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## Drug quantitation on a benchtop liquid chromatography–tandem mass spectrometry system<sup>1</sup>

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### Abstract

The specificity and selectivity of LC–MS–MS is illustrated to explain why LC–MS–MS has become the method of choice for quantitation within the pharmaceutical industry. Two assays are described that demonstrate the facility with which new ion trap technology can utilize the selectivity and sensitivity of LC–MS–MS to quantitate trace level components within complex matrices, in particular human plasma. One assay undergoes a validation procedure and demonstrates the utility of this new technology for drug quantitation within a regulated environment.

*Keywords:* Liquid chromatography–mass spectrometry; Detection, LC; Steroids; SR 27417

### 1. Introduction

In recent years the application of liquid chromatography–tandem mass spectrometry (LC–MS–MS) has been used to an increasing degree for quantitative analysis of drug compounds at trace levels. The reason for this is the facility with which LC–MS–MS is able to analyze trace components in complex matrices such as plasma. A comparison with LC–UV serves to illustrate the unique capabilities of tandem MS [1,2] and explains why its use is so prevalent within the pharmaceutical industry (see Fig. 1).

An LC–UV analysis affords retention time data based on the elution characteristics of the compound

of interest and this data will change if the eluent used is altered or if a different LC column is used. If a photodiode array detector is used the spectral information affords increased confidence in the conclusions derived as the complete UV spectrum is generated. If only partial separation is achieved, quantitation of the two components can be problematic, particularly if they are analogues with similar absorbance characteristics. When analyzing complex matrices such as plasma, the non-specific nature of UV detection means that extensive sample clean-up and LC separation is often required.

An LC–MS analysis affords retention time data based on a summation of all the ions that are detected, referred to as the total ion chromatogram (TIC). Each data point along this chromatogram consists of a mass spectrum affording molecular mass data. A change of eluent or LC column will result in a different retention time but will not alter

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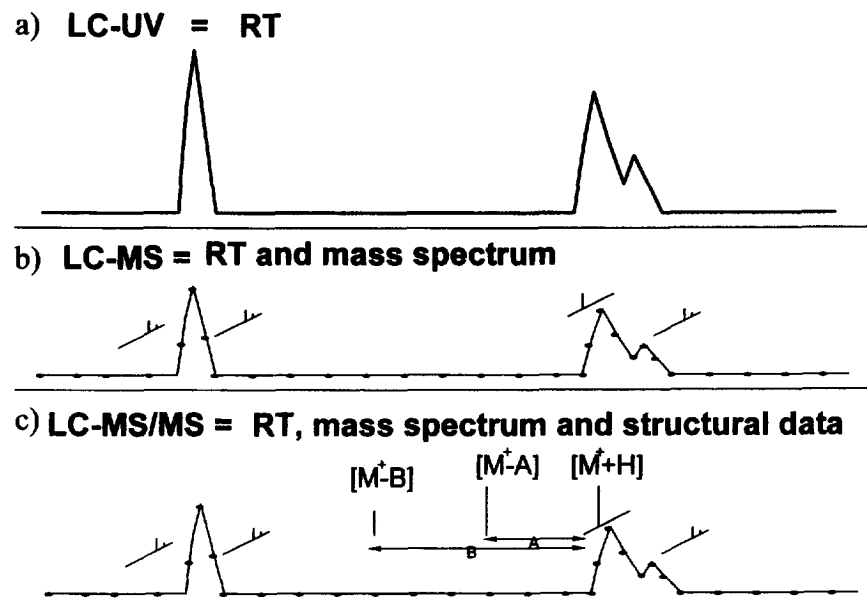


Fig. 1. Schematic depiction of the information content within (a) LC-UV, (b) LC-MS and (c) LC-MS-MS data.

the mass spectrum obtained. Therefore LC-MS methods are inherently more robust. If two analogues are partially resolved or even co-elute, the mass spectra obtained will be different for the two species (as most species have a different molecular mass). The mass spectra can be used to generate a reconstructed ion chromatogram (RIC) which will enable quantitation of two co-eluting analogues to be carried out. LC-MS analyses are still subject to interference from complex matrices and this means that it is often necessary to carry out sample clean-up and LC separation to afford meaningful data.

An LC-MS-MS analysis, in common with LC-UV and LC-MS, affords retention time data based on a summation of all the ions that are detected. In addition to molecular mass information the MS-MS spectrum yields structurally specific data based on the collisionally induced dissociation (CID) fragments that are generated. These fragments are characteristic of the structural moieties present in the analyte. As a result, the MS-MS spectrum ensures highly specific data affording a very high confidence in the results obtained and is therefore inherently less susceptible to interference from complex matrices.

CID is a process by which translational energy is imparted to an ionized molecule so that the molecule fragments into two or more ions [3]. CID is carried

out by accelerating selected (parent) ions into a region that has a higher density of gas molecules. The resultant collisions with the gas induce dissociation of the parent molecule. Because the fragment (daughter) ions, reflect the structure of the parent ion, CID affords structurally specific data.

Complex matrices often afford interference from the matrix across a wide retention time range. In an MS-MS experiment the mass for the parent ion of interest is specifically selected with the concomitant elimination of all ions of differing molecular mass. If there is a contaminant with the same molecular mass (isobaric) that co-elutes with the parent of interest, then only those two ions are selected. After CID, only fragment ions derived from the analyte of interest are selected, thus eliminating the CID fragments from the contaminant. This two stage filtering process allows very selective data to be derived from complex matrices in the presence of co-eluting, isobaric contaminants.

Because LC-MS-MS is able to extract specific, quantitative data from gross sample mixtures the development of high-resolution LC methods has not been necessary. For the same reason method development and validation times for an LC-MS-MS assay are typically 1–2 weeks and analysis times are typically 2–5 min, resulting in a very high sample

throughput. These are the core reasons why the application of LC–MS–MS, particularly in the pharmaceutical industry, has become the method of choice. The high cost of instrumentation capable of LC–MS–MS has been the reason for it not being utilized more widely.

The recent developments in ion trap technology, including the use of external ionization, trivial MS and MS–MS experimental setup and interfacing to atmospheric pressure ionization sources, now offers all the advantages and facility of LC–MS–MS [4–6] at approximately half the cost of triple quadrupole systems. Also triple quadrupole systems have typically required extensive training to operate optimally whereas that is not the case with modern ion traps. They are physically smaller, truly benchtop, instruments and that serves to make the acceptance of this technology less daunting.

It is the intention of this paper to demonstrate that this new technology is able to provide reliable validated quantitative data. To that end two analyses are described demonstrating quantitation of human plasma samples and validation of one of those methods at the 25 pg/ml level.

## 2. Experimental

### 2.1. Steroid assay

#### 2.1.1. Reagents

HPLC-grade methanol and water (Burdick and Jackson, Muskegon, MI, USA), acetic acid (Curtin Matheson Scientific, Houston, TX, USA), testosterone, hydrocortisone, [ $^2\text{H}_3$ ]hydrocortisone (Sigma, St Louis, MO, USA) and [ $^2\text{H}_3$ ]testosterone (Cambridge Isotope Labs., Andover, MA, USA) were used in these analyses.

#### 2.1.2. Sample preparation

Human plasma (1-ml aliquots) were extracted using SPECPLUS 3ML  $\text{C}_8$  extraction columns (ANSYS, Irvine, CA, USA). The disc was conditioned with 0.2 ml of methanol followed by elution of the steroids with 1 ml of ethyl acetate. The eluate was evaporated to dryness, spiked with steroids (testosterone and hydrocortisone were spiked at a

level of 94 pg/ml to 12 088 pg/ml and the internal standards, [ $^2\text{H}_3$ ]testosterone and [ $^2\text{H}_3$ ]hydrocortisone, at a level of 10 000 pg/ml) and reconstituted with 200  $\mu\text{l}$  of mobile phase and 100  $\mu\text{l}$  injected on-column.

#### 2.1.3. LC–MS–MS

A Hewlett-Packard (HP) 1050 modular LC system was used with a flow of methanol–water (5% acetic acid) (60:40) at 0.5 ml/min through a Supelcosil LC-18-DB (33 $\times$ 4.6 mm, 5  $\mu\text{m}$ ) column. The LCQ (Finnigan MAT, San Jose, CA, USA) was operated using the atmospheric pressure chemical ionization (APCI) probe in the positive ion selection mode, with the vaporizer set to 450°C and the heated capillary to 175°C. Full scan MS–MS data were obtained for the four components as follows: testosterone  $m/z$  289 $\rightarrow$ 80–305; [ $^2\text{H}_3$ ]testosterone  $m/z$  292 $\rightarrow$ 80–305; hydrocortisone  $m/z$  363 $\rightarrow$ 100–305 and [ $^2\text{H}_3$ ]hydrocortisone  $m/z$  366 $\rightarrow$ 100–305.

Under these LC conditions hydrocortisone and testosterone have retention times of 2.04 and 4.97 min, respectively. Therefore the mass spectrometer was set to analyze hydrocortisone and [ $^2\text{H}_3$ ]hydrocortisone between 0–3.5 min and then switched to analyze testosterone and [ $^2\text{H}_3$ ]testosterone between 3.5–6.0 min.

### 2.2. SR 27417 assay

#### 2.2.1. Reagents

HPLC-grade acetonitrile, hexane and water (Burdick and Jackson), formic acid, ammonium acetate (J.T. Baker, Phillipsburg, NJ, USA) and SR 27417 and the deuterated internal standard (obtained in-house) were used throughout these analyses.

#### 2.2.2. Sample preparation

A 1-ml volume of human plasma was spiked with internal standard (100 pg/ml) and extracted with 6 ml of hexane by rotary mixing for 20 min, followed by centrifugation for 10 min. The organic layer was transferred to a conical tube and evaporated to dryness under nitrogen at 45°C. The extract was reconstituted with 200  $\mu\text{l}$  of acetonitrile–water (50:50) and 50  $\mu\text{l}$  injected on-column.

### 2.2.3. LC–MS–MS

A Hewlett-Packard (HP) 1050 modular LC system was used with a flow-rate 0.5 ml/min through a Keystone BDS Hypersil C<sub>18</sub> (30×4.6 mm, 5 μm) column. Gradient elution was performed with the first 2.8 min diverted to waste.

Mobile phase A consisted of 2 mM ammonium acetate–0.2% formic acid in HPLC-grade water and mobile phase B consisted of 2 mM ammonium acetate–0.2% formic acid in HPLC-grade acetonitrile–water (98:2, v/v). Gradient was 50:50 A–B to 100% B in 3.0 min, 1.0 min hold at 100% B, return to 50:50 at 4.5 min, 7.0 min run time.

The LCQ (Finnigan MAT) was operated using the electrospray (ESI) probe in the positive ion selection mode, with the spray voltage set to 4.5 kV and the heated capillary set to 250°C. Selected reaction monitoring (SRM) data were obtained by monitoring the two transitions: SR 27417 *m/z* 465→420 and [<sup>2</sup>H<sub>4</sub>]SR 27417 *m/z* 469→424.

This assay was previously validated on a TSQ-7000 triple quadrupole mass spectrometer and the same samples were analyzed on the LCQ in order to determine how the LCQ compares with the TSQ-

7000. The results of this comparison have been published [5,7]. This is the reason why SRM and not full scan MS–MS was performed.

## 3. Results and discussion

### 3.1. Steroid assay

A calibration curve was generated by making five injections of a series of nine standards over a period of 5 h. Thus at each of the nine levels there were five replicates each injected 1 h apart. The zero level standards contained endogenous levels of hydrocortisone and testosterone (Figs. 2 and 3) which caused an offset from zero to be observed in the calibration curves. The calibration curve obtained for hydrocortisone gave a  $R^2=0.9934$  ( $y=0.0001x+0.0917$ ), while the curve for testosterone afforded a  $R^2=0.9986$  ( $y=0.00001x+0.1535$ ).

The peak at a retention time of 1.50 min observed in the hydrocortisone chromatogram is clearly not simply an analogue as the full scan MS–MS spec-

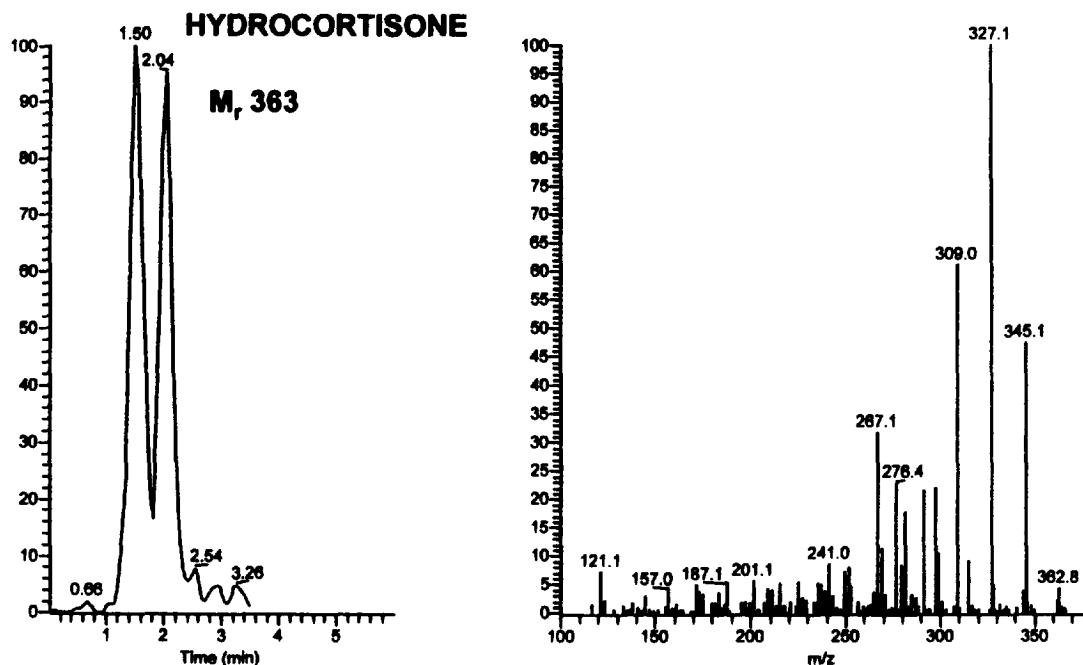


Fig. 2. Chromatogram and full scan MS–MS spectrum for zero level standard of hydrocortisone (peak at 2.04 min).

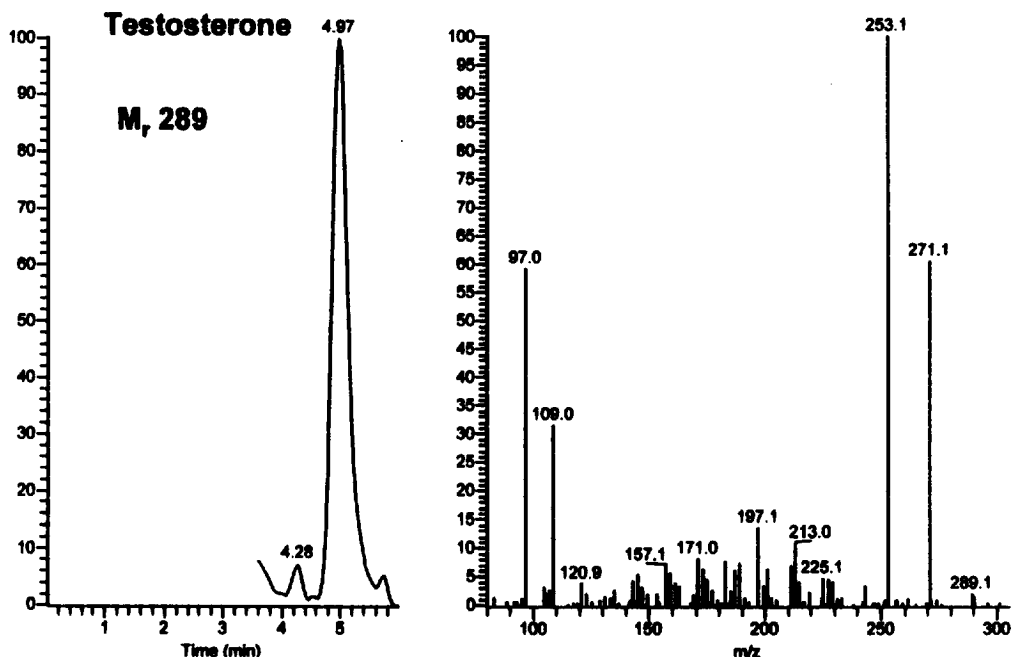


Fig. 3. Chromatogram and full scan MS–MS spectrum for zero level standard of testosterone (peak at 4.97 min).

trum obtained is significantly different from that obtained for hydrocortisone (see Fig. 4).

The use of full-scan MS–MS aids method development in that it allows the decision as to which product ion(s) to use for quantitation to be made after a calibration curve has been acquired. It is then possible to perform the quantitation using different ions or combinations of ions so as to afford the best results. In the case of hydrocortisone, the ions at  $m/z$  309 and 327 were used as the quantitation masses and  $m/z$  97, 109 and 253 were used for testosterone. Thus several ions can be monitored simultaneously by the use of full-scan MS–MS. In addition full-scan MS–MS allows matrix contaminants to be identified immediately, as in the case of the peak at 1.50 min within the hydrocortisone chromatogram, based on the spectral differences observed.

### 3.2. SR 27417 assay

A calibration curve for SR 27417 (Fig. 5) was generated using standards at 10, 25, 50, 100, 200, 400, 600, 800 and 1000 pg/ml. A single injection was made at each concentration except for the 10

and 1000 pg/ml levels which were injected in triplicate. Fig. 6 reproduces the mass chromatogram for the 10 pg SR 27417 standard and the internal standard. Since one quarter of the reconstituted plasma extract was injected, this chromatogram represents a 2.5-pg on-column injection.

The data obtained afforded a quadratic  $1/X^2$  calibration curve with a correlation coefficient of 0.9973.

Having obtained the results certain criteria were tested to determine if the LCQ generated data that can be validated. These criteria were divided into two areas, within-day variability and between-day variability.

The within-day variability was tested by use of quality control (QC) samples at levels of 10, 25, 400 and 1000 pg/ml. Six replicates of the QC set were analyzed.

The between-day variability was tested by use of QC samples at levels of 10, 400 and 1000 pg/ml. This QC set was used on three separate occasions.

Validations were accepted by the following criteria: no more than 33% of samples within a given concentration level were beyond  $\pm 15\%$  of nominal,

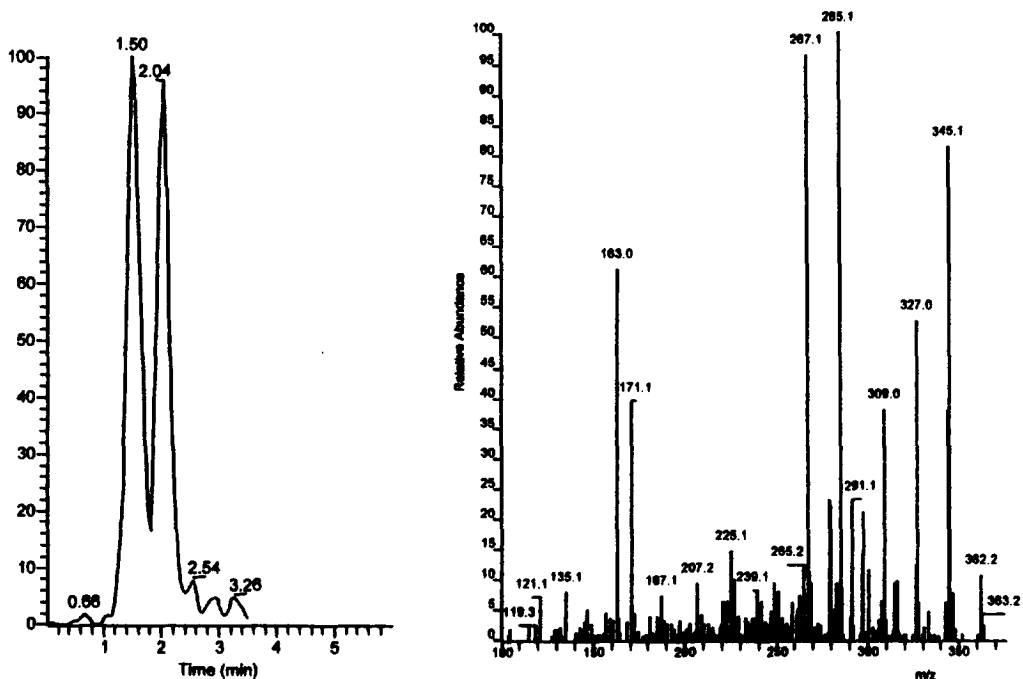


Fig. 4. Chromatogram and full scan MS-MS spectrum for matrix contaminant within the zero level standard (peak at 1.50 min).

except at the minimum quantifiable level (MQL), where the acceptance criteria was  $\pm 20\%$  of nominal. The accuracy and precision for each level were not greater than  $\pm 15\%$  of nominal, except at the MQL, where values were not beyond  $\pm 20\%$  of nominal.

Accuracy and precision were calculated as follows:

$$\text{Accuracy} = \frac{(\text{observed conc.} - \text{expected conc.})}{\text{expected conc.}} \cdot 100\%$$

$$\text{Precision} = \frac{\text{standard deviation}}{\text{mean}} \cdot 100\%$$

The validation data are given in Tables 1 and 2.

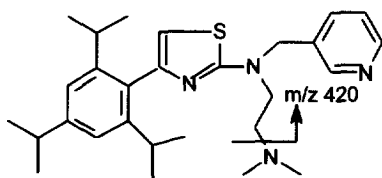


Fig. 5. Structure of SR 27417.

### 3.2.1. Within-day validation

The 10 pg/ml level afforded accuracy and precision data of  $\pm 15.54\%$  and  $\pm 13.36\%$ , respectively. Though these data strictly speaking meet the criteria, we prefer data sets to be no more than  $\pm 10\%$  for both precision and accuracy during the method development stage to leave a sufficient margin so that any minor variations during assay analysis are still within validated criteria.

### 3.2.2. Between-day validation

The 10 pg/ml level afforded accuracy and precision data of  $\pm 12.69\%$  and  $\pm 7.86\%$ , respectively. Again this data set met the criteria but the accuracy data are above the  $\pm 10\%$  level we prefer.

The within-day and between-day validation data, taken in combination, demonstrate that the LCQ is capable of validating this assay with a MQL of 25 pg/ml.

## 4. Conclusions

The intention of this paper was to demonstrate that

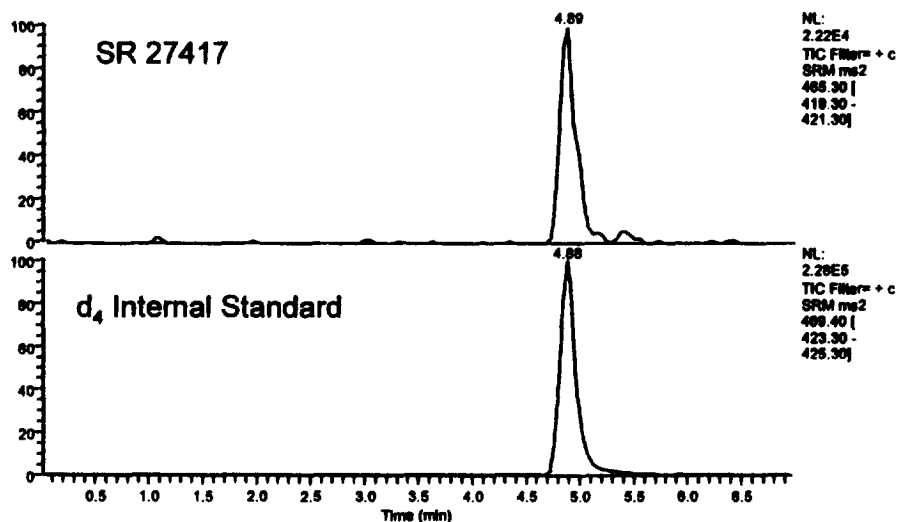


Fig. 6. Mass chromatograms of SR 27417 10 pg/ml (2.5 pg on-column) and SR 27417 100 pg/ml (25 pg on-column).

Table 1  
Within-day validation data for SR 27417

	Nominal concentration (pg/ml) ( <i>n</i> = 6)			
	10	25	400	1000
Mean	11.5	25.37	408.02	1006.22
S.D.	1.58	2.13	17.16	44.03
R.S.D. (%)	13.36	8.41	4.21	4.38
M%D <sup>a</sup>	15.54	1.47	2.00	0.62

<sup>a</sup> mean percent deviation.

the LCQ offers a lower-cost alternative for quantitative LC-MS-MS analysis of trace level components within complex matrices. The data are unequivocal, clearly this new technology affords the sensitivity, accuracy and precision necessary to generate validated quantitative data.

Table 2  
Between-day validation data for SR 27417

	Nominal concentration (pg/ml) ( <i>n</i> = 3)		
	10	400	1000
Mean	11.27	430.66	999.63
S.D.	0.89	27.48	45.86
R.S.D. (%)	7.86	6.38	4.62
M%D <sup>a</sup>	12.69	7.67	-0.84

<sup>a</sup> Mean percent deviation.

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