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Quantitative determination of pravastatin and its biotransformation products in human serum by turbo ion spray LC/MS/MS

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Abstract

A sensitive, specific, accurate and reproducible analytical method was developed and validated to quantify pravastatin (Prav), pravastatin-d_5 (Prav-d_5), SQ-31906, SQ-31906-d_5, and pravastatin lactone (Prav–Lac) in human serum samples. Serum samples (0.5 ml) were acidified and extracted by a solid-phase extraction procedure to isolate all five analytes from human serum. Sample extracts were reconstituted and analyzed by turbo ion spray liquid chromatography/tandem mass spectrometry (LC/MS/MS) in the positive ion mode. The total run time was 9 min between injections. The assay demonstrated a lower limit of quantitation (LLQ) of 0.5 ng/ml for all five analytes. The calibration curves were linear from 0.5 ng/ml to 100 ng/ml for all five analytes. The coefficients of determination of all calibration curves were ≥ 0.999. Precision and accuracy quality control (QC) samples were prepared at concentrations of 2, 30, 80, and 500 ng/ml for all analytes. The intra-assay and inter-assay precision calculated from QC samples were within 8% for all analytes. The inter-assay accuracy calculated from QC samples was within 8% for all analytes. The extraction recoveries were $\geq 90\%$ for all analytes. Benchtop stability experiments in an ice-water bath (≤ 10°C) demonstrated that over time, Prav–Lac hydrolyzes to Prav in serum. Prav, Prav-d_5, SQ-31906, and SQ-31906-d_5 were stable under these conditions for up to 24 h. Hydrolysis was minimized by buffering the serum to pH 4.5 and maintaining the serum sample in an ice-water bath. All analytes were stable after three freeze/thaw cycles and in reconstitution solution after 1 week at 4°C. Stability of all analytes in human serum was demonstrated after storage at -70°C for 77 days. The benchtop (≤ 10°C) stability of pooled study samples was also investigated and the results were comparable to those obtained from serum QC samples.

Keywords: Pravastatin; LC/MS/MS; Human serum; Quantitative determination; Lactonization; Hydrolysis

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1. Introduction

Pravastatin (Prav) (Fig. 1) is an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in the biosynthesis of cholesterol [1,2]. Prav is a δ-hydroxyacid, which exists in solution with its lactone equilibrium product (Prav–Lac) in a pH-dependent manner. Additionally, unpublished chemical stability experiments conducted at Bristol–Myers Squibb indicate that Prav can isomerize to SQ-31906 (Fig. 1) at acidic pH. Since these additional compounds are also potential biotransformation products [3,4], it was necessary to develop a method which would minimize these interconversions ex vivo. There currently exist analytical methods for the quantitative determination of Prav only or Prav and SQ-31906, which employ a variety of techniques, including HPLC-UV, LC/MS/MS, ELISA or GC/NCI/MS [5–11]. However, none of these enables the simultaneous LC/MS/MS quantitation of Prav, SQ-31906 and Prav–Lac while monitoring the inter-conversion of the analytes. This paper presents a method which meets these requirements and provides a high degree of accuracy, sensitivity and specificity. The method incorporates solid-phase extraction of the analytes from human serum followed by chromatographic separation using high-performance liquid chromatography and detection by electrospray tandem mass spectrometry. This method also incorporates as analytes the penta-deutero analogs Prav-d_5 and SQ-31906-d_5 and employs the tri-deutero analogs Prav-d_3 and Prav-Lac-d_3 as internal standards. The structures of the target analytes are shown in Fig. 1.

2. Experimental

2.1. Reagents and chemicals

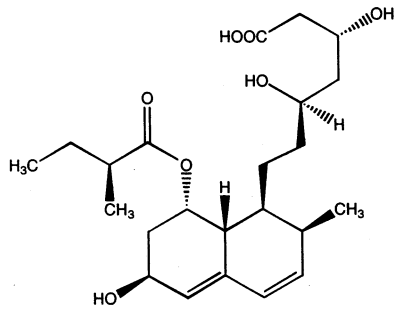
SQ-33469 (the tetramethylbutylamine salt of Prav), Prav-d_5 (sodium salt), SQ-31906 (sodium salt), SQ-31906-d_5 (sodium salt), Prav–Lac, Prav-d_3 (sodium salt; the internal standard for Prav, Prav-d_5, SQ-31906 and SQ-31906-d_5), and Prav–Lac-d_3 (the internal standard for Prav–Lac) were all characterized products of Bristol–Myers Squibb Pharmaceutical Research Institute. For all analytes except Prav–Lac, the concentrations were reported as the sodium salt of the compound. Acetonitrile and methanol were purchased from Burdick and Jackson (Muskegon, MI). Glacial acetic acid was purchased from JT Baker (Danvers, MA, USA). High purity water was prepared in-house using a NANOpure® water purification system obtained from Barnstead (Dubuque, IA). House high-purity nitrogen (99.998%) was used, and argon (99.999%) was purchased from Empire Gas (Elmira, NY). Control human serum was purchased from Biological Specialty (Colmar, PA).

2.2. Equipment

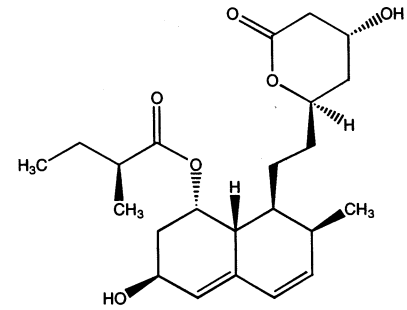
LC/MS/MS analysis was performed using a Sciex API III^{plus} (Toronto, Ontario, Canada) triple quadrupole mass spectrometer equipped with an atmospheric pressure ionization (API) Turbo Ion Spray® interface. The HPLC system consisted of a pair of LC10AD pumps and a SCL-10A controller (Shimadzu, Kyoto, Japan) as well as a Series 200 autosampler (Perkin–Elmer, Norwalk, CT 06859). The HPLC column used was a Keystone Betasil® ODS (5 μm particle size, 2.1 × 100 mm) purchased from Keystone Scientific, Bellefonte, PA 16823. Polypropylene test tubes (13 × 100 mm) were obtained from Elkay Product (Worcester, MA) and polypropylene vials (16 × 57 mm) were purchased from Sarstedt (Newton, SC). Polypropylene microvials (0.25 ml) were purchased from Sun Brokers (Wilmington, NC). Isolute® solid-phase extraction cartridges (100 mg, 1-ml, C_8) were obtained from International Sorbent Technology (Mid Glamorgan, UK). The TurboVap LV Evaporator® was supplied by Zymark (Hopkinton, MA).

2.3. Standard and quality control (QC) preparation

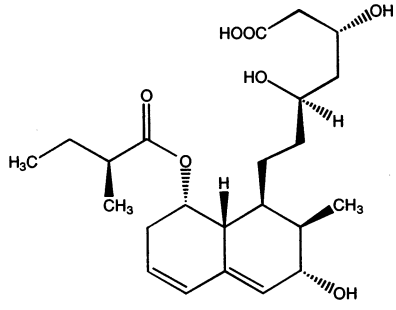
Stock solutions of all compounds except for Prav–Lac were prepared in acetonitrile–water (90:10,v/v) while those for Prav–Lac were prepared in acetonitrile, to minimize potential hy-



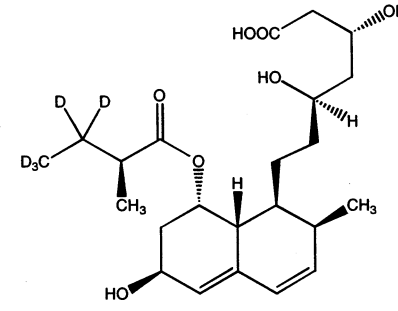
Pravastatin ($C_{23}H_{36}O_7$)
$M_r = 424.25$ $[M+NH_4]^+ = 442.28$



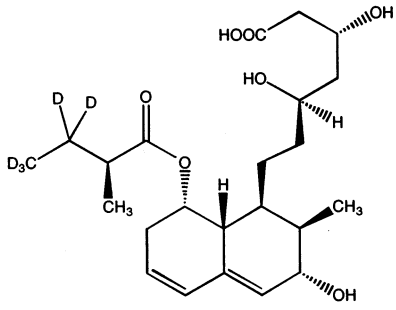
Pravastatin Lactone ($C_{23}H_{34}O_6$)
$M_r = 406.24$ $[M+NH_4]^+ = 424.27$



SQ-31906 ($C_{23}H_{36}O_7$)
$M_r = 424.25$ $[M+NH_4]^+ = 442.28$



Pravastatin-d$_5$ ($C_{23}D_5H_{31}O_7$)
$M_r = 429.29$ $[M+NH_4]^+ = 447.32$



SQ-31906-d$_5$ ($C_{23}D_5H_{31}O_7$)
$M_r = 429.29$ $[M+NH_4]^+ = 447.32$

Fig. 1. Chemical structures of Prav, SQ-31906, Prav–Lac, Prav-d_5 and SQ-31906-d_5.

drolysis. Separate stock solutions were used for the preparation of the standard curve and QC samples. All solutions were kept refrigerated (4°C) when not in use. From these stocks, standard and QC working solutions in acetonitrile–water (90:10,v/v) were prepared which contained all analytes at a concentration of 25 μg/ml. These were used in the preparation of the serum standards and QCs.

All serum samples were maintained in an ice-water bath at a temperature of ≤ 10°C, until they were extracted, to minimize hydrolysis of the lactones. The standard calibration curve for each analyte consisted of seven concentrations prepared in duplicate. Calibration standards were prepared by spiking 10 ml of control serum at a concentration of 100 ng/ml of each analyte (Std 7). Standards 1–6 were prepared by dilution of Std 7 with control serum. The standard curve range in human serum was 0.5–100 ng/ml for each analyte. Two different types of QC samples were used in this validation. Analytical QC samples, used for the assessment of accuracy and precision, were prepared containing all five analytes. Four levels of these QCs (2, 30, 80, 500 ng/ml) were prepared and stored at -70°C until analyzed. The 500 ng/ml QC sample was diluted $10\times$ with control human serum before analysis. The second type of QC used were stability QCs, which were prepared containing either Prav only, SQ-31906 only or Prav–Lac only at a concentration of 50 ng/ml. These QCs were prepared to monitor any analyte inter-conversions that could occur during sample storage and processing. These QCs were included in all stability studies. During routine analysis they were extracted as the last samples in the set.

Table 1
HPLC gradient program

Time (min)	%B
0	10
1	60
5	60
6	90
7	90
7.5	10
8.5	Stop

2.4. Sample preparation

Aliquots (0.5 ml) of human serum standard, QC and blank sample, were added to 13×100 mm polypropylene tubes containing 0.3 ml of ammonium acetate (pH 4.5; 500 mM). This adjusted the serum to pH 4.5. The internal standard solution (50 μl) was then added to each tube. The concentration of the internal standards Prav-d_3 and Prav–Lac-d_3 in the serum was 20 ng/ml. The analytes were then isolated from the matrix by solid-phase extraction using Isolute® C_8 cartridges. The cartridges were conditioned by sequentially adding 1-ml aliquots of methanol and ammonium acetate (pH 4.5, 10 mM). Serum samples were loaded onto the cartridges and the cartridge beds were then consecutively washed with 1-ml portions of water and acetonitrile–water (1:9, v/v). The analytes were eluted from the cartridges with 1 ml of acetonitrile–water (7:3, v/v). After evaporating the eluents to dryness at 45°C under nitrogen, the dry extracts were reconstituted in 50 μl of acetonitrile–methanol–ammonium acetate (pH 4.5;10 mM) (35:35:30) and transferred to polypropylene autosampler vials.

2.5. Chromatographic and mass spectrometric conditions

The analytes were chromatographically separated using high-performance liquid chromatography (HPLC) with gradient elution. The eluents consisted of ammonium acetate (pH 4.5; 5 mM) (Eluent A) and acetonitrile:methanol (1:1; v/v) (Eluent B) at a flow rate of 0.2 ml/min through the analytical column. The gradient program is listed in Table 1. For all analyses, 7 μl of the reconstituted sample was injected. The total run time was 9 min between injections. The mass spectrometer was operated in the turbo ion spray mode with positive ion detection. The turbo ion spray temperature was maintained at 300°C and a voltage of 4.8 kV was applied to the sprayer needle. Nitrogen was used as the turbo ion spray and nebulizing gas. The argon collision gas thickness was set to 2.5×10^{14} atom/cm^2. The typical

Table 2
SRM transitions monitored during analysis[a]

Analyte	Transition monitor	Dwell time
Period 1:		
Prav, SQ-31906	m/z = 442.3 → m/z = 269.2	125 ms
Dummy	m/z = 800 → m/z = 100	125 ms
Prav-d_5, SQ-31906-d_5	m/z = 447.3 → m/z = 269.2	125 ms
Dummy	m/z = 800 → m/z = 100	125 ms
Prav-d3 (ISTD)	m/z = 445.3 → m/z = 269.2	125 ms
Dummy	m/z = 800 → m/z = 100	125 ms
Period 2:		
Prav-Lac	m/z = 424.3 → m/z = 269.2	200 ms
Dummy	m/z = 800 → m/z = 100	200 ms
Prav-Lac-d_3 (ISTD)	m/z = 427.3 → m/z = 269.2	200 ms
Dummy	m/z = 800 → m/z = 100	200 ms

[a] Two different periods were used for increased sensitivity.

declustering potential, controlled by the orifice voltage, was 6 V and the collision energy was 12 eV for all analytes. The analytes were monitored by selected reaction monitoring (SRM) of the collision-induced dissociation (CID) of the precursor ion to its corresponding product ion. The mass spectrometer was operated at unit mass resolution in both Q1 and Q3. For all analytes, the ammonium adduct of the analyte $[M + NH_4]^+$ was monitored in the first quadrupole and the corresponding product ion at m/z 269 was monitored in the third quadrupole, according to Table 2. Due to the fact that the analytes and internal standards all possess a common product ion in their SRM transitions and several coelute, it was important to be aware of and minimize Q2 crosstalk. This was eliminated by the inclusion of 'dummy' SRM transitions in the scanning order and the use of both Q2 settling and a pause time.

2.6. Data processing and regression

The SRM chromatographic peaks were integrated using the PE-Sciex program MacQuan (v. 1.4), after which the peak area ratios of the standards were plotted versus concentration and a linear curve fit, weighted by $1/x$, where x = concentration, was used to produce the regression line.

3. Results and discussion

For all analytes, the predominant ion observed during Q1 scanning was the ammonium adduct $[M + NH_4]^+$ (Fig. 2a–e). Monitoring this adduct as the SRM precursor ion afforded the highest sensitivity for all analytes. Although inducing CID by increasing the orifice voltage [12,13] did cause dissociation of the adducts to the protonated molecular species, comparable sensitivity was not achievable when monitoring $[M + H]^+$ in Q1. Initially, it was expected that optimum sensitivity would be achieved by monitoring the acidic compounds (Prav, SQ-31906 and deuterated analogs) in the negative ion detection mode followed by a subsequent reversal of ionization polarity prior to elution of the neutral lactones. However, no increase in sensitivity was observed when compared to positive ion detection of the analytes under the experimental conditions. The use of turbo ion spray was found to dramatically increase the sensitivity of all compounds. However, at temperatures above 300°C, the sensitivity of SQ-31906 dropped off significantly, while no similar effect was seen for Prav or Prav–Lac. A significant amount of the d_6 analog is apparent in the Q1 spectra for Prav-d_5 (Fig. 2d) and SQ-31906-d_5 (Fig. 2e). Concentrations for these analytes were not corrected for the d_6 since the

clinical batches were known to contain the same level of d_6. The full-scan MS/MS mass spectra of the analytes (Fig. 3a–e) indicate that the most abundant product ion is found at m/z 269 for all analytes. Plausible mechanisms for the CID fragmentation of the parent ions to the product at m/z 269 are presented in Fig. 4. The initial loss of NH_3 from the $[M + NH_4]^+$ ion has not been depicted. Similar mechanisms can be proposed for the remaining analytes that result in product ions consistent with the proposed mechanisms.

3.1. Assay selectivity

While Prav and SQ-31906 share the same SRM transitions, as well as Prav-d_5 and SQ-31906-d_5,

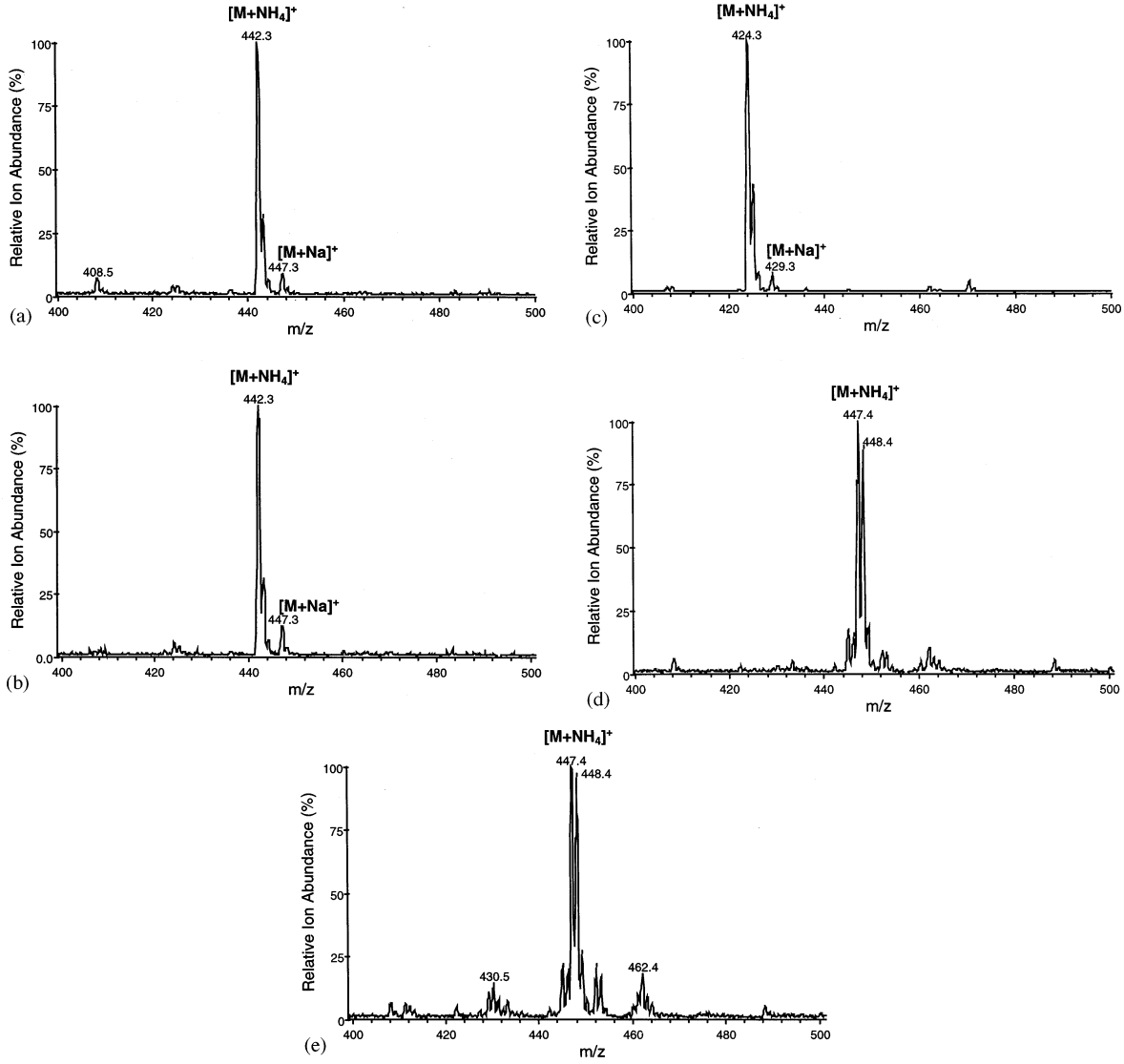


Fig. 2. Electrospray positive ion Q1 mass spectra for Prav (2a, $[M + NH_4]^+ = 442.3$), SQ-31906 (2b, $[M + NH_4]^+ = 442.3$), Prav-Lac (2c, $[M + NH_4]^+ = 424.3$), Prav-d_5 (2d, $[M + NH_4]^+ = 447.4$) and SQ-31906-d_5; (2e $[M + NH_4]^+ = 447.4$. All spectra were obtained by coinfusion of solutions of the individual analytes and the HPLC mobile phase. A significant level of the d_6 analog is evident in the spectra for Prav-d_5 and SQ-31906-d_5.

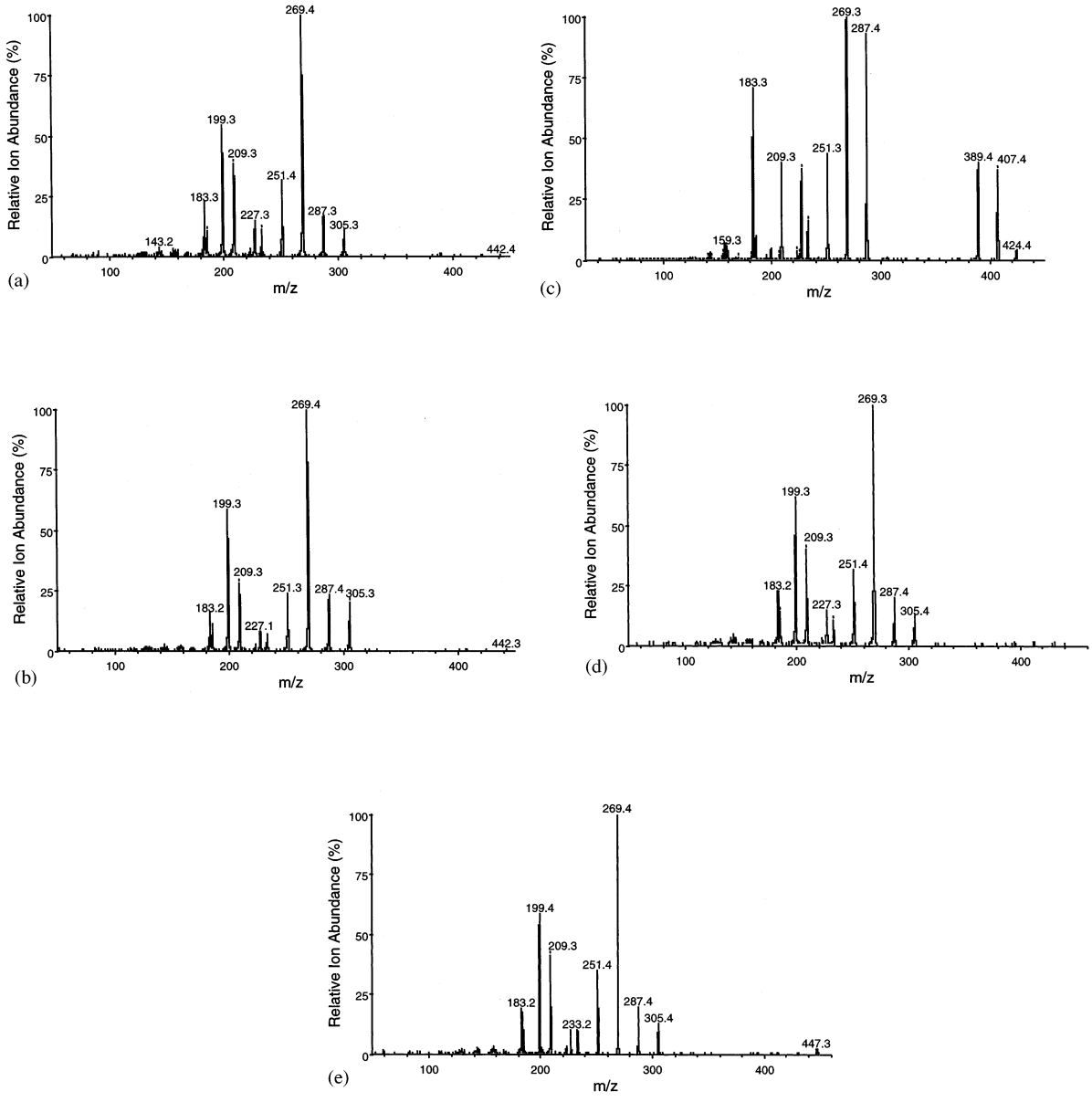


Fig. 3. Electrospray positive ion CID product ion spectra for Prav (3a), SQ-31906 (3b), Prav–Lac (3c), Prav-d_5; (3d) and SQ-31906-d_5; (3e). All spectra were obtained by coinfusion of solutions of the individual analytes and the HPLC mobile phase.

the analytes were differentiated by chromatographic resolution. The chromatographic separation between Prav and SQ-31906 was optimized by using methanol–acetonitrile (1:1) as the organic component of the HPLC eluent. The specificity of the method was demonstrated by monitoring the analyte transitions in processed blank (drug-free) serum. This was performed during routine analytical runs as well as during the validation of the lower limit of quantitation (LLQ). The SRM LC/MS chromatograms of a blank human serum extract are shown in Fig. 5. SRM

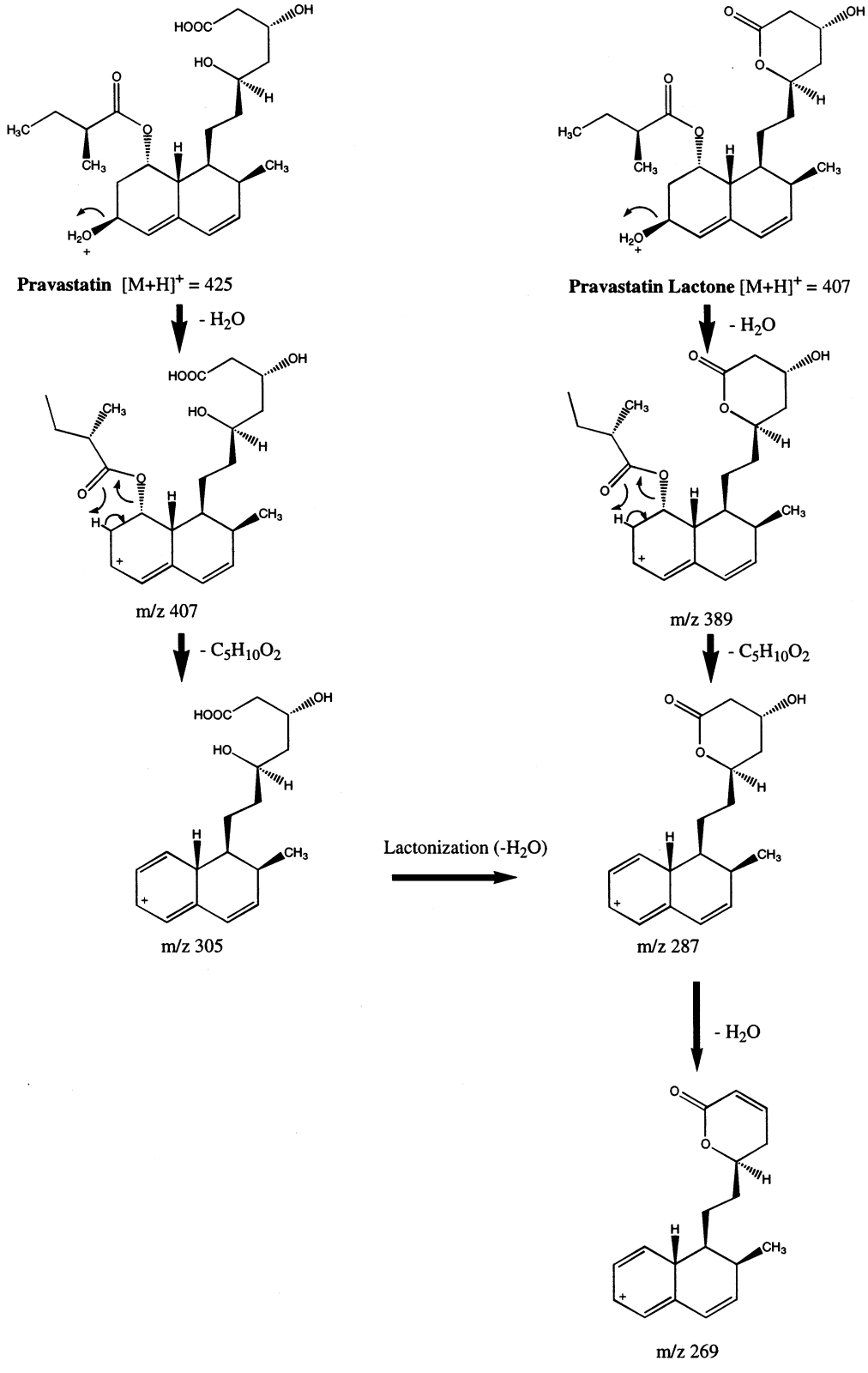


Fig. 4. Plausible CID fragmentation mechanisms for Prav and Prav–Lac.

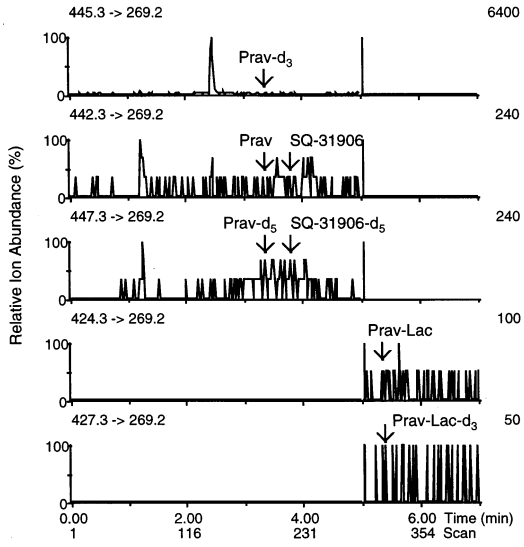


Fig. 5. SRM chromatograms of Prav, Prav-d_5, SQ-31906, SQ-31906-d_5, Prav–Lac and their internal standards in control blank human serum extract.

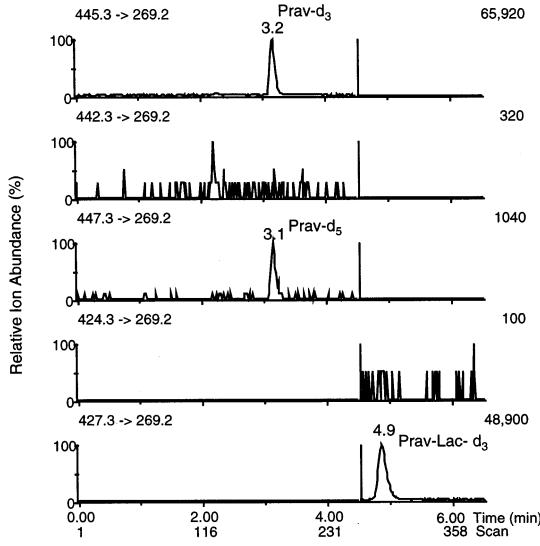


Fig. 6. SRM chromatograms of Prav, Prav-d_5, SQ-31906, SQ-31906-d_5, Prav–Lac and their internal standards in human serum extract containing internal standards only (zero sample).

LC/MS chromatograms from blank human serum extract, containing the internal standards (zero sample), are shown in Fig. 6. It should be noted that a small peak was consistently observed in the

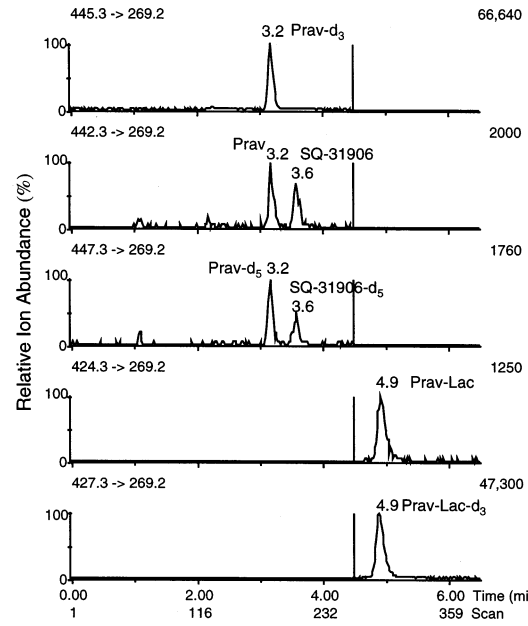


Fig. 7. SRM chromatograms of Prav, Prav-d_5, SQ-31906, SQ-31906-d_5, Prav–Lac and their internal standards in serum spiked at the LLQ (0.5 ng/ml).

Prav-d_5 transition of the zero sample and is a result of the A + 2 isotope peak from the Prav-d_3 internal standard. This interference did not affect the linearity, accuracy, or precision at the LLQ of Prav-d_5, but it did introduce a significant positive y-intercept into the standard curves of Prav-d_5. SRM LC/MS chromatograms from the lowest calibration standard (0.5 ng/ml of each analyte) and the upper limit of quantitation (ULQ;100 ng/ml of each analyte) are shown in Figs. 7 and 8, respectively. The responses for Prav-d_5 and SQ-31906-d_5 are lower than those for Prav and SQ-31906 due to the abundance of d_6 label in the compound. This is not as apparent for Prav-d_5 in the low standard (Fig. 7) due to the A + 2 isotopic contribution from the internal standard Prav-d_3. However, it is readily apparent in the high concentration standard depicted in Fig. 8.

3.2. Linearity

Representative calibration curve data for Prav, SQ-31906 and Prav–Lac are listed in Tables 3–5. Comparable results were also obtained for Prav-

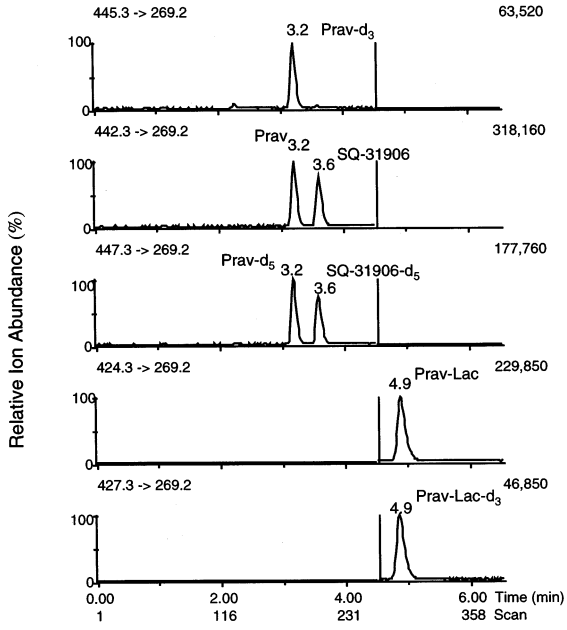


Fig. 8. SRM chromatograms of Prav, Prav-d_5, SQ-31906, SQ-31906-d5, Prav–Lac and their internal standards in serum spiked at the ULQ (100 ng/ml).

d_5 and SQ-31906-d_5. Standard curve summary data for the three accuracy and precision runs are listed for all analytes in Tables 6–10. The coefficients of determination (r^2) were ≥ 0.999 for all analytes and runs.

3.3. Lower limit of quantitation

For validation of the LLQ, seven different lots of serum were spiked with the analytes at the lowest level of the standard curve (0.5 ng/ml) and analyzed versus a standard curve. The seven different lots of blank serum were also analyzed after spiking with the internal standards only, in order to determine whether any endogenous serum constituents coelute or interfere with the analytes. The results obtained for the LLQ experiment are listed in Table 11. The mean accuracies were within 11.4% for all analytes and the precisions were within 10.8% CV.

3.4. Recovery

The extraction recoveries of the analytes from serum were calculated by comparing the response of serum samples spiked with the analytes prior to extraction to those spiked following extraction. The internal standards were spiked after extraction in both cases. Recovery was assessed at 2, 50 and 100 ng/ml and peak area ratios (analyte/internal standard) were used for the calculations. Recovery was not concentration dependent and the recovery was $>90\%$ for all analytes, with precision within 5.8% CV for all analytes and concentrations.

Table 3
Results for a typical calibration curve for Prav

Standard number	Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Deviation from nominal (%)
1	0.5	0.46	−8.0
1	0.5	0.48	−4.0
2	1	0.97	−3.0
2	1	0.93	−7.0
3	5	5.45	9.0
3	5	5.25	5.0
4	25	26.23	4.9
4	25	26.25	5.0
5	50	50.18	0.4
5	50	51.54	3.1
6	75	74.94	−0.1
6	75	74.29	−0.9
7	100	99.40	−0.6
7	100	96.63	−3.4

Table 4
Results for a typical calibration curve for SQ-31906

Standard number	Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Deviation from nominal (%)
1	0.5	0.49	−2.0
1	0.5	0.44	−12.0
2	1	1.02	2.0
2	1	1.05	5.0
3	5	5.36	7.2
3	5	5.18	3.6
4	25	23.27	−6.9
4	25	26.92	7.7
5	50	48.20	−3.6
5	50	46.21	−7.6
6	75	77.70	3.6
6	75	77.27	3.0
7	100	101.36	1.4
7	100	98.52	−1.5

Table 5
Results for a typical calibration curve for Prav–Lac

Standard number	Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Deviation from nominal (%)
1	0.5	0.47	−6.0
1	0.5	0.49	−2.0
2	1	0.99	−1.0
2	1	1.01	1.0
3	5	5.19	3.8
3	5	5.04	0.8
4	25	25.89	3.6
4	25	25.58	2.3
5	50	50.52	1.0
5	50	51.10	2.2
6	75	73.60	−1.9
6	75	74.72	−0.4
7	100	99.59	−0.4
7	100	98.83	−1.2

3.5. Accuracy and precision

Tables 12–16 present the accuracy and intra- and inter-assay precision which was determined by analyzing six replicates of QC samples at four concentrations on three different days. The inter-assay accuracy (deviation from nominal) was within 7.13% for all analytes and QC concentration levels. The intra- and inter-assay precision (% CV) was within 7.97% for all analytes and QC concentrations.

3.6. Stability

The stability of the analytes was assessed in the stock solutions, in serum on the benchtop in an ice-water bath, in serum after repetitive freeze–thaw cycles, in incurred study samples and in serum after long-term storage at −70°C. Stability was considered to have been demonstrated if the mean responses of the test samples were within 15% of the controls.

Table 6
Summary of the calibration curve data for Prav from the three accuracy and precision runs

Run number	Slope	Intercept	*R*-squared
10	0.0503	0.00810	1.00
11	0.0506	0.00671	0.999
12	0.0528	0.00523	1.00
N	3	3	3
Mean	0.0512	0.00668	
S.D.	0.00136	0.00144	

Table 7
Summary of the calibration curve data for Prav-d_5 from the three accuracy and precision runs

Run number	Slope	Intercept	*R*-squared
10	0.0275	0.0160	1.00
11	0.0268	0.0159	0.999
12	0.0280	0.0156	1.00
N	3	3	3
Mean	0.0274	0.0158	
S.D.	0.000604	0.000186	

Table 8
Summary of the calibration curve data for SQ-31906 from the three accuracy and precision runs

Run number	Slope	Intercept	*R*-squared
10	0.0486	0.00530	1.00
11	0.0443	0.00479	1.00
12	0.0423	0.00135	1.00
N	3	3	3
Mean	0.0451	0.00381	
S.D.	0.00322	0.00215	

Table 9
Summary of the calibration curve data for SQ-31906-d_5 from the three accuracy and precision runs

Run number	Slope	Intercept	*R*-squared
10	0.0263	0.00193	1.00
11	0.0232	0.00236	0.999
12	0.0222	0.00157	1.00
N	3	3	3
Mean	0.0239	0.00195	
S.D.	0.00216	0.000400	

Table 10
Summary of the calibration curve data for Prav–Lac from the three accuracy and precision runs

Run number	Slope	Intercept	*R*-squared
10	0.0479	0.00467	1.00
11	0.0474	0.00313	1.00
12	0.0476	0.00197	1.00
N	3	3	3
Mean	0.0476	0.00326	
S.D.	0.000285	0.00136	

3.7. Stock and working solution stability

The stability of the analytes in stock and working solutions was examined by comparing freshly prepared solutions to solutions which had been previously prepared and stored for up to 5 months at 4°C. These solutions were diluted in triplicate with reconstitution solution and internal standard solution, and analyzed by LC/MS/MS. For all analytes, the peak area ratios of all replicates exhibited individual deviations within 10% of the peak area ratios of the freshly prepared solutions. Thus, stability of the stock and working solutions was established for storage at 4°C for 5 months.

3.8. Benchtop stability

The results obtained from the benchtop stability experiment, in which analytical QC samples were stored in an ice-water bath for up to 24 h, showed decreasing concentrations of Prav–Lac over time. When combined with the increasing Prav concentrations that were observed over time, hydrolysis of Prav–Lac to Prav is clearly indicated. These data underscore the importance of minimizing the length of time during which the sample is thawed, as well as the urgency of acidification of the sample during analysis. Acidification has also been found to be important for the stabilization of atorvastatin, another δ-hydroxy-acid which can lactonize [14]. The percent deviation from time 0 (%DEV) for Prav at 24 h was $> +29.8\%$ for each QC level while the %DEV for Prav–Lac at 24 h was $> -29.0\%$ for each QC level. Fortunately, minimal loss ($< 2\%$) of

Prav–Lac was observed at the 1-h time point, the usual amount of time required to aliquot study samples during analysis. SQ-31906 was stable in an ice-water bath for up to 24 h with %DEV within 4% for all levels. The benchtop stability data for stability QC samples, which contained only one of either Prav, SQ-31906 or Prav–Lac at a concentration of 50 ng/ml, indicated that Prav and SQ-31906 were both stable under these conditions for up to 24 h. There was no observable

Table 11
Summary of the data from the lower limit of quantitation (LLQ) experiment

Serum replicate	Prav (0.5 ng/ml)	Prav-d_5 (0.5 ng/ml)	SQ-31906 (0.5 ng/ml)	SQ-31906-d_5 (0.5 ng/ml)	Prav–Lac (0.5 ng/ml)
1	0.548	0.568	0.509	0.551	0.490
2	0.593	0.554	0.481	0.557	0.496
3	0.526	0.623	0.475	0.672	0.469
4	0.515	0.458	0.521	0.474	0.454
5	0.551	0.536	0.521	0.569	0.494
6	0.538	0.491	0.530	0.561	0.462
7	0.524	R[a]	0.447	0.516	0.458
Mean	0.542	0.538	0.498	0.557	0.475
S.D.	0.0260	0.0583	0.0305	0.0604	0.0179
RSD (%)	4.80	10.8	6.12	10.8	3.77
% of theoretical	108	108	99.5	111	95.0
% deviation	8.44	7.67	−0.460	11.4	−5.03

[a] R = sample rejected.

Table 12
Summary statistics from three validation runs for Prav in human serum quality control samples

	QC1	QC2	QC3	QC4[a1]
Nominal concentration (ng/ml)	2	30	80	500
Mean observed concentration (ng/ml)	2.05	30.4	81.5	536
% deviation from nominal	2.27	1.40	1.83	7.13
Between-run precision (%RSD)	0.00*1	0.00*1	0.00*1	4.35
Within-run precision (%RSD)	4.57	1.82	1.88	1.68

[a] QC4 is a 10-fold dilution QC.
* No significant additional variation was observed as a result of performing the assay in different runs.

Table 13
Summary statistics from three validation runs for Prav-d_5 in human serum quality control samples

	QC1	QC2	QC3	QC4[a1]
Nominal concentration (ng/ml)	2	30	80	500
Mean observed concentration (ng/ml)	2.04	30.5	80.0	529
% deviation from nominal	1.87	1.77	−0.0559	5.89
Between-run precision (%RSD)	0.00*1	0.00*1	0.00*1	4.58
Within-run precision (%RSD)	7.97	2.36	1.96	1.61

[a] QC4 is a 10-fold dilution QC.
* No significant additional variation was observed as a result of performing the assay in different runs.

Table 14
Summary statistics from three validation runs for SQ-31906 in human serum quality control samples

	QC1	QC2	QC3	QC4[a1]
Nominal concentration (ng/ml)	2	30	80	500
Mean observed concentration (ng/ml)	2.12	30.2	83.6	532
% deviation from nominal	6.05	0.650	4.48	6.34
Between-run precision (%RSD)	3.55	3.49	3.53	4.68
Within-run precision (%RSD)	5.59	1.80	2.10	1.61

[a] QC4 is a 10-fold dilution QC.

Table 15
Summary statistics from three validation runs for SQ-31906-d5 in human serum quality control samples

	QC1	QC2	QC3	QC4[a1]
Nominal concentration (ng/ml)	2	30	80	500
Mean observed concentration (ng/ml)	2.09	30.0	84.1	531
% deviation from nominal	4.74	0.0964	5.10	6.22
Between-run precision (%RSD)	3.15	2.94	4.17	4.75
Within-run precision (%RSD)	4.95	2.11	1.79	1.76

[a] QC4 is a 10-fold dilution QC.

Table 16
Summary statistics from three validation runs for Prav–Lac in human serum quality control samples

	QC1	QC2	QC3	QC4[a1]
Nominal concentration (ng/ml)	2	30	80	500
Mean observed concentration (ng/ml)	1.95	30.3	80.5	528
& % deviation from nominal	−2.65	0.956	0.686	5.57
Between-run precision (%RSD)	1.46	0.631	0.00[*1]	4.92
Within-run precision (%RSD)	1.15	1.07	1.57	1.37

[a] QC4 is a 10-fold dilution QC.
* No significant additional variation was observed as a result of performing the assay in different runs.

conversion of SQ-31906 to Prav–Lac and no observable conversion of Prav to SQ-31906 or Prav–Lac. There was, however, 4.40, 5.94, 16.7 and 36.2% conversion of Prav–Lac to Prav after 0, 1, 6, and 24 h, respectively. This is consistent with the data obtained for the analytical QCs. There was minimal ($\leq 1.61\%$) conversion of SQ-31906 to Prav at all of the timepoints.

Although the benchtop stability of the analytes in buffered (pH 4.5) serum was not investigated, data obtained from the stability QCs which were included in each run and extracted as the last samples in the set, indicated that all conversions were $\leq 5\%$ during the validation experiments. In effect, the benchtop stability was investigated in each analytical run through the inclusion of these QCs. Benchtop stability was also performed for incurred study samples stored in an ice-water bath for up to 24 h. No significant difference was seen in the stability of the analytes in these samples when compared to analytical QCs.

3.9. Freeze–thaw stability

All analytes were found to be stable in the analytical QCs after three freeze–thaw cycles. The

mean deviations of the freeze–thaw QCs were within 9% of the zero-cycle QCs for all cycles and concentrations. The precision values were all within 5.49% CV. The freeze-thaw data from the stability QCs also indicated acceptable freeze-thaw stability. There was no observable conversion to SQ-31906 or Prav–Lac in any of the QC samples. There was $\leq 1.68\%$ conversion of SQ-31906 to Prav after two freeze–thaw cycles. There was 3.94, 5.63 and 7.20% conversion of Prav–Lac to Prav after 0, 1, and 2 freeze–thaw cycles, respectively, which was deemed acceptable, although some hydrolysis of the lactone is apparent.

3.10. Long-term storage stability

The long-term storage (-70°C) stability of the analytes was examined in analytical and stability QCs, as well in incurred study samples. This was performed by analysis of stored serum samples versus a freshly prepared calibration curve. For analytical and stability QCs stored for 77 and 81 days, respectively, all mean deviations from nominal were $\leq 10.1\%$ with precision of $\leq 4.7\%$ CV. All conversions in the stability QCs were $\leq 1.6\%$. The mean predicted concentrations of all analytes in a pooled incurred study sample stored at -70° C for 81 days deviated from the Day 0 values by $\leq 12.6\%$ with precision of $\leq 8.44\%$ CV.

3.11. Reinjection reproducibility

The reproducibility of reinjecting an analytical run was examined by recapping the autosampler vials from the freeze–thaw stability run and storing them in a refrigerator at 4°C. After 7 days, the standards and QCs were reinjected and the results were compared. No decrease in signal-to-noise was observed and all standard curve slopes were within 10% of those from Day 0. The mean deviations of the analytical QCs from Day 0 were $< 7\%$ and precision ($n = 5$) was $\leq$ 6.24% CV. No additional conversion was observed in the freeze–thaw QCs, which indicates stability in the reconstituted state at 4°C for 1 week.

4. Conclusions

A liquid chromatographic electrospray tandem mass spectrometric method for the quantitative determination of Prav, SQ-31906, Prav–Lac, Prav-d_5, and SQ-31906-d_5 in human serum was developed and validated. Development and validation of the method were analytically challenging due to the fact that Prav can reversibly lactonize to Prav–Lac or reversibly isomerize to SQ-31906. These interconversions were minimized under the optimized experimental conditions employed. The method was been shown to provide good sensitivity, accuracy and precision. Additionally, the interconversions of the analytes have been minimized by pH adjustment and temperature and documented by the use of stability QCs. The analytical method has been used for the analysis of approximately 7000 clinical samples.

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