

Research Article

A General LC-MS/MS Method for Monitoring Potential β -Lactam Contamination in Drugs and Drug-Manufacturing Surfaces

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Abstract. Penicillins and some non-penicillin β -lactams may cause potentially life-threatening allergic reactions. Thus, possible cross contamination of β -lactams in food or drugs can put people at risk. Therefore, when there is a reasonable possibility that a non-penicillin product could be contaminated by penicillin, the drug products are tested for penicillin contamination. Here, a sensitive and rapid method for simultaneous determination of multiple β -lactam antibiotics using high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed and validated. Mass spectral acquisition was performed on a Q-Exactive HF mass spectrometer in positive ion mode with parallel reaction monitoring (PRM). The method was validated for seven β -lactam antibiotics including one or two from each class and a synthetic intermediate. The quantification precision and accuracy at 200 ppb were in the range of ± 1.84 to ± 4.56 and -5.20 to 3.44% , respectively. The limit of detection (LOD) was 0.2 ppb, and the limit of quantitation (LOQ) was 2 ppb with a linear dynamic range (LDR) of 2–2000 ppb for all eight β -lactams. From various drug products, the recoveries of eight β -lactams at 200 and 2 ppb ranged from 93.8 ± 3.2 to $112.1 \pm 4.2\%$ and 89.7 ± 4.6 to $110.6 \pm 1.9\%$, respectively. The application of the method for detecting cross contamination of trace β -lactams (0.2 ppb) and for monitoring facility surface cleaning was also investigated. This sensitive and fast method was fit-for-purpose for detecting and quantifying trace amount of β -lactam contamination, monitoring cross contamination in manufacturing processes, and determining potency for regulatory purposes and for quality control.

KEY WORDS: cross contamination; LC-MS/MS; parallel reaction monitoring; penicillin; β -lactams.

INTRODUCTION

Penicillin or other non-penicillin drugs containing a β -lactam moiety are antibiotics with a long history in the treatment of a wide range of infectious diseases in both human and veterinary applications. While these antibiotics play an important role in disease treatments, they pose a potential risk for individuals who are hypersensitive to them (1–4). In fact, penicillin allergy is the most common cause of drug-induced anaphylaxis, and the allergy accounts for up to 1000 deaths per year (5–7). Hundreds of thousands of tons of antibiotics are consumed each year, and 30–60% pass through animals and humans completely unchanged. The different compounds then reach the ocean via hospital waste, municipal sewage, fish farms,

or run-off from agricultural use and landfills. Cases of allergic reactions after consumption of foods containing antibiotic residues have been widely reported in the literature (8–11). Thus, antibiotic monitoring is an essential quality control measure in safe food or drug production.

The massive production of antibiotics has created another risk—cross contamination. Cross contamination of β -lactams, especially exposure of non-penicillin products to penicillins, has been shown to be a safety concern for patients (6,12–14). Recent cases involving potential cross contamination in repackaging operations, loss of containment in segregated facilities, potential undeclared penicillin in homeopathic products, and contaminated compounded products have required the use of test methods stipulated by regulations. However, the current FDA's guidance for industry (i.e., “Non-Penicillin Beta-Lactam Drugs: A CGMP Framework for Preventing Cross-Contamination”) (15) references a test method which was published in 1965 and that lacks sufficient sensitivity to low levels of β -lactams (16). Because the threshold dose at which allergenic response could occur is extremely low and difficult to define (11,17), a more sensitive and robust analytical method is a critical need in the food and

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pharmaceutical industries for regulatory purposes or for product quality control.

Liquid chromatography (LC) with UV detection has been used to determine antibiotics (18–20), but such methods often lack the sensitivity and specificity required for β -lactam detection. Thus, these LC-UV techniques have been replaced by methods that use mass spectrometry (MS) detection to provide more specific determination leading to unequivocal confirmation of the compounds studied. However, most of the LC-MS methods have been developed for the analysis of residue penicillin and related antibiotics in food products (21–25). In addition, these methods are not optimized or validated for trace amount detection in drug formulations. For example, Bekoe *et al.* used an LC-MS/MS method to investigate the quality of antibiotic drug products with potency measurements on 13 antibiotic active pharmaceutical ingredients (26). However, the Bekoe *et al.* method was developed for the assay of antibiotics not for trace level determination.

While working with an FDA compliance for-cause assignment in 2015, a sensitive LC-MS/MS method for the detection and quantification of penicillin G (PNG) and 6-aminopenicillanic acid (6-APA) in homeopathic products was developed in our laboratory. In the current study, more extensive method development and validation were conducted to extend the LC-MS/MS method for the determination of all five types of β -lactam antibiotics (27) found in different formulations, including tablets, capsules, suspensions, and injections. Using this validated method, cross contamination and facility surface cleaning were also investigated for selected drugs. This LC-MS/MS approach is a single method that can be used for simultaneous detection of multiple β -lactams at levels as low as 0.2 ppb. Moreover, since a wide LC gradient was used and the drugs tested here were dispersed throughout the chromatogram, the method should be easily adapted to other β -lactams and antibiotics.

MATERIALS AND METHODS

Materials, Reagents, and Standards

The β -lactam standards of amoxicillin (AMX), ampicillin (AMP), penicillin G (PNG), cefuroxime (CFX), imipenem (IMP), loracarbef (LRC), and aztreonam (AZT) were purchased from USP (Rockville, MD). The 6-aminopenicillanic acid (APA) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). The d₇-penicillin G (dPNG) and d₄-amoxicillin (dAMX) were purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). All standards had purities greater than 99%. Drug products were purchased through a pharmacy (Table I). Optima formic acid and

Optima LC-MS grade solvents were purchased from Fisher Scientific (Pittsburgh, PA). Milli-Q water (Millipore, MA) was used for preparation of all solutions.

Preparation of Samples, Calibration Standards, and Validation Samples

Stock solutions of each β -lactam standard were prepared at 400 ppm in the mobile phase mixture without formic acid (A/B, 10:90, v/v, see chromatographic conditions). The solutions were sonicated for 5–30 min to ensure the solid particulates fully dissolved. A 5% acetonitrile solution was used as the diluent for the preparation of the calibration mixtures from the individual standard stocks. A stock mixture containing 20,000 ppb of each β -lactam standard and 200 ppb dPNG as an internal standard was prepared. Prior to LC-MS/MS analysis, a series of dilutions were made from the 20,000 ppb standard mixture to obtain concentrations of 2000, 200, 20, 2, and 0.2 ppb for each standard. The final concentration of the internal standard dPNG was 200 ppb in all standard solutions. These standard mixtures were used to obtain calibration curves and to determine the method's limit of detection (LOD) and limit of quantitation (LOQ) for β -lactams. Two separate mixtures at 200 and 400 ppb were prepared for all standards and used for instrument precision and accuracy tests.

The recovery test samples were prepared by spiking eight β -lactam standards and the internal standard dPNG into solutions made from the drug products. For tablets and capsules, composites were generated using the contents of ten capsules or the powder from ten ground tablets. Samples having an equivalent amount of the active compound (i.e., 10 mg according to the label claim) were transferred to a 10-mL volumetric flask and dissolved in 5 mL of 5% acetonitrile. Samples were sonicated for 10 min and diluted to volume with 5% acetonitrile. An aliquot of the test sample was filtered using a 0.20- μ m filter. For injection and suspension formulations, the drug containers were shaken vigorously for a minute, an amount equivalent to 10 mg of the active compound by volume was transferred to a 10-mL volumetric flask and mixed with 5 mL 5% acetonitrile. Samples were sonicated for 10 min and diluted to 10 mL with 5% acetonitrile. An aliquot of the test sample was filtered using a 0.20- μ m filter. Filtrates were diluted 1000 times while adding desired amounts of the standards. Three samples were prepared for each drug filtrate: a blank containing the internal standard only and two samples with 2 and 200 ppb β -lactam standards, respectively, in addition to the internal standard.

Table I. Drug Products Used in this Study

Names	Formulations	Dosages	Manufacturer
Penicillin G potassium	Injection	5,000,000 units	SANDOZ
Amoxicillin	Capsules	500 mg	SANDOZ
Ampicillin	Suspension for oral	125 mg/5 mL	DAVA
Cefuroxime	Injection	750 mg	SAGENT
Penicillin V potassium	Tablets	500 mg	AUROBINDO
Lovastatin	Tablets	40 mg	LUPIN

Table II. Name, Structure, Molecular Weight (Mw), Water Solubility (Sw), PRM Transition, and Collision Energy for Standards

Name	Chemical structure	Mw (g/mol)	Sw* (g/L)	Precursor ion →quantifier	HCD Energy (eV)
(+)-6-Aminopenicillanic acid (APA)		216.26	4.0	217.0640→189.0692	35
Imipenem (IMP)		317.36	10.0	300.1009→126.0374	30
Amoxicillin (AMX)		419.45	3.93	366.1115→114.0012	30
Aztreonam (AZT)		435.43	0.043	436.0589→313.0599	30
Loracarbef (LRC)		367.78	0.325	350.0898→174.0550	30
Ampicillin (AMP)		349.40	0.605	350.0898→106.0656	30
Penicillin G potassium (PNG)		372.48	100	335.1060→176.0706	35
Cefuroxime sodium (CEF)		446.37	Freely soluble	442.1024→211.0173	30
Penicillin G-d7 (internal standard)		379.52		342.1496→183.1146	35
Amoxicillin-d4 (internal standard)		369.43		370.0373→212.0682	25

*Solubility references: 1. ChemIDplus (US National Library of Medicine). 2. DrugBank database

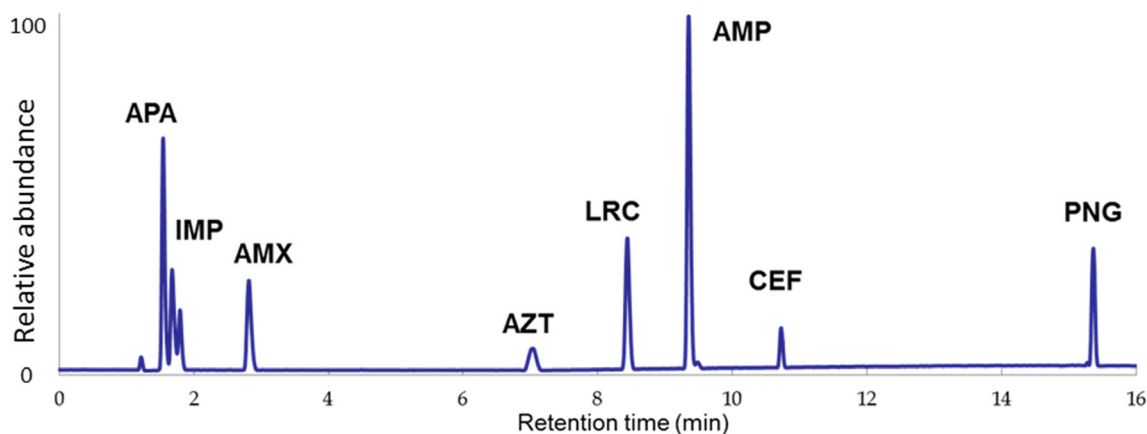


Fig. 1. Chromatogram of standard solution containing eight β -lactams at 200 ppb with MS detection

For the cross contamination test, samples were prepared in highly concentrated drug solutions. A 5% acetonitrile solution was added to the powder from tablets/capsules to form a slurry containing 100 mg/mL of the active compounds. The slurries were sonicated for 5 min followed by centrifugation at 3000 g for 30 min. The supernatants and injections (100 mg/mL active compounds) were filtered using 0.20- μ m filters. Two samples were prepared for each drug filtrate: a blank containing the internal standard only and a sample with 0.2 ppb β -lactam standard mixture and an internal standard of 200 ppb.

To mimic possible contamination in a manufacturing facility, a surface contamination test was designed. These

samples were prepared by depositing 10 and 100 μ L of solutions containing 2 and 20 ng of PNG separately on two thoroughly cleaned bench areas. After air drying, the bench surfaces were wiped five to eight times with small pieces of filter papers ($\sim 2 \times 2$ cm) wetted with 5% acetonitrile. The filter papers were then soaked in 10 mL of 5% acetonitrile solution to extract out the PNG. A control sample was prepared on a clean bench area where no PNG was deposited. All three solutions were filtered with 0.20- μ m filters, and the internal standard dPNG of 200 ppb was added to each testing sample prior to LC-MS/MS analysis.

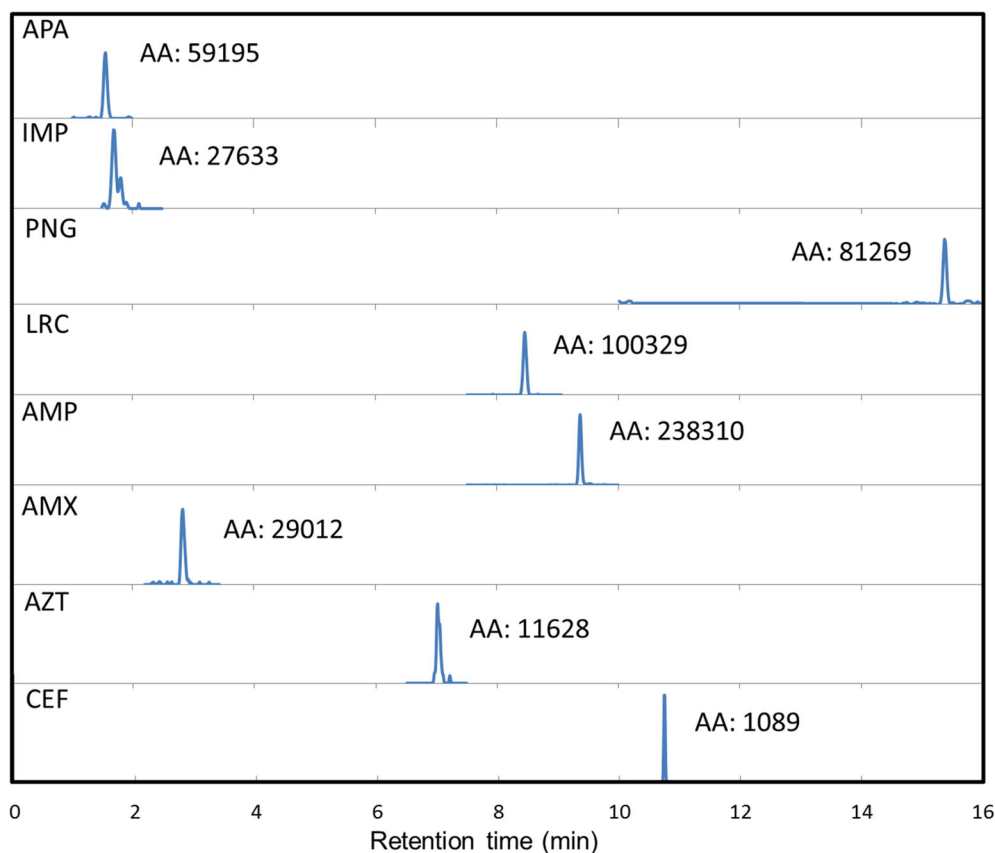


Fig. 2. Chromatograms of all standards at 0.2 ppb extracted from PRM acquisition of a standard mixture, with the count number labeled by each peak. AA refers to autointegrated area

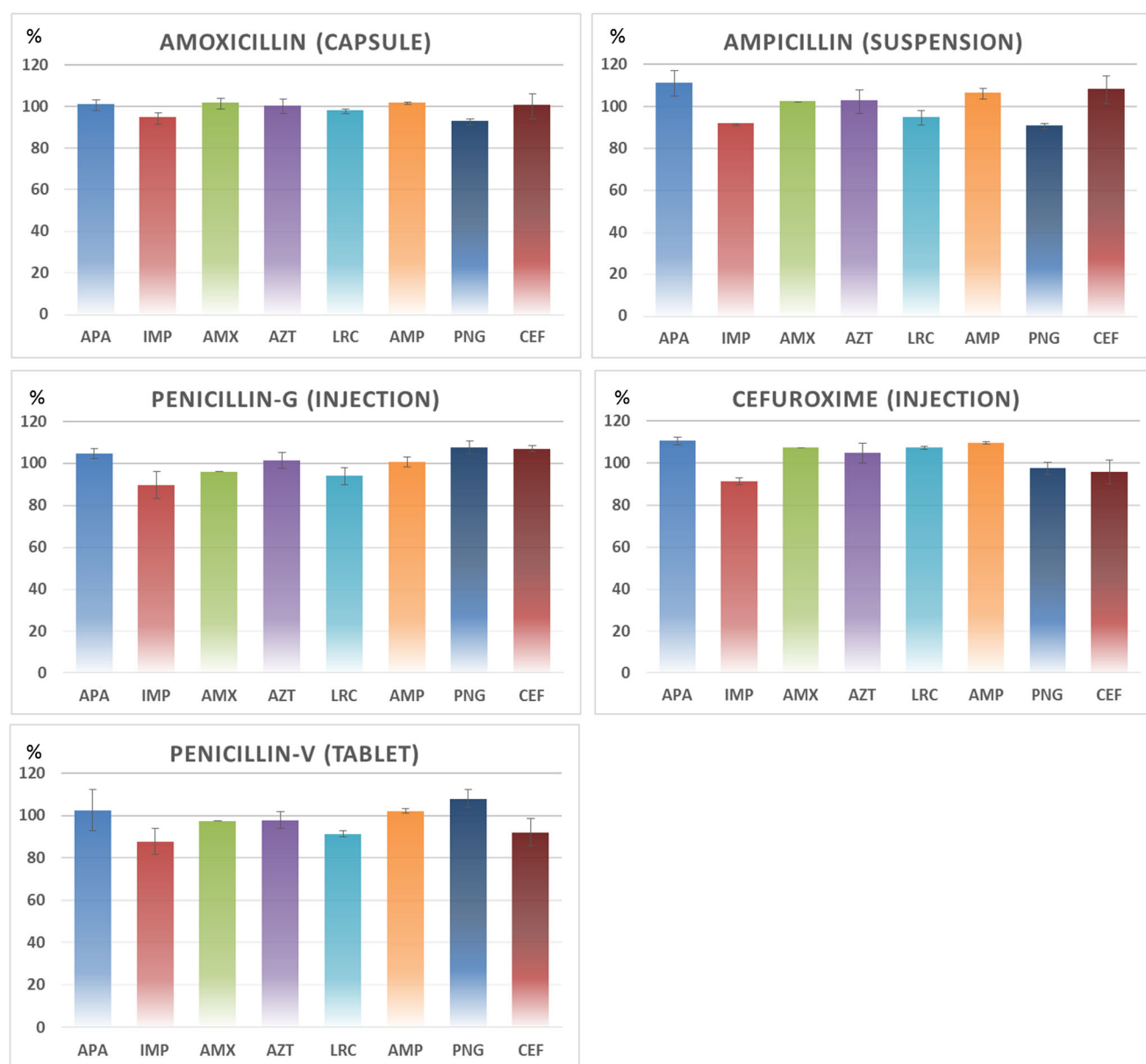
Table III. Validation Parameters Summarized for Eight Investigated β -Lactams Determined Using Calibration Curves Within Linear Dynamic Range (LDR) of 2–2000 ppb

		APA	IMP	AMX	AZT	LRC	AMP	PNG	CEF
r^2 (2–2000 ppb LDR)		0.9878	0.9997	1	0.9999	0.9999	0.9998	0.9997	1
Accuracy (%)	200 ppb	−5.12	−5.20	1.04	0.05	2.03	3.44	3.07	−0.89
	400 ppb	0.87	−1.50	5.84	5.54	0.92	−0.80	3.25	0.67
Precision (std%)	200 ppb	1.84	3.34	4.56	2.69	3.86	2.19	2.71	3.72
	400 ppb	5.34	4.48	3.01	4.21	2.86	2.63	3.24	4.39

Chromatographic and Mass Spectrometric Analyses

LC-MS/MS analysis was performed on a Thermo Q-Exactive HF mass spectrometer coupled with a Vanquish LC

system. A Waters Acquity UPLC BEH C18 (1.7 μ m, 2.1 $\times$ 150 mm) column at 35°C was used for sample separation. Mobile phase A was water-acetonitrile-methanol at 90:6:4 (v/v/v), and mobile phase B was water-acetonitrile-methanol at 5:65:30 (v/v/v)

**Fig. 3.** Recoveries of eight β -lactams at 2 ppb from five different drug matrixes. The recoveries and standard errors were calculated based on three injections

v). Both mobile phases contained 0.1% formic acid (98%). The LC gradient consisted of several steps starting with 1% (B) holding for 5 min (0–5 min) and increasing to 75% (B) during 15 min (5–20 min). Isocratic elution at 75% (B) for 1 min (20–21 min) was followed by mobile phase A increasing back to 99% during 1 min (21–22 min) and equilibrating for another 8 min (22–30 min). Flow rate was $250 \mu\text{L min}^{-1}$, and the injection volume was $10 \mu\text{L}$ with the autosampler set at 4°C .

The mass spectrometer was operated in the positive ion mode and calibrated using an LTQ Positive Ion Calibration Mix (Pierce). Xcalibur™ instrument control software (version 4.0) was used for data acquisition. The key parameters were set as follows: ionization voltage at 3.5 kV, sheath gas at 4 and auxiliary gas at 1 arbitrary unit, and capillary temperature at 325°C . Mass scan range was 100 to 500 m/z with resolution of 60,000 for MS^1 and 30,000 for MS^2 . Data acquisition time was 23 and 18 min for MS^1 and MS^2 , respectively. Since all compounds eluted before 16 min, the MS^2 acquisition was stopped at 18 min. LC elute was diverted to waste when not at data acquisition. For cross contamination test samples, because the concentrations of the drug APIs were very high

and samples were normally “dirty,” to protect the instrument MS data was only acquired within short time windows when the targeted compounds (e.g., PNG) eluted.

For each standard, collision-induced dissociation products of precursors were detected in the positive ion parallel reaction monitoring (PRM) mode. To set up the PRM experiment, selected precursor ion for each standard was added to “inclusion list” with corresponding detection time window. PRM transitions and collision energies for each compound are listed in Table II. Peak area integration was performed using the Thermo QualBrowser software.

RESULTS AND DISCUSSION

Method Development and Optimization

For this work, the challenge in sample preparation was the solubility differences of the eight β -lactam compounds. To make stock solutions at the highest concentration possible and the sample solvent closest to mobile phase A possible, various mixtures of water, methanol, and acetonitrile were investigated.

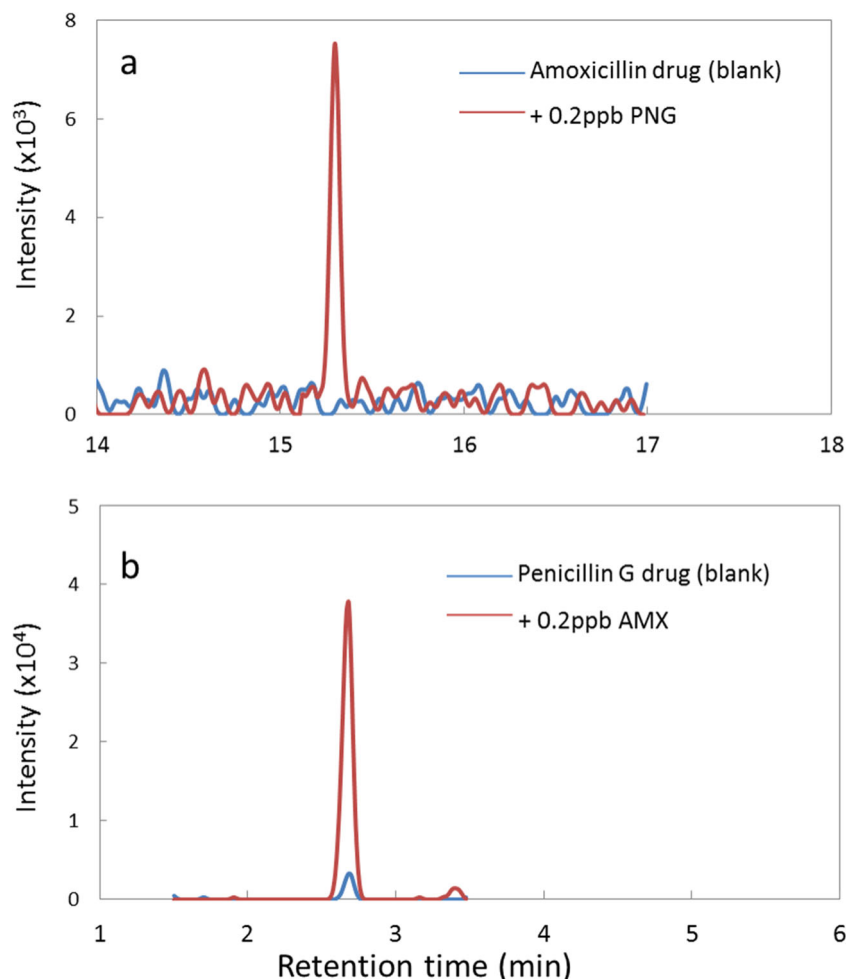


Fig. 4. Detection of trace amounts of β -lactams in drug products. **a** 0.2 ppb penicillin G spiked into amoxicillin capsules: blue trace represents amoxicillin drug only, red trace represents 0.2 ppb penicillin G spiked into amoxicillin drug. **b** 0.2 ppb amoxicillin spiked into penicillin G injection: blue trace represents penicillin G only, red trace represents 0.2 ppb amoxicillin spiked into penicillin G

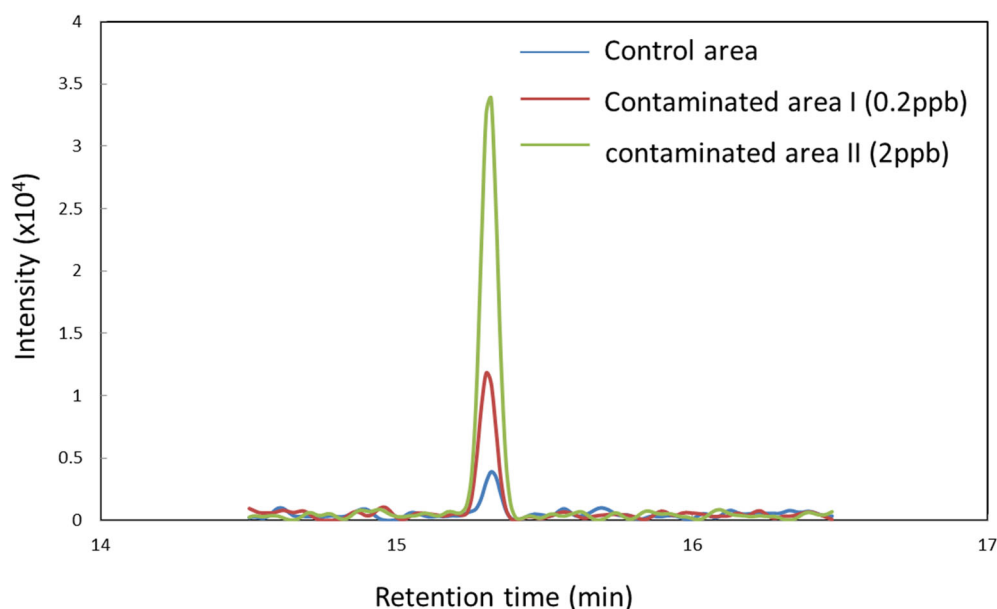


Fig. 5. Detection of penicillin G from a contaminated facility surface. Blue line represents the control area, red line represents contamination of 0.2 ppb, and green line represents contamination of 2 ppb penicillin G

The mixture of mobile phases A and B at 10 to 90 was found to give clear solutions for all compounds at 400 ppm. Sonication was needed for compounds with low solubility.

Although chemical structures, acid-base properties, and solubility of these targeted β -lactams are different, they are all polar and reverse phase liquid chromatography was suitable for separation on column. The best possible resolution was desired, because the LC-MS/MS method was to simultaneously detect and quantify all five classes of β -lactams. Technically, these β -lactams can be analyzed with both positive and negative ionization modes, or by switching between two modes for different lactams. Here, to simplify the mobile phase composition and to make method transfer to a different LC-MS system more feasible, only the positive mode was chosen in the method development.

Eight β -lactam standards were injected separately for LC-MS to optimize the chromatographic and mass spectrometric conditions and to obtain individual MS spectra. Then, a mixture of eight standards was injected together for further optimization and for appropriate selection of the precursor/quantifier for each β -lactam standard (Table II). A typical chromatogram of the mixture is shown in Fig. 1. Figure 1 shows that most of the standards were well separated and dispersed throughout the chromatogram except APA and

IMP. However, because APA and IMP have distinct precursor/quantifier ions (Table II), accurate quantification can be achieved despite non-baseline separation. IMP was split into two peaks that have the same mass and fragments. These peaks represent the presence of the imine and secondary amine in equilibrium. Thus, two peak areas were summarized for quantification of IMP.

Method Validation

Through properly designing the PRM experiment by assigning each precursor in a specific time window in the MS² inclusion list, the high specificity of detection for each β -lactam was achieved. Comparing with selected ion monitoring (SIM), the low noise of the selective PRM method improved the sensitivity of the method. Figure 2 shows chromatograms of all eight β -lactams at a concentration of 0.2 ppb. All peaks had similar full width at half height and peak shape with sufficient ion counts for identification and quantitation. The lowest concentration investigated in this study was 0.2 ppb which was defined as limit of detection (LOD).

The peak area ratios of analytes to the internal standard were used for quantification to minimize the variations caused by instrument conditions, possible degradation or day to day

Table IV. Potency of β -lactams in Drug Products

Drug products	Concentrations determined by assay test (ppb)
Amoxicillin (capsules)	1111.3 $\pm$ 33.4
Ampicillin (suspension)	899.0 $\pm$ 23.2
Penicillin G (injection)	1145.4 $\pm$ 31.5
Cefuroxime (injection)	1093.1 $\pm$ 25.8

differences. Calibration curves for all eight β -lactams were obtained from standard concentrations between 2 to 2000 ppb and coefficients of determination (r^2) were greater than 0.99 for all β -lactams in linear fits of the data across this range. All results were the average of three injections. The lowest concentration of calibration curves, 2 ppb, was defined as the limit of quantitation (LOQ). Instrument precision was investigated using data from six injections of β -lactam standard mixtures at 200 and 400 ppb. Precision was expressed as a percentage of standard deviation at two concentrations and was between 1.84 and 5.34 (Table III). Method accuracy at 200 and 400 ppb for each β -lactam was also determined with six injections using calibration curves. These results indicate the method was suitable for simultaneous quantification of all β -lactams from different classes.

The effect of drug excipients (matrix effect) was examined by spiking β -lactam standards into different drug formulations including tablets, capsules, suspensions, and injections (both intravenous and muscular). The standard mixture at concentrations of 2 and 200 ppb each along with the internal standard dPNG was added to five drug matrix preparations, and the recovered amount was determined using calibration curves. For each drug preparation, a blank sample spiked with dPNG only (no other β -lactam) was analyzed with LC-MS/MS and the background concentration was subtracted from the spiked pool concentration to obtain the recovery. For example, to determine spiked AMX recovery in an amoxicillin injection, the amount of amoxicillin (active ingredient) was determined from the blank sample. This amount was subtracted from the spiked pool concentration for AMX, and the resulting concentration was compared with a nominal concentration to obtain AMX recovery. At the concentration of 200 ppb, the recoveries were between 93.8 ± 3.2 and $112.1 \pm 4.2\%$ for all eight β -lactams in all five drug matrixes (data not shown). Figure 3 shows the recoveries of each β -lactam standard from five drug matrixes at 2 ppb spiking, ranging from 89.7 ± 4.6 to $110.6 \pm 1.9\%$. These results demonstrated that the existence of drug excipients did not affect the quantification of β -lactams using this method.

Application of the Method

Detection of Trace Amounts of β -Lactams in Drug Products

The validated method was applied to detect trace amounts of β -lactams in drug products and cross contamination between different β -lactam drugs. Two β -lactam drugs, amoxicillin capsules and a cefuroxime injection, at 100 mg/mL API (as label stated) were spiked with 0.2 ppb PNG and 200 ppb dPNG as an internal standard. A symmetric sample, penicillin G injection at 100 mg/mL API spiked with 0.2 ppb AMX and 200 ppb dAMX as an internal standard, was also analyzed to examine the method's flexibility. In addition, a non β -lactam drug, Lovastatin tablets at 100 mg/mL API, was tested by spiking with 0.2 ppb PNG and 200 ppb dPNG to demonstrate the capability of the method to detect trace penicillin contamination in a non β -lactam drug product. For all these four samples, a blank drug preparation spiked with dPNG or dAMX only was analyzed for comparison. In all cases, spiked β -lactams at 0.2 ppb were

undoubtedly detected and identified. Figure 4a shows the detection of 0.2 ppb PNG in amoxicillin capsules, and Fig. 4b shows the detection of 0.2 ppb AMX in a penicillin G injection. Because the method was validated with a LOQ of 2 ppb, quantification to 0.2 ppb spiking was not attempted. If desired and the observed peaks have sufficient signal to noise, the amount of β -lactam present can be estimated using calibration curves. These results demonstrate that the method was capable of detecting and quantifying trace levels of β -lactams in various therapeutic formulations. Thus, the method can be used for both limit and quantification tests. To detect trace levels of contaminants, the sample concentration of the API must be very high, to the point where the API does not need to be completely dissolved. After sonication of the highly concentrated sample, the trace amount of contaminants present should all be in solution. Filtering of the saturated sample removed any remaining API particulates from the sample before analysis. Clear injectable drugs can be analyzed "as is" after spiking with the internal standard and filtering. For quantification purpose, the deuterated internal standard solution should be made freshly to reduce possible H/D exchange. Minor undeuterated compound may appear if the stock internal standard solution is not fresh.

Detection of Facility Surface Contamination

Therapeutics are generally manufactured in large production plants and production lines for a range of different active pharmaceutical ingredients are often run in parallel or share the same equipment. The facility should be tested for cleanliness when switching to a different production line to prevent cross contamination and to ensure the safety of the finished products. Two cleaning test samples along with a blank sample were prepared and analyzed with the β -lactam validated method. As shown in Fig. 5, 0.2 ppb of penicillin G was clearly detected from the contaminated surface. For the 2 ppb contamination sample, the experimental concentration of penicillin G was 2.24 ± 0.12 ppb from three LC-MS/MS injections. The small peak in the control sample is the minor un-deuterated impurity in deuterated penicillin G which was added as an internal standard at 200 ppb.

Assay of β -Lactam APIs in Drug Products

Although this method was developed for the determination of trace levels of β -lactams, the method can also be used to measure the potency of β -lactam drug products since it was validated for quantification in the range of 2–2000 ppb. As stated in the recovery study section, for each of the five drug preparations, a blank sample spiked with an internal standard only (no other β -lactam) was analyzed using LC-MS/MS and the background concentration was subtracted from the spiked pool concentration to obtain the recovery. The background concentrations were API concentrations in the drug products. Those drug preparations were made at 1000 ppb according to label claims. The concentration of penicillin G was estimated because the label claim was 5,000,000 units per vial and therefore had no weight claim. Table IV shows the experimentally determined concentrations of the β -lactam API using the corresponding

calibration curves. The potency for penicillin V was not determined, because its standard curve was not pursued.

CONCLUSIONS

The method developed and validated in this study highlights the advantages of using liquid chromatography in combination with mass spectrometry for drug contaminant analysis. The approach was designed for simultaneous analysis of multiple β -lactams and liquid chromatography provided the primary separation. Then mass spectrometric detection, especially using PRM and deuterated internal standards, provided high sensitivity, selectivity, accuracy, and precision for identification and quantitation.

Because the method was developed with eight standards representing all five classes of β -lactam antibiotics, the approach as presented can analyze drugs containing these β -lactams. While different formulations may require minor modifications to the method, overall, the method is robust to the differences in excipients across the antibiotics tested with good recovery. An advantage of the mass detector in this protocol is that both known and unknown impurities can be identified. Drugs can be tested as is, or may require minimal sample preparation. With simplified mobile phases and LC-MS settings, up to 16 samples can be run within 24 h.

Potential applications of the method include detection and quantification of trace level contaminants, monitoring of cross contamination in manufacturing processes or final products, and potency determination for quality control. In addition, consumed antibiotics passing through animals and humans and entering the food supply chain have also been a concern. Although the sample preparation procedures would vary, this method may also be used for the detection of antibiotic residues in food products. The key to successful use of this approach is the use of an internal standard to obtain reliable quantitative results with mass spectrometry analysis.

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