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Development of a high sensitivity bioanalytical method for alprazolam using ultra-performance liquid chromatography/tandem mass spectrometry

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A rapid, specific, assay was developed for the benzodiapine alprazolam in rat plasma using sub-2 μm particle liquid chromatography (LC) and tandem quadrupole mass spectrometry (MS/MS). The limit of quantification using protein precipitation was determined to 10 pg/mL, whereas the limit of quantification using solid-phase extraction (SPE) was determined to be 1.0 pg/mL. The assay was optimized for throughput and resolution of the analyte of interest from the hydroxy metabolite. During the method development process the plasma matrix signal was monitored, for lipids and other endogenous metabolites, to maximize signal response and minimize ion suppression. This was achieved by using a tandem quadrupole mass spectrometer equipped with a novel collision cell design which allowed for the simultaneous collection of full scan MS and multiple reaction monitoring (MRM) data. The lipid profile from the SPE process was significantly less than obtained with the protein precipitation approach.

Keywords: bioanalysis; alprazolam; UPLC; MS/MS; SPE

Introduction

Bioanalysis plays a critical role in providing data for both drug discovery and development. The data developed is used to support the proof of exposure in safety assessment studies and provides information to determine the effect of increasing dosage on the pharmacokinetic exposure of the systemic system to the drug under test. The data produced is also used to scale between animals and humans to determine dosage in human clinical trials and finally to provide supporting data for human clinical trials.[1–3] Over the last 15 years liquid chromatography/tandem mass spectrometry (LC/MS/MS) using multiple reaction monitoring (MRM) mode has become the technique of choice for bioanalysis; this is due to the specificity, sensitivity and selectivity of the MRM process.[4–7] This dominance of LC/MS/MS is due to the high resolving power of modern liquid chromatography, the ease of method development and the compatibility of the HPLC system with the sample solution,[8] facilitated by the development of the atmospheric pressure chemical ionization (APcI) source.[9] The use of short columns and high flow rates has become the most common mode of operation using isocratic methods.[10–14] Recently, the development of sub-2 μm particle LC has improved both the productivity and sensitivity of bioanalytical assays,[15,16] which is due to the elevated flow rates and increased chromatographic efficiency of the sub-2 μm particle materials.

During the development of a bioanalytical assay it is necessary to produce a sample preparation process, a mass spectrometry MRM method and chromatography separation that resolves the analyte(s) of interest from the exogenous drug metabolites formed *in vivo*, the endogenous metabolites and other compounds present in the matrix. To optimize these methods it is necessary to monitor and evaluate both the MRM signal for the analyte of interest and also the full scan, non-specific, data of the background endogenous signal. Of these endogenous analytes the phospholipids present in the plasma/serum samples have been widely reported as the major reason for ion suppression in bioanalytical assays. In the study published by Chambers *et al*.[17] the authors showed that by using solid-phase extraction (SPE) the background endogenous signals in LC/MS/MS bioanalysis could be significantly reduced. To illustrate some of the opportunities for increasing assay sensitivity and monitoring matrix effects in bioanalysis we show the development of an assay for the common benzodiazapine alprazolam.

Alprazolam is a short-acting benzodiapine used for treatment of moderate to severe anxiety disorders, panic attacks, and as an adjunctive treatment for anxiety associated with clinical depression. Alprazolam is readily absorbed from the gastrointestinal tract with the peak plasma concentration being achieved in 1–2 h. Most of the drug is bound to plasma protein, mainly serum albumin. Alprazolam is hydroxylated in the liver to α-hydroxyalprazolam, which is also pharmacologically active. Some of the drug is also excreted in its dosed form. The compound is typically dosed at 1–3 mg/day resulting in a C_{max} level of 8–37 ng/mL with a typical

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half-life of 12 h.[18,19] Thus to correctly define the pharmacokinetics of the compound a high sensitivity assay was required.

In this paper we show the development of a bioanalytical assay for alprazolam in rat plasma in the range of 0.5–500 pg/mL. The sensitivity of the assay with respect to the mobile phase pH and organic modifier nature was evaluated as was the cleanliness of the sample extract using SPE and protein precipitation. Finally, the sensitivity of the assay using protein precipitation, conventional SPE and micro-scale SPE was evaluated.

Experimental

Chemicals

Methanol, acetonitrile, formic acid, and ammonium hydroxide were obtained from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Rat plasma was purchased from Equitech-Bio (Kerrville, TX, USA) and stored frozen at 20 °C prior to use. Alprazolam, deuterated internal standard and the hydroxy metabolite were purchased from Cerilliant (Round Rock, TX, USA).

Sample preparation

Alprazolam was supplied dissolved in methanol as a 1 mg/mL solution. This solution was volumetrically diluted in methanol/water (1 : 1) solution to produce a dilution series. A calibration line was prepared by spiking alprazolam from the standard dilution series into control rat plasma over the range 0.1–10 000 pg/mL and quality control (QC) samples were prepared at concentrations of 25, 250, 625, 2500 and 6250 pg/mL. The plasma samples were extracted by loading 375 µL of plasma onto an Oasis HLB SPE plate (Waters, MA, USA) which had been previously prepared by washing with methanol and water. Samples were washed with 25% methanol and eluted with 1000 µL 40 : 60 isopropanol/acetonitrile. The sample was collected in a 2 mL microtitre plate and diluted with an equal volume of water prior to analysis. The samples were also prepared by µElution SPE, whereby 375 µL of plasma was added to a µElution OASIS HLB plate, the plate was washed as before and eluted with 50 µL of 40 : 60 isopropanol/acetonitrile followed by dilution to 100 µL with water. Finally, the samples were prepared by protein precipitation of 375 µL of plasma with 750 µL of acetonitrile.

In the matrix and sensitivity recovery experiment, 375 µL of rat plasma were processed in all modes of sample preparation. For protein precipitation 750 µL of acetonitrile were added to the sample, giving a final volume of 1125 µL, the 30 mg SPE analysis was eluted with 1.0 mL of organic solvent and the low sorbent bed process was eluted with 1000 µL of organic solvent. As the final volume of the sample varied from process to process, all the samples were diluted to 2000 µL with water prior to analysis by LC/MS. A 10 µL aliquot of each sample was injected onto the LC/MS system.

Liquid chromatography

The chromatographic separations were performed on an ACQUITY® Ultra-Performance LC® separations module (Waters Corporation, MA, USA). Chromatography was performed on a 2.1 × 50 mm ACQUITY BEH C8 column. The column was maintained at 45 °C and eluted under gradient conditions over 2.0 min at a flow rate of 0.6 mL/min. Mobile phase consisted of methanol or acetonitrile as the organic modifier and formic acid (0.1% v/v) or ammonium hydroxide (0.1% v/v).

Table 1. Optimal MRM transitions, cone and capillary voltages for Alprazolam, D5 internal standard and OH metabolite

Compound	Transition (m/z)	Cone (V)	Collision Energy (V)
Alprazolam	309.2 > 281.0	40	25
Alprazolam D5	314.2 > 286.1	40	25
Alprazolam OH	325.0 > 297.0	40	25

Mass spectrometry

Mass spectrometry was performed on a Waters Xevo™ TQ mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization (ESI) interface. Settings were as follows: capillary voltage, 1 kV; source temperature, 150 °C; desolvation temperature, 600 °C; desolvation gas flow rate, 1200 L/h; and cone gas flow, 40 L/h. The mass spectrometer was operated in MRM mode, MRM mode with precursor ion scan or MRM mode with full scan MS (dual scan MRM mode). Table 1 illustrates the transitions and conditions utilized for each of the compounds analyzed in this assay.

Validation protocol

The assay was validated using the procedures defined in the FDA Guidance for Industry Bioanalytical Method Validation. Two replicate accurate weighings of approximately 10 mg alprazolam were made and then diluted to 10 mL in methanol to give a 1 mg/mL solution. From this two separate dilution series were created in 50 : 50 methanol/water and these solutions were used to create a calibration line in plasma over the concentration range of 0.5–10 000 pg/mL and four QC samples covering the range of 0.5–10 000 pg/mL. A series of four validations runs were carried out on four separate occasions using two calibration lines and six replicates of each of the five QC samples. The matrix factor was calculated using the injection of extracted plasma blank, plasma blank spiked with authentic standard and authentic standard spiked in solvent. A freeze/thaw validation was not performed.

Results and Discussion

Mass spectrometry MRM

The development of a LC/MS/MS method requires the selection and optimization of the mass spectrometry and chromatography conditions. Alprazolam belongs to the benzodiazepine class of compounds having an elemental composition of $C_{17}H_{13}ClN_4$, giving a monoisotopic molecular mass of 308.0829. Alprazolam was found to give the best response in positive ion mode using ESI. The cone voltage, collision energy and most selective and sensitive fragment ion were optimized using both manual and automatic tuning. The best fragment transition in terms of specificity and sensitivity was determined to be $m/z = 309.2 > 281$ using a cone voltage of 40 V and a collision energy of 25 eV. The comparison of the MS/MS sensitivity of alprazolam at two different pH values (pH 3 and 10) showed a 5- to 10-fold increase in sensitivity with the pH 10 mobile phase compared with the one of pH 3 (Fig. 1). This may be viewed as an unusual finding as the general opinion is that solutions with low pH generally provide more protons and better ionization; in this case the opposite is observed. However, there is more than enough excess of protons provided by the ESI process within the source of the mass spectrometer; thus the

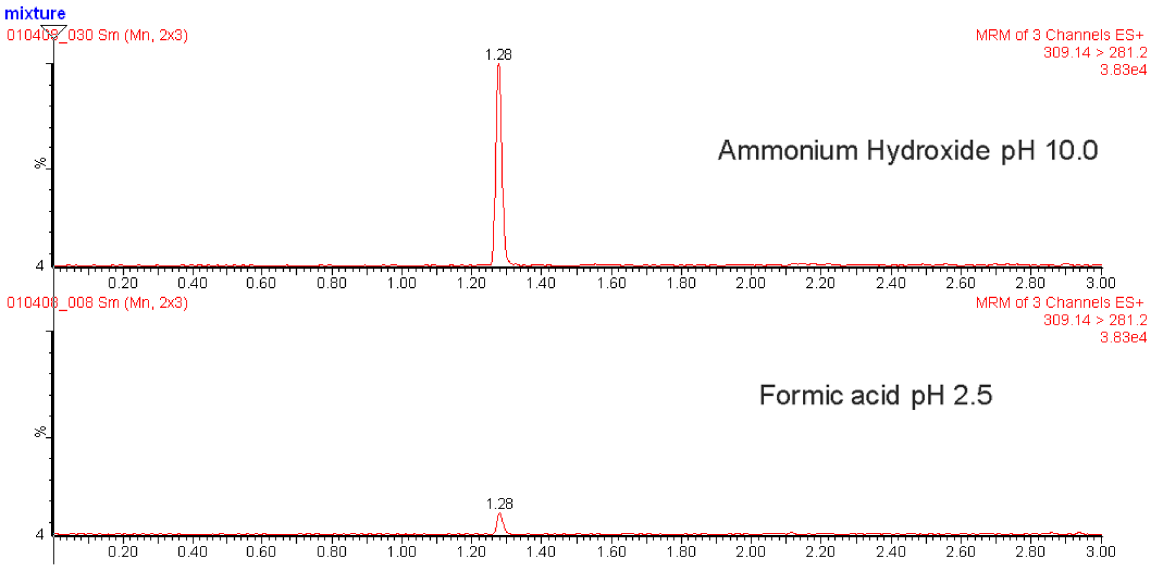

Figure 1. Comparison of MS peak response for alprazolam in basic (top) and acidic (bottom) mobile phases.

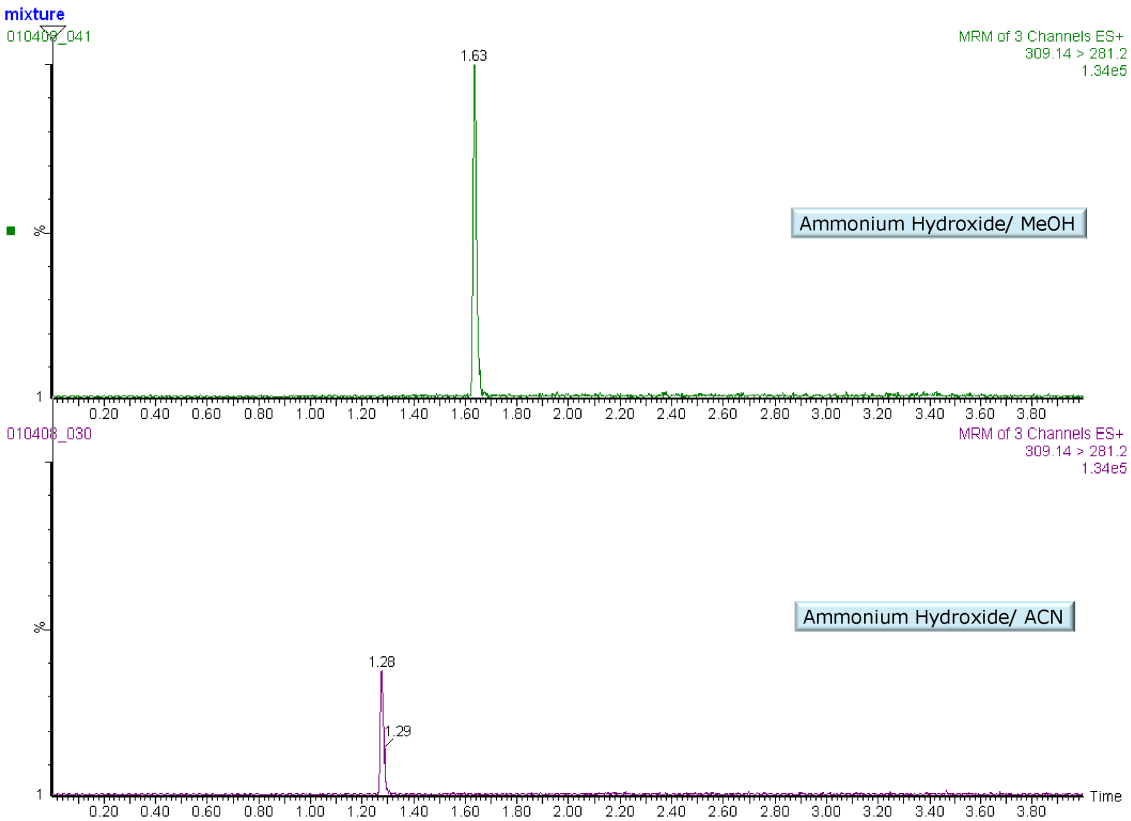

Figure 2. Comparison of MS peak response for alprazolam in basic - methanol (top) and basic - acetonitrile (bottom) mobile phases.

low concentration of proton ions in the mobile phase does not adversely effect the MS ionization process. This fact has also been observed for several other compounds, which did not contain amine groups, and will be the subject of further work to further explain this observation.

The initial chromatographic evaluation was performed using acetonitrile as the organic modifier; the effect of changing the organic modifier to methanol on the retention time and MS/MS detector response is displayed in Fig. 2. The data acquired showed that when using methanol the retention time of alprazolam was increased from 1.28 min to 1.63 min, and the signal response increased by a factor of 2.5. This increase in sensitivity can be attributed to three factors: (i) the superior desolvation properties of methanol in the mass spectrometer; (ii) the later elution of the peak in the methanol gradient causing it to be eluted in a greater percentage of organic solvent which also increases the desolvation efficiency of the electrospray process; and (iii) greater proton donor capacity of methanol.

Chromatography

The chromatographic conditions were developed using a reversed-phase chromatography system, using sub-2 µm particle LC. Previous work by many authors has illustrated the benefits of these sub-2 µm particle LC materials.[20–23] Most importantly, this extra resolution allows for greater confidence in resolution of the analyte of interest from the endogenous components in the sample and from the metabolites of the dosed candidate pharmaceutical.[24] The LC/MS method for aprazolam in rat plasma was developed to address the following major factors; resolution of the analyte from endogenous components in plasma, resolution from metabolites, sensitivity of the assay to lower than 50 pg/mL, and high throughput. Previous work had identified hydroxylation as a major route of metabolism for aprazolam, with hydroxyaprazolam being the major metabolite.[18,19] The retention of alprazolam and resolution from hydroxyalprazolam was evaluated on organo-silica hybrid C8, C18, and polar embedded C18 columns as well as a high-strength C18 silica column. The chromatography was performed using 2.1 × 50 mm columns, to facilitate short analysis times and hence rapid evaluation, and a generic 5–95% gradient over 2 min was utilized. The data generated showed that the silica-based C18 column gave the greatest retention of the compound and metabolite, whilst the C8 and silica columns gave the best resolution between the hydroxy metabolite and alprazolam itself. Therefore, the two best columns to progress with in the method development process were the organo-silica hybrid C8 and high-strength silica C18 material.

The acidity or alkalinity of the mobile phase will change the ionization state of the analytes, e.g. acids will be in their ionic form with high pH mobile phases whereas they will be neutral at low pH. This change in ionization state will directly effect the chromatography; in reversed-phase mode neutral compounds will be retained longer and generate less peak tailing. Thus operation at a pH where the analytes are in their neutral form is preferred from a chromatographic point of view, from an MS point of view it is more beneficial to have the analyte in a charged state as it will enhance the sensitivity. The effect of mobile phase pH was investigated to effect resolution and sensitivity. The resolution of the 5-hydroxy metabolite from the alprazolam metabolite was unaffected by the move from pH 3 to pH 10. Therefore, as the sensitivity was substantially greater with the high pH mobile phase, this was selected for use. This selection meant that the high-strength silica column could not be used as it is not chemically stable at this high pH. However, the chemical stability of the organo-silica hybrid C8 column allows this column to be operated at high pH, and thus this column was selected for this analysis.

Using protein precipitation the assay was shown to be linear over the range of 10–10 000 pg/mL. To demonstrate the usability and robustness of the methodology the assay was subject to partial validation over the concentration range of 10–10 000 pg/mL. The calculated concentration relative standard deviation (RSD) of six replicate analyses of the 25, 250, 625, 2500 and 6250 pg/mL QC samples is displayed in Table 2. Here we can see that the RSD for the 25 pg/mL sample is 3.01.

Table 2. Alprazolam QC samples nominal average and % RSD values

QC	Nominal Value (ng/mL)	Average	% RSD
QC 0.025	0.025	0.0230	3.01
QC 0.25	0.25	0.2460	5.29
QC 0.625	0.625	0.5961	2.09
QC 2.5	2.5	2.4414	2.46
QC 6.25	6.25	6.1139	2.00

Sample preparation

When developing a bioanalytical assay it is critical to resolve the analyte(s) from the endogenous material in the sample matrix. Chambers *et al.*[17] showed how the presence of phospholipids when co-eluting with an analyte compound causes matrix effects, reducing sensitivity and creating greater variability in the sample analysis. Therefore, during the LC method development process it is necessary to monitor and measure the background signal in the LC/MS trace. Phospholipids have previously been measured using the MRM transition m/z 184 > 184;[23] this analysis is specific for only the choline head group of the phospholipids, but provides no information on the mass ion of the lipid, nor does it provide any information on the non-phospholipid endogenous material. The lipid fraction and the endogenous small molecule interference were monitored simultaneously in the same analytical run using full scan MS and MS/MS data acquisition. This simultaneous data acquisition of MS and MS/MS data in the time frame of a narrow chromatographic peak (1–3 s wide at base) is normally not possible on a conventional tandem quadrupole mass spectrometer as these instruments require the collision cell to be emptied and refilled with collision gas when switching between MS and MS/MS analysis. This normally takes several hundreds of milliseconds, resulting in the acquisition of just a few points across a narrow, high-resolution, LC peak. The mass spectrometer used here is equipped with a novel collision cell design that allows for the rapid switching between MS and MS/MS mode as the collision cell is continuously filled with the collision gas. This functionality was investigated for the collection of MRM, MS and precursor ion scanning data in one analytical run to monitor the plasma matrix in the sample. The data displayed in Fig. 3 shows the data for simultaneous collection of MRM and precursor ion scanning MS for the product ion m/z 184 for an acetonitrile protein-precipitated rat plasma sample. The top chromatogram shows the lipid profile for the precursors of m/z 184 in positive ion mode. The middle chromatogram shows the MRM response for the D5 internal standard of alprazolam (m/z 314 > 286) and the bottom chromatogram shows the MRM response for alprazolam (m/z 309 > 281). In this data we can see that the alprazolam signals (standard and D5 internal standard) are clearly resolved from the lipids in the sample. The data in Fig. 4 shows the full scan MS and MRM data for the same protein-precipitated plasma sample. The upper chromatogram shows the full scan MS data for the plasma sample, the middle chromatogram illustrates the MRM chromatogram for the internal standard of alprazolam and the hydroxyl metabolite and the lower chromatogram shows the MRM profile for alprazolam. In this data we can see that there is significant co-elution of endogenous material when the alprazolam peak is eluting. This may cause significant ion suppression in the MRM analysis of the analyte.

During the course of a preclinical and clinical development process both the lipid profile and the non-lipid endogenous profile may vary significantly. Factors such as gender, polymorphic variation, age, diet, health, and co-administered medication will result in a variable plasma profile from subject to subject or species to species or even day to day. Therefore, to ensure that the assay will be robust and reliable it may be necessary to further improve the chromatography or sample preparation process. The

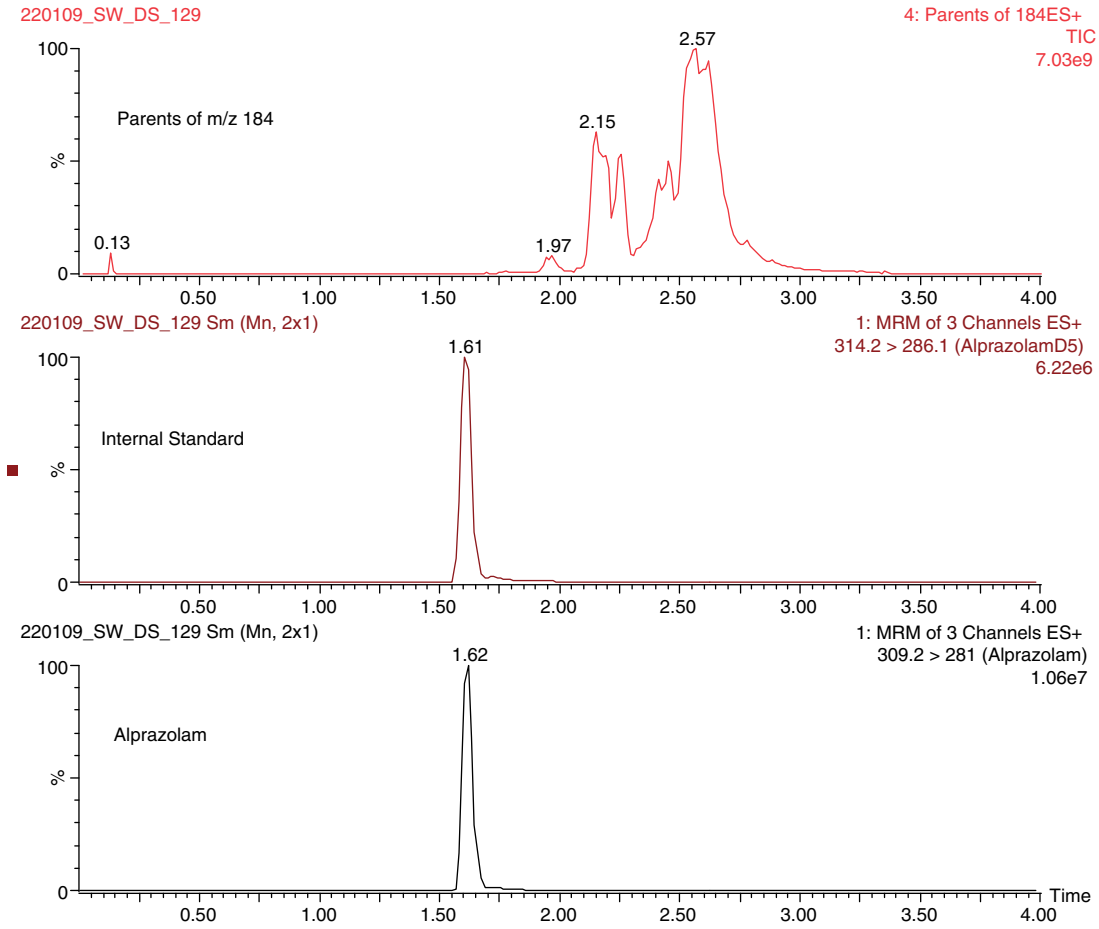

Figure 3. Positive ion LC/MS/MS chromatogram of alprazolam and D5-alprazolam internal standard in protein-precipitated plasma. Top chromatogram: precursor ion scan LC/MS chromatogram (precursor *m/z* 184); middle chromatogram: D5 internal standard MRM analysis (*m/z* 314 > 286); lower chromatogram: alprazolam MRM analysis (*m/z* 309 > 281).

two major options for improved sample cleanup are liquid-liquid extraction (LLE) and solid-phase extraction (SPE).

The rat plasma sample was subjected to SPE. The effect of the SPE elution concentration on the LC/MS profile of the lipid fraction was investigated, using the LC/MS/MS transition *m/z* 184 > 184. In this experiment various concentrations of organo-aqueous eluent were evaluated (Fig. 5). The data illustrates that as the organic concentration of the eluent is increased, more lipid material is washed from the column. Thus it would be ideal to use a lower concentration wash if possible as long as acceptable analyte recovery is achieved. The elution of analyte was also monitored using the same wash concentrations as used to evaluate the lipid fraction elution profile. The results showed that it was necessary to utilize 100% organic solvent to elute all of the alprazolam analyte and hence give a reproducible recovery. Thus it was not possible to use a lower concentration eluent to minimize the lipid fraction eluted in the SPE process. However, if the MS response for the signal from the precursors of *m/z* 184 from the SPE analysis (Fig. 5) is compared with that obtained from the signal from the protein precipitation analysis (Fig. 3) it can be seen that the SPE process gave a response of 8 e[7] whilst the protein precipitation process gave a response of 7 e,[9] almost a 100-fold improvement. Thus it is clear that the SPE process results in less lipid fraction in the sample and hence less chance for matrix effects.

The starting volumes and final volumes of the protein precipitation and SPE processes were different, making comparisons of the relative sensitivity and lipid profile difficult and subject to assumptions. To accurately compare the two approaches an experiment was performed where the volume of the starting plasma volume and final eluting sample from each process was normalized to the same fixed volumes. The LC/MS/MS-generated data is shown in Fig. 6. This data illustrates the lipid profile of samples prepared by protein-precipitated plasma, SPE and SPE using a low sorbent bed SPE plate (μElution plate). The data shown in Fig. 6 illustrates that SPE material resulted in the cleanest lipid LC/MS trace whilst the protein-precipitated fraction produced the LC/MS trace with the largest lipid response.

As the same amount of plasma and analyte were used in each extraction process it was possible to measure and compare the analyte signal for each of the processes. The data showed that the μElution plate gave the best signal response and protein precipitation the lowest signal response. Thus for this assay the best methodology is the low sample bed μElution plate. With the protein precipitation approach, described previously, the assay limit of quantification (LOQ) was 10 pg/mL (Fig. 7a) whilst with the SPE method the LOQ was determined to be 5 pg/mL (Fig. 7b).

Although the SPE process gave an improved signal response when compared to protein precipitation it was noted during the use of the assay that the SPE plasma extract contained a

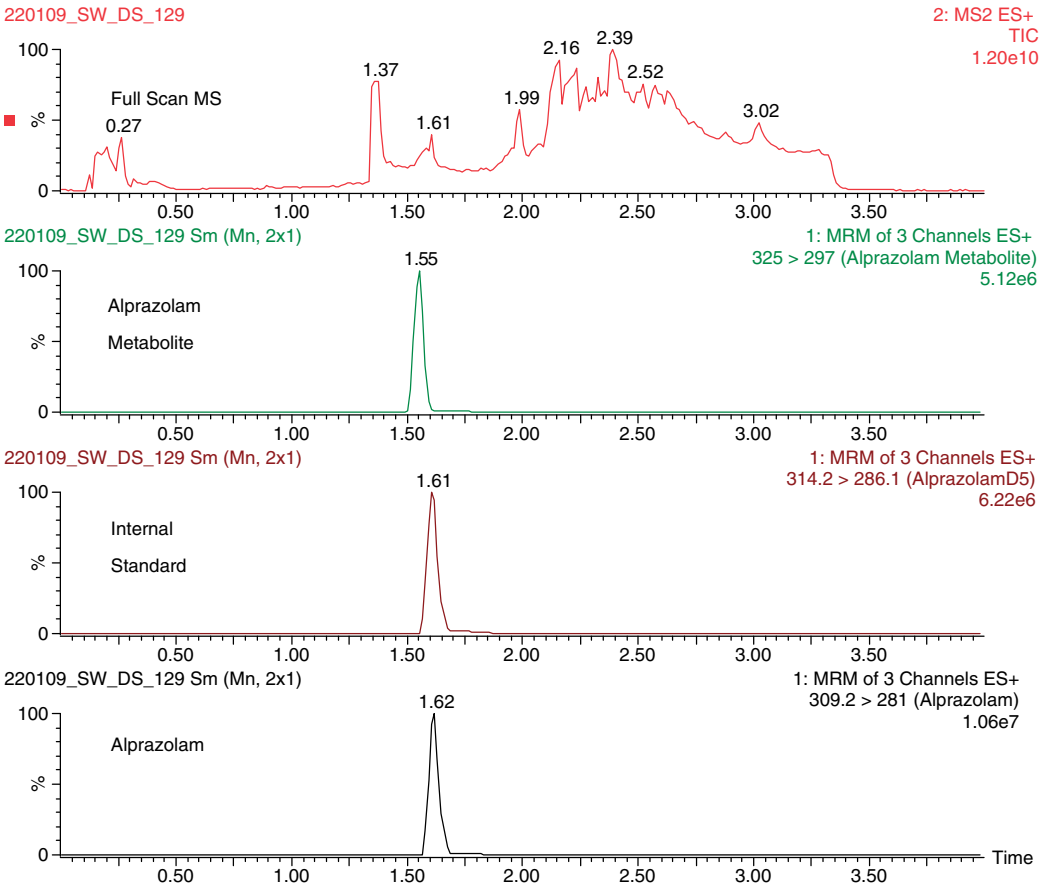

Figure 4. Positive ion LC/MS/MS chromatograms of alprazolam and D5-alprazolam internal standard in protein-precipitated plasma. Top chromatogram: full scan MS chromatogram; middle chromatogram: D5 internal standard MRM analysis (*m/z* 314 > 286); lower chromatogram: alprazolam MRM analysis (*m/z* 309 > 281).

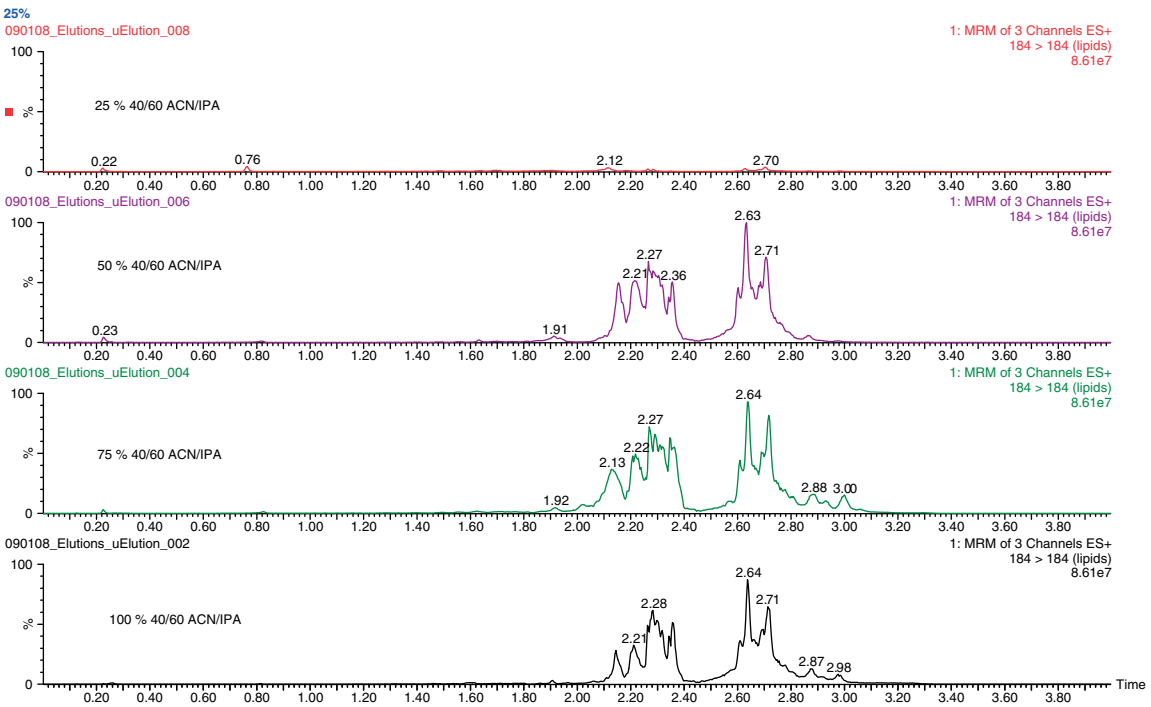

Figure 5. Comparison of lipid fraction LC/MS/MS chromatogram from SPE analysis of blank rat plasma MRM (184 > 184).

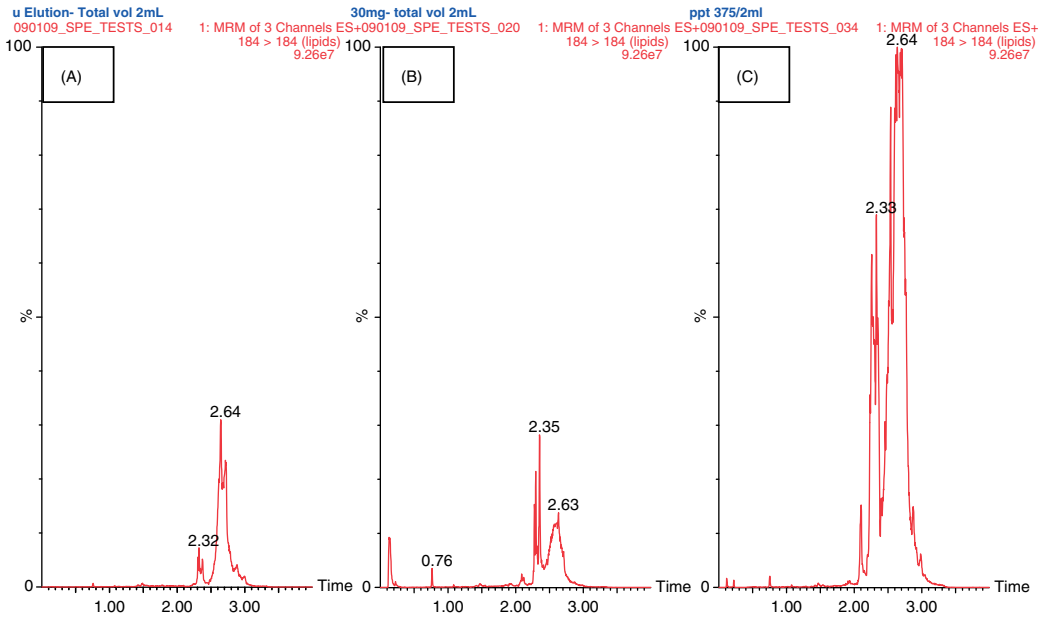

Figure 6. LC/MS/MS analysis, MRM 184 > 184, from rat plasma extract from A) 10 mg μElution SPE plate, B) 30 mg SPE plate, C) protein precipitation.

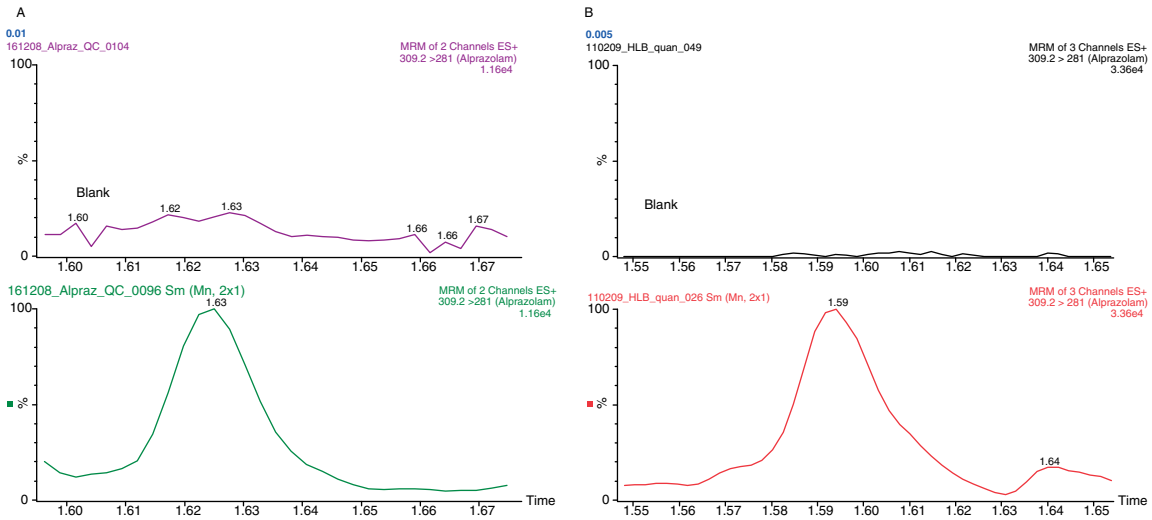

Figure 7. Limit of quantification (LOQ) for alprazolam in rat plasma extract A) 10 pg/mL using protein precipitation (upper chromatogram blank, lower chromatogram spike standard), B) 5 pg/mL using solid phase extraction (upper chromatogram blank, lower chromatogram spike standard).

co-eluting endogenous peak which deleteriously affected the limit of quantification (LOQ). This endogenous peak could not be resolved using the methanol/basic aqueous mobile phase on the C8 column, by either adjusting the column temperature or gradient slope. However, when methanol was replaced by acetonitrile as the organic modifier the endogenous peak was completely resolved and the LOQ was determined to be 1.0 pg/mL, with a signal-to-noise value 5 time greater than a blank. In this example we can see the overlay of the 1 pg/mL standard with the blank sample which represents the lower limit of quantification for the assay. The assay was linear over the range of 1.0 to 500 pg/mL. Thus on this occasion, despite the fact that neat standard methanol gave the superior response, the ability of acetonitrile to resolve the endogenous peak and analyte resulted in a lower limit of quantification. The matrix factor, calculated using the convolution approach,[25] was determined to be 1.01 with a coefficient of variance (CV) of 0.45 with internal standard normalization and 0.99 with a CV of 1.2 using the non-convolution approach.

Conclusion

Using sub-2 µm particle LC a rapid, simple specific assay was developed for alprazolam in rat plasma. The limit of quantification using protein precipitation was determined to be 10 pg/mL, whereas the lower limit of quantification using solid-phase extraction was determined to be 1 pg/mL. The variability of the assay using protein precipitation was determined to be less than 6% at the 10 pg/mL level. The lipid profile from the protein precipitation and SPE processes showed that significantly less, 100-fold, plasma signal was obtained with SPE compared to protein precipitation. A comparison of 30 mg SPE and reduced

sorbent bed SPE showed that the μElution SPE plate gave the best analyte response.

The use of a new novel tandem quadrupole mass spectrometer with a collision cell continuously filled with argon allowed MRM, full scan MS and precursor ion scanning to be used simultaneously. Using this multi-acquisition capability the plasma matrix signal was monitored and a SPE process developed to maximize signal response and minimize ion suppression.

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