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# Bioanalytical method validation design for the simultaneous quantitation of analytes that may undergo interconversion during analysis

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**Abstract**

In the analysis of post-dose biological samples for quantitative determination of two analytes that can potentially undergo interconversion, it is essential to minimize the interconversion during the multiple steps of the bioanalytical method. However, even after optimizing the conditions of each step, some interconversion may be unavoidable. Even then, a method can be developed for the accurate simultaneous determination of the two analytes in post-dose biological samples if the composition, in terms of the ratio of the concentrations of the two analytes, of the calibration standards and quality control (QC) samples are selected judiciously, in relation to the composition of the unknown samples to be analyzed. As an example of such interconverting analytes, a δ-hydroxy acid compound (analyte 1) and its δ-lactone (analyte 2) were selected as model compounds that can potentially undergo interconversion. The effects of changing the relative concentrations of the two analytes in QC samples vis-à-vis the calibration standards on the performance of the method under conditions were investigated where: (a) the interconversion between the two analytes was minimized; (b) the conversion of analyte 2 to analyte 1 was enhanced; (c) the interconversion between the two analytes was enhanced. The results showed that the method performance, as measured by the accuracy and precision of the QC samples, was not acceptable when the ratio of concentration of analyte 1 to that of analyte 2 in the QC samples was different from that in the calibration standards and the conditions used facilitated the conversion of one analyte to the other. However, when the relative concentration of the two analytes in the QC samples was identical to that of the calibration standards, the method performance was acceptable under all three conditions of interconversion. This was because the same degree of interconversion took place in the QC samples and calibration standards. The purpose of QC samples in bioanalytical methods is to gauge how the method will perform for the analysis of post-dose test samples and hence, ideally, the relative concentrations of the analytes in QC samples should be selected to mimic the anticipated concentrations in the test samples. However, the relative concentrations of the analytes in test samples may not be known a-priori, or may change from sample to sample; therefore, it is not always possible to construct QC samples that exactly mimic the relative concentrations of analytes in the test samples. Thus, in order to cover the variety of test samples, the method should

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include, in addition to QC samples that contain the analytes at the same relative concentration as in the calibration standards, QC samples with relative concentrations that are different from those in the calibration standards, including those that contain only analyte 1 and only analyte 2. In addition, the conditions adopted for the method should favor the minimization of the conversion of the analyte that is expected to be the major component in the post-dose test samples. 

*Keywords:* Method validation design; Bioanalysis; Interconversion; Interconverting analytes; Relative concentration in QC samples; Relative concentration in calibration standards

## 1. Introduction

Frequently, bioanalysts are asked to analyze biological samples for two analytes that can potentially convert from one to the other during any of the numerous steps of the bioanalytical method. Such interconversion may occur in the biological matrix on the bench before taking aliquots for analysis, during extraction, during evaporation to dryness, or in the reconstitution solution in the injection vial. A number of classes of drugs could give post-dose samples which may contain analytes that can potentially undergo interconversion. Such post-dose samples may be obtained with 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, such as lovastatin and simvastatin [1]. These drugs are administered in their lactone forms, but the post-dose samples contain both the lactones and the open-ring hydroxy acids. On the other hand, atorvastatin is administered in the open-ring hydroxy acid form and the post-dose samples contain both the acid and the lactonized form [2]. For samples that contain hydroxy acids and the corresponding lactones, maintaining the pH of the samples around 4–5 minimizes interconversion [3–5]. Increasing the pH above 6 facilitates the conversion of the lactone to the acid (in the ionized form); on the other hand, lowering the pH facilitates the conversion of the acid to the lactone or the lactone to the acid (in the non-ionized form). Another category of samples that may undergo ex-vivo conversion are samples that contain a carboxylic acid drug and its acylglucuronide. The acylglucuronide will revert to the drug due to hydrolysis. The hydrolysis rate can be minimized by adjusting the pH of the sample to 3–4; outside this pH region, the rate increases as the pH is increased or decreased [6]. Samples that contain a thiol drug and its disulfide may also cause analytical challenges due to the potential for the conversion of the thiol to the disulfide, which is pH dependent; the lower the pH, the better is the stability of the thiol compound [7]. A fourth category of potentially problematic samples are those which contain a drug and its isomeric biotransformation product that can undergo ex-vivo interconversion. An example of this category are samples that contain an E-isomer methyloxime drug and its Z-isomer biotransformation product [8], where ex-vivo conversion occurs at pH below 6.0. Another category of samples that should be mentioned is those samples that contain a drug and the prodrug. Minimizing the ex-vivo conversion of the prodrug will depend on the functional group used to form the prodrug.

During method development for the quantitative analysis of post-dose samples containing two analytes that can undergo ex-vivo interconversion, conditions must be optimized to minimize such interconversion. However, even the optimal conditions adopted may not totally prevent interconversion. It is thus essential to design the composition (in terms of the ratio of the concentration of one analyte to that of the other) of the calibration standards and quality control (QC) samples so that the accuracy and precision obtained for the QC samples realistically reflect the accuracy and precision that will be obtained for the anticipated post-dose samples. Ideally, the composition of the QC samples should be identical to that of post-dose samples. However, in the early phase of drug development the composition of the post-dose samples will not be known and, in addition, the composition will likely change from sample to sample, depending on the time point at which the samples were taken following drug administration. Under this circumstance, the

appropriate design of the composition of the calibration standards and QC samples is essential. Surprisingly, in published methods for the simultaneous determination of compounds that can potentially undergo interconversion [1,9–12], this problem has not been discussed and was not taken into consideration in the design of the QC samples vis-à-vis the calibration standards. The work presented here gives the results of an investigation, using a model δ-hydroxy acid compound (analyte 1) and its δ-lactone (analyte 2), undertaken to illustrate the effects of using varying compositions of QC samples under different conditions of treatment of the plasma samples in order to effect different degrees of interconversion.

## 2. Experimental

### 2.1. Reagents and chemicals

Pravastatin and pravastatin lactone (Fig. 1), the model δ-hydroxy acid and δ-lactone compounds selected for the investigation, are characterized products of the Bristol-Myers Squibb Pharmaceu-

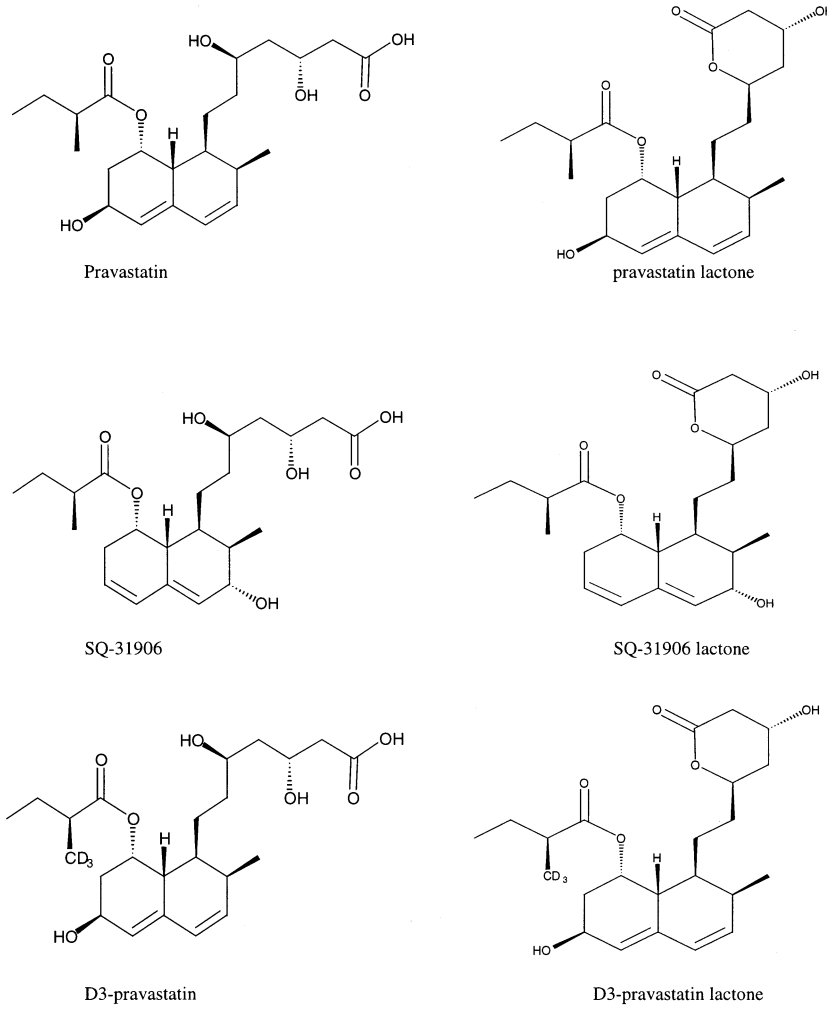



Fig. 1. Chemical structures of pravastatin, pravastatin lactone, SQ-31906, SQ-31906 lactone, D3-pravastatin and D3-pravastatin lactone.

tical Research Institute. D3-pravastatin and D3-pravastatin lactone (Fig. 1), used as internal standards, are also characterized products of the Bristol-Myers Squibb Pharmaceutical Research Institute. Acetonitrile (HPLC grade) and formic acid (98%) were purchased from EM Science (Gibbstown, NJ). Ammonium acetate (HPLC grade) was from Fisher Scientific (Fair Lawn, NJ). Ammonium formate (97%) was from Aldrich (Milwaukee, WI). House deionized water, further purified with a Milli-Q water purifying system (Millipore Corporation, Bedford, MA), was used. Ammonium acetate solutions (0.1 M and pH 3.8, and 0.2 M and pH 6.8) were prepared by adding 0.77 and 1.54 g ammonium acetate into 100 ml Milli-Q water and adjusting the pH to 3.8 and 6.8 with acetic acid or ammonium hydroxide, respectively. Ammonium formate solution (10 mM, pH 4.5) was prepared by adding 1.26 g ammonium formate into 2000 ml Milli-Q water and adjusting the pH to 4.5 with formic acid. Hydrochloric acid/sodium chloride solution (2 N HCl/0.5 M NaCl) was prepared by adding 1.76 ml of concentrated HCl and 2.9 g of NaCl into 100 ml of water.

### 2.2. Equipment

The mass spectrometer used was a Finnigan (San Jose, CA) TSQ-7000 mass spectrometer equipped with an atmospheric pressure ionization (API-2) electrospray interface. A Waters 2690 Separations Alliance (Milford, MA) liquid chromatographic system equipped with an autosampler was used, together with a Perkin-Elmer series 200 LC pump. Two Waters symmetry C18 5-μm columns were used: 3.9 × 50 and 4.6 × 150 mm. A Waters Qasis HLB extraction column (30 μm, 1 × 50 mm) was also used.

### 2.3. Standards and QC samples

Two stock solutions (0.5 mg $ml^{-1}$) of pravastatin, one for standard and the other for QC samples, and one stock solution of D3-pravastatin were prepared separately in water/acetonitrile (10:90, v/v). Two stock solutions (0.5 mg $ml^{-1}$) of pravastatin lactone, one for standard and the other for QC samples, and one stock solution of D3-pravastatin lactone were prepared separately in acetonitrile. Internal standard working solution I, used in interconversion conditions 1–2, was prepared by adding appropriate portions of D3-pravastatin and D3-pravastatin lactone stock solutions to 0.1 M ammonium acetate solution (pH 3.80) to obtain 500 ng $ml^{-1}$ for each internal standard. Internal standard working solution II, used in the intercoversion condition 3, was prepared by adding appropriate portions of D3-pravasatin and D3-pravastatin lactone stock solutions to 0.2 M ammonium acetate solution (pH 6.80) to obtain 500 ng $ml^{-1}$ for each internal standard.

The calibration standard curve in human plasma ranged from 5 to 500 ng $ml^{-1}$ for both pravastatin and pravastatin lactone with each standard concentration point containing equal amounts of the two analytes. The QC samples, prepared in human plasma, contained only pravastatin, or only pravastatin lactone, or both in varying ratios of pravastatin to pravastatin lactone: 1:1, 1:10, 10:1, 1:3 and 3:1. All the concentrations of the QC samples fell within the calibration curve range. The composition of the standard and QC samples are summarized in Table 1. Each concentration point of the calibration standard curve was run in duplicate and each QC sample was run in five replicates. For the calibration standard curve, least-squares linear regression, weighted to $1/x^2$, was utilized.

### 2.4. Interconversion conditions and sample preparation

The standard and QC samples were analyzed by direct injection of the plasma samples without prior extraction, as described under Section 2.5, after they were subjected to three different interconversion conditions. Under condition 1, all the standard and QC samples were prepared in an ice-bath and analyzed immediately after preparation. For analysis, a 50-μl portion of the internal standard working solution I was added to 100 μl of each calibration standard and QC sample, which lowered the pH of the samples to 4.2, where interconversion was minimal. Under condi-

Table 1
Composition of calibration standards and quality control (QC) samples[a]

| | Pravastatin (ng ml$^{-1}$) | Pravastatin lactone (ng ml$^{-1}$) |
|---|---|---|
| Standard 1 (1:1) | 5.00 | 5.00 |
| Standard 2 (1:1) | 10.0 | 10.0 |
| Standard 3 (1:1) | 25.0 | 25.0 |
| Standard 4 (1:1) | 50.0 | 50.0 |
| Standard 5 (1:1) | 100 | 100 |
| Standard 6 (1:1) | 200 | 200 |
| Standard 7 (1:1) | 300 | 300 |
| Standard 8 (1:1) | 400 | 400 |
| Standard 9 (1:1) | 500 | 500 |
| QC1 (1:1) | 15.0 | 15.0 |
| QC2 (1:1) | 200 | 200 |
| QC3 (1:1) | 420 | 420 |
| QC4 (1:3) | 5.00 | 15.0 |
| QC5 (1:10) | 20.0 | 200 |
| QC6 (1:10) | 42.0 | 420 |
| QC7 (0:15) | 0 | 15.0 |
| QC8 (0:200) | 0 | 200 |
| QC9 (0:420) | 0 | 420 |
| QC10 (3:1) | 15.0 | 5.00 |
| QC11 (10:1) | 200 | 20.0 |
| QC12 (10:1) | 420 | 42.0 |
| QC13 (15:0) | 15.0 | 0 |
| QC14 (200:0) | 200 | 0 |
| QC15 (420:0) | 420 | 0 |

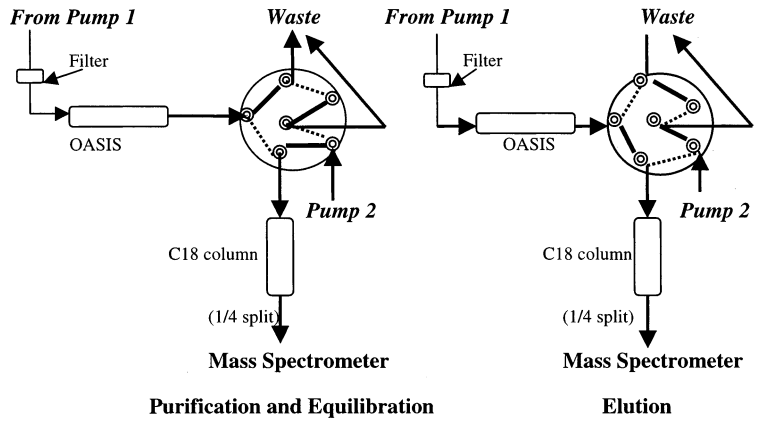
[a] The numbers in parentheses show the ratios of the pravastatin concentrations to the pravastatin lactone concentrations.

tion 2, all the standard and QC samples were analyzed after keeping them as is (i.e. at pH 7.3, without adjusting the pH) at room temperature for 4 h to promote the hydrolysis of pravastatin lactone to pravastatin. For analysis, a 50-μl portion of the internal standard working solution I was added to 100 μl of each calibration standard and QC sample, which lowered the pH of the samples to 4.2, where interconversion was minimal. Under condition 3, all the standard and QC samples were adjusted to pH 1.8 by adding 80 μl of the hydrochloric acid/sodium chloride solution to 1.0 ml of each calibration standard and QC sample. The samples were then vortexed at room temperature for 2 h to promote interconversion between the two analytes. For analysis, a 50-μl portion of the internal standard working solution II was added to 100 μl of each sample, which raised the pH to 4.2. These processed samples from conditions 1–3 were kept at 4°C on the HPLC autosampler during analysis.

### 2.5. Chromatographic and mass spectrometric conditions

The on-line purification system, depicted in Fig. 2, was used for the direct injection of processed plasma samples without any prior extraction. The basis of on-line purification following direct injection of plasma samples has been described previously [13]. For processed plasma samples obtained under conditions 1 and 2, the samples (50 μl) were injected into the Oasis HLB extraction column (1 × 50 mm and 30 μm) with a mobile phase of 100% aqueous 10-mM ammonium formate (pH 4.5) at a flow rate of 3.0 ml min$^{-1}$, with the column effluent directed to waste. This was the purification step (Fig. 2). The flow rate was then reduced to 0.8 ml min$^{-1}$, the mobile phase was changed to a mixture of 10-mM ammonium formate (pH 4.5) and acetonitrile (60:40, v/v), and the C18 analytical column (3.9 × 50 mm, 5 μm) was connected to Oasis HLB column, with the column effluent directed to the mass spectrometer. This was the elution step (Fig. 2). The flow rate was then increased back to 3 ml min$^{-1}$ with 100% acetonitrile first and then 100% aqueous 10-mM ammonium formate (pH 4.5) and the column effluent was directed to waste. This was the equilibration step (Fig. 2). The total run time was 6.0 min. The mobile phase gradient scheme, which was used in conjunction with Fig. 2, is shown in Table 2. For processed plasma samples obtained under condition 3, the same purification, elution and equilibration steps were applied using a different analytical column (4.6 × 150 mm), gradient scheme (Table 3) and valve switching program (Fig. 2). The total run time was 8.0 min.

The Finnigan TSQ-7000 triple quadrupole mass spectrometer was operated in both negative and positive electrospray ionization (ESI) modes by the application of within-run polarity switching, starting in the negative mode and then switching




Note: Pump 1 is Alliance 2690 system. Pump 1 is operated in a gradient mode as shown in Tables 2 and 3. Pump 2 is a Perkin-Elmer 200 series LC pump operated at 0.8 ml $min^{-1}$ with an isocratic mobile phase of 10 mM ammonium formate (pH 4.5) and acetonitrile: 60/40 for conditions 1 and 2; 50/50 for condition 3.

| Steps of on-line purification | Time range | Divert valve position |
|---|---|---|
| Purification step | 0-1 min | Divert valve switched to waste |
| Elution step | 1-4.9 (1-7.0) min | Divert valve switched to MS |
| Equilibration step | >4.9 (>7.0) min | Divert valve switched back to waste |
| Total run time | 6 (8) min | |

Data in parentheses are for condition 3.

Fig. 2. A schematic representation of the on-line purification system.

Table 2
HPLC gradient program for conditions 1 and 2

| Time (min) | Flow rate | 10 mM ammonium formate (pH 4.5) | Acetonitrile |
|---|---|---|---|
| 0.0 | 3.0 | 100 | 0 |
| 0.6 | 3.0 | 100 | 0 |
| 0.8 | 0.8 | 100 | 0 |
| 0.9 | 0.8 | 60 | 40 |
| 5.0 | 0.8 | 60 | 40 |
| 5.1 | 3.0 | 0 | 100 |
| 5.4 | 3.0 | 0 | 100 |
| 5.5 | 3.0 | 100 | 0 |
| 6.0 | 3.0 | 100 | 0 |

Table 3
HPLC gradient program for condition 3

| Time (min) | Flow rate | 10 mM ammonium formate (pH 4.5) | Acetonitrile |
|---|---|---|---|
| 0.0 | 3.0 | 100 | 0 |
| 0.6 | 3.0 | 100 | 0 |
| 0.8 | 0.8 | 100 | 0 |
| 0.9 | 0.8 | 50 | 50 |
| 7.0 | 0.8 | 50 | 50 |
| 7.1 | 3.0 | 0 | 100 |
| 7.4 | 3.0 | 0 | 100 |
| 7.5 | 3.0 | 100 | 0 |
| 8.0 | 3.0 | 100 | 0 |

to the positive mode. For the sample preparation conditions 1 and 2, the mass spectrometer was switched from negative to positive mode at 3.8 min and the total acquisition time was 5.0 min. For the sample preparation condition 3, the mass spectrometer was switched from negative to positive mode at 5.5 min and the total acquisition time was 7.0 min.

In the negative mode, the selected reaction monitoring (SRM) transition involved the $[M-H]^-$ precursor ion to product ions: $m/z$ 423 to $m/z$ 321 and $m/z$ 423 to $m/z$ 303 (the sum of the two transitions) for pravastatin; $m/z$ 426 to $m/z$ 321 and $m/z$ 426 to $m/z$ 303 (the sum of the two transitions) for D3-pravastatin. In the positive mode, the SRM transition involved the $[M+NH_4]^+$ precursor ion to product ion: $m/z$ 424 to $m/z$ 183 for pravastatin lactone and $m/z$ 427 to $m/z$ 183 for D3-pravastatin lactone. Fragmentation occurred in the collision cell (Q2) using argon as the collision gas with the collision energy set at 20 eV at both negative and positive ESI mode. The collision gas pressure was 2.3–2.5 mTorr. The scan rate was set at 0.2 s scan$^{-1}$ for pravastatin and D3-pravastatin, and at 0.5 s scan$^{-1}$ for pravastatin lactone and D3-pravastatin lactone. Mass peak width was 1.0 mass unit at half height for both Q1 and Q3 and the mass span was 0.6 mass unit for all the compounds. The spray voltage was set to 4.5 kV. The sheath and auxiliary gas (nitrogen) pressure was set at 80 psi and 40 U, respectively. The heated capillary temperature was set at 300°C. The data were acquired on the mass spectrometer using Finnigan Interactive Chemical Information System (ICIS) 8.3.0 software and the chromatographic peaks for each ion channel were integrated using Finnigan LCQuan/LCQ1.2 software.

## 3. Results and discussion

The principal objective of this study was to demonstrate the effects of interconversion between the analytes during the numerous steps of bioanalysis on the accuracy and precision of the method. The model compounds used here are pravastatin, a carboxylic acid with a δ-hydroxyl group, and pravastatin lactone, the δ-lactone form of the acid. The full-scan Q1 and Q3 electrospray spectra of pravastatin and pravastatin lactone are shown in Fig. 3. Pravastatin gave an intense $[M-H]^-$ signal at $m/z$ 423 and pravastatin lactone gave an intense $[M+NH_4]^+$ signal at $m/z$ 424. Pravastatin gave intense product ions at $m/z$ 321 and 303 using $m/z$ 423 as the precursor ion, and pravastatin lactone gave an intense product ion at $m/z$ 183 using $m/z$ 424 as the precursor ion. Within-run polarity switching was used because the negative mode was optimum for pravastatin and the positive mode was optimum for pravastatin lactone. A longer C18 analytical column ($4.6 \times 150$ mm) was used for samples obtained under condition 3 because of the need for the separation of pravastatin from its isomer (SQ-31906), and pravastatin lactone from its isomer (SQ-31906 lactone). The two isomers were produced under the acidic environment of condition 3.

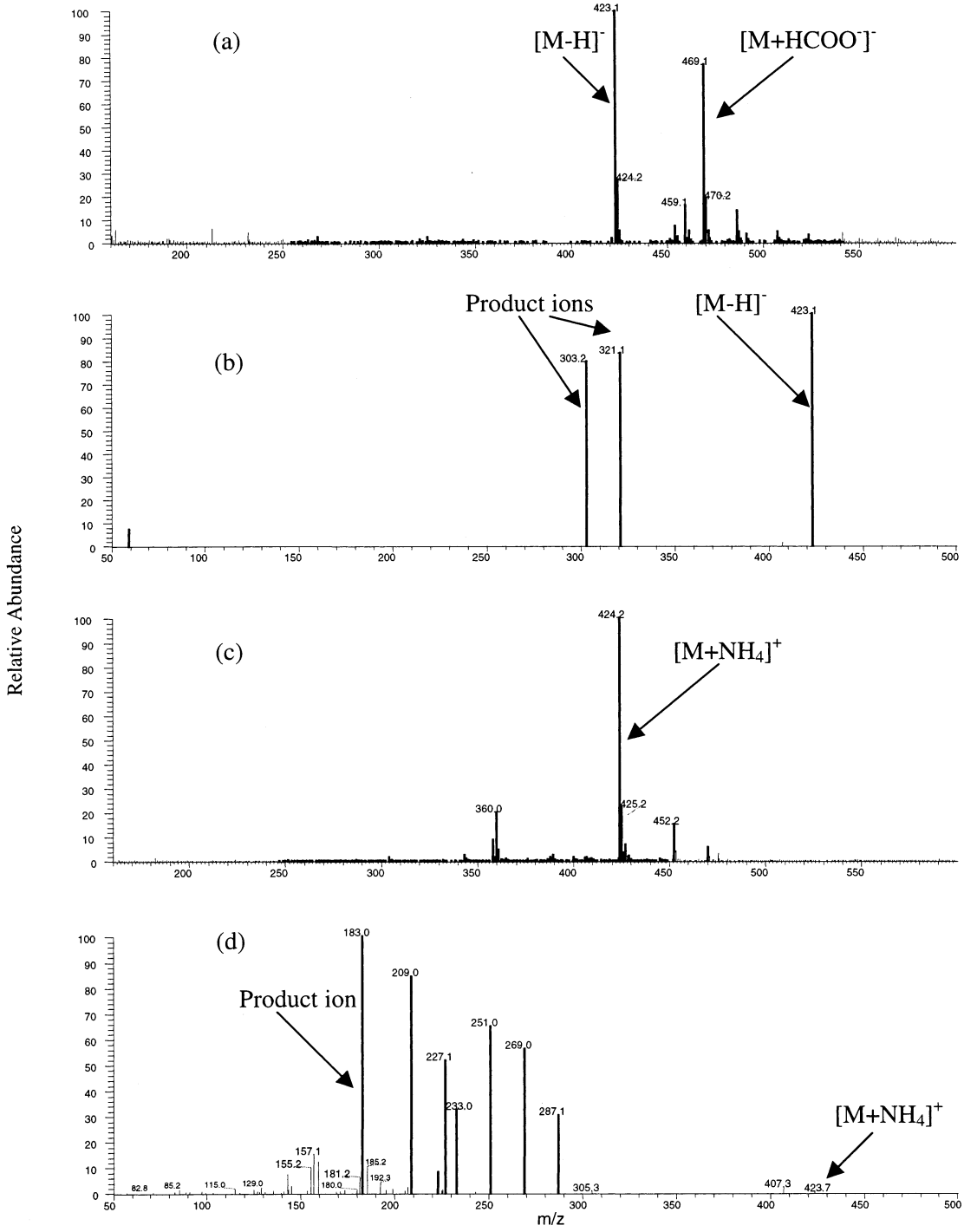



Fig. 3. Negative electrospray full-scan (a) and product ion MS/MS mass spectra (b) (precursor ion: $m/z$ 423) of pravastatin; positive electrospray full-scan (c) and product ion MS/MS mass spectra (d) (precursor ion: $m/z$ 424) of pravastatin lactone.

Figs. 4 and 5 show SRM chromatograms of pravastatin-only and pravastatin lactone-only QC samples treated under the three conditions of sample preparation. Under condition 1, there was no conversion of pravastatin to pravastatin lactone or vice versa since the prepared plasma

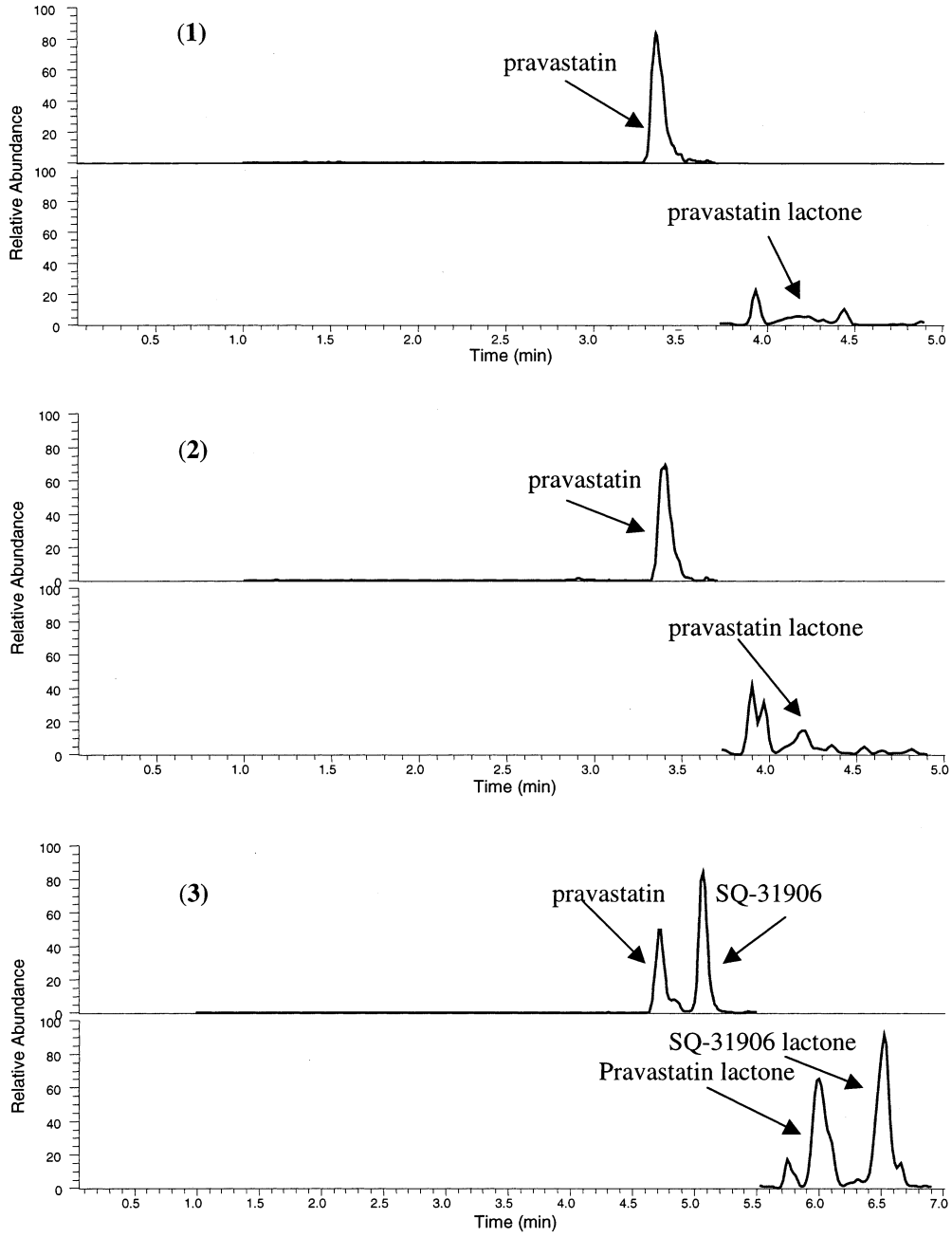



Fig. 4. Selected reaction monitoring (SRM) chromatograms of QC14 (pravastatin-only quality control (QC) sample at 200 ng $ml^{-1}$) under conditions (1), (2) and (3).

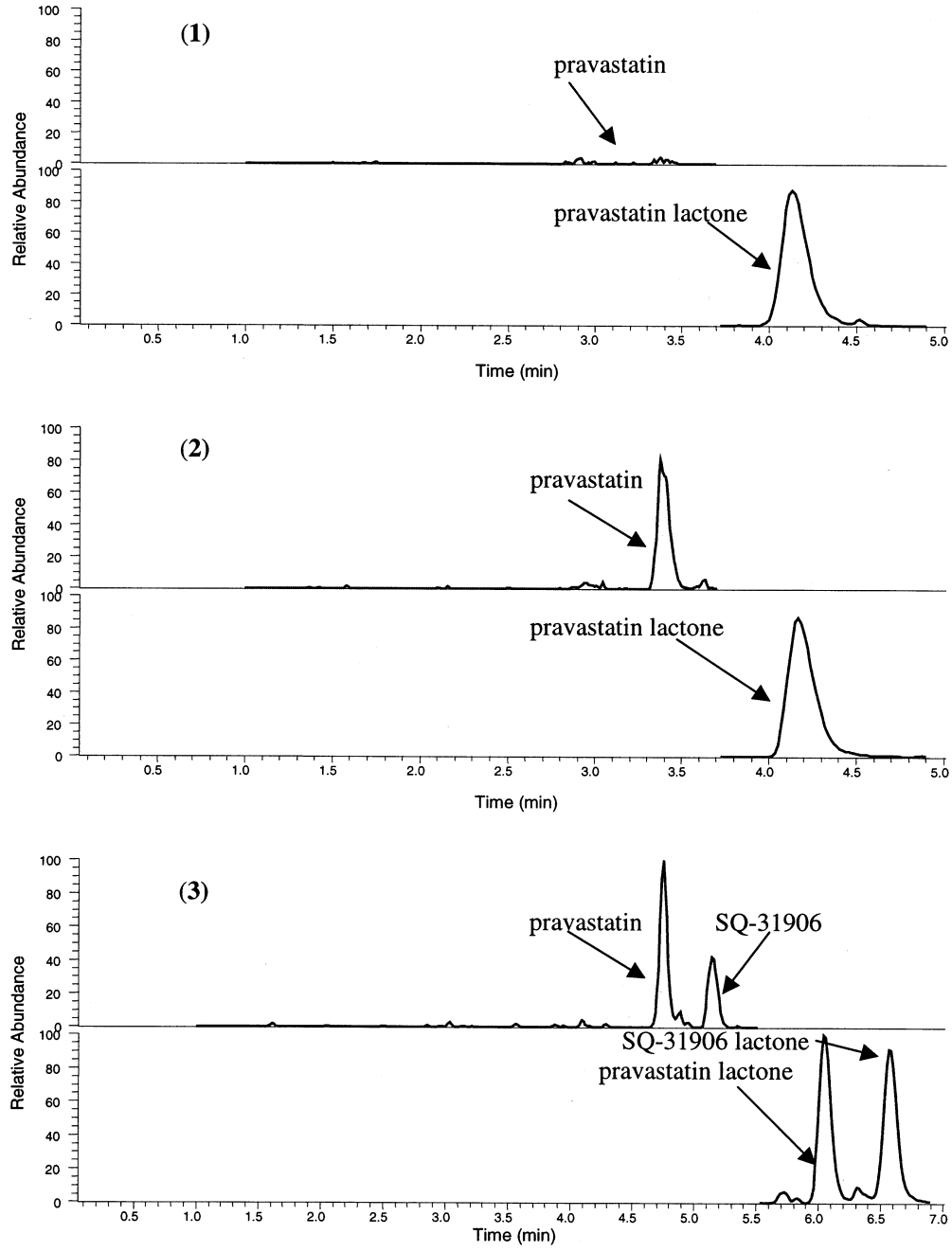



Fig. 5. Selected reaction monitoring (SRM) chromatograms of QC8 (pravastatin lactone-only quality control (QC) sample at 200 ng $ml^{-1}$) under conditions (1), (2) and (3).

solutions were maintained at pH 4.2 to minimize the interconversion. Under condition 2, pravastatin lactone was hydrolyzed to produce pravastatin, but the conversion from pravastatin to pravastatin lactone was not observed. Under acidic condition 3, there were several conversion

pathways. Pravastatin not only underwent intramolecular esterification to yield pravastatin lactone, but also underwent isomerization to produce positional isomers of pravastatin (SQ-31906) (Fig. 1) and pravastatin lactone (SQ-31906 lactone). Meanwhile, pravastatin lactone produced not only pravastatin but also SQ-31906 and SQ-31906 lactone. Thus, the same number of products were obtained whether the starting material was pravastatin or pravastatin lactone. This is in agreement with previous findings [14].

The results of analysis of samples from condition 1, where there was no interconversion between pravastatin and pravastatin lactone, are shown in Table 4. The performance of the standard curve for both pravastatin and pravastatin lactone was excellent as the back-calculated concentrations of the individual standards were within 5.2% of the nominal concentrations. The accuracy and precision for both pravastatin and pravastatin lactone were excellent in all QC samples, as the measured concentrations were within 8.6% of the nominal concentrations and the % RSD values were within 8.7.

Table 5 summarizes the results of analysis of samples from condition 2, which allowed the conversion of pravastatin lactone to pravastatin in both the standards and QC samples. This condition was adopted to mimic a real-life situation where one of the two analytes converts to the

Table 4
Accuracy and precision under condition 1[a]

| | Pravastatin | | | Pravastatin lactone | | |
|---|---|---|---|---|---|---|
| | Nominal conc. (ng ml$^{-1}$) | RSD (%) | Dev. (%) | Nominal conc. (ng ml$^{-1}$) | RSD (%) | Dev. (%) |
| Standard 1 (1:1) | 5.00 | 7.3 | −1.7 | 5.00 | 7.9 | +0.1 |
| Standard 2 (1:1) | 10.0 | 5.2 | +2.5 | 10.0 | 1.3 | −0.5 |
| Standard 3 (1:1) | 25.0 | 6.4 | +1.2 | 25.0 | 5.2 | −2.1 |
| Standard 4 (1:1) | 50.0 | 6.6 | +3.4 | 50.0 | 2.8 | +5.2 |
| Standard 5 (1:1) | 100 | 5.5 | −0.1 | 100 | 1.9 | +2.6 |
| Standard 6 (1:1) | 200 | 3.0 | −2.1 | 200 | 1.7 | +0.3 |
| Standard 7 (1:1) | 300 | 0.5 | −1.5 | 300 | 3.4 | −1.9 |
| Standard 8 (1:1) | 400 | 1.4 | −0.7 | 400 | 1.7 | −2.8 |
| Standard 9 (1:1) | 500 | 0.5 | −0.9 | 500 | 0.7 | −0.9 |
| QC1 (1:1) | 15.0 | 5.9 | +1.3 | 15.0 | 4.7 | −2.2 |
| QC2 (1:1) | 200 | 2.8 | −7.1 | 200 | 1.6 | −7.9 |
| QC3 (1:1) | 420 | 0.6 | −8.6 | 420 | 3.9 | −5.8 |
| QC4 (1:3) | 5.00 | 3.0 | −6.6 | 15.0 | 4.3 | −1.7 |
| QC5 (1:10) | 20.0 | 3.9 | −4.8 | 200 | 3.3 | −5.3 |
| QC6 (1:10) | 42.0 | 5.1 | −2.6 | 420 | 2.0 | −6.3 |
| QC7 (0:15) | 0 | N/A | N/A | 15.0 | 5.8 | −3.5 |
| QC8 (0:200) | 0 | N/A | N/A | 200 | 3.7 | −6.2 |
| QC9 (0:420) | 0 | N/A | N/A | 420 | 1.8 | −1.3 |
| QC10 (3:1) | 15.0 | 3.1 | −7.8 | 5.00 | 8.7 | +4.3 |
| QC11 (10:1) | 200 | 2.4 | −7.4 | 20.0 | 6.0 | +3.2 |
| QC12 (10:1) | 420 | 5.0 | −4.8 | 42.0 | 2.4 | −0.3 |
| QC13 (15:0) | 15.0 | 8.2 | −2.6 | 0 | N/A | N/A |
| QC14 (200:0) | 200 | 3.0 | −8.0 | 0 | N/A | N/A |
| QC15 (420:0) | 420 | 2.3 | −6.5 | 0 | N/A | N/A |

[a] Dev., deviation of the mean concentration from the nominal concentration ($n = 2$ for standards; $n = 5$ for quality control (QC) samples). N/A, not applicable, because the measured concentration was below LLQ. The numbers in parentheses show the ratios of the pravastatin concentrations to the pravastatin lactone concentrations.

Table 5
Accuracy and precision under condition 2[a]

| | Pravastatin | | | Pravastatin lactone | | |
|---|---|---|---|---|---|---|
| | Nominal conc. (ng ml$^{-1}$) | RSD (%) | Dev. (%) | Nominal conc. (ng ml$^{-1}$) | RSD (%) | Dev. (%) |
| Standard 1 (1:1) | 5.00 | 2.0 | −7.6 | 5.00 | 4.6 | +4.7 |
| Standard 2 (1:1) | 10.0 | 0.2 | +10.7 | 10.0 | 11.0 | −6.3 |
| Standard 3 (1:1) | 25.0 | 3.3 | +9.6 | 25.0 | 5.7 | −6.4 |
| Standard 4 (1:1) | 50.0 | 1.1 | +3.4 | 50.0 | 4.0 | −3.5 |
| Standard 5 (1:1) | 100 | 1.9 | +5.4 | 100 | 6.2 | −4.0 |
| Standard 6 (1:1) | 200 | 2.1 | −2.8 | 200 | 1.8 | +5.0 |
| Standard 7 (1:1) | 300 | 2.5 | −4.9 | 300 | 0.2 | +5.0 |
| Standard 8 (1:1) | 400 | 3.7 | −8.9 | 400 | 3.2 | +2.4 |
| Standard 9 (1:1) | 500 | 1.5 | −4.8 | 500 | 0.2 | +3.2 |
| QC1 (1:1) | 15.0 | 7.0 | +2.7 | 15.0 | 6.7 | +3.5 |
| QC2 (1:1) | 200 | 5.8 | +4.8 | 200 | 2.6 | −6.3 |
| QC3 (1:1) | 420 | 0.7 | −10.6 | 420 | 2.1 | −4.2 |
| QC4 (1:3) | 5.00 | 6.0 | +27.6 | 15.0 | 3.5 | +5.0 |
| QC5 (1:10) | 20.0 | 7.7 | +158.2 | 200 | 2.9 | −6.4 |
| QC6 (1:10) | 42.0 | 4.4 | +65.4 | 420 | 2.3 | +2.1 |
| QC7 (0:15) | 0 | 8.8 | 4.72[b] | 15.0 | 6.7 | −5.6 |
| QC8 (0:200) | 0 | 5.1 | 58.4[b] | 200 | 2.9 | −6.4 |
| QC9 (0:420) | 0 | 5.6 | 74.7[b] | 420 | 5.9 | −6.1 |
| QC10 (3:1) | 15.0 | 1.3 | −20.2 | 5.00 | 5.0 | +7.2 |
| QC11 (10:1) | 200 | 1.1 | −19.4 | 20.0 | 6.3 | +6.5 |
| QC12 (10:1) | 420 | 2.5 | −17.9 | 42.0 | 2.0 | +6.4 |
| QC13 (15:0) | 15.0 | 3.5 | −19.2 | 0 | N/A | N/A |
| QC14 (200:0) | 200 | 1.7 | −19.8 | 0 | N/A | N/A |
| QC15 (420:0) | 420 | 1.4 | −18.4 | 0 | N/A | N/A |

[a] Dev., deviation of the mean concentration from the nominal concentration ($n = 2$ for standards; $n = 5$ for quality control (QC) samples). N/A, not applicable, because the measured concentration was below LLQ. The numbers in parentheses show the ratios of the pravastatin concentrations to the pravastatin lactone concentrations.
[b] Measured concentration in ng ml$^{-1}$.

other during one or more of the multiple steps of a bioanalytical method, perhaps unknown to the bioanalyst. The performance of the standard curve for both pravastatin and pravastatin lactone was excellent as the back-calculated concentrations of the individual standards were within 10.7% of the nominal concentrations. The accuracy and precision for both pravastatin and pravastatin lactone were excellent in the QC samples in which the ratio of pravastatin to pravastatin lactone was identical to that in the calibration standards (ratio of 1:1). The measured concentrations of these QC samples were within 10.6% of the nominal concentrations and the % RSD values were within 7.0. The accuracy for pravastatin was unacceptable for all QC samples in which the ratio of pravastatin to pravastatin lactone was different from the 1:1 ratio in the calibration standards. The lactone-only and 1:10 QC samples showed positive deviations and the 10:1 and pravastatin-only QC samples showed negative deviations. On the other hand, the accuracy and precision for pravastatin lactone were excellent in all QC samples in which the ratio of pravastatin to pravastatin lactone was different from the 1:1 ratio in the calibration standards. The measured concentrations were within 7.2% of the nominal concentrations and the % RSD

values were within 6.7%. Thus, under condition 2, where the conversion was one-way, with pravastatin lactone, as the substrate analyte, producing pravastatin, as the product analyte, acceptable accuracy for the product analyte was obtained only in QC samples in which the ratio of the two analytes was identical to the ratio in the calibration standards. On the other hand, acceptable accuracy and precision for the substrate analyte were obtained for all QC samples of varying ratios of the two analytes.

Table 6 summarizes the results of analysis of samples from condition 3, which allowed interconversion between pravastatin and pravastatin lactone in both the standards and QC samples. The performance of the standard curve for both pravastatin and pravastatin lactone was excellent as the back-calculated concentrations of the individual standards were within 9.5% of the nominal concentrations. The accuracy and precision for both pravastatin and pravastatin lactone were excellent in the QC samples in which the ratio of pravastatin to pravastatin lactone was identical to that in the calibration standards (ratio of 1:1). The measured concentrations of these QC samples were within 10.2% of the nominal concentrations and the % RSD values were within 8.2. The accuracy and precision for pravastatin were ex-

Table 6
Accuracy and precision under condition 3[a]

| | Pravastatin | | | Pravastatin lactone | | |
|---|---|---|---|---|---|---|
| | Nominal conc. (ng ml$^{-1}$) | RSD (%) | Dev. (%) | Nominal conc. (ng ml$^{-1}$) | RSD (%) | Dev. (%) |
| Standard 1 (1:1) | 5.00 | 11.8 | +1.2 | 5.00 | 0.4 | +3.3 |
| Standard 2 (1:1) | 10.0 | 10.7 | −3.2 | 10.0 | 1.0 | −3.5 |
| Standard 3 (1:1) | 25.0 | 6.1 | −3.1 | 25.0 | 1.0 | −9.5 |
| Standard 4 (1:1) | 50.0 | 0.4 | +8.5 | 50.0 | 1.1 | +0.4 |
| Standard 5 (1:1) | 100 | 0.0 | +6.4 | 100 | 2.9 | +3.3 |
| Standard 6 (1:1) | 200 | 1.9 | −1.8 | 200 | 2.9 | +0.2 |
| Standard 7 (1:1) | 300 | 0.7 | −0.8 | 300 | 0.4 | +5.1 |
| Standard 8 (1:1) | 400 | 5.1 | −0.8 | 400 | 0.0 | +5.0 |
| Standard 9 (1:1) | 500 | 1.3 | −6.3 | 500 | 0.1 | −4.4 |
| QC1 (1:1) | 15.0 | 8.2 | −3.6 | 15.0 | 2.5 | −3.4 |
| QC2 (1:1) | 200 | 2.7 | +8.4 | 200 | 1.2 | +10.2 |
| QC3 (1:1) | 420 | 5.9 | +5.3 | 420 | 1.8 | +7.2 |
| QC4 (1:3) | 5.00 | 24.2 | +11.9 | 15.0 | 2.9 | +0.9 |
| QC5 (1:10) | 20.0 | 8.0 | +87.8 | 200 | 2.5 | −1.1 |
| QC6 (1:10) | 42.0 | 7.6 | +89.6 | 420 | 1.7 | +4.1 |
| QC7 (0:15) | 0 | N/A | N/A | 15.0 | 3.7 | −3.9 |
| QC8 (0:200) | 0 | 12.2 | 20.4[b] | 200 | 2.5 | −1.1 |
| QC9 (0:420) | 0 | 5.0 | 41.3[b] | 420 | 1.7 | +6.4 |
| QC10 (3:1) | 15.0 | 5.2 | −9.6 | 5.00 | N/A | N/A |
| QC11 (10:1) | 200 | 2.3 | +1.2 | 20.0 | 3.6 | +79.5 |
| QC12 (10:1) | 420 | 4.3 | −4.1 | 42.0 | 2.6 | +73.4 |
| QC13 (15:0) | 15.0 | 5.7 | −10.0 | 0 | | N/A |
| QC14 (200:0) | 200 | 6.8 | +3.6 | 0 | 6.7 | 7.91[b] |
| QC15 (420:0) | 420 | 3.9 | −2.4 | 0 | 2.7 | 28.1[b] |

[a] Dev., deviation of the mean concentration from the nominal concentration ($n = 2$ for standards; $n = 5$ for quality control (QC) samples). N/A, not applicable, because the measured concentration was below LLQ. The numbers in parentheses show the ratios of the pravastatin concentrations to the pravastatin lactone concentrations.

[b] Measured concentration in ng ml$^{-1}$.

cellent in the pravastatin-only and 10:1 QC samples. The deviations of the measured concentrations from the nominal concentrations were within 10% and the percentage of RSD values were within 6.8. In these QC samples, the effect of intercon version under condition 3 was a net decrease in the concentration of pravastatin. Thus, in these QC samples, pravastatin is a substrate analyte; therefore no adverse effect is expected on the accuracy of the substrate analyte, as discussed above under condition 2. However, the accuracy for pravastatin was unacceptable in the pravastatin lactone-only and 1:10 QC samples. The measured concentrations of these QC samples showed positive deviations from the nominal concentrations. In these QC samples, the effect of interconversion under condition 3 was a net increase in the concentration of pravastatin. Thus, in these QC samples, pravastatin is a product analyte; therefore an adverse effect is expected on the accuracy of the product analyte, as discussed above under condition 2. The accuracy results of pravastatin lactone in the QC samples in which the pravastatin to pravastatin lactone ratios were different from the 1:1 ratio in the calibration standards were the reverse of those of pravastatin. Thus, the accuracy for pravastatin lactone was excellent for the pravastatin lactone-only and 1:10 QC samples. The deviations of the measured concentrations from the nominal concentrations were within 6.4% and the % RSD values were within 3.7. However, the accuracy for pravastatin lactone was unacceptable for the pravastatin-only and 10:1 QC samples. The measured concentrations of these QC samples showed positive deviations from the nominal concentrations.

It is clear from the above results that QC samples that contain the two analytes in the same ratio as in the calibration standards give acceptable accuracy for both analytes under all conditions, including those conditions that allow the conversion of one analyte to the other. On the other hand, QC samples that contain the analytes in ratios different from that in the calibration standards give acceptable accuracy for both analytes only under conditions where there is no interconversion during analysis. Under conditions that allow conversion between the analytes, such QC samples will fail the accuracy criteria for at least one of the analytes. Thus, a method validated for the quantitation of the two analytes using calibration standards with 1:1 analyte concentrations and QC samples with the same 1:1 analyte concentrations can be used for accurate measurement of the analytes in post-dose samples only if such samples contain the two analytes in the 1:1 ratio. Therefore, to obtain an accurate indication of the performance of the method for all the post-dose samples, QC samples that cover the entire spectrum of the composition of the post-dose samples should be used.

When developing a method for quantitation of two analytes that can potentially interconvert during any of the multiple steps of bioanalysis, the first step is to select conditions that will eliminate or minimize the interconversion. When optimizing the conditions for the elimination or minimization of conversion of the one analyte to the other, the conditions adopted should be more favorable (in terms of eliminating conversion) toward the analyte that is expected to be the major component in the post-dose samples. The second step is to judiciously select the composition of the QC samples vis-à-vis the composition of the calibration standards. The following set of calibration standards and QC samples are recommended during method validation: 1:1 calibration standards throughout the curve range; 1:1 low-QC samples, 1:1 mid-QC samples; 1:1 high-QC samples; analyte 1-only QC samples; 10:1 QC samples; analyte 2-only QC samples; and 1:10 QC samples. Analyte concentration ratios different from 1:10 and 10:1 may be used depending on the expected analyte concentration ratios in the post-dose samples.

## 4. Conclusions

It is essential for the bioanalysts to be aware that some analytes can potentially undergo interconversion during any of the multiple steps of bioanalysis. The conditions adopted for a bioanalytical method for quantitation of potentially interconverting analytes should eliminate or minimize the interconversion. In addition, the composition, in terms of the concentration ratio

of the two analytes, of the QC samples should be selected judiciously vis-à-vis the composition of the calibration standards. Since the purpose of QC samples is to predict the performance of the method for the post-dose samples, the QC samples should cover the spectrum of the composition of the post-dose samples.

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