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# Monitoring bacterial resistance to chloramphenicol and other antibiotics by liquid chromatography electrospray ionization tandem mass spectrometry using selected reaction monitoring

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**Antibiotic resistance is a growing problem worldwide. For this reason, clinical laboratories often determine the susceptibility of the bacterial isolate to a number of different antibiotics in order to establish the most effective antibiotic for treatment. Unfortunately, current susceptibility assays are time consuming. Antibiotic resistance often involves the chemical modification of an antibiotic to an inactive form by an enzyme expressed by the bacterium. Selected reaction monitoring (SRM) has the ability to quickly monitor and identify these chemical changes in an unprecedented time scale. In this work, we used SRM as a technique to determine the susceptibility of several different antibiotics to the chemically modifying enzymes β-lactamase and chloramphenicol acetyltransferase, enzymes used by bacteria to confer resistance to major classes of commonly used antibiotics. We also used this technique to directly monitor the effects of resistant bacteria grown in a broth containing a specific antibiotic. Because SRM is highly selective and can also identify chemical changes in a multitude of antibiotics in a single assay, SRM has the ability to detect organisms that are resistant to multiple antibiotics in a single assay. For these reasons, the use of SRM greatly reduces the time it takes to determine the susceptibility or resistance of an organism to a multitude of antibiotics by eliminating the time-consuming process found in other currently used methods.** 

**Keywords:** Selected Reaction Monitoring; Antibiotics; Resistance; Bacteria; Chloramphenicol

## Introduction

Antibiotic resistance is a growing problem.[1] The overuse of antibiotics and the adaptability of bacteria have created a growing number of resistant bacterial strains.[2,3] In 2005, there were over 11,000 deaths from methicillin-resistant *Staphylococcus aureus* alone.[4] *Acenitobacter baumannii*, an inherently multidrug resistant organism, has recently emerged as one of the most common infectious organisms of wounds in military service members serving in Iraq and Afghanistan.[5–8] *Enterococcus* sp. have long been associated with nosocomial infections and are often multidrug resistant.[9] Because of the high mortality rate caused by these organisms, rapidly determining which antibiotic an organism is susceptible to is imperative to improving clinical outcomes.

Current methods to determine the susceptibility or resistance of bacteria to a particular antibiotic typically involves isolating the organism from an individual specimen (blood, spinal fluid, tissue, etc.). The isolated bacteria are grown on culture media, and then one of two methods employed for analyzing antibiotic resistance, Kirby–Bauer and minimal inhibitory concentration dilution method (MIC). Kirby–Bauer is a qualitative method whereby the bacteria to be tested are inoculated over the entire surface of an agar plate. Then, disks containing a standardized concentration of different antibiotics are placed on the plate. Susceptibility is determined from a standardized range of the resulting diameter of inhibition of bacterial growth around the disk. Conversely, the MIC dilution method is a quantitative method that allows determination of the lowest concentration of an antibiotic that inhibits the visible growth of a microorganism. Although these two methods are effective, susceptibility testing is a slow process, often requiring 48–72 h for results.[10] Because of this delay, a physician will often treat the patient with broad spectrum antibiotics until the susceptibility results are known. Treating with an ineffective antibiotic not only may compromise the health of the patient but also can contribute to the selection of antibiotic-resistant organisms.[11] Therefore, it is necessary to develop a

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method to determine the organism's susceptibility to numerous antibiotics in as short of a time span as possible, thus preventing increased resistance and also reducing the morbidity and mortality among patients.

One common mechanism used by bacteria to enable their resistance to antibiotics is the elaboration of enzymes that can inactivate the antimicrobial activity of antibiotics by chemically modifying their structure. β-lactamase, an enzyme expressed by bacteria resistant to the β-lactam class of antibiotics, hydrolyzes the β-lactam ring of these antibiotics, which include penicillins and cephalosporins.[12,13] However, more recently approved cephalosporins are less susceptible to hydrolysis by many types of β-lactamase. Because of selective pressure on bacteria through antibiotic use, mutations leading to structural changes in the β-lactamase protein allow it to hydrolyze these newer cephalosporins.[14] In the case of chloramphenicol, resistant organisms produce chloramphenicol acetyltransferase that acetylates the hydroxyl moiety, thereby preventing the drug from binding to the bacterial ribosome.[15] Chemical modifications can either reduce or eliminate the effectiveness of an antibiotic. Therefore, methods to identify chemical changes to an antibiotic allow one to immediately determine organism resistance to that specific antibiotic. This is particularly true if an enzymatic chemical modification is already known and has been correlated to resistance by the organism.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has recently been reported as a rapid technique for monitoring the chemical modifications of antibiotics via enzymes expressed by antibiotic-resistant organisms.[16–18] A number of different antibiotic-resistant organisms were investigated including *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. Antibiotic resistance was detected in these organisms by observing changes in the β-lactam antibiotics, particularly hydrolysis. A number of different β-lactam antibiotic were investigated including penicillins, cephalosporins, and carbapenems. Although these results demonstrate the effectiveness of MALDI-TOF MS as a qualitative technique for determining antibiotic resistance, it suffers from being a superior quantitative technique compared with other current mass spectrometric techniques used for quantitation, such as selected reaction monitoring (SRM).

Selected reaction monitoring is a mass spectrometric technique that has a long history of being able to detect compounds with very high sensitivity and selectivity.[19,20] SRM has already been used for the detection and quantification of antibiotics in the human body, including the determination of cefuroxime in plasma and the analysis of β-lactam antibiotics in kidney tissue.[21,22] SRM has also been used to monitor the level of antibiotics in the environment, such as identifying and quantifying antibiotics in waste water or determining the levels of tetracycline in manure compost.[23,24] However, there has been little-to-nothing published on the use of SRM for determining antibiotic resistance.[25]

By monitoring the presence or absence of an antibiotic and/or its chemically modified form after exposure to a bacterial culture, SRM has the potential to be a rapid and accurate screening technique for determining the susceptibility or resistance of such bacterial organisms. This would allow one to screen the effectiveness of numerous antibiotics to a particular bacterial organism in a single assay. Moreover, the technique would not be limited to antibiotic resistance based on a single chemical modification pathway but rather also applicable to any known chemical modification pathways used by the organism. Therefore, the presence or absence of unmodified antibiotics by SRM could determine whether an organism is expressing an antibiotic-inactivating protein.

## Materials and methods

### Chemicals and reagents

Amoxicillin, ampicillin, nafcillin, cloxacillin, chloramphenicol, and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO). Dibasic sodium phosphate, monobasic potassium phosphate, ammonium chloride, sodium chloride, magnesium sulfate, calcium chloride, glucose, sodium azide, thiamine, and tryptophan were purchased from Fischer (Waltham, MA). Casamino acids were obtained from Difco (Sparks, MD). Ethanol was obtained from Pharmco-AAPER (Brookfield, CT) and high-performance liquid chromatography (HPLC) grade acetonitrile (ACN) from J.T Baker-Mallinckrodt (St. Louis, MO). Water was obtained in-house from a Milli-Q UF Plus filtration system and further purified by distillation. 3-*O*-acetylchloramphenicol and 1,3-*O*,*O*-diacetylchloramphenicol were manufactured on campus by the University of Texas Medical Branch (UTMB) Organic Synthesis Core facility.

Antibiotic stock solutions for HPLC optimization and quality control were prepared by dissolving the corresponding antibiotic in water and serially diluting to obtain a concentration of 500 ng/ml. For cultures, ampicillin stock solution was prepared as a 50 mg/ml solution in water, and chloramphenicol stock solution was prepared as a 30 mg/ml solution in ethanol. Because of its low solubility, chloramphenicol was first dissolved in ethanol (5 mg/ml) and then diluted to 500 ng/ml with water. Because of the limited lifespan of β-lactam antibiotics in solution, stock solutions were prepared daily.

M9 broth was prepared as follows: 5.3 g $Na_2HPO_4$, 3 g $KH_2PO_4$, 0.5 g NaCl, 1.0 g $NH_4Cl$, and 2.5 g casamino acids were dissolved in water to a volume of 500 ml. The solution was autoclaved for 20 min, after which 0.5 ml 1M $MgSO_4 \cdot 7H_2O$, 0.1 ml 0.5 M $CaCl_2$, 2.5 ml 40% (w/v) glucose, 5 mg thiamine, and 10 mg of tryptophan were added.

### Sample preparation

Ampicillin-resistant *E. coli* (pUC18) and chloramphenicol-resistant *E. coli* (pACYC184) were separately cultured overnight in M9 broth. Overnight cultures (20 h) resulted in $1.0 \times 10^8$ colony-forming units/ml (cfu/ml). To 150 ml of fresh M9 broth, 250 μl of pUC18-transformed *E. coli* and 15 μl of ampicillin stock solution for culture were added. To another 150 ml of fresh M9 broth, 150 μl of pACYC184 transformed *E. coli* and 15 μl of chloramphenicol stock solution for culture were added. The samples were then mixed and incubated at 37 °C. Next, 1.5 ml of broth was removed each hour (including time = 0) and centrifuged at 3000 rpm. The supernatant was removed, and 15 μl of 0.02% (w/v) $NaN_3$ was added. The supernatant solutions were then frozen (−80 °C) until ready for analysis by mass spectrometry.

### Chromatography

HPLC separation was performed by using an LC Packings Nano-LC system, consisting of an autosampler, a binary gradient pump, and a loading pump. The column and trap column were made in-house. The column was made from 75 μm ID polyimide-coated fused silica capillary (Polymicro Technologies, Phoenix, AZ) packed with 5 μm



  

Zorbax SB-C18 reversed-phase packing (Agilent, Santa Clara, CA) to a length of 5 cm by using a Pressure Injection Cell (NextAdvance, Averill Park, NY). The trap column was prepared in the same manner but to a length of 1 cm.

The aqueous mobile phase (A) consisted $H_2O$ : ACN : FA (95 : 4.9 : 0.1 v/v/v) and the organic mobile phase (B) of ACN : FA (99.9 : 0.1 v/v). Column flow was 250 nl/min. Next, 2 μl of antibiotic stock solution was injected onto a trap column at 2 μl/min for 5 min and then eluted onto the column. The column elution gradient was optimized and was as follows: started from 100% A (5 min isocratic) to 70% B over 25 min; ramp to 90% B for 1 min and hold for 4 min; ramp back to 100% A for 1 min and hold for 5 min to re-equilibrate. The mass spectrometer was used as the detector in all of the SRM experiments.

### Mass spectrometry

Tandem mass analysis was performed on a Thermo Scientific (San Jose, CA) LTQ-Velos Orbitrap mass spectrometer using higher energy collision dissociation (HCD). Antibiotic samples were dissolved in $H_2O$ : ACN : FA (95:4.9:0.1 v/v/v) at a concentration of 50 μg/ml and infused at a rate of 5 μl/min. The spray voltage was 3 Kv, and the isolation width was 1 Da. Spectra were obtained in the positive ion mode at 100 k resolution.

Selected reaction monitoring was performed on an AB/SCIEX (Framingham, MA) 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer with a nanoflow source. The mass spectrometer was operated in the positive ion mode for all experiments under the following conditions: curtain gas: 15 psi; collision gas: high ($4 \times 10^{-5}$ torr); spray voltage: 2.5 kV; ion source gas 1: 15 psi; ion source gas 2: 0 psi; interface heater temperature: 70 °C; Q1 and Q3 resolution: low; scan time: 500 ms. The declustering potential, entrance potential, collision energy, and collision exit potential were tuned for each individual antibiotic (5 μg/ml in $H_2O$ : ACN : FA, 50 : 49.9 : 0.1 v/v/v) with an infusion flow of 5 μl/min to obtain the highest sensitivity for each antibiotic. The instrument was calibrated by using AB/SCIEX PPG calibration standard and tuned to the manufacturer's specifications. Data were acquired and processed with Analyst® Software, version 1.4.1 (AB/SCIEX), and the data were plotted for publication by using Sigma Plot, version 11.0 (Systat Software, Chicago, IL).

## Results and discussion

Different antibiotic classes were evaluated in this study. Because β-lactam antibiotics are one of the most common varieties of antibiotics, we chose several antibiotics with different susceptibility to a particular β-lactamase. Ampicillin is an early β-lactam antibiotic of the penicillin class, whereas piperacillin is a more recent form that is active against a broader range of bacteria and more resistant to certain β-lactamases. Nafcillin and cloxacillin are also of the penicillin class but exhibit an even higher degree of resistance to certain β-lactamases. Cefoperazone is a β-lactam antibiotic of the cephalosporin class that is susceptible to only very limited types of β-lactamase. The hydrolytic action of β-lactamase on penicillins and cephalosporins is represented in Fig. 1(a). In the case of β-lactam antibiotics, ring opening occurs at the nitrogen–carbonyl site via hydrolysis.

Chloramphenicol is a protein synthesis inhibitor susceptible to acetylation by chloramphenicol acetyltransferase, as illustrated in Fig. 1(b). Acetylation first occurs at the C-3 hydroxyl moiety. This acetyl moiety then shifts to the C-1 hydroxyl group via a nonenzymatic rearrangement. This product is then again acetylated at the C-3 site to form a diacetylated product. The hydrolyzed versions of β-lactam antibiotics and the acetylated versions of chloramphenicol have no antimicrobial activity. Therefore, the presence or absence of these antibiotics (modified or unmodified) by SRM can determine whether an organism is expressing the corresponding antibiotic-inactivating protein (in this case β-lactamase or chloramphenicol acetyltransferase).

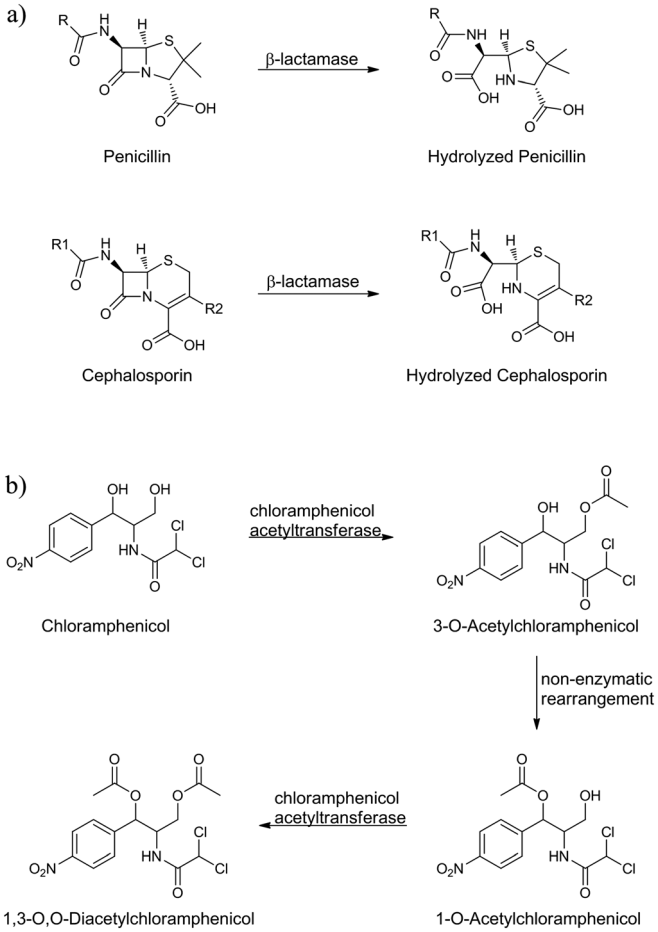


**Figure 1.** (a) Hydrolysis of β-lactam antibiotics by β-lactamase and (b) the acetylation of chloramphenicol by chloramphenicol acetyltransferase. The hydrolysis products of β-lactam antibiotics exhibit no antibacterial activity. Both 3-*O*-acetylchloramphenicol and 1,3-*O,O*-diacetylchloramphenicol also exhibit no antibacterial activity.

Table 1 lists the SRM transitions for the antibiotics used in this study. Each transition was optimized by infusing the sample into the mass spectrometer and adjusting the MS parameters to obtain the highest sensitivity. Although several unique transitions for each antibiotic were investigated, we list those that resulted in high sensitivity, greater signal-to-noise ratio, and no overlap in transition *m/z* values among different antibiotics.

3-*O*-acetylchloramphenicol and 1,3-*O,O*-diacetylchloramphenicol, the acetylated products from the interaction of chloramphenicol with chloramphenicol acetyltransferase-expressing bacteria, were also investigated to refine this SRM approach. Chloramphenicol and its acetylated forms were analyzed on an Orbitrap mass spectrometer to obtain structural and empirical formulas for the product ions in the MS/MS spectra. For chloramphenicol

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**Table 1.** Table of selected reaction monitoring (SRM) settings

| Antibiotic | SRM transition (*m/z*) Q1 Q3 | DP[a] | EP[b] | CXP[c] | CE[d] |
|---|---|---|---|---|---|
| Ampicillin | 350.1 160.1 | 60 | 11 | 11 | 19 |
| Cefoperazone | 646.1 143.1 | 70 | 10 | 10 | 50 |
| Cloxacillin | 436.1 277.0 | 70 | 7 | 7 | 23 |
| Nafcillin | 415.1 199.1 | 70 | 10 | 10 | 30 |
| Piperacillin | 518.2 143.1 | 70 | 8 | 7 | 30 |
| Chloramphenicol | 323.0 275.0 | 45 | 8 | 8 | 21 |
| 3-*O*-acetylchloramphenicol | 365.0 275.0 | 45 | 8 | 8 | 25 |
| 1,3-*O,O*-diacetylchloramphenicol | 365.0 347.0 | 45 | 8 | 8 | 22 |

[a]Declustering potential voltage.
[b]Entrance potential voltage.
[c]Collision exit potential voltage.
[d]Collision energy voltage.

(Fig. 2(a)), *m/z* 275 and *m/z* 305 were the major product ions with the *m/z* 305 peak resulting from the loss of the C1 hydroxyl moiety and the *m/z* 275 peak from the cleavage of the C3 carbon, resulting in the loss of methanol in addition to the previous C1 hydroxyl loss. The loss of the C1 hydroxyl moiety was also observed for 3-*O*-acetylchloramphenicol, which yielded a product ion at *m/z* 347 (Fig. 2(b)). In the case of 1,3-*O,O*-diacetylchloramphenicol where the hydroxyl group had been acetylated to form an acetoxy moiety, its loss from the C1 carbon is instead observed (Fig. 2(c)) and also resulted in the product ions at *m/z* 347.

For chloramphenicol, SRM transitions 323 > 305 and 323 > 275 both had similar sensitivities on the QTRAP after tuning. However, during the chromatography, the 323 > 305 transition had a higher background than did the 323 > 275 transition. Therefore, we chose the 323 > 275 transition for monitoring chloramphenicol. For 3-*O*-acetylchloramphenicol, the transition 365 > 275 was used, as it had better sensitivity and signal-to-noise after tuning than did the 365 > 347 transition. The transition 407 > 347 was used for 1,3-*O,O*-diacetylchloramphenicol. Other product ions observed in the product ion spectrum did not yield acceptable sensitivity when used as an SRM transition.

The elution times also varied on the basis of the amount of acetylation. The addition of acetyl moieties to chloramphenicol resulted in increased retention time on the column. The acetylation of the C1 hydroxyl group increased the retention time from 12 to 14 min. The addition of the second acetyl group on the C3 hydroxyl group increased it further to 15.5 min. All three forms of chloramphenicol were, therefore, easily separated by chromatography.

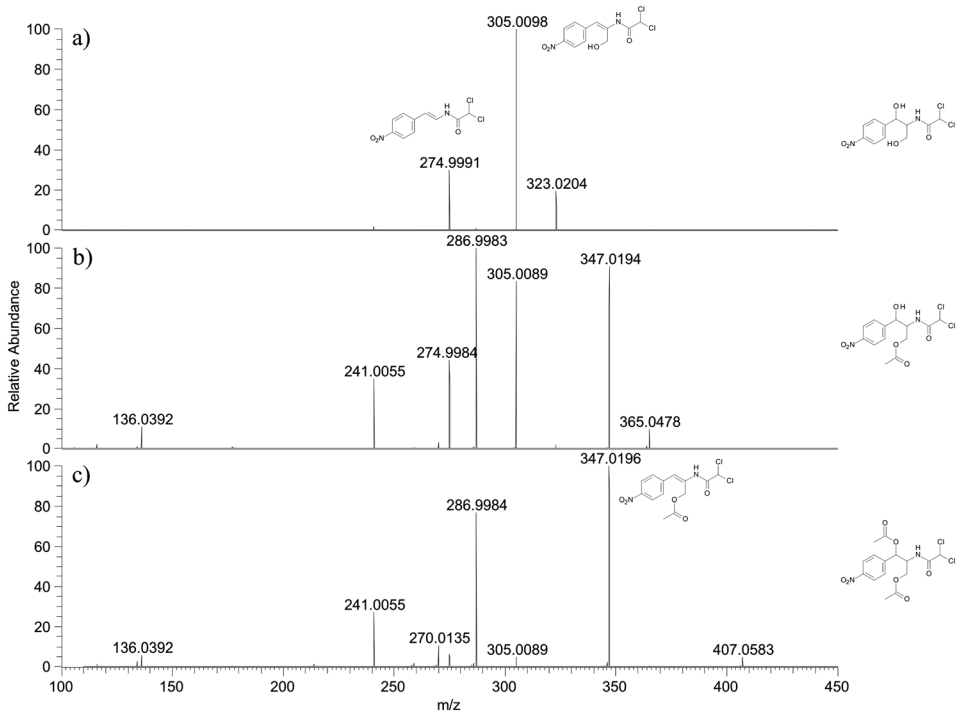


**Figure 2.** Product ion spectrum of (a) chloramphenicol, (b) 3-*O*-acetylchloramphenicol, and (c) 1,3-*O,O*-diacetylchloramphenicol. Moieties on the C1 carbon were found to be the most volatile and lead to cleavage. Loss of the hydroxyl group on the C1 carbon (and subsequent dehydration) is observed for both chloramphenicol and 3-*O*-acetylchloramphenicol. The loss of the acetyl group in the C1 position is observed for 1,3-*O,O*-diacetylchloramphenicol.



  

All β-lactam antibiotics in this study exhibited good ionization efficiencies with limits of detection in the low picogram range. Previous authors have performed SRM for chloramphenicol in the negative ion mode.[26,27] However, when using the SRM assay described herein, we found that chloramphenicol, and its acetylated forms, ionized very efficiently in positive ion mode. A linear regression of varying concentrations of chloramphenicol is given in Fig. 3, which illustrates both good linearity and sensitivity of the antibiotic in positive ion mode. In addition, the acetylated forms of chloramphenicol were found to be close to the same limits of detection and sensitivity of native chloramphenicol. Of the β-lactam antibiotics, there was approximately one order of magnitude greater sensitivity for cloxacillin and nafcillin by SRM than for the other β-lactam antibiotics.

Because ampicilloic acid is the major product from the hydrolysis of ampicillin with β-lactamase, we wanted to develop an SRM for its detection. However, we were not able to easily observe ampicilloic acid by mass analysis, possibly because of the high volatility of the amine group and its possible degradation in the ion source. When we performed a parent ion scan ($m/z = 160.1$) of hydrolyzed ampicillin, we identified a doubly charged ion at $m/z = 358.7$ that directly correlated with the amount of hydrolyzed ampicillin. We did not investigate the identity of this ion, but it may be the result of a dimerization of ampicillin and/or one of its hydrolyzed products, a process observed by others.[28,29]

We also investigated the ability of SRM to determine the specificity of a particular β-lactamase enzyme to hydrolyze the selected group of antibiotics mentioned earlier. The type of β-lactamase that a bacterium expresses will determine which β-lactam antibiotic the enzyme is able to hydrolyze. More recent Food and Drug Administration (FDA)-approved antibiotics are designed to make them less susceptible to the actions of β-lactamase. However, because of selective pressure on the organism by newer and more recently approved antibiotics, changes in the enzyme allow it to also hydrolyze many, if not all, of the later classes of antibiotics.

An SRM method was developed that would allow us to determine the effect of a specific β-lactamase enzyme to a number of antibiotics. A β-lactamase obtained from the antibiotic-resistant bacterium *Bacillus cereus* was chosen for this experiment because of its well documented specificity to hydrolyze certain β-lactam antibiotics.[30] Ampicillin, piperacillin, and nafcillin are susceptible to hydrolysis by this β-lactamase. This is not the case with cloxacillin and cefoperazone, which are both resistant to the hydrolyzing effects of this β-lactamase. Chloramphenicol should also be unaffected by this enzyme because it is not of the β-lactam class of antibiotics. The ability of this particular β-lactamase enzyme to hydrolyze the aforementioned antibiotics is represented in Fig 4. Prior to introducing the enzyme to a solution comprising the six antibiotics, each antibiotic was clearly observed by SRM (Fig. 4(a)). After adding the enzyme and incubating for 2 h, the antibiotics ampicillin, piperacillin, and nafcillin were completely hydrolyzed and therefore no longer observed (Fig. 4(b)). However, cloxacillin, cefoperazone, and chloramphenicol appeared to be unhydrolyzed by the enzyme as predicted. SRM was therefore able to simultaneously monitor the susceptibility of a number of different antibiotics to a β-lactamase in a single analysis.

We also wanted to ascertain whether SRM can determine if an organism growing in culture is resistant to a particular antibiotic. For example, by monitoring the concentration of chloramphenicol and/or its acetylated forms in the growth media, one can determine not only if an organism is resistant to chloramphenicol but also the rate at which the antibiotic is modified. For this reason, we decided to observe the effects of a chloramphenicol-resistant strain of *E. coli* grown in M9 broth-containing chloramphenicol. *E. coli* in this experiment was transformed by the plasmid pACYC184, which encodes a chloramphenicol resistance gene.[31] The plasmid allows *E. coli* to express the enzyme chloramphenicol

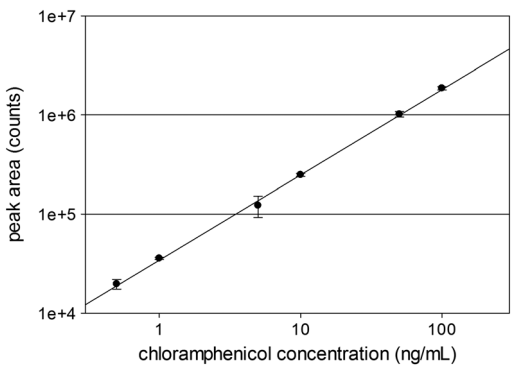


**Figure 3.** Linear regression for the detection of chloramphenicol in the ranges of 2.5–500 ng/ml (12.5 pg to 2.5 ng total chloramphenicol/injection) using transition 323.0 > 275.0. Coefficient of determination ($R^2$) = 0.9954.

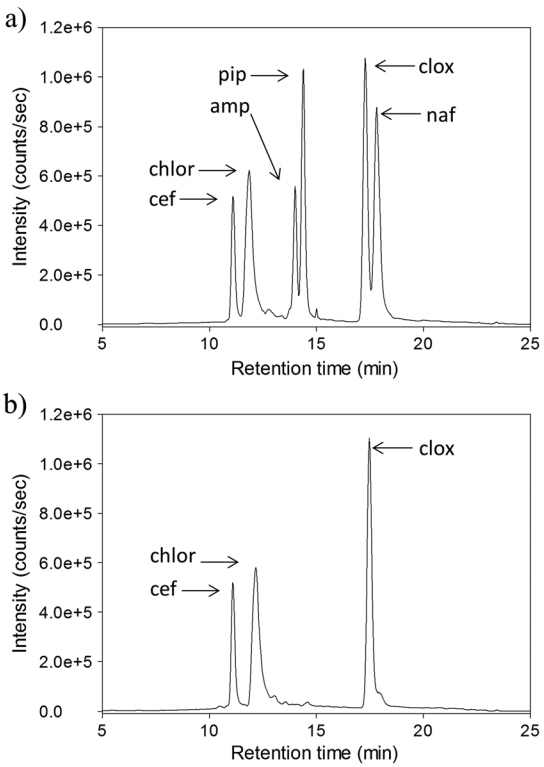


**Figure 4.** Selected reaction monitorings of six different antibiotics (amp = ampicillin, cef = cefoperazone, chlor = chloramphenicol, clox = cloxacillin, naf = nafcillin, and pip = piperacillin): (a) before the introduction of a β-lactamase enzyme from *B. cereus* and (b) after the reaction of the enzyme with the antibiotics. All of the antibiotics hydrolyzed by β-lactamase are known to be susceptible to the enzyme. Cloxacillin and cefoperazone, both known to be resistant to this specific β-lactamase, are observed in the spectrum. Chloramphenicol was also unaffected, as expected, as it is not a β-lactam antibiotic and therefore not hydrolyzed by β-lactamases.

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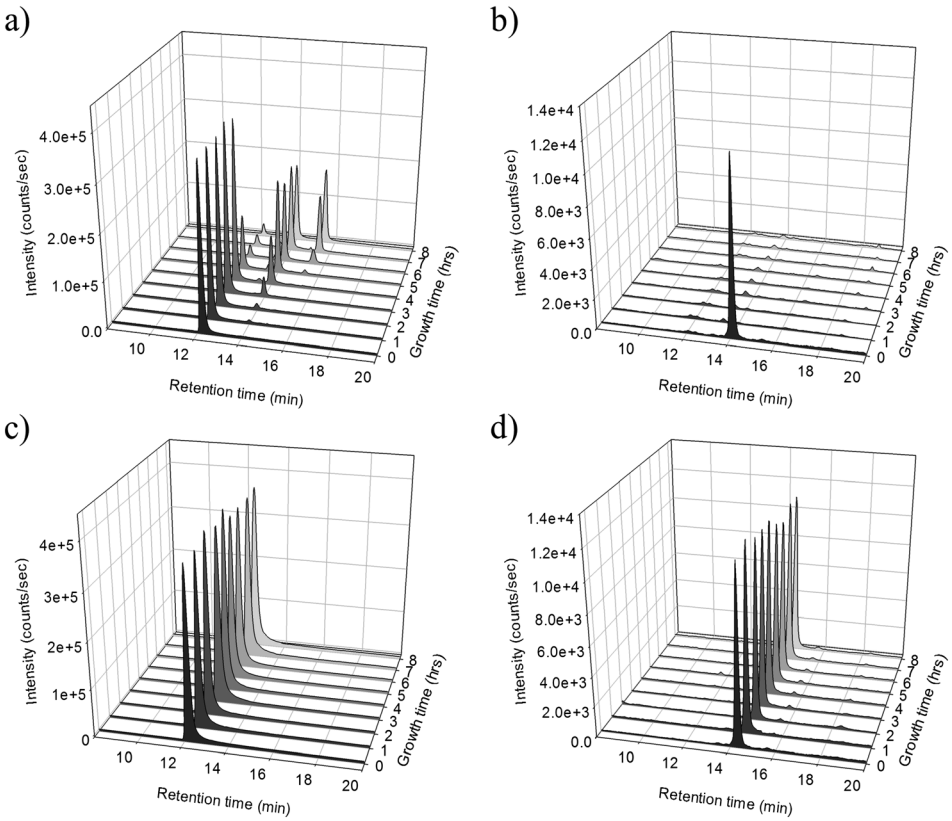


**Figure 5.** Selected reaction monitorings of M9 broth containing either chloramphenicol or ampicillin were analyzed at 1-h intervals. (a) At time = 0, *Escherichia coli* (pACYC184) was both added to broth-containing chloramphenicol and incubated at 37 °C. Samples were reanalyzed after every hour to monitor the reduction of chloramphenicol and the formation of both 3-*O*-acetylchloramphenicol and 1,3-*O*,*O*-diacetylchloramphenicol. At 0 h, only chloramphenicol was observed (rt = ~12 min). However, at 3 h, a significant amount of 3-*O*-acetylchloramphenicol (rt = ~14 min) was observed, indicating that the bacteria were resistant to chloramphenicol. At 6 h, almost all chloramphenicol had been acetylated. (b) The same experiment but using β-lactamase expressing *E. coli* (pUC18) and ampicillin. After 1 h, all ampicillin was hydrolyzed by the organism. (c) *E. coli* (C600), which is susceptible to chloramphenicol, did not result in any change in chloramphenicol concentration. (d) E. coli (C600) also did not result in the hydrolysis of ampicillin and is therefore susceptible to this antibiotic.

acetyltransferase, which inactivates chloramphenicol via acetylation. The decrease in available unmodified chloramphenicol and the increase in the concentration of acetylated forms would indicate that the organism is resistant to chloramphenicol. This is illustrated in Fig. 5(a) where the growth media was sampled every hour and assayed by SRM. At the starting point (time = 0) when the bacteria were first introduced into the growth media, only unmodified chloramphenicol (retention time 12 min) was observed in the growth media. However, after only 3 h of bacterial growth, a significant amount of 3-*O*-acetylchloramphenicol (retention time 14 min) was observed, indicating that the *E. coli* was expressing chloramphenicol acetyltransferase and therefore acetylating the chloramphenicol. After 6 h, almost no chloramphenicol was observed, as it had been entirely converted to the acetylated form. Also at 6 h, there was a significant amount of the diacetylated chloramphenicol observed (retention time 15.5 min). Because the analysis time for performing SRM was less than 30 min, the rate-limiting step for determining chloramphenicol resistance in *E. coli* was the time necessary for bacterial growth, a factor beyond our immediate control.

The same experiment was repeated, but instead *E. coli* was transformed with the ampicillin resistance plasmid pUC18, which allows the organism to express a β-lactamase enzyme that specifically hydrolyzes ampicillin.[32] Figure 5(b) represents the SRM assay specific for ampicillin sampled after every hour of bacterial growth. At initial conditions, only ampicillin was observed, but after 1 hr, no ampicillin was observed in the growth media. By using this method, the time required to determine ampicillin resistance in *E. coli* was only 1 h because of the rapid hydrolysis of ampicillin by the enzyme. As a side note, when ampicillin was added to a broth that had a 1 μM concentration of the *B. cereus* β-lactamase, all ampicillin was hydrolyzed within minutes.

The experiments were also performed using a control group, consisting of *E. coli* (C600) that did not have any of the antibiotic resistance plasmids. Figure 5(c) is the SRM of *E. coli* (C600) that is susceptible to chloramphenicol. We did not observe any decrease in the concentration of chloramphenicol over an 8-h period, indicating that the *E. coli* did not express any chloramphenicol acetyltransferase and was therefore susceptible to chloramphenicol. This was also the case when the organism was grown with ampicillin present, as seen in Fig. 5(d). The concentration of ampicillin remained constant over the 8-h period, and no hydrolysis was observed (Fig. 5(d)), thus indicating the susceptibility of *E. coli* (C600) to ampicillin.

## Conclusions

We have shown that SRM is both a rapid and effective technique for determining the susceptibility or resistance of an organism to



  

antibiotic treatment. The Kirby–Bauer assay remains the gold standard for determining antibiotic resistance; however, this technique has drawbacks. It requires one to grow the organism before antibiotic sensitivity can be determined. It also requires the isolated organism to be recultured and a zone test performed. This adds to the time required to perform the assay and thereby delays effective antibiotic therapy. Therefore, reducing the time necessary to perform a susceptibility test is one of the most important benefits to performing the analysis by SRM.

We have found that SRM can detect modifications of antibiotics that occur in a short period. In the case of β-lactam antibiotics, the hydrolysis occurs almost immediately, and therefore, the limiting factor is the time required to perform the SRM analysis. Current susceptibility testing can take anywhere from 48 to 72 h to perform. However, SRM analysis can be performed in less than an hour. This reduces the time necessary to determine the susceptibility of an organism to any given antibiotic.

The duty cycle of current mass spectrometers allows the screening of multiple antibiotics in a single run. SRM has already been reported capable of screening dozens of pharmaceutical compounds simultaneously. By applying this technique to analyze antimicrobial resistance, nearly all of the current FDA-approved antibiotics could be screened in a single assay. Also, because bacterial resistance is determined by observing the presence or absence of an antibiotic in the SRM spectrum, one can determine in a single assay not only to which antibiotics an organism is resistant but also those which may be effective against it. Whereas some antibiotic resistance mechanisms do not lead to structural changes to the antibiotic (i.e., efflux pumps and mutation), this SRM approach would still represent a major step forward in antibiotic resistance detection.

The application of SRM also has many advantages in terms of sensitivity. By using a nanoflow interface, the amount of sample needed per injection is small (~1 μl). Therefore, only minute amounts of biological sample are needed. Sample collection vials as small as 100 μl containing a multitude of antibiotics can be used. This would be of great importance if there is only a small amount available of a biological sample or pathogenic bacteria in a clinical sample. Another benefit of this improved sensitivity is that analysis can be performed with a low concentration of antibiotic, far below the MIC for antibiotic susceptible bacteria. When this occurs, the microbe will continue to grow regardless of susceptibility or resistance to the antibiotic. This allows the bacteria to be unaffected by the antibiotics during culturing and growth. Thus, modification of the antibiotics that they are resistant to will continue to take place, whereas leaving unchanged the antibiotics that the bacteria are susceptible to.

Although the SRM transitions to the chemically modified forms of antibiotics (i.e., acetylated chloramphenicol in our case) have been investigated, such transitions do not need to be developed to detect antibiotic resistance. Because any chemical modification of an antibiotic can be a sign of bacterial resistance, simply observing the decrease in the concentration of the antibiotic is enough to determine that the organism is resistant to that antibiotic. This is advantageous if a new and unknown chemical modification pathway is developed by the organism to a given antibiotic. One does not need to know what chemical modification is occurring, because any chemical change may result in the elimination of the antibiotic in the spectrum.

The purchase of the required mass spectrometer and liquid chromatography instrumentation to perform this experiment has a high initial cost. However, this initial cost would eventually be offset by alleviating the need for the other time-consuming chemical protocols and outweighed by the multiple advantages of SRM listed previously. Future work will focus on using this method with real biological samples and investigating its use in a clinical setting.

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