JOURNAL OF MASS SPECTROMETRY, VOL. 32, 152–158 (1997)

Quantitative Ionspray Liquid Chromatographic/Tandem Mass Spectrometric Determination of Reserpine in Equine Plasma

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A method based on ionspray liquid chromatography/tandem mass spectrometry (LC/MS/MS) was developed for the determination of reserpine in equine plasma. A comparison was made of the isolation of reserpine from plasma by liquid–liquid extraction and by solid-phase extraction. A structural analog, rescinnamine, was used as the internal standard. The reconstituted extracts were analyzed by ionspray LC/MS/MS in the selected reaction monitoring (SRM) mode. The calibration graph for reserpine extracted from equine plasma obtained using liquid–liquid extraction was linear from 10 to 5000 pg ml^{-1} and that using solid-phase extraction from 100 to 5000 pg ml^{-1}. The lower level of quantitation (LLQ) using liquid–liquid and solid-phase extraction was 50 and 200 pg ml^{-1}, respectively. The lower level of detection for reserpine by LC/MS/MS was 10 pg ml^{-1}. The intra-assay accuracy did not exceed 13% for liquid–liquid and 12% for solid-phase extraction. The recoveries for the LLQ were 68% for liquid–liquid and 58% for solid-phase extraction. © **1997 by John Wiley & Sons, Ltd.**

J. Mass Spectrom. **32**, 152–158 (1997)
No. of Figures: 6 No. of Tables: 3 No. of Refs: 25

KEYWORDS: liquid chromatography/tandem mass spectrometry; reserpine; equine plasma

INTRODUCTION

Reserpine, an alkaloid isolated from the root of *Rauwolfia serpentiana*, is used for the treatment of hypertension and pyschoses in humans. Reserpine was one of the first effective drugs used on a large scale in the treatment of hypertension.[1] It is also used as a tranquilizer in horses. Small doses of narcotics, stimulants and other drugs have been used in attempts to alter the performance of racing dogs and horses.[2] Few data are available on the pharmacokinetic properties of reserpine, owing in part to the lack of assay capabilities for detecting low concentrations of the drug and its metabolites. For clinical analyses where a high volume of samples may need to be analyzed, rapid methods for sample preparation and analysis are needed.

A number of methods for the determination of reserpine in plasma have been reported, including thin-layer chromatography (TLC),[3,4] gas chromatography (GC)[5] and high-performance liquid chromatography (HPLC) with ultraviolet and fluorescence detection.[6–14] For instance, detection limits of 100 pg ml^{-1} (from 2 ml samples) in equine plasma[15] and 300 pg ml^{-1} (from 3 ml samples) in human plasma[9] have been reported using HPLC with fluorescence detection. Although GC/mass spectrometry (GC/MS) is the most widely accepted technique for the identification of drug metabolites, it has generally been unsuccessful for the determination of low-volatility and thermally labile compounds such as reserpine. This basic drug is an ideal candidate for modern atmospheric pressure ionization (API)[16] techniques such as ionspray[17] that do not require analyte volatility. Additionally, HPLC, the method of choice for the separation of polar, low-volatility drugs, is an ideal separation technique for these compounds when coupled with detection by ionspray mass spectrometry.[17,18]

Sample preparation is usually required to remove interfering matrix components and to trace enrich the target analytes prior to analysis. Trace quantities of target compounds must be extracted efficiently from complex sample matrices including plasma, urine and tissue samples to obtain accurate analytical results.[19,20] Traditionally, liquid–liquid extraction has been used for sample clean-up, but this method can be time consuming and tedious and it uses large quantities of solvent.[21] Solid-phase extraction (SPE) has replaced liquid–liquid procedures in many instances because it is fast, efficient and easily automated.[21,22] Also, SPE requires minimal quantities of solvent, and its principles are similar to liquid chromatography, which facilitates HPLC analyses. SPE is growing in popularity for these reasons and also because of the current availability of a wide variety of bonded silica sorbents.

Most current SPE sample preparation procedures are performed using SPE cartridges. The cartridges are comprised of a medical-grade plastic syringe barrel packed with porous particles that have an average diameter of 40 μm. These cartridges contain packed beds with high permeability; therefore, they are useful in handling biological matrices without clogging. However, these packed beds tend to channel randomly, thus reducing the efficiency of the adsorbent to capture analytes.[23]

SPE disks closely resemble membrane filters. They are flat, usually 1 mm or less in thickness, with diameters ranging from 4 to 96 mm. These characteristics

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CCC 1076–5174/97/020152–07 $17.50

Received 2 August 1996
Accepted 2 October 1996

allow higher flow rates, thereby increasing extraction speed while decreasing the solvent elution volume.[21,22] Some disks have filters that are supported above the extraction disk to prefilter samples containing particulates without clogging the extraction disk. With the extraction disk configuration, the channeling observed with packed cartridges may be eliminated.[24]

This paper is concerned with rapid, high-throughput analyses coupled with good sample preparation using LC with MS/MS as the final clean-up and separation step. In this study, liquid–liquid and solid-phase extraction techniques were compared for the efficient isolation of reserpine from equine plasma. This work is intended to provide a means of assisting with the forensic determination of reserpine at ultra-trace levels (10 pg/ml to 5 ng/ml) in equine plasma. LC/MS/MS results from both sample preparation techniques are presented and compared with respect to recovery, accuracy and detection limits.

EXPERIMENTAL

Reagents and materials

Reserpine (methyl reserpate 3,4,5-trimethoxybenzoic acid ester) and rescinnamine (methyl reserpate 3,4,5-trimethoxycinnamic acid ester) (internal standard) were obtained from Sigma Chemical (St Louis, MO, USA). Heptane (99%, HPLC grade) used in the liquid–liquid extraction was obtained from Sigma–Aldrich (St Louis, MO and Milwaukee, WI, USA). Other solvents such as 3-methylbutan-1-ol and *tert*-butyl methyl ether, were obtained from Aldrich Chemical (Milwaukee, WI, USA). HPLC mobile phases and solid-phase extraction reagents, which included methanol, glacial acetic acid, 85% phosphoric acid and ethyl acetate were obtained from Fisher Scientific (Rochester, NY, USA). Ammonia solution was obtained from Sigma Chemical. All equine plasma was kindly supplied by Dr E. Dubovi (Diagnostic Laboratory, New York State College of Veterinary Medicine at Cornell University, Ithaca, NY, USA).

Sample preparation

Two extraction techniques were used. First, a three-step liquid–liquid extraction procedure[9] was used to isolate reserpine and rescinnamine from 2 ml of fortified equine plasma. The eluent was concentrated to dryness in a SpeedVac SVC100 (Savant Instruments, Farmingdale, NY, USA) and reconstituted in 10 µl of 50% acetonitrile–50% 5 mM NH_4OAc in water (pH 7.12). All pH measurements were taken before the acetonitrile was added. This extraction technique provides clean extracts with acceptable recovery (60%) at low pg ml^{-1} levels, e.g. 10 pg ml^{-1}. However, this method is tedious and time consuming.

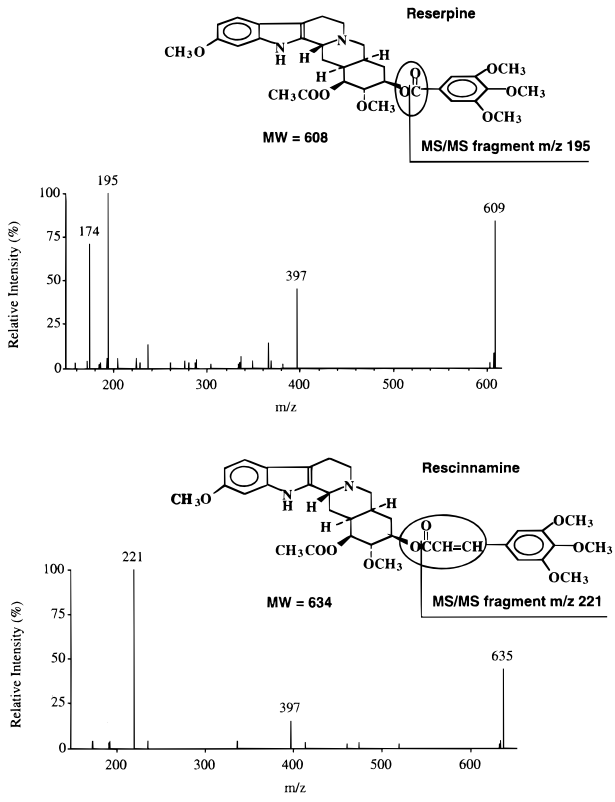

Figure 1. Infusion CID product ion mass spectra for reserpine and rescinnamine. Acquisition parameters: step size, 0.2 u; collision energy, 55 eV; collision gas thickness, 380×10^{12} atoms cm^{-2}. Infusion of 80:20 acetonitrile–water solution of reserpine and rescinnamine (400 pg μl^{-1}) at 4 µl min^{-1}.

An alternative sample preparation procedure, solid-phase extraction, was developed using Ansys (Irvine, CA, USA) 3 ml MP1 disks. Sample pretreatment for this method included adding 1 ml of 0.1 M phosphate (KH_2PO_4) buffer (pH 4.0, adjusted with 85% phosphoric acid) to 1 ml of equine plasma and the specified amount of analyte and internal standard. The extraction procedure included conditioning the disks in sequence with 200 µl of methanol and phosphate buffer, loading the sample, washing in sequence with 550 µl each of 1.0 M acetic acid and methanol, and eluting with two portions (500 and 200 µl) of 2% ammonia solution in ethyl acetate. This method was automated with a Gilson (Middleton, WI, USA) ASPEC (automated solid phase extraction system with extraction columns) 233 XL system. The eluate was concentrated to dryness in a SpeedVac SVC100 and reconstituted in 10 µl of 50% acetonitrile–50% 5 mM NH_4OAc in water (pH 5.51, adjusted with glacial acetic acid). These samples were then transferred to low-volume autosampler inserts for analysis, 5 µl from 10 µl being injected on-column.

The determination of the recovery of reserpine from equine plasma was carried out by spiking (post-extracted) control equine plasma extracts. After extraction, the analyte and internal standard were added to the plasma extracts and the reconstituted extracts were assayed by LC/MS/MS. The area ratios obtained were compared with those of the corresponding pre-extraction spike of equine plasma to give a measure of the percentage recovery (Table 1).

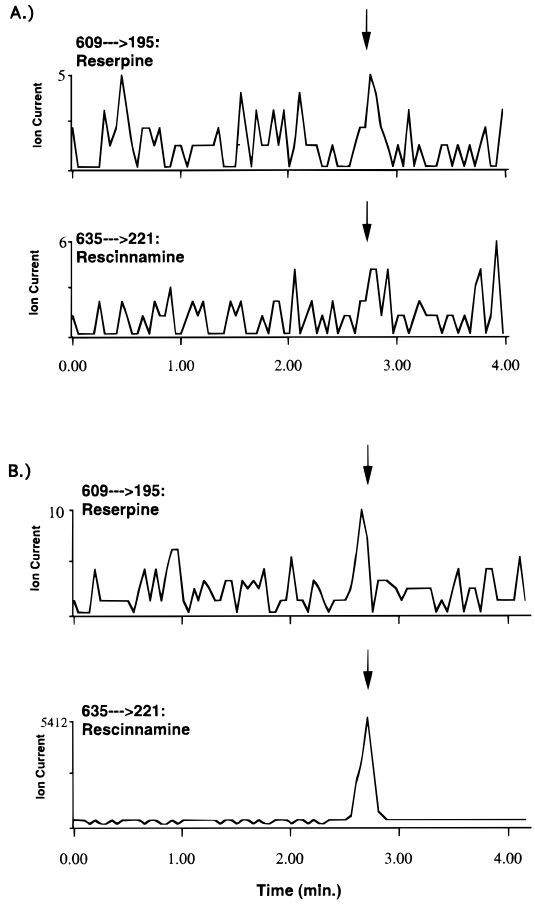

Figure 2. Liquid–liquid extraction. SRM-LC/MS ion current profile of (A) double blank extract of 2 ml of control equine plasma (no analyte or internal standard) and (B) blank extract of 2 ml of control equine plasma (internal standard but no analyte).

Chromatography

Analyses were performed with the following HPLC systems: a Waters Model 616 solvent-delivery system (Milford, MA, USA) combined with a Rheodyne (Cotati, CA, USA) Model 7725i injector equipped with a 20 μl external loop and an Applied Biosystems (Foster City, CA, USA) Model 140A solvent-delivery system combined with a Waters Model 717 autosampler equipped with a 250 μl loop and a 25 μl syringe. The column was a Betasil C_{18} (100 mm × 1 mm i.d.) packed with 5 μm particles (Keystone Scientific, Bellefonte, PA, USA). The HPLC eluent for the liquid–liquid extraction study was maintained under isocratic conditions with a flow rate of 50 μl min^{-1} using 80% acetonitrile–20% 5 mM NH_4OAc in water (pH 7.12), while 80% acetonitrile–20% 5 mM NH_4OAc in water (pH 5.47, adjusted with glacial acetic acid) was used for the SPE study. The injection volume in these experiments was 5 μl, 50% of the reconstitution volume.

Table 1. Recoveries for the determination of reserpine in plasma by LC/MS/MS[a]

	Recovery (%)	
Reserpine (pg ml^{-1})	Determined by liquid–liquid extraction	Determined by solid-phase extraction
10	76.3	—[b]
50	68.0	—[b]
100	—[b]	58.2
300	—[b]	52.6
500	67.6	57.3
2000	69.3	43.8
5000	70.2	not reported

[a] Data are averages of two measurements.
[b] Concentrations not used in specified extraction procedure.

Mass spectrometry

A Sciex (Thornhill, Ontario, Canada) TAGA 6000E atmospheric pressure ionization (API) triple-quadrupole mass spectrometer upgraded to an API-III was used. Nitrogen was used as the nebulizing gas and was maintained at 50 psi for the ionspray LC/MS interface. Polypropylene glycol (PPG, average $M_r = 425$) in 66:34 CH_3CN–H_2O (3 mM NH_4OAc) was used for tuning and mass-axis calibration for each mass-resolving quadrupole (Q1 and Q3). Both mass analyzers were operated under unit mass resolution conditions. The declustering energy for both MS and MS/MS experiments was set at 30 eV. Ultrapure argon was used as the collision gas in the collision cell (Q2). For MS/MS experiments, the mass spectrometer was programmed to focus the protonated molecule ions $[M + H]^+$ at m/z 609 and 635 for reserpine and the internal standard, respectively, via the first quadrupole mass filter (Q1) with collision-induced fragmentation in Q2 and monitoring of the product ions at m/z 195 (reserpine) and 221 (internal standard) in Q3 using the selected reaction monitoring (SRM) mode. The collision gas thickness was maintained at (350–380) × 10^{12} atoms cm^{-2}, which produced a collision energy of 55–59 eV. The dwell time for each monitored transition was 1000 ms. Data were collected with PE-Sciex RAD (routine acquisition and display) software. Peak area ratios obtained from SRM of the analyte (m/z 609 → 195) and the internal standard (m/z 635 → 221) were computed using Sciex's MacQuan software from the corresponding chromatographic peak areas.

RESULTS AND DISCUSSION

The product ion mass spectra obtained for reserpine and the internal standard, rescinnamine, are shown in Fig. 1. No product ions were observed below m/z 174. The transitions used in SRM for the quantitation of the analyte resulted form cleavage at the carbon–oxygen bond alpha to the carbonyl group. Analytical standards of reserpine were analyzed by selected reaction monitoring LC/MS/MS (SRM-LC/MS). The m/z 609 → 195 transition was used to monitor for reserpine, which

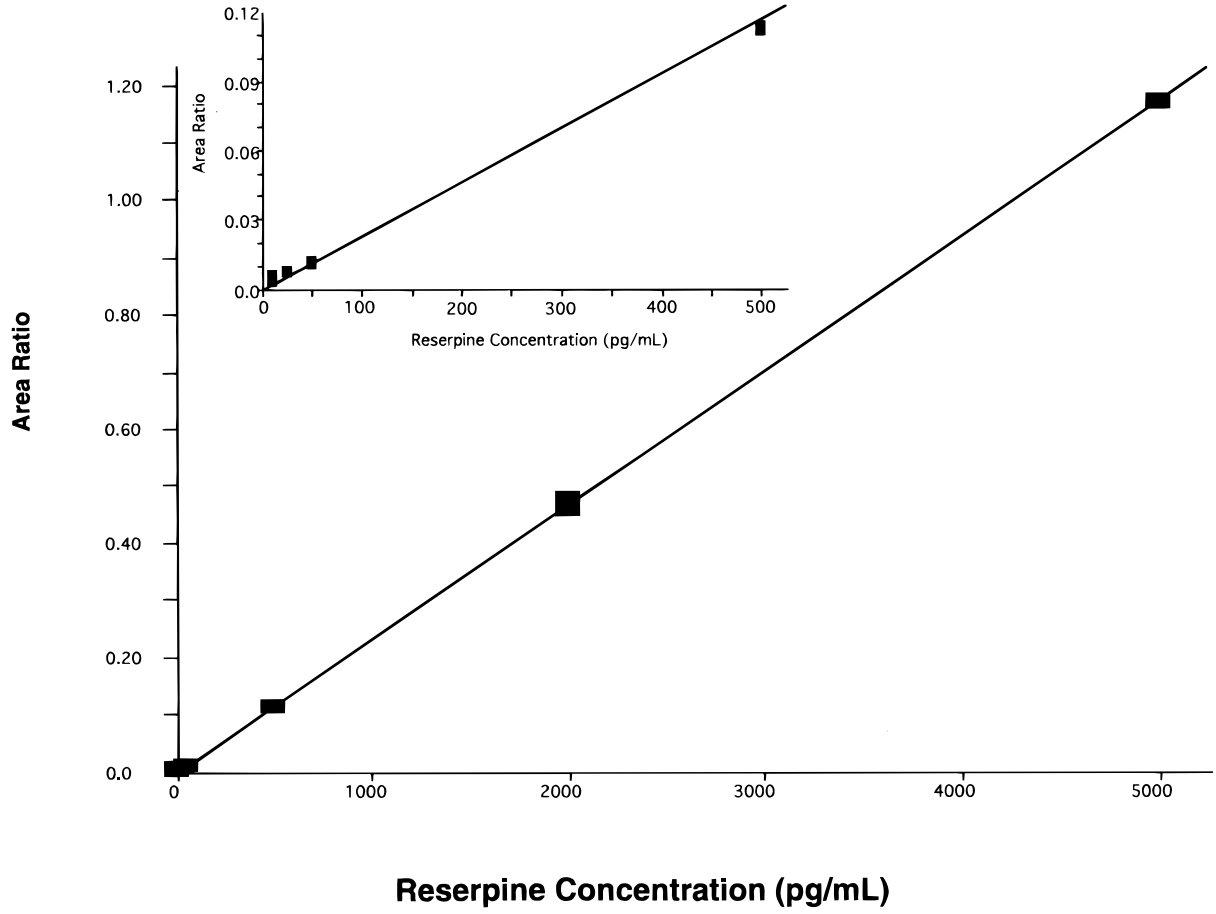

Figure 3. Liquid–liquid extraction: calibration graph for reserpine in equine plasma fortified from 10 to 5000 pg ml^{-1}. Inset: calibration graph from 10 to 500 pg ml^{-1}.

elutes at 2.74 min and the m/z 635 → 221 transition was monitored for the internal standard, rescinnamine, which eluted at the same retention time as reserpine under the HPLC conditions used in this work.

Liquid–liquid extraction

The SRM-LC/MS ion current profiles for the double blank and blank of equine plasma samples are shown in Fig. 2. The trace shown in Fig. 2(A) was obtained from a control blank equine plasma and that in Fig. 2(B) from an equine plasma sample spiked with rescinnamine at 1800 pg ml^{-1}. Figure 2(A) and (B) illustrate that the extracts of plasma containing no analyte showed peak area ratios that displayed essentially no interference from endogenous plasma components.

Calibration standards were prepared in duplicate by adding aliquots of reserpine and the internal standard to 2 ml of control equine plasma. The concentrations of the standard for the calibration graph were 10, 50, 500, 2000 and 5000 pg ml^{-1} (Fig. 3). The concentration of the internal standard in each sample was 1800 pg ml^{-1}. The intra-assay accuracy [(mean observed concentration/theoretical concentration) × 100] results represent the accuracy observed within an analysis. Accuracy was assessed from the results of both calibration standards (10, 50, 500, 2000 and 5000 pg ml^{-1}) and quality control samples at 20, 200 and 3000 pg ml^{-1}. Duplicates of each calibration standard and quality control level were used in the intra-assay analysis. Acceptable accuracy was observed over the range 50–5000 pg ml^{-1} with a correlation coefficient of 0.999 (Table 2). The accuracy at 10 pg ml^{-1}, the lowest concentration analyzed for the calibration

Table 2. Liquid–liquid extraction method-intra-accuracy results

Reserpine concentration in equine plasma (pg ml^{-1})	Accuracy % DEV
10	104.8
20[a]	57.8
50	3.7
200[a]	5.4
500	3.5
2000	2.0
3000[a]	12.3
5000	0.2

[a] Quality control samples.

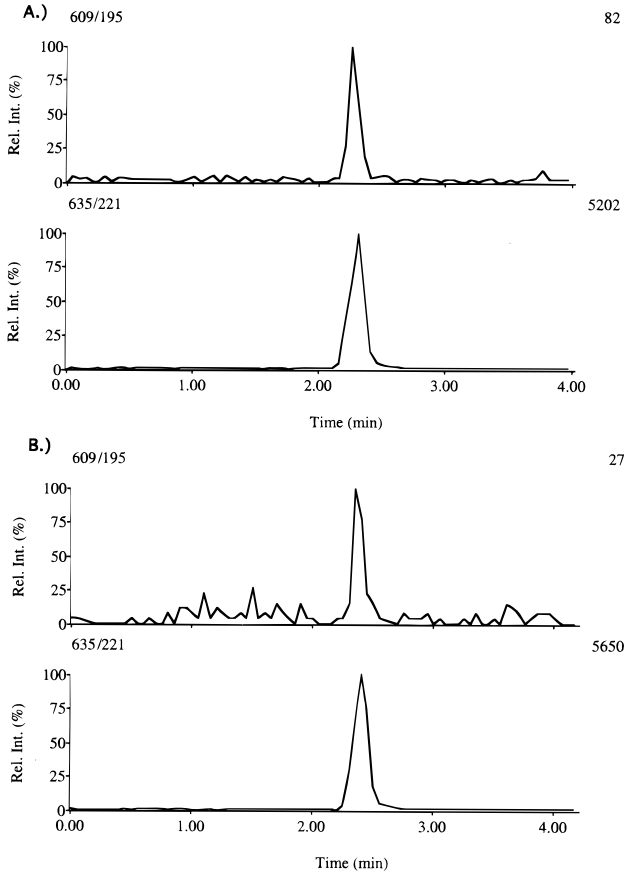

Figure 4. Liquid–liquid extraction: (A) lower level of quantitation, 50 pg ml^{-1} or reserpine and 1800 pg ml^{-1} of rescinnamine; (B) limit of detection, 10 pg ml^{-1} of reserpine and 1800 pg ml^{-1} of rescinnamine.

graph, exceeded the value of 20% used for defining the lower level of quantitation (LLQ) (Table 2).[25] The LLQ was defined as the lowest concentration on the calibration graph for which an acceptable accuracy of 100 ± 20% was obtained.[25] Therefore, this assay has an LLQ of 50 pg ml^{-1} [shown in Fig. 4(A)] based on 2 ml aliquots of equine plasma. The relative standard deviation (RSD) at the LLQ concentration was 5.2. The limit of detection (LOD) for this extraction technique shown in Fig. 4(B) was 10 pg ml^{-1}. The ratio of peak (signal) intensity to noise intensity (S/N) for liquid–liquid extraction is 3:1 at 10 pg ml^{-1}. The quantitation calculations were computed with Sciex MacQuan software.

Solid-phase extraction

In the SPE experiments, reserpine was extracted from 1 ml of equine plasma *vs.* 2 ml in the liquid–liquid extraction method discussed above. Since this method was automated with the Gilson ASPEC (see Experimental section for details) and the chromatography was rapid, high volumes of samples could be analyzed.

Calibration standards were prepared in duplicate by adding reserpine and the internal standard, rescinnamine, to 1 ml of phosphate buffer (used to adjust the solution to a pH of 4) in 1 ml of control equine plasma. Figure 5 shows the calibration graph for the concentrations of the standards at 100, 300, 500, 2000 and 5000 pg ml^{-1}, where the concentration of the internal standard for the standard graph is 1800 pg ml^{-1}

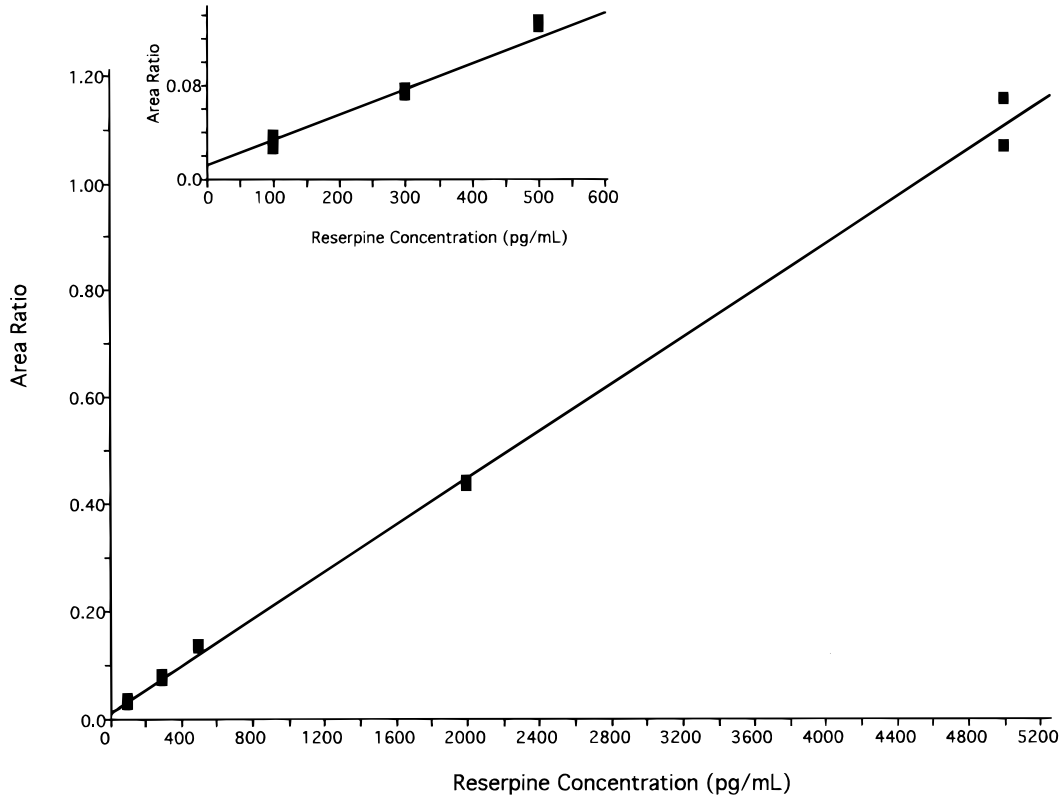

Figure 5. Solid-phase extraction: calibration graph for reserpine in equine plasma fortified from 100 to 5000 pg ml^{-1}. Inset: calibration graph from 100 to 500 pg ml^{-1}.

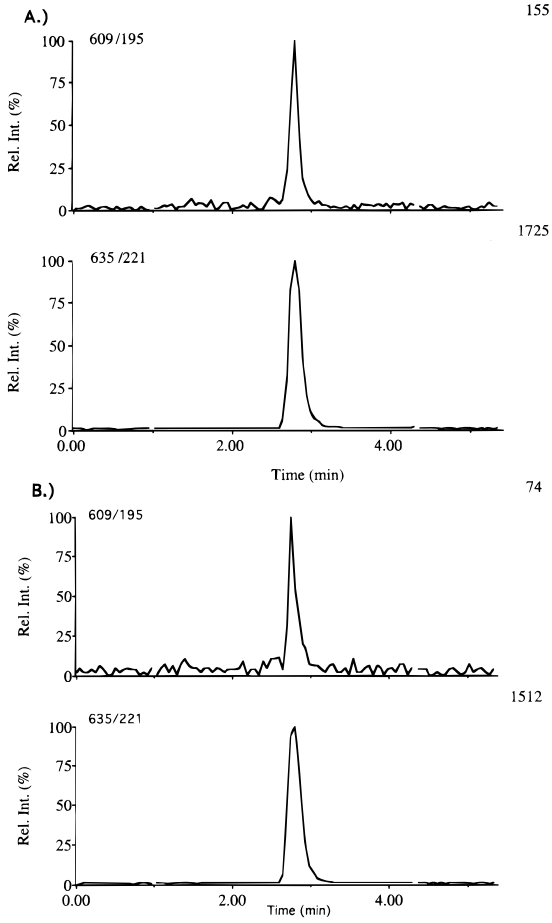

Figure 6. Solid-phase extraction: (A) lower level of quantitation, 200 pg ml^{-1} of reserpine and 1800 pg ml^{-1} of rescinnamine; (B) limit of detection, 100 pg ml^{-1} of reserpine and 1800 pg ml^{-1} of rescinnamine.

(correlation coefficient = 0.998). Accuracy was determined as in the liquid–liquid LC/MS/MS results by the calibration standards and quality control samples. The quality control concentrations were 200, 1000 and 4000 pg ml^{-1}. Acceptable accuracy was observed over the range 200–5000 pg ml^{-1} (Table 3). Therefore, the LLQ of reserpine in equine plasma for this method was defined as 200 pg ml^{-1}, shown in Fig. 6(A), and the LOD of 100 pg ml^{-1} is shown in Fig. 6(B). The S/N is 7:1 for SPE at 100 pg ml^{-1}.

Table 1 shows that the recoveries for liquid–liquid extraction were higher than those for SPE. Also, it shows that the recovery was extremely low for SPE at high concentration levels. It is suggested that this resulted from the order in which the samples were prepared (low to high concentration) on the ASPEC system. It was observed that when the last samples were prepared, the transfer line of the ASPEC was very cloudy, indicating that perhaps some of the plasma had undergone precipitation, even though a 1:1 mixture of methanol and water instead of 100% methanol was used as the needle wash solvent to prevent this type of problem.

Table 3. Solid-phase extraction: intra-accuracy results

Reserpine concentration in equine plasma (pg ml^{-1})	Accuracy % DEV
100	19.0
200[a]	8.5
300	3.2
500	11.7
1000[a]	11.1
2000	3.1
4000[a]	7.6
5000	4.1

[a] Quality control samples.

CONCLUSIONS

Liquid–liquid extraction provided the best lower level of quantitation of the two extraction techniques for reserpine. It takes ~2 days to prepare 60 plasma samples via liquid–liquid extraction whereas the same 60 samples can be prepared easily overnight using the Gilson robotic approach. Thus higher volume sample analyses may be significantly improved by the latter method, albeit with higher LOQs. Although this method is more tedious and time consuming, it provides very clean extracts for LC/MS/MS analysis. This method, excluding the centrifugation, can be automated, thus reducing errors that could occur in a multiple-step procedure.

The SPE procedure for the SRM-LC/MS quantitation of reserpine afforded a relatively simple method. No time-consuming derivatization steps were required. The use of Ansys disks reduced the time required for SPE, and the automation of this method provided a rapid, high-throughput analysis for LC/MS/MS. Information on precision and reproducibility for this method are not available from the results presented. The method was not fully validated pursuant to full validation guidelines, so multiple replicates (e.g. six replicates at each quality control level) were not available to determine precision, etc.

HPLC coupled with ionspray mass spectrometry offers enhanced capabilities for the detection of reserpine in biological samples. This combination of analytical techniques provides an invaluable bioanalytical tool because of its high specificity, including chromatographic retention times and mass spectrometric characteristics of the reserpine and rescinnamine. This analytical approach could provide improved detection and quantitation capabilities for incurred equine plasma samples.

Acknowledgements

Financial support for this project was provided by Monsanto Corporate Research, St Louis, MO, USA. We acknowledge Gilson for the loan of the ASPEC XL and PE-Sciex for continued financial support. Also, thanks are due to Dr Dennis Blevins of Ansys for his technical support with the SPEC disk technology. This work was presented in part at the 43rd Conference on Mass Spectrometry and Allied Topics, Atlanta, GA, May 1995.

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