CHAPTER 5

Mass spectrometry and quantitative bioanalysis

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5.1 *Introduction*

What is mass spectrometry? Mass spectrometry (MS) is based on the ionisation of molecules so that they become charged particles and can then be sorted according to their mass to charge ratio (m/z). The mass spectrometer is the instrument that provides the requisite functionality for this technique; however, there are several different types of mass spectrometers. The key to understanding advances in mass spectrometry is to appreciate the problem-solving nature of technological developments. Most of these advances are driven by the instrument manufacturers and have concentrated on improving methods of ionising molecules and the selectivity and sensitivity of detection. (For more detailed reading on mass spectrometery, see Bibliography.)

MS was originally used in conjunction with gas chromatography (GC) for the quantitative analysis of drugs. When liquid chromatography (LC) replaced GC as the preferred technique for bioanalysis, GC–MS continued to be used because of the difficulties encountered interfacing LC and MS. GC was used for volatile compounds

or compounds which had been reacted with derivatising agents to render volatile products. These molecules were readily ionised by techniques such as electron impact (EI), and gas flow into the mass detector was advantageous for the technique. LC, however, introduced new problems, the molecules analysed were generally non-volatile and were not readily ionised. The mobile phase was liquid and present in more significant quantities than the analyte and was also incompatible with the high vacuum of a mass spectrometer. How to interface LC with MS provided a technological challenge which was successfully overcome by changing the ionisation techniques and the design of the mass spectrometer. In recent years these technological advances have made LC–MS the method which is now regarded as the standard method used for bioanalysis in pharmaceutical laboratories and clinical contract organisations. In this chapter we will review these developments and focus on the use of MS in quantitative analysis. MS is also a powerful tool for structural and molecular information and is used extensively in qualitative analysis and identification of metabolites. This is discussed in Chapter 17.

5.2 *The instruments*

There are a number of different mass spectrometers which are characteristic of the instrument manufacturer, ionisation technique or the instrument design (some instruments are designed so that they are optimised for particular applications). There are also technological differences in the mass analyser. For example, in sector instruments, ions are produced in the source of a mass spectrometer that is operating under vacuum. They are then accelerated by an electric field into a magnetic region. Scanning the magnetic field of the electromagnet sequentially focuses ions of differing mass at the detector. In contrast, quadrupole mass spectrometers operate by filtering masses through a radio frequency voltage field. Whilst there are differences in the instruments, the scientific principles and overall components are the same for all mass spectrometers. These are illustrated in Figure 5.1.

In each case there is a need to introduce the analysed sample into the mass spectrometer; in practice this is the interface with the chromatographic separation technique (primarily LC). The analyte molecule is then converted into an ionic compound by the ionisation process; following ionisation the ions are progressed to the mass analyser and ion detector. Each of these components is discussed in more detail in the following sections.

5.3 *Analytical interfaces*

There are a number of possible interfaces with LC and the main purpose is to evaporate the mobile phase and transfer the analytes to a gaseous phase suitable

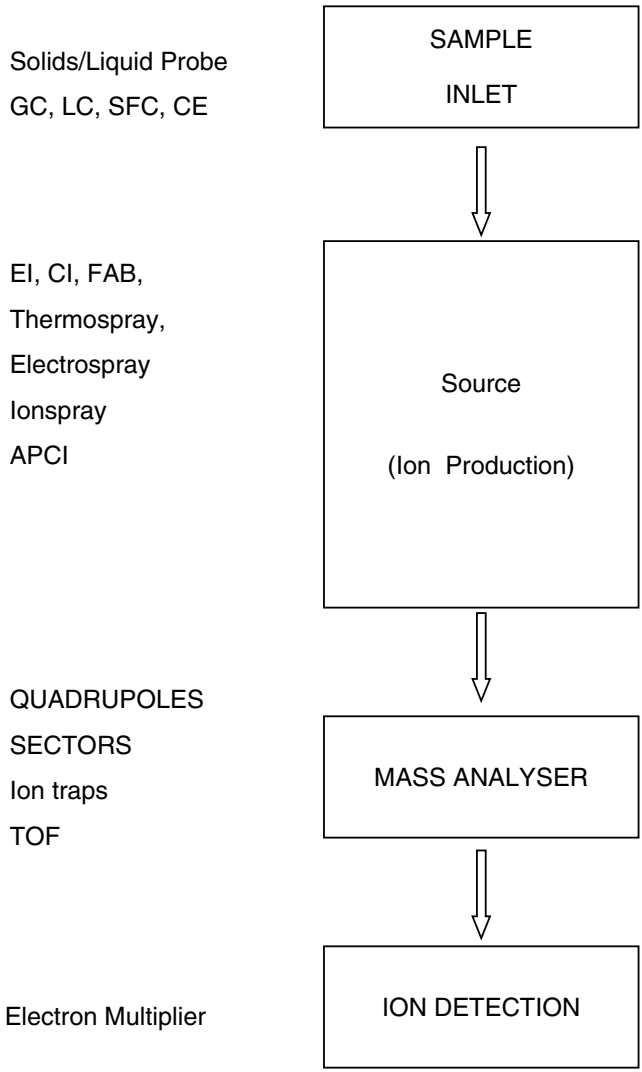

FIGURE 5.1 *Components of a mass spectrometer.*

for ionisation and to switch from the high or atmospheric pressures at which chromatographic separation was achieved to the lower pressures required for mass analysis. The main interfaces are described below.

5.3.1 ATMOSPHERIC PRESSURE–CHEMICAL IONISATION (APCI)

APCI creates gas-phase ions at atmospheric pressure. The ions produced pass through a series of channels into successive vacuum chambers. The eluant from

the LC is sprayed into a heated chamber at a temperature of approximately 400 °C. The heat rapidly evaporates the liquid and solutes contained therein and does not appear to degrade most drugs and metabolites.

5.3.2 Electrospray (ES)

The ES interface produces gas-phase ions at atmospheric pressure. These pass into the vacuum system through a series of apertures separating successive vacuum stages. ES is composed of a hollow needle with a high electric potential through which the eluant flows. The high field at the tip of the needle produces a cone-shaped liquid meniscus from which a spray of highly charged droplets emerges. Subsequent evaporation of the droplets results in ion formation. Conventional ES operates at flow rates of 1–10 μL; higher flow rates can be achieved with alternative ES, ionspray (pneumatic assisted), ultraspray (ultrasonic assisted) and turbospray (thermally assisted).

5.3.3 Particle beam (PB)

The PB interface is designed to remove the mobile phase whilst transferring the majority of the analyte to the mass analyser. The PB conducts several functions: an aerosol generator serves to disperse the liquid eluant into a high surface area spray which passes into the heated desolvation chamber in which solvent-depleted solute particles are produced. The momentum separator serves to direct the aerosol through a series of apertures at atmospheric pressure into the low-pressure ion source.

5.3.4 Continuous flow fast atom bombardment (flow FAB)

The column eluant is directly introduced into the vacuum region of the MS through a probe at a very low flow rate of 5–10 μL. The eluant is mixed with a matrix material (glycerol, thioglycerol or nitrobenzyl alcohol) to facilitate the ionisation process. This mixture passes over the flattened end of the probe to form a very thin layer from where evaporation occurs and the low flow rates ensure that most of the solvent mixture evaporates in the ion source.

5.3.5 Thermospray

The thermospray vapouriser is a heated capillary tube through which the LC eluant passes and by controlling the temperature the complete eluant can be evaporated at

the tip of the vapouriser. The thermospray is capable of controlling the evaporation of a wide variety of mobile phases. The vapourised solute is introduced into a reduced pressure spray chamber from which it passes into the high vacuum of the mass analyser.

5.4 *Ionisation*

There are several methods for producing ions in the source of a mass spectrometer. EI is a long established ionisation technique; however, a number of 'soft' ionisation techniques such as chemical ionisation (CI), fast atom bombardment (FAB), atmospheric pressure ionisation or ES are used. The latter two are the most important for modern quantitative LC–MS.

5.4.1 Electron impact (EI)

This was the commonest method of ionisation for GC–MS, as a great number of organic compounds were suitable for EI but it has limited use in LC–MS. To give an EI spectrum the compound must be volatile; specifically it must have a vapour pressure of at least 10^6 Torr. Ions are formed when a beam of electrons hits the sample molecules in the gas phase. This gives the sample molecules high energy, and fragment ions are formed. Unfortunately some compounds will fragment entirely and not produce molecular ions. EI can be performed by direct probe and GC–MS. PB is the only commercial LC–MS interface that produces EI spectra. A beam of solute particles enters the source of the mass analyser and impinges on the surface of the ion source. The ion source walls are heated to several hundred degrees facilitating the 'flash vapourisation' of the solute particles. Once in vapour phase the solute molecules are ionised by EI.

5.4.2 Chemical ionisation (CI)

CI can produce molecular ions for some volatile compounds that do not give molecular ions in EI. CI uses a reagent gas to transfer protons to the sample, usually producing $(M + H)^+$ quasimolecular ions. These ions have a tendency to fragment because they are even-electron species and excess energy is imparted to them. The reagent gas (methane, isobutane or ammonia) is present in the ion source, it is ionised by an electron beam and the resulting ions undergo a series of ion–molecule reactions to produce species such as CH_5^+ in methane. These reagent gases in a ratio of 106:1 to the sample then collide with sample molecules producing ions by proton transfer. CI has been used with PB and API interfaces for LC–MS.

5.4.3 Fast atom bombardment (FAB)

FAB is the most popular ionisation technique for non-volatile and/or thermally labile molecules. FAB has provided spectra for compounds which were unsuccessful for EI or CI. It works best for polar and higher molecular weight compounds such as peptides and ionic species. FAB utilises a beam of neutral fast moving atoms (xenon, argon) which impinge on a metal target coated with liquid matrix in which the sample has been dissolved. The impact of these atoms on the liquid surface transfers kinetic energy to the sample in a manner that results in the desorption of the sample ions into the gaseous phase. This process is gentle and little fragmentation occurs. FAB spectra are complex showing $(M + H)^+$ quasimolecular ions, fragments and cluster groups of the matrix and the sample plus matrix. Trifluroacetic acid can be added to the matrix to encourage formation of protonated ions. Some compounds give better negative ion FAB spectra where the quasimolecular ion is $(M - H)^-$. FAB has been used with the PB interface but is more commonly associated with the continuous flow FAB interface.

5.4.4 Electrospray ionisation (ESI)

Unlike most ionisation processes in MS which occur in the gaseous phase, ESI is the transfer of ions present in the liquid phase into the gas phase. A pre-requisite for ion production with ES is that the analyte exists in solution as an ion. ES can produce ions from 100 per cent aqueous solutions with no organic modifiers. Mobile phases may have volatile buffers such as ammonium acetate in moderate concentration. Many buffers especially those containing alkali metals will decrease sensitivity by competing for ionisation. ESI can also produce ions from 100 per cent solutions in organic solvents such as methanol and acetonitrile.

5.4.5 Atmospheric pressure chemical ionisation (APCI)

The LC eluant passes into a heated nebuliser or ionspray where it is mixed with a nebuliser gas; both these inlets are designed to provide soft ionisation of polar molecules. Ions are created at atmospheric pressure with little or no heating (API). Ionisation is accomplished with a source of electrons introduced with the heated spray. The electrons are supplied by a discharge source or 63 Ni beta emittor. These sources produce a rich stream of reagent ions resulting from interaction with the electrons. The reagent ions are produced by electron ionisation of the source gases; usually air or nitrogen is used. The ionisation of O_2 and N_2 by EI leads to the formation of hydronium ion water cluster $H_3O(H_2O)_n$. At atmospheric pressure there is significant interaction between reagent ions and

analyte ions produced by the heated nebuliser. The gas-phase analytes will become and remain protonated if their proton affinity is greater than that of water (amines, for example, have a high proton affinity). The ionisation process is one of the most efficient with almost 100 per cent ionisation obtained under ideal conditions.

APCI is the method of choice for drugs and metabolites and has become the most widely used technology for high-throughput bioanalysis. The sensitivity, robustness and reliability of APCI are greater than those obtained with ES.

5.5 *Mass analysers*

All mass analysers determine the mass of an ion and mass to charge ratio and measure gas-phase ions. There are four main types of mass analysers:

5.5.1 Sector mass analyser

This is the traditional magnetic sector mass spectrometer in which ions created in the ion source are accelerated with high voltages into the analyser magnetic field. The radius of curvature in a given magnetic field of the sector is a function of m/z (mass to charge ratio). Ions of differing masses can be separated and detected by a single detector by varying the magnetic field or the source voltage to scan the mass range. Sector MS is capable of separating all ions all the time but can only detect one mass at a time.

5.5.2 Quadrupoles

A quadrupole MS consists of four parallel rods (quads) equally spaced around a central axis. Ions are introduced along the axis of the poles. The ions are accelerated at low voltages, and by applying different voltages to the different quads, conditions can be established in which only ions with a particular m/z ratio can pass through to the ion detector. Effectively only one ion is monitored; this is known as single ion monitoring (SIM) and is the most sensitive method for a single quad.

5.5.3 Ion trap MS

This mass analyser works by trapping ions and then detecting them based on their m/z ratios. The ion trap is a variation of the quad mass filter and uses the same principles

to trap the ions. After trapping, the ions are detected by placing them in unstable orbits causing them to leave the trap. The ion trap is used in drug metabolism but are not as suitable for high-throughput bioanalysis as quads in a tandem MS.

5.5.4 Time of flight (TOF) analysers

These analysers are based on the fact that ion velocity is mass dependant. They consist of an ion source, a 'flight' tube and a detector. Each mass enters the flight tube at different velocities, small mass ions having a higher velocity. The mass ions separate as they pass down the tube and arrive at the detector. TOF has ideal characteristics for structural analysis and are often combined in modern MS with quads.

5.5.5 Tandem mass spectrometry

Tandem mass spectrometry also called mass spectrometry–mass spectrometry (MS–MS) because the instrument contains two mass spectral analysers in tandem; between the two analysers is a collision gas cell. Generally soft ionisation techniques do not cause fragmentation of the ionised particles. The basic approach of MS–MS is the measurement of mass to charge ratios of ions before and after fragmentation of the selected ion by collision with a high-pressure gas (normally helium). This collision process is called collision-induced dissociation (CID).

5.5.6 Mass detection

There are two main methods for ion monitoring: single ion monitoring (SIM) and selective reaction monitoring (SRM) or multiple reaction monitoring (MRM).

5.6 *Use of MS in quantitative LC–MS*

The improvements in LC–MS have been largely driven by instrument manufacturers and the characteristics are based on the commercially available instruments. In the past few years the main instruments used for high-throughput bioanalysis have been supplied by two vendors: PE Sciex and Micromass. These represent the API and ESI interfaces respectively. In addition to the technical developments, the cost and size of instruments including the required services have been significantly reduced with instruments becoming cheaper and smaller. Sciex MS caused a revolution in high-throughput quantitative bioanalysis by reducing significantly

the time taken to analyse a single sample from 10–15 to 2–5 minutes. In addition the use of the tandem MS arrangement increased the specificity so that only minimal chromatographic separation was required.

The tandem MS arrangement allows the *m*/*z* relating to the analyte to be selected at the first quad and the *m*/*z* characteristic of the main fragment or daughter ion to be selected at the second quad. This provides for good selectivity and sensitivity without significant chromatographic separation required from endogenous material.

Although it is a soft ionisation technique if higher temperatures are used it can affect the decomposition of thermally labile molecules such as N-oxide metabolites and conjugates. If the spectrum indicates a loss of 16 amu (atomic mass units) this can indicate an N-oxide; however, if the conjugate reverts to the aglycone this can be difficult to detect. Molecular weight information is obtained from quasimolecular ions $(M + H)^+$ (positive mode) or $(M - H)^-$ (negative mode).

5.6.1 Optimising physical parameters for APCI

- Vary probe temperature – the formation of adduct ions may be reduced; however, high temperatures may cause problems if compound is thermally labile.
- Drying gas pressure – dependent on LC flow rates.
- Source temperature – dependent on flow rates and solvent composition.
- Cone voltage – high voltage will effect fragmentation and may lead to decreased molecular ion signal.
- More independent of buffer concentration than ESI.

Under APCI conditions a component with a molecular weight similar to the parent compound but at a different retention time is indicative of an N-oxide or conjugate.

5.7 *Developing an LC–MS assay method*

5.7.1 Example 1: a semi-automated method for the determination of fluticasone propionate (CCI187811) in human plasma using solid phase extraction and liquid chromatography tandem mass spectrometry

Fluticasone propionate (FP) is quantified in human plasma by automated solid phase extraction using a Packard MultiPROBE robotic sample handler and analysed by high-performance liquid chromatography (HPLC) with tandem mass spectrometric detection (LC–MS–MS) using selective reaction monitoring (SRM). Samples

are spiked with internal standard, ^{13}C-labeled FP, extracted using C18 solid phase sorbent packed into columns in the 96-well plate format and eluted with methanol (Table 5.1). Extracts are reconstituted with methanol/buffer (50:50) after evaporation and submitted for analysis. Protonated molecules, MH^+, were used as precursor ions with the following SRM transitions being monitored for FP and [$^{13}C_3$]-FP, respectively: m/z 501 → m/z 313 and m/z 504 → m/z 313. A chromatogram of an extract of blank plasma is shown in Figure 5.2; compare with the chromatograms of the lowest and highest standards presented in Figures 5.3 and 5.4, respectively. A typical chromatogram obtained from a plasma sample taken from a patient receiving FP is illustrated in Figure 5.5. The calibration range for this method is 20–1,500 pg/mL from 0.5 mL plasma and the concentrations in calibration samples, quality control (QC) samples and study samples are determined using least-squares linear regression with a 1/X weighting factor (Figure 5.6).

HPLC Conditions	
Mobile phase	25 mM ammonium formate/methanol 20:80, 1 mL/min
Column	150 × 4.6 mm i.d. ResElut C8 BDS operated at 40 °C
Injection volume	80 µL
Split ratio	Between 5 and 10
Mass spectrometer parameters	
Mass spectrometer	PE Sciex API III+
Ionisation mode	Turbo ionspray
Polarity	Positive ion
Scan mode	Selected reaction monitoring (SRM)
Collision gas	Argon
Collision gas thickness (CGT)	250
Nebuliser gas	Zero grade nitrogen
Pause time	20 m
Acquisition time	4.5 minutes
Q2 settling	ON (1000 amu Q3 park mass)
The following transitions were monitored	
FP	m/z 501 → m/z 313 Dwell time: 250 ms
[$^{13}C_3$]-FP	m/z 504 → m/z 313 Dwell time: 250 ms

5.7.2 Example 2: sumatriptan (GR43175X) and rizatriptan (GR289537X) in human plasma

To support clinical trials, a generic SPE 'Triptan' liquid chromatographic tandem mass spectrometric method has been developed for the determination of Sumatriptan (GR43175X) and Rizatriptan (GR289537X) in human plasma.

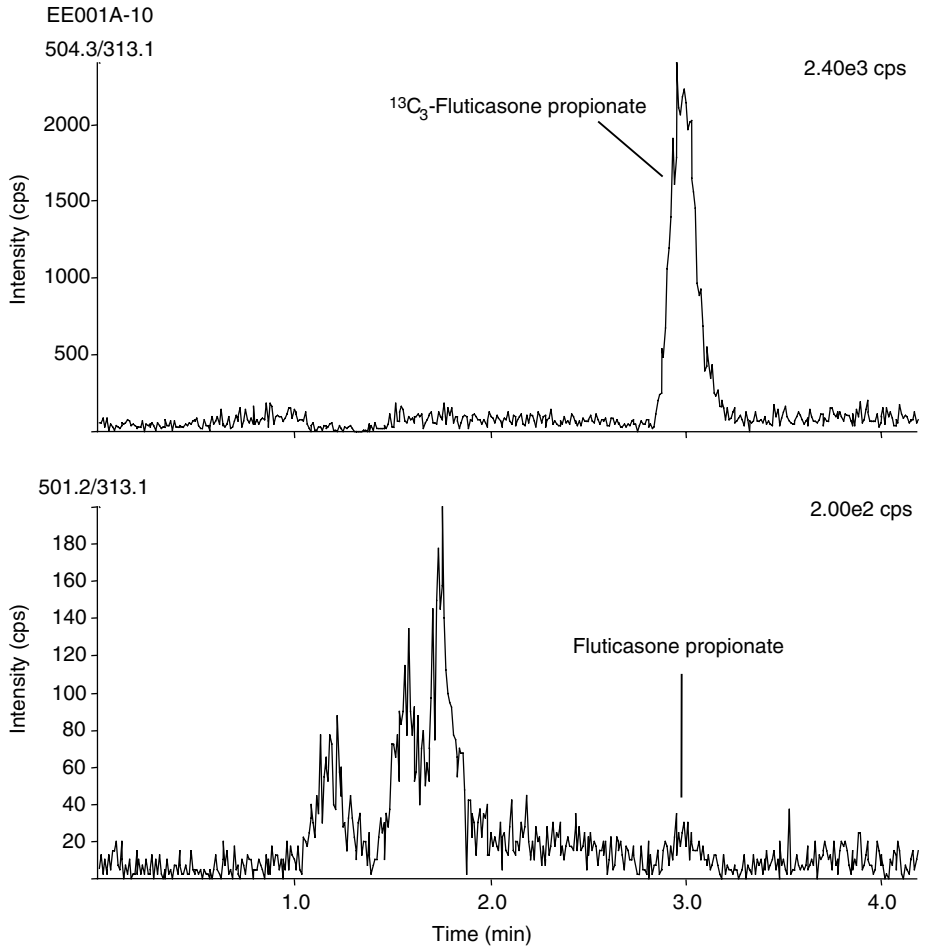

FIGURE 5.2 *Representative chromatogram of blank matrix.*

TABLE 5.1 *Sample preparation conditions*

Step	SPE process	Solvent	Volume (mL)	Bleed factor	Vacuum (s)
1	Condition	10% MeOH (v/v) aq.	0.1	50	10
2	Load	Sample ISTD	0.4	30	180
3	Wash	Water	0.2	10	30
4	Wash	Water	0.2	10	60
5	Elute	MeOH	0.2	30	90

Samples are spiked with internal standard, ^{13}C-labeled FP, extracted using C18 solid phase sorbent packed into columns in the 96-well plate format and eluted with methanol.

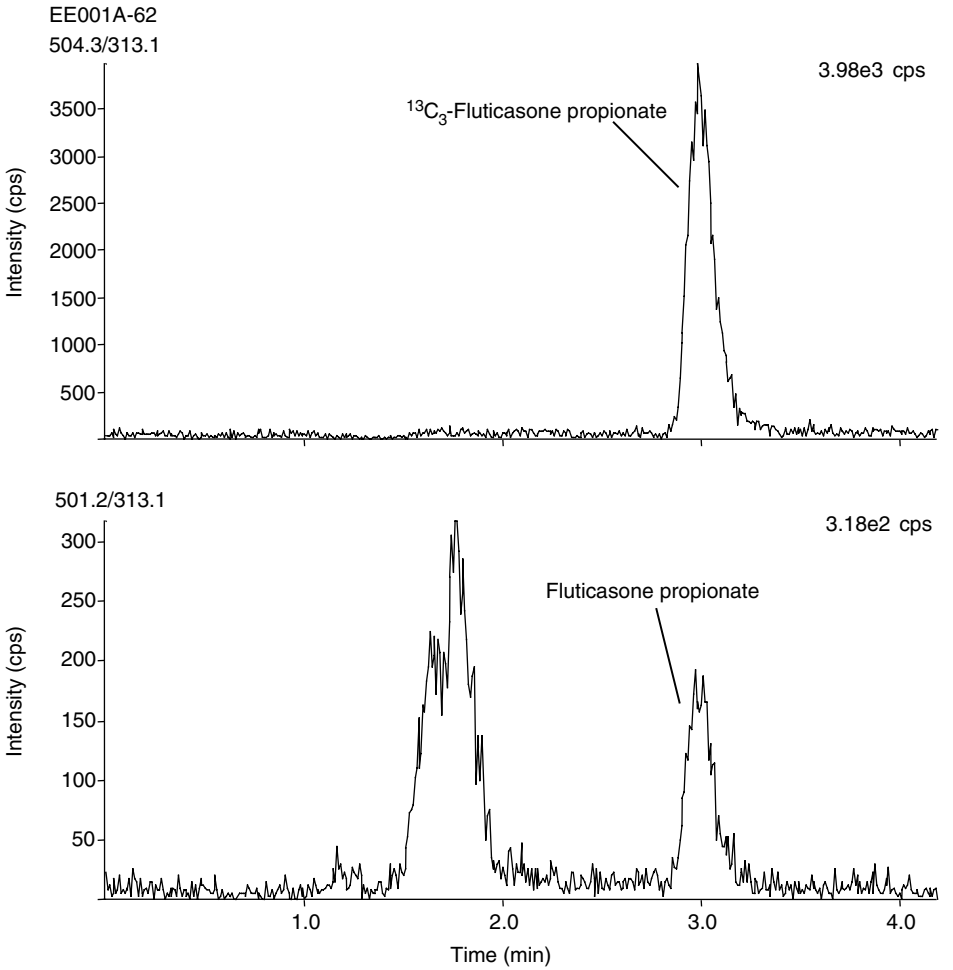

FIGURE 5.3 *Representative chromatogram of standard at LLOQ.*

A Sciex API 365 Plus mass spectrometer with a turbo ionspray interface was used to identify the precursor and product ions for each of the compounds. Infusion of 1 μg/mL solutions and background subtraction were used to collect full scan and MS–MS spectra.

Sumatriptan produces a precursor ion of 296 and the most dominant product ion of 58. The deuterated internal standard produces a precursor ion of 299 and a product ion of 61 (Figure 5.7).

Rizatriptan produces a precursor ion of 270 and the most dominant product ion of 201. The deuterated internal standard produces a precursor ion of 276 and a product ion of 207 (Figure 5.8).

An automated solid phase extraction (SPE) is performed using a zymate XP robot with a series of customised workstations (4). The method is internally standardised;

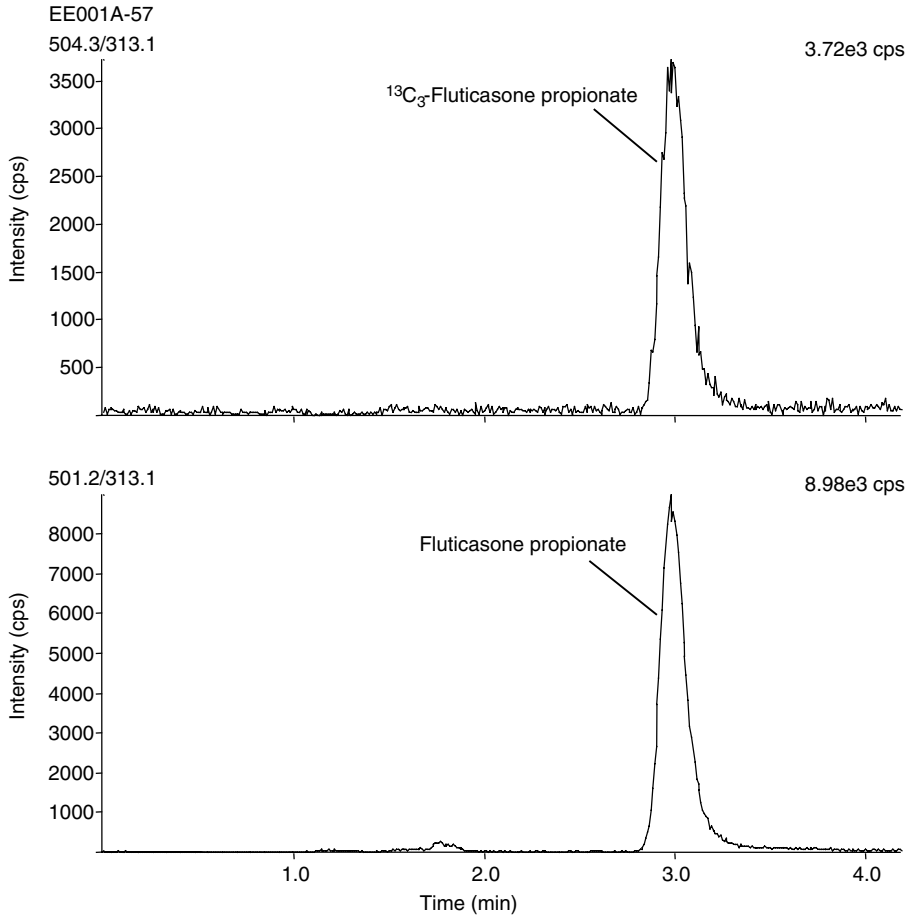

FIGURE 5.4 *Representative chromatogram of standard at ULOQ.*

for GR43175X, ISTD [2H3]-GR43175H is used and for GR289537X, ISTD [2H6]-GW289537C is used.

To start with, a 5 mg Oasis extraction block and 96-well collection plate are housed in the carousel and this is accessed by a zymate XP robot for sample preparation. The SPE station conditions the Oasis block ready for sample loading. A Multiprobe (a four-probe aspirating and dispensing station) transfers the internal standard and sample to the Oasis block. The SPE station then washes the block and elutes the samples into a 96-well collection plate (Figure 5.9).

The resulting extracts are evaporated to dryness under a stream of heated nitrogen and reconstituted with 90:10 0.1 per cent formic acid (aq):acetonitrile. The plate is then transferred to the automated plate sealer where it is sealed with aluminium and polypropylene plate ready for mass spectrometric analysis.

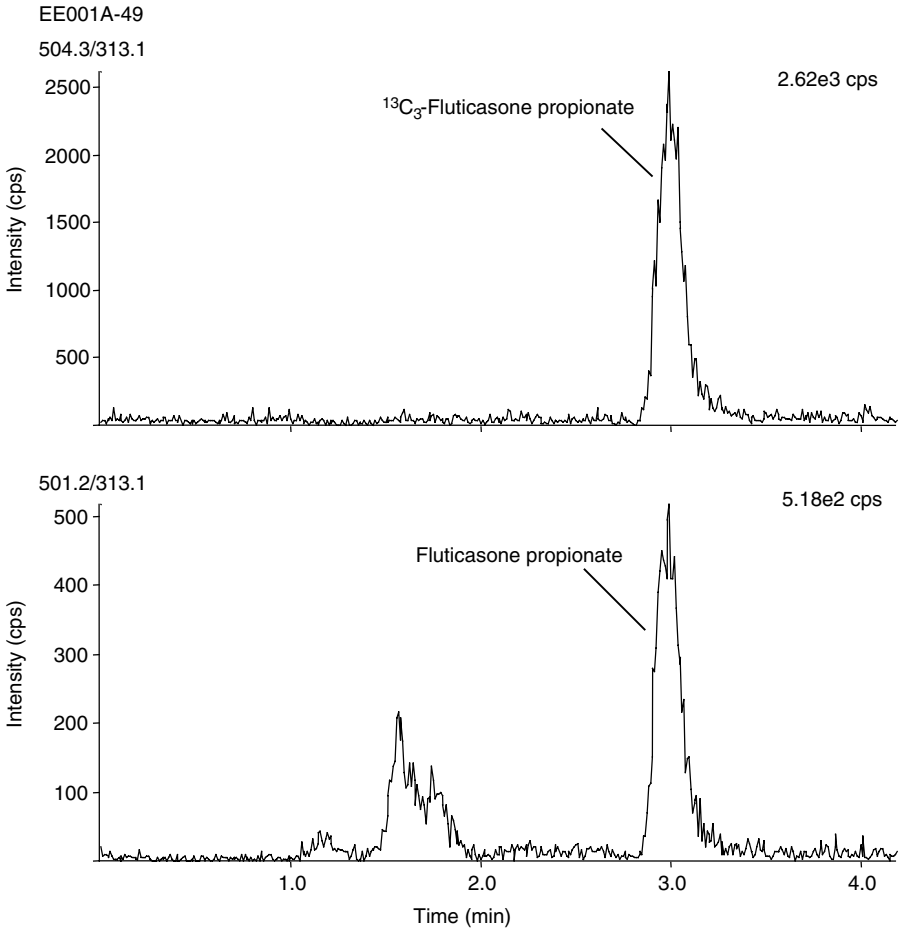

FIGURE 5.5 *Representative chromatogram of subject 27683.*

Samples are analysed using HPLC under gradient conditions. 20 µL of sample is injected onto a 5 cm × 2.1 mm i.d. ODS3 column (5 µm) and the analytes are eluted using a combination of two mobile phases: A = 0.1% formic acid; B = 95% acetonitrile: 4.9% water: 0.1% formic acid (Table 5.2).

Typical chromatograms for blank plasma, lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) are illustrated in Figure 5.10. Data is acquired using Multview and processed using Macquan. Peak area ratios are used to construct a calibration line with a linear regression fit and a $1/X$ weighting factor. Typical calibration lines, range 0.1–20 ng/mL, are shown in Figures 5.11 and 5.12 for Sumatriptan and Rizatriptan, respectively.

The accuracy and precision of Sumatriptan and Rizatriptan in human plasma was determined using spiked validation control samples spiked at 20, 10, 1.0, 0.25 and 0.1 ng/mL. These were analysed in replicates of six on four separate occasions and

the agreement between the measured and nominal concentrations of validation control samples assessed. Results are shown in Tables 5.3 and 5.4. This method has successfully been used to support several clinical studies.

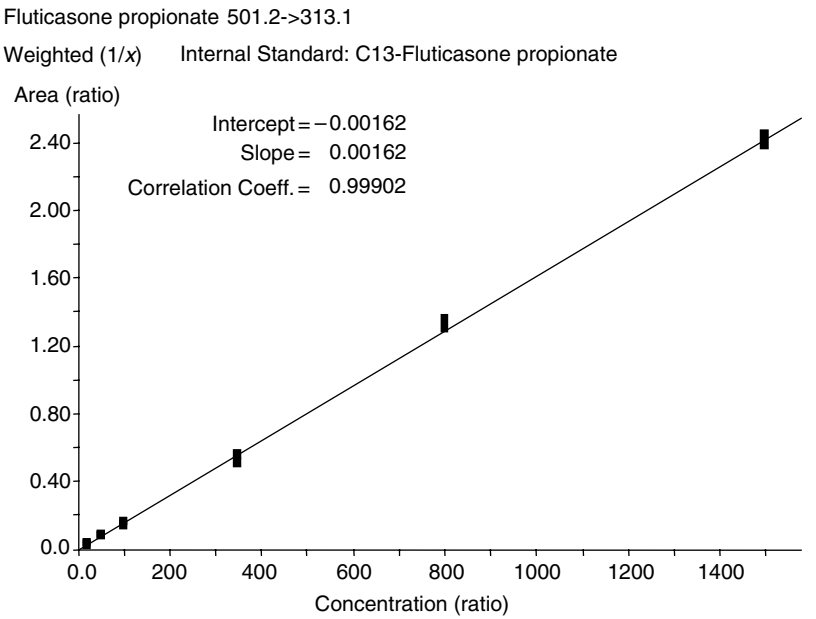

FIGURE 5.6 *Representative calibration curve for fluticasone propionate.*

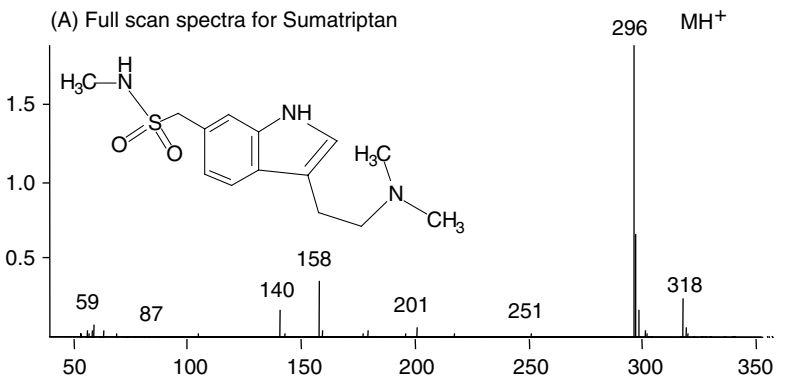

FIGURE 5.7 *MS–MS spectra for Sumatriptan.*

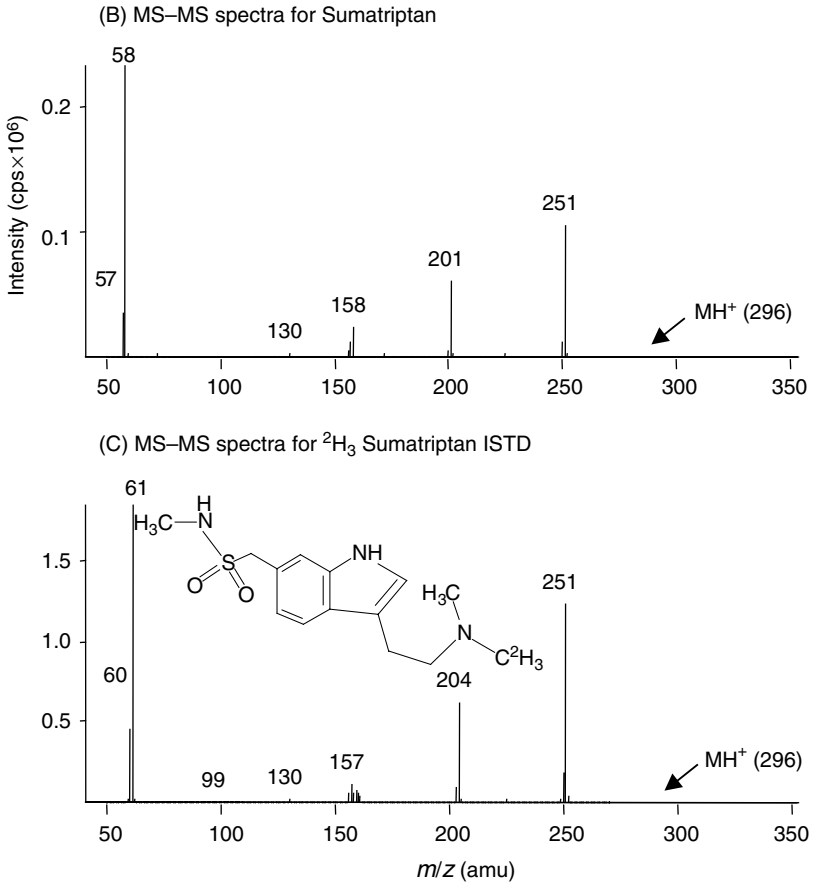

FIGURE 5.7 *(Continued).*

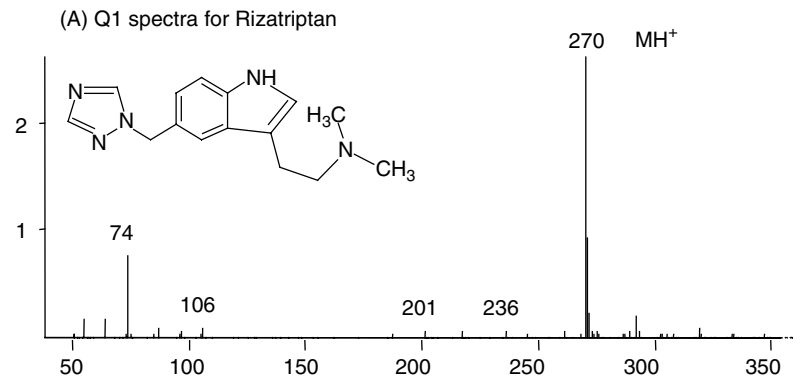

FIGURE 5.8 *MS–MS spectra for Rizatriptan.*

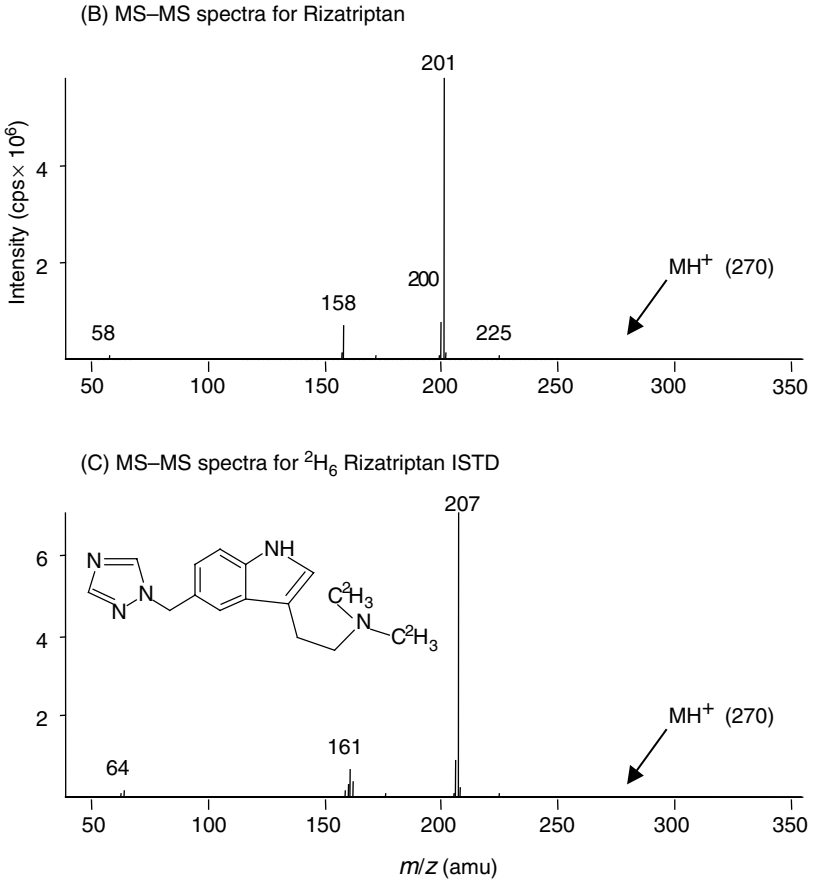

FIGURE 5.8 (*Continued*).

TABLE 5.2 *Samples are analysed using HPLC under gradient conditions. 20 μL of sample is injected onto a 5 cm 2.1 mm i.d. ODS3 column (5 μm) and the analytes are eluted using a combination of two mobile phases: A = 0.1% formic acid; B = 95% acetonitrile:4.9% water:0.1% formic acid*

Start (min)	Duration (min)	Flow (μl/min)	Profile	% Solvent A	% Solvent B
−0.1	0.1	800	0	100	0
0	2.0	800	−2	0	100
2.0	0.1	800	−10	100	0
2.1	1.9	800	0	100	0

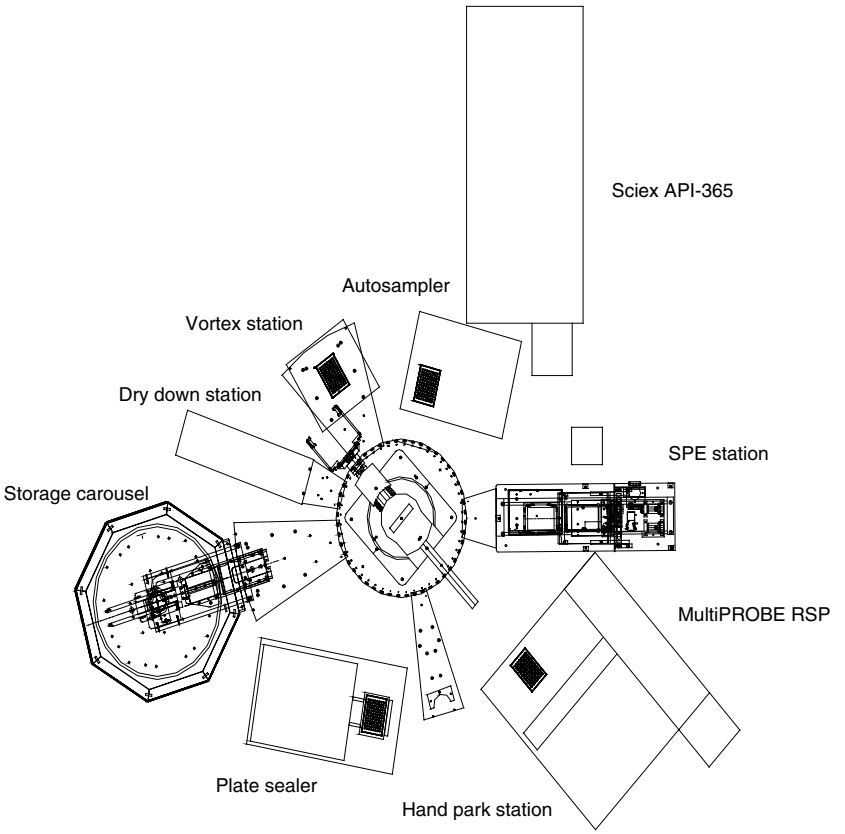

FIGURE 5.9 *Schematic representation of Zymark Zymate sample preparation system.*

TABLE 5.3 *Assay validation results for Sumatriptan assay*

	VC1	VC2	VC3	VC4	VC5
Target and actual VC concentrations for Sumatriptan					
Target VC	20	10	1	0.25	0.1
Number	24	24	24	23	22
Mean	20.5066	9.6593	1.0078	0.2235	0.0933
Standard deviation	0.4048	0.2538	0.0372	0.0176	0.0088
Accuracy					
%Bias	2.5%	−3.4%	0.8%	−10.6%	−6.7%
Precision: 1-way ANOVA on log-transformed data					
%CV					
Intra-assay	2.0%	2.6%	3.8%	7.1%	8.5%
Inter-assay	Negligible	0.7%	Negligible	2.4%	4.7%
Overall	2.0%	2.6%	3.8%	7.5%	9.8%

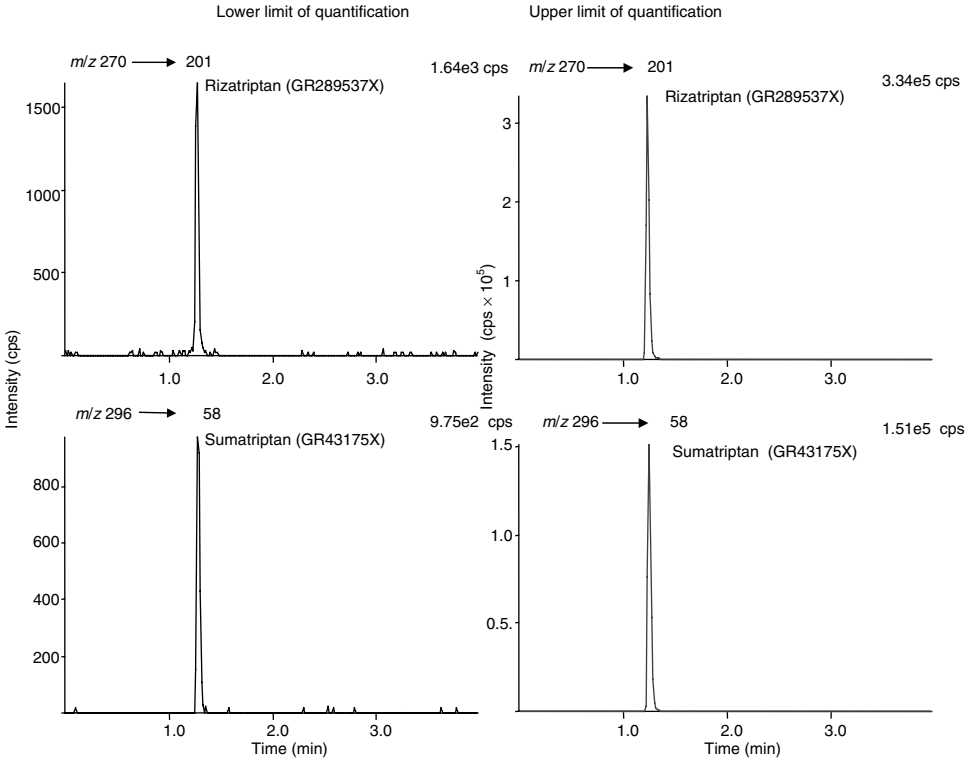

FIGURE 5.10 *Lower and upper limits for Sumatriptan and Rizatriptan assays.*

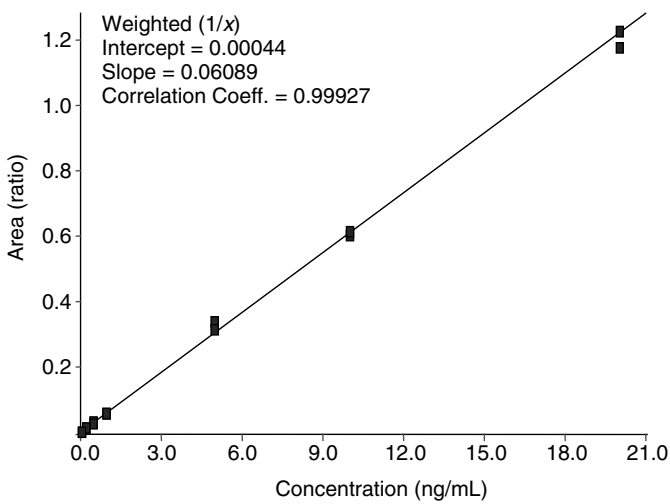

FIGURE 5.11 *Calibration line for Sumatriptan.*

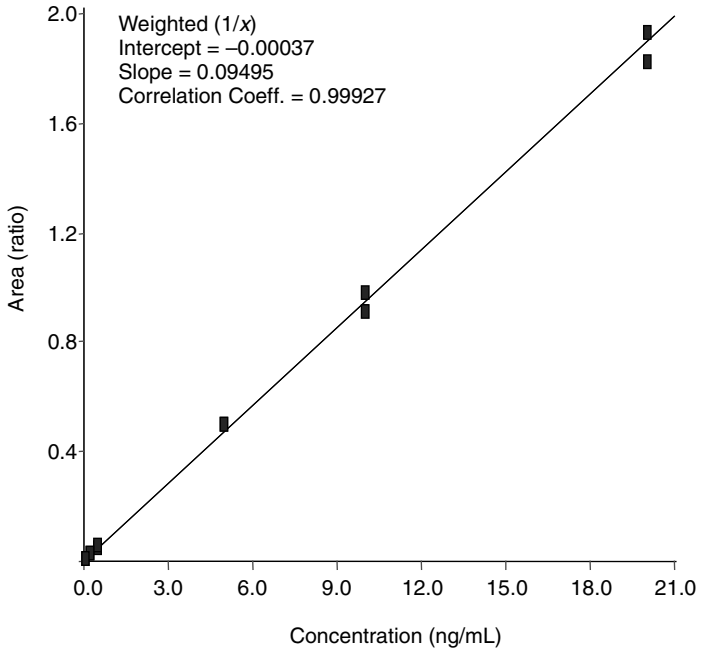

FIGURE 5.12 *Calibration line for Rizatriptan.*

TABLE 5.4 *Assay validation results for Rizatriptan assay*

	VC1	VC2	VC3	VC4	VC5
Target and actual VC concentrations for Rizatriptan					
Target VC	20	10	1	0.25	0.1
Number	24	24	24	23	22
Mean	19.570	9.781	1.068	0.222	0.103
Standard deviation	0.772	0.301	0.037	0.015	0.009
Accuracy					
%Bias	−2.1%	−2.2%	6.8%	−11.3%	2.7%
Precision: 1-way ANOVA on log-transformed data					
%CV					
Intra-assay	2.2%	1.7%	3.3%	6.6%	8.1%
Inter-assay	3.7%	2.9%	1.5%	Negligible	3.6%
Overall	4.3%	3.4%	3.6%	6.6%	8.9%

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