              |              |               |
| 24      | M171/15   | chicken muscle |         |                |             |              |              |               |
| 25      | M176/15   | chicken muscle |         | 3.28           |             |              |              | 66            |
| 26      | M177/15   | chicken muscle |         |                |             |              |              | 190.5         |

Table 8. Drug Concentration Present per Gram of Soil (ng/g)

| entry | sample ID | ampicillin | cefpodoxime | clotrimazole | norfluoxetine | fluconazole | ketoconazole | ornidazole | tetracyclines |
|-------|-----------|------------|-------------|--------------|---------------|-------------|--------------|-------------|---------------|
| 1     | X1(AP)    | 2.54       | 0.06        |              |               |             |              |             | 0.95          |
| 2     | X2(AP)    |            |             |              |               |             |              |             |               |
| 3     | X3(AP)    |            | 1.70        | 0.76         | 1.7           |             |              |             |               |
| 4     | X4(AP)    | 3.56       |             | 0.71         | 1.41          |             |              |             |               |
| 5     | X5(AP)    |            |             |              |               |             |              |             |               |
| 6     | X6(AP)    |            |             | 0.21         |               |             |              |             |               |
| 7     | L1(AP)    |            |             |              |               |             |              |             |               |
| 8     | L2(AP)    |            |             | 4.53         |               |             |              |             |               |
| 9     | L3(AP)    |            |             | 0.32         |               |             |              |             |               |
| 10    | L4(AP)    |            |             |             |               |             |              |             |               |
| 11    | L5(AP)    |            |             |             |               |             |              |             |               |
| 12    | L6(AP)    |            |             |             |               |             |              |             |               |
| 13    | L7(AP)    |            |             |             |               |             |              |             |               |
| 14    | L8(AP)    |            |             |             |               |             |              |             |               |
| 15    | L1(EP)    |            |             |             |               |             |              |             |               |
| 16    | L1(PF)    |            | 0.42        | 2.78         |               |             |              |             |               |
| 17    | L2(PF)    | 0.32       |             |             |               |             |              |             |               |
| 18    | L2A(PF)   |            |             |             |               |             |              |             |               |
| 19    | 2L1(PF)   | 3.5        | 0.01        | 0.1          | 0.48          | 0.35        | 1.9          | 0.79        |
| 20    | 2L2(PF)   | 1.43       |             | 0.02         | 0.39          | 0.28        | 1.04         | 0.74        |
| 21    | 3L1(PF)   |            |             | 0.73         | 0.03          | 0.30        |              |             |
| 22    | 4L1(PF)   |            |             | 0.61         | 2.02          | 0.05        | 0.42          | 1.04        |
| 23    | 4L2(PF)   |            |             | 0.21         | 0.8           | 0.3         | 0.74          |             |
| 24    | P1(DF)    | 0.61       | 2.02        | 0.34         |              | 0.43        | 0.41         |             |
| 25    | P2(DF)    | 0.7        |             | 0.8          | 3             | 0.3        | 0.74         |             |
| 26    | P3(DF)    | 4.43       |             | 0.43         |              | 0.61        |              |             |
| 27    | P4(DF)    | 0.82       |             | 0.43         |              | 0.41        |              |             |
| 28    | P5(DF)    | 0.27       |             |             |              |             |              |             |
| 29    | C1(DF)    |            |             |             |              |             |              |             |
Analysis of raw meat samples collected from different retail meat shops was carried out successfully, and the results were correlated with standard tolerance limits set by the Food Safety and Standards Authority of India (FSSAI).\textsuperscript{47,48} FSSAI amended the Food Safety and Standards (contaminants, toxins, and residues) Regulations, 2011, to include new tolerance limits for 103 antibiotics and other veterinary drugs in meat and meat products, poultry, and fish.\textsuperscript{47} The amended regulations specify tolerance limits for an additional 76 antibiotics at 0.01 mg/kg; these antibiotics are either prohibited or not intended for use in food-producing animals.\textsuperscript{48} Tables 7 and 8 represent residual levels of antibiotics in raw meat/animal tissue and soil samples, respectively. We found up to 19-fold higher limits of amphotericin in pork and chicken muscle tissues, 8-fold higher limits of cefpodoxime and 3-fold higher limits of ketoconazole in pork tissue, and 6-fold higher limits of clotrimazole and 2-fold increased residual levels of pyrimethamine in chicken tissue samples as shown in Table 7 and Figure 3.

The soil samples were analyzed, and it was found that of the 29 samples, 15 contain antimicrobial residues in the range of 0.01–4.53 ng/g per sample. Ampicillin, cefpodoxime, clotrimazole, norfluoxetine, fluconazole, ketoconazole, ornidazole, and tetracyclines traces were found in the majority of samples. Cedaxrol, cefotaxime, ofloxacin, and griseofulvin were not detected in soil samples listed in Table 8 and shown in Figure 4.

2.4. Applications. At present, antimicrobial agents have been exploited for their benefits in the clinical as well as nonclinical field. Antibiotics are a predominant category, and their irrational use results in drug resistance and the emergence of superinfection in human population. AMAs are being extensively used in animal husbandry, which is indirectly associated with human health. Besides, the biocompost fertilizers prepared from their manure also contain the excreted antibiotic traces, and the same can be found in the crops whose ultimate consumption indirectly affects human health.\textsuperscript{1}

Hence, we have designed a rugged, robust, and universal all-in-one method for the detection of various AMA residues in the human/animal plasma, raw meat, and soil. In the case of any adverse events such as hypersensitivity or drug intoxication caused after consuming the food residues containing AMAs or a combination of multiple drugs, the developed method will serve as a solution for simultaneous analysis of various agents in the blood samples, and this will help in reducing the number of samples processing, time of analysis, and cost. We have analyzed veterinary and soil samples as shown in Tables 7 and 8, which represent remnants of antimicrobials. We were unable to perform experiments on clinical samples due to human ethical approval and unavailability of Biosafety Level-III (BSL-III) facility. However, the detection limit of the method was within the blood levels of AMAs as reported in Table 6. The analysis of such kind of clinical samples can also be performed using a single robust analytical method, which was earlier accomplished by multiple analytical methods. Thus, it could be used for the measurement of AMAs at different time intervals to maintain a constant concentration of medication (TDM) in patients.

The established method can also prove its application in understanding the development of environmental antimicrobial resistance and detection and quantitation of residual AMAs in animal-derived food products as a standard quality control test for human consumption. Numerous reports have shown the effect of antimicrobials on soil and food samples and hence our quantitation method will be helpful to determine practical concentrations and their impact in the field.

3. CONCLUSIONS

In this study, we reported a sensitive, selective, and reproducible LC-MS/MS-based method for simultaneous estimation of 20 multiple classes of antimicrobial drugs in human plasma. In addition, the methods for the quantitation of raw meat and soil samples were also reported. The established method was rapid with approximately 10 min of sample preparation time and 5 min of LC-MS/MS run time; therefore, it has a high-throughput capability. The performance of the validated assay including precision, linearity, and sensitivity was suitable for routine pharmacokinetic and clinical analyses. The method offers a feasible approach for potential pharmacokinetic studies, routine clinical applications, and high-throughput and simultaneous TDM of these drugs.

4. EXPERIMENTAL SECTION

4.1. Materials and Reagents. Acyclovir (ACY), amphotericin-B (AMB), ampicillin trihydrate (AMP), artemisinin (ART), cefadroxil (CDR), cefpodoxime proxetil (CPD), cephalaxin (CEP), clotrimazole (CLO), fluconazole (FLU), fluoxetine hydrochloride (FLX), griseofulvin (GRI), ketoconazole (KET), natamycin (NAT), norfluoxetine hydrochloride...
(NFLX), ofloxacin (OFL), ornidazole (ORN), phenacetin (PHC), pyrimethamine (PYR), sulfadoxine (SDX), tetracycline (TET), formic acid (purity ~98%) for mass spectrometry, high performance liquid chromatography (HPLC)-grade methanol and Discovery DSC-18 cartridges (1 mL tubes, 100 mg, Lot no. 3721501), and Waters Oasis HLB cartridges (1 mL tubes, 30 mg, Lot no. 117A33036A) were purchased from Sigma-Aldrich (St. Louis, MO). Cefotaxime acid (CFT) was procured from LKT Labs, Inc. (USA). HPLC-grade acetonitrile (LiChrosolv) was supplied by Merck Specialities Pvt. Ltd., India. Ultrapure water was obtained from the Milli-Q Plus PF water purification line (Millipore, Billerica, MA). Remaining chemicals were of analytical standard.

4.2. Preparation of Standards and Quality Control Samples. Stock solutions (1 mg/mL) of ACY and AMP were prepared in ultrapure water. Stock solutions (1 mg/mL) of ART, PYR, SDX, CFT, CEP, NAT, ORN, and NFLX were prepared in methanol. The standards (1 mg/mL) of FLX and TET were prepared in acetonitrile (ACN), whereas AMB, FLU, CEP, CPD, CDR, CLO, GRI, KET, and OFL were prepared in dimethyl sulfoxide (DMSO). A mixed stock solution of 20 μg/mL in ACN was prepared from respective mother stock solutions. Regularly, fresh calibration standards (CSS) and QC samples were prepared by adding 10 μL of the mixed stock solution into 190 μL of blank matrix containing PHC (100 ng/mL) as IS to obtain the desired calibration range as mentioned in Table 2.

Three different QC samples, that is, LQC, MQC, and HQC, were prepared daily in multiples of five. All of the mother and mixed stocks were kept at 2–8 °C and allowed to attain room temperature before use.

4.3. LC-MS/MS Conditions. A Shimadzu (Japan) SIL-HTc autosampler was used to inject 20 μL of processed sample aliquots onto a Waters Symmetry Shield C18 column (150 × 4.6 mm², 5 μm) preceded by a guard column. The instrument was equilibrated using an isocratic solvent system consisted of methanol and 0.5% FA in ultrapure water (80:20, v/v) at a flow rate of 0.75 mL/min. The autosampler temperature was kept at 6 ± 2 °C. The mobile phase was filtered through a 0.22 μm membrane filter (Millipore) and ultrasonically degassed 15 min before use. The column oven temperature was kept at 30 °C, and the total run time for LC was 5 min. The rinsing solution was methanol/ultrapure water (80:20, v/v). Certain LC parameters like rinsing amount, rinsing speed, needle stroke, sampling speed, purging time, and rinse dip period were kept at 300 μL, 25 μL/s, 52 mm, 3.0 μL/s, 1.0 min, and 10 s, respectively. To ensure insignificant carry-over effect, the washing mode was set to before and after suction.

The detection of AMAs was carried out on an API 4000 Q-trap mass spectrometer (Applied Biosystems, Canada) using an ESI source in positive mode with gas 1, gas 2, and curtain gas supply set at 60, 50, and 15 psi, respectively. The source temperature and ion spray voltage were set at 500 °C and 5500 V, respectively. The dwell time was 200 ms for the analytes and IS, and nitrogen gas was used as both curtain and collision gas. The MS/MS method was operated at unit resolution in the MRM, using precursor ion—product ion combinations shown in Table 1. Analyte-based parameters such as declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) are given in Table 1. All of the raw data were analyzed with PE SCIEX Analyst software from Applied Biosystems (version 1.4.2).

4.4. Extraction Procedure. 4.4.1. Human Plasma. The extraction of antibiotics from human plasma samples was carried out with SPE using Discovery DSC-18 cartridges. Before extraction, cartridges were preconditioned twice with 2 mL of methanol accompanied by 2 mL of 0.5% v/v FA in ultrapure water aspirating with a Supelco Visiprep equipment. All of the CS and QC samples were diluted with 600 μL of 0.5% v/v FA in ultrapure water, vortexed on a cyclomixer (Spinix Tarsons, India) for 5 min, and loaded on preconditioned cartridges. To suppress the matrix effect, the cartridges were then washed with 1 mL of 5% v/v methanol in ultrapure water, and the solution accumulated in the tubes was discarded. Analytes were eluted in fresh tubes with 2 mL of pure methanol and then dried using a nitrogen dryer (TurboVap) at 50 °C for 30 min. Dried samples were reconstituted with 100 μL of methanol, vortex-mixed, and injected (10 μL) into LC-MS/MS for analysis.

4.4.2. Animal Tissue. Each tissue sample was weighed and diluted with ultrapure water (1:4) to obtain a tissue homogenate using a tissue homogenizer (IKA T18 digital ULTRA-TURRAX, Germany). The extraction of AMAs from animal tissue samples was carried out with SPE using Oasis HLB (1 cc, 30 mg) extraction cartridges. Prior to the extraction, cartridges were preconditioned twice with 2 mL of methanol followed by equilibration with 2 mL of 0.5% FA in ultrapure water. The samples (100 μL) containing IS were mixed with 400 μL of 0.5% FA, vortex-mixed for 5 min on a cyclomixer (Spinix Tarsons, India), and loaded into preconditioned cartridges. Then, cartridges were washed with 1 mL of 5% v/v methanol in ultrapure water to suppress the matrix effect, and the solution collected in the tubes was discarded. Analytes were eluted with 2 mL of pure methanol in fresh tubes and subsequently dried at 50 °C using a nitrogen dryer (TurboVap) for 30 min. Dried samples were reconstituted with 100 μL of methanol, vortex-mixed, and loaded into LC-MS/MS for analysis. We have received 26 raw meat samples, and their estimation for antibiotics is given in Table 7.

4.4.3. Soil. The antimicrobials were extracted from soil samples by dissolving the soil in methanol (1:3 v/v) and kept for overnight shaking (Genei Rocker-100, Bangalore, India). Briefly, 10 g of solid was weighed and dissolved in 30 mL of methanol. The soil was dissolved thoroughly by mixing on a vortex shaker (Spinix vortex shaker, Tarsons, Mumbai, India) for 10 min followed by overnight shaking. After that, soil samples were subjected to centrifugation at 13 752g for 30 min. Approximately, 30 mL of organic solvent was collected from each soil sample and transferred to six glass, Vensil, tubes (5 mL each) for drying using nitrogen gas (TurboVap). Post drying, 500 μL of methanol was added to all Vensil tubes, vortexed, and mixed for 5 min. Finally, concentrated samples were dried and reconstituted with 200 μL of methanol. To remove the presence of any particulate matter that may contaminate the LC-MS/MS system, reconstituted samples were transferred into a 1.5 mL microcentrifuge tube and subjected to centrifugation at 16 502g for 30 min. After that, 50 μL of the supernatant was carefully collected and transferred into LC-MS/MS vials. From this, 10 μL of the sample was injected into the mass spectrometer for analysis. We have received 29 soil samples, and analyte estimates are given in Table 8.

4.5. Method Validation. The developed method was validated according to the US Food and Drug Administration (FDA) bioanalytical method validation guidelines. The
experiments related to human plasma were performed according to the standard protocol approved by the Institutional Ethical Committee vide approval no. XLIX ECM A-/P6. The method was validated on (a) human plasma, (b) meat (the samples containing no traces of antibiotics are considered as blank and tissue validation is performed), and (c) soil; the composition of soil varies with the different geographical locations and hence to prevent the variable matrix effect we cannot validate the soil samples using a specific type of soil; therefore, the method for soil analysis was validated using ultrapure water and methanol.

4.5.1. Selectivity and Specificity. Selectivity was assessed by analyzing six blank matrix samples (each of plasma and raw meat) spiked with analytes and IS. For specificity, six separate lots of blank plasma and meat were tested for interferences at analyte and IS retention times. They were processed as per the extraction method mentioned in Section 4.4.

4.5.2. Sensitivity. The method’s sensitivity was determined in calibration standards from the signal-to-noise (S/N) ratio of analyte response. The S/N ratio should be greater than 3 for a lower limit of detection (LOD) and greater than 10 for LLOQ. The calibration curves were recorded by plotting the ratio of peak area (analytes/IS) versus concentration.

4.5.3. Accuracy and Precision. The intra- and interday accuracy and precision were assessed from replicate analysis (n = 5) of quality control samples containing analytes at different concentrations (LQC, MQC, and HQC). The accuracy was expressed in terms of % bias. The precision was determined with respect to % RSD. The data acceptability requirements included accuracy of ±15% standard deviation (SD) from the nominal values and precision of ±15% RSD except for LLOQ, where accuracy and precision should not exceed ±20%.

\[
\text{%bias} = \left(\frac{\text{observed concentration} - \text{nominal concentration}}{\text{nominal concentration}}\right) \times 100
\]

4.5.4. Recovery and Matrix Effect. The absolute recovery of analytes and IS was calculated by comparing the peak area ratio of QC samples (LQC, MQC, and HQC, n = 5) prepared in plasma and meat with subsequent post-extracted plasma standard QC samples. The recovery was considered to be acceptable if the % coefficient of variation (%CV) was ±20% among the mean recoveries at LQC and HQC levels. During the development of bioanalytical methods, it is difficult to determine the matrix effect of multiple compounds in a single run.\(^{31-35}\) The matrix effect of human plasma components on analyte ionization and IS was determined by comparing the responses of the post-extracted plasma standard QC samples (LQC, MQC, and HQC, n = 5) with respect to the response of analytes from neat standard samples at equivalent concentrations. If the peak area ratio is below 85% or above 115%, a matrix effect is considered.\(^{56}\)

4.5.5. Calibration Curve. The linearity of the method was assessed using plasma, raw meat, and a water/methanol mixture for soil samples spiked with analytes in the concentration range mentioned in Table 2. Five of those linearity curves have been analyzed using the least-squares method. Each calibration curve consisted of a blank sample, a zero sample (blank + IS), and eight nonzero concentrations. The calibration curves were evaluated by best fit for peak area ratio (analytes/IS) versus standard nominal concentrations using \(y = mx + c\) with the 1/x² weighting factor. The calibration curve had to have a correlation coefficient \((r^2)\) of 0.997 or better for analytes. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal values except at LLOQ, which was set at ±20%.

4.5.6. Carry-Over. The carry-over was assessed by injecting two processed blank samples followed by an ULOQ sample (500 ng/mL). The response of the first blank sample should not be more than 20% of the response of a processed LLOQ sample and 5% of the IS.

4.5.7. Stability. Antibiotic stability in plasma and meat was assessed at 4 °C for 72 h. The benchtop stability was evaluated at ambient temperature (20 °C) for 24 h using QC samples in six replicates. The storage stability at -70 ± 10 °C over 15 days was also evaluated. The stability of freeze/thaw was determined after three cycles of freeze/thaw (room temperature to -70 ± 10 °C).

4.5.8. Detection of Antimicrobials Present in Animal Tissue and Soil Samples. Antibiotics are widely used in veterinary medicine; therefore, drug residues can also persist in foods derived from animals, which might also result in destructive effects on human health.\(^{57,58}\) Experimental studies were performed on a variety of animal tissues and soil samples collected by our collaborators from local areas of the northeast regions of India certified by the domestic regulatory agencies. Analysis of each soil sample and the random mixture of the soil samples was performed and, finally, quantitative estimation of the group of AMAs was carried out on the above samples using the highly selective and sensitive LC-MS/MS technique.

4.5.9. Preparation of Standards and Quality Control Samples. A standard stock solution of all of the antibiotics was prepared using the same method as mentioned above in Section 4.2. The CS and QC samples were prepared daily by spiking 5 μL of the mixed stock solution into 95 μL of ACN containing phenacitin (100 ng/mL) as an IS. The range of CS and QC was the same as given in Table 2.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c03863.

MS/MS ion spectra of 20 AMAs showing prominent precursor and product ion peaks (Figures S1–S20) (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**

Rabi Sankar Bhatta — Pharmaceutics & Pharmacokinetics Division, CSIR-Central Drug Research Institute, Lucknow 226031, India; orcid.org/0000-0003-4844-2388; Phone: +91-522 2772974; Email: rabi_bhatta@cdri.res.in, rabi.cdri@gmail.com

**Authors**

Anjali Mishra — Pharmaceutics & Pharmacokinetics Division, CSIR-Central Drug Research Institute, Lucknow 226031, India; Academy of Scientific & Innovative Research (AcSIR), New Delhi 110 001, India

Yashpal Singh Chhonker — Pharmaceutics & Pharmacokinetics Division, CSIR-Central Drug Research Institute, Lucknow 226031, India

Amol Chhatrapati Bisen — Pharmaceutics & Pharmacokinetics Division, CSIR-Central Drug Research Institute, Lucknow 226031, India
Yarra Durga Prasad – Pharmaceutics & Pharmacokinetics Division, CSIR-Central Drug Research Institute, Lucknow 226031, India
Sachin Laxman Tulsankar – Pharmaceutics & Pharmacokinetics Division, CSIR-Central Drug Research Institute, Lucknow 226031, India; Academy of Scientific & Innovative Research (AcSIR), New Delhi 110 001, India
Hardik Chandasana – Pharmaceutics & Pharmacokinetics Division, CSIR-Central Drug Research Institute, Lucknow 226031, India; Academy of Scientific & Innovative Research (AcSIR), New Delhi 110 001, India
Tushar Dey – Division of Animal Health, ICAR Research Complex for North Eastern Hill Region, Meghalaya 793103, India
Sarvesh Kumar Verma – Pharmaceutics & Pharmacokinetics Division, CSIR-Central Drug Research Institute, Lucknow 226031, India; Jawaharlal Nehru University, New Delhi 110001, India
Veenu Bala – Academy of Scientific & Innovative Research (AcSIR), New Delhi 110 001, India; Medicinal & Process Chemistry Division, CSIR-Central Drug Research Institute, Lucknow 226031, India
Sanjeev Kanjoiya – Sophisticated Analytical Instruments Facility, CSIR-Central Drug Research Institute, Lucknow 226031, India
Sandeep Ghatak – Division of Animal Health, ICAR Research Complex for North Eastern Hill Region, Meghalaya 793103, India

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c03863

Author Contributions
◆A.M. and Y.S.C. contributed equally to this work.

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Notes
The authors declare no competing financial interest.

This study was performed in line with the principles of the Declaration of Helsinki. All experiments were performed in agreement with the standard protocol approved by the Institutional Ethical Committee vide approval no. XLIX ECM A-/P6.

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