Article

Development of an Liquid Chromatography–Tandem Mass Spectrometry Method for the Determination of Amoxicillin in Broth Medium and its Application to an In Vitro Pharmacokinetic and Pharmacodynamic Model

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Abstract

A simple, rapid and highly sensitive liquid chromatographic–tandem mass spectrometry (LC–MS-MS) method has been developed and validated for the quantification of amoxicillin in broth—a liquid bacterial culture medium. After appropriate dilution with ultrapure water, broth samples containing amoxicillin and an internal standard (IS) were extracted by acetonitrile and dichloromethane. The extract was injected into the system. The analyte and the IS were separated by a prepacked Atlantis C_18 column using acetonitrile–0.1% formic acid as a mobile phase and detected by selected reaction monitoring in electrospray ionization positive ion mode. The calibration curve of amoxicillin was linear over the concentration range of 0.05–20.00 µg/mL. The mean recovery of amoxicillin from broth was 71.7%, and the intra- and interday precision and accuracies of the assay were within 10%. Amoxicillin was stable in broth for 12 h at room temperature (24°C), for 6.5 months at −80°C and for 24 h after preparation in an autosampler at room temperature. It has been successfully applied to an in vitro pharmacokinetic (PK) and pharmacodynamic (PD) model in which the broth is used for bacterial growth. The method provides high-throughput biological analysis to facilitate the in vitro PK and PD model of amoxicillin.

Introduction

Amoxicillin and amoxicillin–clavulanate are widely used for the treatment of community-acquired respiratory tract infections, especially for those caused by Streptococcus pneumoniae (1–3). We attempted to establish an in vitro pharmacokinetic (PK)/pharmacodynamic (PD) model simulating the PK profile of amoxicillin in humans, determined the effect of amoxicillin on the growth of different resistant strains of S. pneumoniae in the model, thus to assess the fitness of bacteria under the selective pressure of the antibiotic. To validate the concentration of amoxicillin in the model, an accurate, sensitive and repeatable analytic method to determine amoxicillin concentration in broth is required. The concentrations of amoxicillin in broth samples were mainly measured by the microbioassay, which is limited by its time-consuming and labor-intensive (4, 5) measures. Liquid chromatographic–tandem mass spectrometry (LC–MS-MS) provides a highly sensitive, selective, accurate, precise and high-throughput technique to quantitatively measure an analyte. The methods of determining amoxicillin in human plasma, bovine milk and bovine or pig tissue have been reported (6–12). To the best of our knowledge, there is no publication of LC–MS-MS methods on the measurement of
amoxicillin in broth. In our study, we found a poor recovery in determining amoxicillin in broth by applying current LC–MS–MS approaches reported in the literature (6–12). Therefore, we intended to develop a new, simple, rapid and high recovery LC–MS–MS method to measure amoxicillin in broth and apply it to an in vitro PK/PD model.

**Experimental**

**Instrumentation and reagents**

Standards of amoxicillin (85.8% purity) and ampicillin (85.7% purity) sodium salt were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (the lot numbers were 130409–201011 and 130410–200706, respectively). Acetonitrile and formic acid (“high-performance liquid chromatography” grade) were obtained from Sigma-Aldrich Laborchemikalien GmbH (Germany). Dichloromethane (analytical grade) was commercially obtained from Ling-Feng Chemical Reagent Co., Ltd (Shanghai, China). A Milli-Q (Millipore, USA) water purification system was used to obtain ultrapure water for the “high-performance liquid chromatography” analysis. Todd Hewitt broth was purchased from Becton, Dickinson (BD) and Company (USA), and yeast extract was from Oxoid (Hampshire, UK).

**Preparation of stock solution, calibration curves and quality control samples**

Stock solutions of amoxicillin and ampicillin were prepared individually at 1000.00 µg/mL in potassium phosphate buffer (50 mM, pH 6) and stored at −80°C. The working solution of amoxicillin (200.00, 100.00, 50.00, 10.00, 5.00, 1.00 and 0.50 µg/mL) and ampicillin (2.00 µg/mL) as an internal standard (IS) was prepared by diluting the stock solution with ultrapure water. The broth calibration curve samples were prepared by diluting the working solution in the blank broth (Todd Hewitt broth supplemented with 0.5% yeast extract) to achieve concentration levels of 20.00, 10.00, 5.00, 1.00, 0.50, 0.10 and 0.05 µg/mL.

A stock solution for quality control (QC) samples was separately weighted and prepared in the same pattern as the standard stock solution. QC samples were prepared similarly as the calibrator at 16.00, 4.00, 0.15 and 0.05 µg/mL and stored at −80°C.

**Sample preparation**

Samples were diluted 50-fold with ultrapure water. A total of 25 µL of the IS working solution (2.00 µg/mL) was added to 500 µL of the diluted sample. The mixture was deproteinized with 500 µL of acetonitrile. Following being vortexed for 1 min and centrifuged for 5 min at 12,000 rpm, 700 µL of supernatant was transferred into another Eppendorf tube and extracted with 700 µL of dichloromethane. After vortex-mixing for 1 min and centrifugation for 5 min at 12,000 rpm, the upper aqueous layer was transferred to an autosampler vial. The injection volume of the supernatant was 5 µL in all experiments.

**LC–MS–MS system**

A Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer equipped with an electrospray ionization source (San Jose, CA, USA) and an Alliance 2690 “high-performance liquid chromatography” system (Waters, Milford, MA, USA) consisting of a quat pump and an autosampler were used for LC–MS–MS analysis.

Chromatographic separation was achieved using an Atlantis C18 column (50 mm × 2.1 mm i.d.; 3 µm) and protected with a pre-column of the same type (10 mm × 2.1 mm i.d.; 3 µm). The mobile phase was 0.1% formic acid in water with acetonitrile (87:13, v/v) at a flow rate of 0.2 mL/min, and the analytic time was 3.5 min. The autosampler was set at 24°C and the column was maintained at room temperature.

The mass spectrometer was operated in the positive ion mode with selected reaction monitoring (SRM). Nitrogen was used as sheath gas and auxiliary gas, and argon as collision gas. The mass conditions were shown as follows: spray voltage, 3,500 V; capillary temperature, 320°C; sheath gas, 40 arbitrary units; auxiliary gas, 6 arbitrary units; collision pressure, 1.5 mTorr; collision energy, 15 eV for amoxicillin, 18 eV for IS; ion transition, m/z 366.1 → 114.0 for amoxicillin, m/z 350.1 → 192.0 for the IS. Quantitative analysis was performed using the Finnigan LCQuan software (version 2.5.6, Thermo Finnigan, USA).

**Method validation**

The method for the quantitative determination of amoxicillin was validated for its selectivity, linearity, matrix effect, recovery, accuracy, precision and stability according to the FDA guidelines (13).

**Selectivity**

The selectivity was assessed by comparing the SRM chromatograms among six samples of broth prepared individually: blank broth samples and the corresponding broth spiked with amoxicillin at the lowest limit of quantification (LLOQ) concentration to test the potential interference at the retention time of the analyte and IS.

**Linearity and LLOQ**

The linearity of the assay method was determined by plotting the peak area ratios of amoxicillin and the IS versus the amount of amoxicillin in broth on 6 consecutive days. Calibration curves were fitted using least-squares linear regression with a 1/z weighting factor. The LLOQ was defined as the lowest concentration of calibration standards that could be quantitatively determined with precision ±20.0% and accuracy within ±20.0%.

**Matrix effect**

Matrix effect from broth was investigated via comparing the mean peak areas of amoxicillin and the IS versus the amount of amoxicillin in broth at four QC levels (A) with that of aqueous samples (B). Matrix effect was expressed as the ratio that is A/B × 100%.

**Recovery**

The recovery of amoxicillin from the extraction procedure was assessed by comparing the mean peak areas of the extracted broth samples spiked at four QC levels and the samples spiked in the mobile phase. The procedure was run in six replicates.

**Accuracy and precision**

The intraday accuracy and precision were evaluated by quantifying amoxicillin at four QC levels in six replicates on the same day. The interday accuracy and precision were evaluated by testing four QC samples on 6 different days. The accuracy was expressed as relative error (RE, %) of the tested concentration over the nominal concentration and the precision was defined as the relative standard deviation (RSD, %). The intra- and interday accuracies from ±15.0% for QC samples at three levels and ±20.0% for LLOQ samples were accepted.
criteria. While for QC samples at three levels and LLOQ samples, the accepted criteria for intra- and interday precision were not above 15.0 and 20.0%, respectively.

Stability
The stability of amoxicillin stock solution was evaluated at −80°C for 6.5 months. Amoxicillin stability in broth at room temperature for 12 h, post-preparative stability for 24 h in the autosampler at 24°C, freeze–thaw stability (three cycles) and long-term stability at −80°C were investigated by using four QC samples. The stability was evaluated by the comparison with the response of freshly prepared samples, and the analyte with a recovery ranging from 85.0 to 115.0% was regarded as stable.

Application of the method to an in vitro PK/PD model
Eventually, we established the in vitro PK/PD infection model as described by Liang et al. (14). The system consists of a fresh medium reservoir, absorption compartment, central compartment and liquid

Figure 1. Chromatograms of amoxicillin in broth samples. (A) Blank broth sample and (B) spiked broth sample with AMO (0.05 µg/mL) and IS (AMP, 0.10 µg/mL). (C) A 2-h broth sample from an in vitro PK/PD model after administration of 500 mg of AMO. AMO, amoxicillin; AMP, ampicillin. This figure is available in black and white in print and in color at JCS online.
waste storage compartment. Amoxicillin PK profiles after a single oral dose of 500 and 1,000 mg of amoxicillin in healthy volunteers were simulated in the model (15, 16). The new analytic method was applied to validate the simulation in vitro. The model was placed in a 35°C incubator. Amoxicillin was added into the absorption compartment, freshly prepared bacterial suspension was injected into the central compartment and the broth was used as the growth medium for bacteria exposed to the simulated infection site concentration. The broth samples were collected from the central compartment at 0, 0.5, 1, 2, 3, 4, 6 and 8 h following the administration of amoxicillin. The samples were centrifuged for 5 min at 12,000 rpm, and the supernatant was transferred into Eppendorf tube and stored at −80°C for analysis.

Results

Optimization of LC–MS-MS conditions

The LC–MS-MS conditions were optimized to achieve a maximum signal response, high sensitivity, good resolution and symmetric peak shape of amoxicillin and the IS. To optimize the MS parameters, the standard solution was infused into the MS instrument. Both the positive and negative ion modes were evaluated and the stronger response of these two analytes was acquired in the positive ion mode. The SRM transitions were selected at m/z 366.1 → 114.0 and m/z 350.1 → 192.0 for amoxicillin and IS, respectively. The separation was tested for various reverse phase columns (Waters Symmetry Shield RP18 50 mm × 2.1 mm i.d., 5 µm; Waters SunFire C18 100 mm × 2.1 mm i.d., 5 µm; Waters X Terra MS C18 50 mm × 2.1 mm i.d., 5 µm and Waters Atlantis C18 columns 50 mm × 2.1 mm i.d., 3 µm). An Atlantis C18 column (50 mm × 2.1 mm i.d., 3 µm) was chosen as a result of the good retention of amoxicillin and the IS as the relative polar compounds on column. After comparison of 0.1% formic acid in water and acetonitrile at different compositions (90:10, 88:12, 87:13 and 85:15, v/v) in isocratic and gradient elutions, formic acid (0.1%) in water with acetonitrile (87:13, v/v) in isocratic elution was used as the mobile phase, achieving an excellent sharp peak and short analytic time. The retention time was found to be 1.29 min for amoxicillin and 2.62 min for the IS.

Method validation

Selectivity

To test the selectivity, blank broth and broth spiked with amoxicillin samples were compared. Figure 1 shows that the typical SRM chromatograms of a blank broth sample (A), a broth spiked with amoxicillin (0.05 µg/mL) and the IS (ampicillin, 0.10 µg/mL) (B), and a broth sample recovered from a PK/PD model (C). There was no substantial interference observed from broth matrix at the retention time of the analyte and IS.

Table I. Matrix Effect of Amoxicillin and Ampicillin (IS)

<table>
<thead>
<tr>
<th>Nominal concentration (µg/mL)</th>
<th>Blank broth</th>
<th>Bacterial suspension in broth</th>
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<tbody>
<tr>
<td>Amoxicillin (n = 6)</td>
<td>16.00</td>
<td>83.9 ± 3.5 80.0 ± 2.5</td>
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<tr>
<td>4.00</td>
<td>79.1 ± 7.6 83.4 ± 7.4</td>
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<tr>
<td>0.15</td>
<td>77.7 ± 8.7 79.4 ± 5.9</td>
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<tr>
<td>0.05</td>
<td>88.4 ± 6.3 77.8 ± 3.9</td>
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<tr>
<td>Mean</td>
<td>82.3 ± 6.5 80.2 ± 4.9</td>
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</tr>
<tr>
<td>Ampicillin (n = 6)</td>
<td>2.00</td>
<td>99.4 ± 4.5 96.7 ± 2.5</td>
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</table>

Linearity and LLOQ

Linearity was observed in the range of 0.05–20.00 µg/mL by employing six calibration standards. A good linear relationship between a peak area ratio (the analyte/IS) and amoxicillin concentration was found based on the best fit and least-squares residuals with a weighting factor of 1/x². The linear regression equation was obtained from the mean of six calibration curves for amoxicillin: y = −0.002986 + 0.160667x, R² = 0.9988.
The LLOQ was 0.05 µg/mL for amoxicillin. The typical chromatogram from the LLOQ sample is presented in Figure 1B.

Recovery and matrix effect
The mean extraction recovery of amoxicillin at the four different concentrations of the QC samples was 71.7 ± 4.5% (RSD = 6.7%). Matrix effects from blank broth and bacterial broth were both evaluated (Table I). There was no strong impact of two different matrices on amoxicillin and the IS.

Accuracy and precision
The intra- and interday precisions were performed. The RSD (%) did not exceed 15.0%. The intra- and interday precisions were in the range of 2.2–8.2 and 0.9–4.5%, respectively, at the four QC levels. The intra- and interday accuracies evaluated by the RE (%) were within acceptable limits of quantitative analysis, ranged from 2.2 to 8.3% and from 0.9 to 4.4%, respectively (Table II).

Stability
The stock solution of amoxicillin was kept stable after stored at −80°C over 3 months. Amoxicillin was stable in broth for 12 h at room temperature and 24 h at room temperature after preparation. It was also kept stable after three freeze–thaw cycles and stored frozen at −80°C for 6.5 months. The results of the stability of amoxicillin under various storage conditions are given in Table III.

Application for an in vitro PK/PD model
The peak concentration (C\text{max}) and the time to reach C\text{max} (T\text{max}) were obtained directly. Other PK parameters were calculated using compartmental analysis by WinNonlin 5.2.1 program (Pharsight, CA, USA). The area under the concentration–time curve during 0–8 h interval (AUC0–8) was estimated by the trapezoidal method. The area under the concentration–time curve from time 0 to infinity (AUC0–\infty) was calculated as the sum of AUC0–8 and C\text{last}/K\text{e}, where C\text{last} is

<table>
<thead>
<tr>
<th>Table II. Intra- and Interday Precision and Accuracy of Amoxicillin</th>
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<tr>
<td>Nominal concentration (µg/mL)</td>
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<tr>
<td>Intraday (n = 6)</td>
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<tr>
<td>16.00</td>
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<td>4.00</td>
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<td>0.15</td>
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<tr>
<td>0.05</td>
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<tr>
<td>Interday (n = 6)</td>
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<tr>
<td>16.00</td>
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<tr>
<td>4.00</td>
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<td>0.15</td>
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<td>0.05</td>
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<th>Table III. Stability of Amoxicillin in Broth Samples</th>
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<tr>
<td>Nominal concentration (µg/mL)</td>
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<tr>
<td>Long-term stability (6.5 months, −80°C)</td>
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<td>---------------------------------</td>
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<tr>
<td>16.00</td>
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<td>4.00</td>
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<td>0.15</td>
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<td>0.05</td>
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<th>Table IV. PK Parameters of Amoxicillin Recovered from an In Vitro PK and PD Model</th>
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<td>Dose (mg)</td>
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<td>C\text{max} (µg/mL)</td>
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<td>AUC0–8 (µg h/mL)</td>
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<td>AUC0–\infty (µg h/mL)</td>
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<tr>
<td>t\text{1/2} (h)</td>
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<tr>
<td>Vd/F (L)</td>
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<tr>
<td>CL/F (L/h)</td>
</tr>
</tbody>
</table>

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Figure 2. Typical in vitro simulated concentration–time profile of amoxicillin after a single oral dosing of 500 mg (filled square box) and 1,000 mg (open circle), respectively. The inserted graph was a partially enlarged view presented at 0–2 h following the administration of AMO.
the last observed concentration and \( K_e \) is the terminal elimination rate constant. The terminal half-life (\( t_{1/2} \)) was calculated from \( \ln 2/K_e \) and the apparent volume of distribution (\( V_d/F \)) was calculated from \( \text{Dose} / (K_e AUC_{0-\infty}) \). The apparent plasma clearance (\( CL/F \)) was calculated from \( \text{Dose} / (AUC_{0-\infty}) \). All simulated concentrations of amoxicillin in broth were within the linear range. The PK parameters are given in Table IV, and Figure 2 shows the mean concentration–time curve of amoxicillin in an in vitro PK/PD model simulating clinical PK profiles after a single oral dose of 500 and 1,000 mg, respectively.

**Discussion**

To the best of our knowledge, there is no publication on the measurement of amoxicillin in broth. We describe a simple, excellent sensitivity, precision and accuracy LC–MS–MS assay for the amoxicillin in broth. An Atlantis C18 column (50 mm x 2.1 mm i.d., 3 μm) was chosen as a result of the good retention of amoxicillin and the IS as the relative polar compounds on column. Formic acid (0.1%) in water with acetonitrile (87 : 13, v/v) in isocratic elution was used as the mobile phase achieving an excellent sharp peak and short analytic time. The same mobile phase composition was reported by De Baere and De Backer (12) for amoxicillin concentration determined in animal feed. Both the positive and negative ion modes were evaluated and the stronger response of these two analytes was acquired in the positive ion mode, which was similar to the results reported by Straub and Vojvdker (17) that amoxicillin had a low signal in the negative mode.

In our study, we tried some sample preparation approaches reported in the literature, but the recoveries of amoxicillin were ≤10.0%. Liquid–liquid extraction methods were tested using diverse organic solvents with ethyl acetate–acetonitrile (3 : 1, v/v), ethyl acetate–isopropanol (2 : 1, v/v), ethyl acetate–methanol (3 : 1, v/v), ethyl acetate, acetonitrile and methanol, respectively. After shaking for 10 min, the samples were centrifuged at 12,000 rpm for 5 min. The upper layer was evaporated to dryness under a gentle stream of nitrogen gas and reconstituted with 200 μL of the mobile phase. After centrifugation at 12,000 rpm for 5 min, the supernatant was injected into the LC–MS–MS system for analysis. The recovery of amoxicillin for each extraction method mentioned above was ≤10.0%. Given the possible impact of the pH value on the extraction efficiency, the samples were acidified or alkalinated before the extraction. However, the recovery could not be enhanced in the either acidified or alkalinated broth sample when using ethyl acetate–isopropanol (2 : 1, v/v), ethyl acetate–methanol (3 : 1, v/v) or ethyl acetate as the extraction solvents.

The methods of the solid phase extraction and protein precipitation by acetonitrile were also tested, respectively, which resulted in the similarly poor recovery. Although it was reported that extraction solvents like the acetonitrile and solid phase extraction can yield satisfactory recovery of amoxicillin from plasma and milk media (8, 11, 18), using protein precipitation with acetonitrile followed by purified with dichloromethane was also reported for bovine muscle tissue and human plasma samples (6, 19).

Considering the influence on the ion response from the nutrient content (mineral salt like sodium chloride) in broth, the broth samples were diluted by 10-, 20-, 50- and 100-fold with ultrapure water, followed by extraction with acetonitrile and dichloromethane. After the optimization of the sample preparation approach, the recovery of amoxicillin was improved to 48.5, 50.2, 73.3 and 87.9%, correspondingly. Liquid–liquid extraction using ethyl acetate–methanol (3 : 1, v/v) was also assessed after the appropriate dilution of the broth samples with ultrapure water. However, the recovery via this extraction was not improved as much as that via the extraction with acetonitrile and dichloromethane. Taking into account the sensitivity, it was demonstrated that a sample diluted by 50-fold was the most favorable. Therefore, the preparation of a broth sample diluted by 50-fold with ultrapure water and extracted by acetonitrile and dichloromethane not only resulted in the high and stable recovery, but also was considered less time-consuming.

Our analytic method was linear in the range of 0.05–20.00 μg/mL. Using 1/\( x^2 \) as the weighting factor, the RE of accuracy of the calibration samples was acceptable, and the accuracy in the low concentration was superior to that with 1/\( x \) as a weighting scheme. So, all the calibration curves in the validation and sample analysis batches were fitted using least-squares linear regression with 1/\( x^2 \) as a weighting factor. The method for the quantitative determination of amoxicillin in broth—a liquid bacterial culture medium—was validated for its selectivity, linearity, matrix effect, discovery, accuracy, precision and stability. And, it was applied to an in vitro PK/PD model.

**Conclusion**

An LC–MS–MS method for quantitative analysis of amoxicillin in broth was developed. The established method fulfills the validation requirements for biological sample analysis with excellent selectivity, precision and accuracy, high extraction recovery and short running time. It was also successfully applied to an in vitro PK/PD model for the quantitative determination of amoxicillin in broth. The method provides high-throughput biological analysis to facilitate the in vitro PK and PD model of amoxicillin.

**Funding**

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**Conflict of interest statement.** None declared.

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