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Quantitative liquid chromatographic–tandem mass spectrometric determination of reserpine in FVB/N mouse plasma using a ''chelating'' agent (disodium EDTA) for releasing protein-bound analytes during 96-well liquid–liquid extraction

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Abstract

A sensitive, specific, accurate and reproducible analytical method employing a divalent cation chelating agent (disodium EDTA) for sample treatment was developed to quantitate reserpine in FVB/N mouse plasma. Samples pretreated with 40 μl of 2% disodium EDTA in water were extracted by a semi-automated 96-well liquid–liquid extraction (LLE) procedure to isolate reserpine and a structural analog internal standard (I.S.), rescinnamine, from mouse plasma. The extracts were analyzed by turbo ionspray liquid chromatography–tandem mass spectrometry (LC–MS–MS) in the positive ion mode. Sample preparation time for conventional LLE was dramatically reduced by the semi-automated 96-well LLE approach. The assay demonstrated a lower limit of quantitation of 0.02 ng/ml using 0.1-ml plasma sample aliquots. The calibration curves were linear from 0.02 to 10 ng/ml for reserpine. The intra- and inter-assay precision of quality control (QC) samples ranged from 1.75 to 10.9% for reserpine. The intra- and inter-assay accuracy of QC samples ranged from −8.17 to 8.61%. Reserpine and the I.S. were found to be highly bound to FVB/N mouse plasma protein. This is the first report of disodium EDTA employed as a special protein-bound release agent to recover protein-bound analytes from plasma. These matrix effects and the effects of pH in the HPLC mobile phase on the sensitivities of LC–MS–MS are discussed in this paper.

Keywords: 96-Well liquid–liquid extraction; Reserpine; Disodium EDTA; Protein-denaturing reagent

1. Introduction

Reserpine (Fig. 1) is extensively used for the treatment of hypertension in humans [1]. The determination of reserpine in plasma has been difficult due to the lack of specific and sensitive analytical methods [2,3]. Suckow et al. developed an improved liquid chromatographic method coupled with fluorescence detection [4]. The method employed liquid–liquid extraction (LLE) of reserpine from plasma with 1.5% isoamyl alcohol in *n*-heptane and 1.0 ml carbonate buffer. The organic layer was back-extracted with 0.1 *M* HCl. The aqueous layer was then alkalinized and extracted with methyl-*tert*.-butyl ether (MTBE). The extracts were oxidized to their

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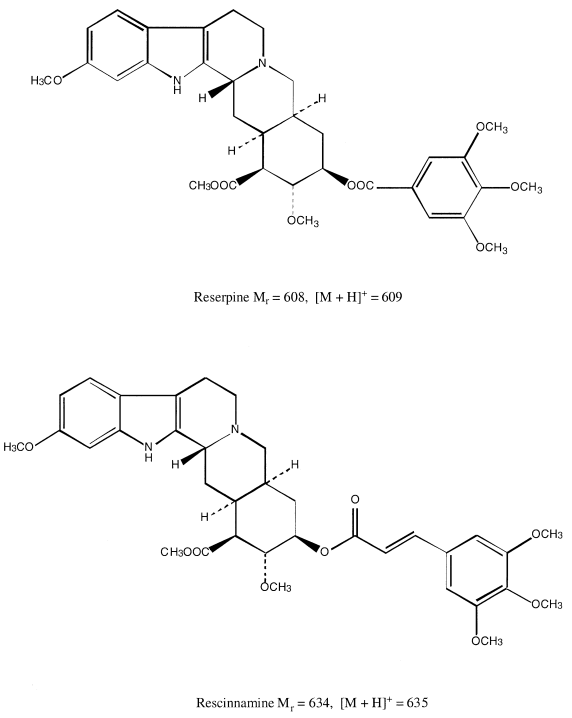

Fig. 1. Chemical structures of reserpine and rescinnamine.

respective fluorophors for fluorescence detection. The achievable lower limit of quantitation (LLQ) was 0.3 ng/ml using 3 ml of human plasma [4]. Anderson et al. adapted the three-step LLE sample preparation procedure to determine reserpine in equine plasma using ionspray liquid chromatography–tandem mass spectrometry (LC–MS–MS) [5]. The LC–MS–MS analysis was sensitive and specific. The LLQ was 0.05 ng/ml using 2 ml of equine plasma. However, the LLE sample preparation was extensive and time consuming. It required approximately 2 days to prepare 60 plasma samples by the LLE procedure. A solid-phase extraction (SPE) procedure was reported for the LC–MS–MS quantitation of reserpine afforded a relatively simple method, but it was offset by the loss of sensitivity in which the achievable LLQ was only 0.2 ng/ml because of adsorptive losses of this ''sticky'' compound and required a relatively large volume of plasma (1 ml) [5].

An LLQ of 0.02 ng/ml using a 0.1-ml mouse plasma aliquot was required to quantitate reserpine in our current study. A factorial design was used to compare multiple experimental treatments with the three-step LLE procedure previously used for equine plasma. The result was a single-step LLE procedure for mouse plasma. This improved LLE procedure was then transferred to the 96-well plate format since this format has proven to yield high throughput by reducing sample preparation time [6–9]. The 96-well procedure requires less than 3 h to prepare 96 plasma samples.

The pH of the LC mobile phase was identified as a critical parameter for optimizing the sensitivity for LC–MS–MS determination of this compound. According to the commonly accepted theory for optimal electrospray ionization, a pH below the pK_a should increase protonation of reserpine and hence the electrospray sensitivity for the target analyte under the positive ion mode electrospray conditions. It has been reported that the pH of the LC mobile phase should be adjusted to two pH units below the pK_a of analytes for optimal response for electrospray [10–12]. The results of optimizing the pH of the LC mobile phase in the current study are counter to these theories.

A structural analog (rescinnamine, Fig. 1) was used as the internal standard (I.S.) for the described analysis. Rescinnamine and reserpine were found to be bound to the protein of FVB/N mouse plasma used in this study. The use of a divalent cation chelating agent, ethylene diaminetetraacetic acid disodium salt (disodium EDTA), for sample treatment in this study was guided by the following considerations. First, conventional protein-denaturing reagents used in sample preparation resulted in a relatively poor LC–MS–MS responses for rescinnamine and reserpine after LLE of treated plasma standards. Second, many of the major serum proteins have an affinity for metal ions, particularly divalent cations. Third, there is growing evidence which suggests the binding of divalent cations to the metal-free form (apoprotein) of metalloproteins or removal of divalent cations from metal-bound metalloproteins induces a change of conformation that involves the altered tertiary structure with the partially unfolded state of the molecule [13–15]. The most striking finding reported here is that disodium EDTA was found to be more effective than the commonly used protein-denaturing reagents to release the bound analytes from protein in the FVB/N mouse plasma

for the quantitative determination of reserpine by LC–MS–MS. We report the use of disodium EDTA as an effective protein-denaturing reagent to release bound small molecules. To the best of our knowledge this phenomenon has not been reported previously. The significant impact upon the trace determination of reserpine in mouse plasma is described in this report.

2. Experimental

2.1. Reagents

Reserpine and rescinnamine were obtained from Sigma (St. Louis, MO, USA). Disodium EDTA, trichloroacetic acid (TCA) and polypropylene glycol (PPG) were obtained from Aldrich (Milwaukee, WI, USA). Ammonium hydroxide, formic acid and MTBE were obtained from EM Science (Gibbstown, NJ, USA). HPLC-grade methanol and acetonitrile were obtained from Burdick & Jackson (Muskegon, MI, USA). Ammonium acetate, glacial acetic acid, trifluoroacetic acid (TFA) and sodium carbonate were obtained from J.T. Baker (Phillipsburg, NJ, USA). Control mouse plasma (anticoagulant: 0.225% dipotassium EDTA in plasma) was obtained from Lampire Biological Labs. (Piperville, PA, USA). FVB/N mouse plasma samples (anticoagulant: 0.18% dipotassium EDTA in plasma) were provided by Pharmacia & Upjohn (Kalamazoo, MI, USA).

2.2. Supplies and equipment

The Personal Pipettor (96-Channel, PP-550) and EVAPOREXTM 96 Channel Sample Concentrator were obtained from Apricot Designs (Monrovia, CA, USA). 96-Well, deep well blocks (1.1-ml) and cap mats were obtained from Matrix Technology (Hudson, NH, USA). A Sorvall RT-6000D refrigerated centrifuge was obtained from DuPont (Wilmington, DE, USA). The polypropylene 96-well collection blocks were obtained from Beckman Instruments (Fullerton, CA, USA). Scotch sealing tapes used for sealing the 96-well blocks when placed on the autosampler were obtained from 3M (St. Paul, MN, USA).

The LC–MS–MS system consisted of a LC-10AD pump and a SCL-10A pump controller (Shimadzu, Columbia, MD, USA), an HTS PAL autosampler (LEAP Technologies, Carrboro, NC, USA), and a Betasil C_{18} (100×2 mm, 5 μm) column (Keystone Scientific, Bellefonte, PA, USA). Three quadrupole mass spectrometer systems were used which included a PE Sciex API III^{Plus}, an API 365 and an API 3000 (PE Sciex, Concord, Canada). The validation and initial sample analyses were carried out on an API III^{Plus}. In subsequent experiments reserpine and the I.S. were found to bind to FVB/N mouse plasma which resulted in reduced recoveries. To achieve improved detection limits, an API 3000 which is well-known for the best sensitivity performance, was used for the later stages of these studies.

LC elution employed linear gradients with a mobile phase A: 5 m*M* ammonium acetate, pH 7.6, and B: acetonitrile [t=0 min, A–B (35:65); t=1.0 min, A–B (10:90); t=2.6 min, A–B (0:100); t=3.8, A–B (0:100); t=4.0, A–B (35:65) followed by equilibration for 2 min]. The flow-rate was maintained at 0.3 ml/min through the 2 mm I.D. HPLC column with the total effluent directed to the turbo ionspray LC–MS interface.

Mass spectrometer conditions for the API III^{Plus} were as follows: curtain gas and nebulizer gas, ultra high purity (UHP) nitrogen; collision gas, UHP Argon; TurboIonSpray temperature, 550°C; TurboIonSpray auxiliary gas, UHP nitrogen at 8 l/min; ion spray voltage, 4300 V; declustering potential, 50 V; collision energy, 35 eV. Mass spectrometer conditions for the API 3000 were: curtain gas, nebulizer gas, and collision gas, UHP nitrogen; TurboIonSpray temperature, 500°C; declustering potential, 50 V; collision energy, 55 eV; TurboIonSpray auxiliary gas, UHP nitrogen at 8 l/min. The mass axis of the instrument was calibrated by infusion of PPG 425 solution (pursuant to the PE Sciex tuning guidelines) at a flow-rate of 10 μl/min. The mass axis was calibrated at m/z 59.0, 251.2, 326.3, 384.3, 558.4 and 674.5. The response of the instrument was optimized by infusing a 100 ng/ml solution of reserpine and the I.S. at 10 μl/min into a flow of 190 μl/min of mobile phase using A–B (10:90). Ion peak widths were maintained at approximately 0.6 u at half-height in both the single MS and MS–MS modes in both quadrupole mass analyzers. The

following selected reaction monitoring (SRM) transitions of the respective $[M+H]^+$ ions were used to quantify the analytes in mouse plasma: reserpine m/z 609.3→m/z 195.1 and rescinnamine m/z 635.3→m/z 221.1. Calculated concentrations are based on peak area ratios of reserpine relative to the I.S. using PE Sciex software MacQuan (v. 1.4).

2.3. Optimization of pH in LC mobile phase

A standard stock solution of reserpine (10 ng/ml in acetonitrile–water, 50:50) was diluted to 0.05 ng/ml and 0.1 ng/ml with acetonitrile–5 m*M* ammonium acetate (pH 8.0, adjusted with 10% ammonium hydroxide in water) (50:50). The solutions were analyzed by LC–MS–MS with A–B (10:90). Mobile phase A (5 m*M* ammonium acetate in water) was adjusted to four different pH levels (7.1, 7.6, 8.0 and 8.6, respectively, adjusted with 10% ammonium hydroxide in water) to select the optimal pH for maximal sensitivities under LC–MS–MS conditions.

2.4. Sample preparation with 96-well LLE

Control mouse plasma samples were extracted by a semi-automated 96-well LLE procedure to isolate reserpine and the I.S. from 0.1-ml plasma samples. Standards (0.02, 0.04, 0.1, 0.4, 2, 5, 7.5 and 10 ng/ml) and blanks were analyzed in duplicate. Quality control (QC) samples at concentrations of 0.06 ng/ml (QC1), 2 ng/ml (QC2) and 8 ng/ml (QC3) of reserpine were freshly prepared for each run due to adsorptive losses at the low QC if these samples were stored in polypropylene vials at −70°C for more than 3 days. This phenomenon has not been completely characterized, but the preparation of fresh QC samples and standards was deemed necessary. If the clinical study samples were stored under these same conditions, the analytical results for study samples with low concentrations might be biased because of the stability problem at low concentrations under these storage conditions. Each QC level was analyzed in replicates of six. Aliquots of 100 μl of each sample, standard, QC1 through QC3, and control blank were pipetted into wells of a 1.1-ml polypropylene, 96-deep well block using a 200-μl Pipetman pipette. Aliquots of 50 μl of I.S. dissolved in 50% acetonitrile in water were added to each well using an Eppendorf Repeating Pipette, with the exception of the control blank samples. For the control blank samples, aliquots of 50 μl of acetonitrile–water (50:50) were added. Aliquots of 20 μl ammonium acetate [1 *M*, pH 9.5, adjusted with concentrated ammonium hydroxide (28–30% NH_3 in water)] were added to each well. The block was covered with Scotch sealing tape, and vortexed for 20 s on a Baxter vortexer at low speed. The sample block was placed on the 96 Channel Personal Pipettor, and 0.5 ml MTBE was added to each well by the 96 Channel Personal Pipettor. The sample block was carefully sealed with a cap mat, vortex-mixed for 10 min and then centrifuged at 3000 rpm for 10 min. The block was placed into a dry ice–acetone bath (the acetone should reach approximately 1/3 of the height of the block) to freeze the lower, aqueous layer. The organic layer was transferred to a clean 96-well collection block by the 96 Channel Personal Pipettor and was evaporated to dryness at approximately 45°C with an EVAPOREX 96 Channel Sample Concentrator. The dried extracts were reconstituted in 150 μl of acetonitrile–10 m*M* ammonium acetate (pH 7.6, adjusted with 10% ammonium hydroxide in water) (67:33). The collection block was covered with Scotch sealing tape, vortex-mixed for 1 min at moderate speed and centrifuged at 3000 rpm for 10 min. The samples were injected for SRM LC–MS analysis using an HTS PAL autosampler.

The extraction recovery of reserpine from mouse plasma was determined by comparing the peak area ratio of reserpine relative to the I.S. peak area in the samples spiked with 0.06, 2 and 8 ng/ml of reserpine. Peak areas for post-extraction spiked samples were compared with samples spiked pre-extraction for recovery studies. The I.S. was spiked post-extraction for all recovery studied samples. The extraction recovery (% recovery) was determined by dividing the pre-extract peak area ratio (reserpine: I.S.) by the post-extract peak area ratio and expressing the result as a percentage. Six replicates were measured at each concentration level to determine extraction recovery.

2.5. Disodium EDTA as a "protein-denaturing" reagent

During method validation, disodium EDTA was not added to the QCs and standards for sample extraction. The extraction procedure was later modified for analysis of the preclinical study samples due to apparent significant losses of reserpine as well as the I.S. bound to proteins in the FVB/N mouse plasma samples from the preclinical study of Pharmacia & Upjohn. Disodium EDTA was found to be an effective ''protein-denaturing'' reagent to release the analytes from proteins or matrix constituents in the FVB/N mouse plasma. An aliquot of 40 μl of 2% disodium EDTA in water was added to each well. The block was covered with Scotch sealing tape, and mixed on a vortex mixer for 20 s. Subsequently, instead of 20 μl ammonium acetate (1 *M*, pH 9.5), 200 μl of sodium carbonate (0.6 *M*, pH 9.5, adjusted with concentrated glacial acetic acid) was added to each well and MTBE was added with the 96 Channel Personal Pipettor. Other commonly employed denaturing reagents including 40 μl of 2% formic acid in water, 2% acetic acid in water, 2% TCA in water and 2% TFA in water, and 200 μl of acetonitrile were compared in the same manner as 40 μl of 2% disodium EDTA in water.

3. Results and discussion

3.1. Optimal pH of the LC mobile phase

The pH of the LC mobile phase reported previously for the determination of reserpine in equine plasma was 7.12 [5]. When the pH of the mobile phase was changed to 8.0 in this work, the sensitivity increased by approximately 140% (Fig. 2). Among the pH levels tested, the optimal pH was 7.6, which resulted in approximately a four-fold increase in sensitivity compared to the response obtained at pH 7.12.

The effects of ammonium acetate concentration and the pH in the reconstitution solution of sample extracts were also investigated in this study, but the results were not conclusive and are not reported here. Different solvents (e.g., methanol vs. acetonitrile) and other buffers in the mobile phase were not compared in this study since satisfactory chromato-

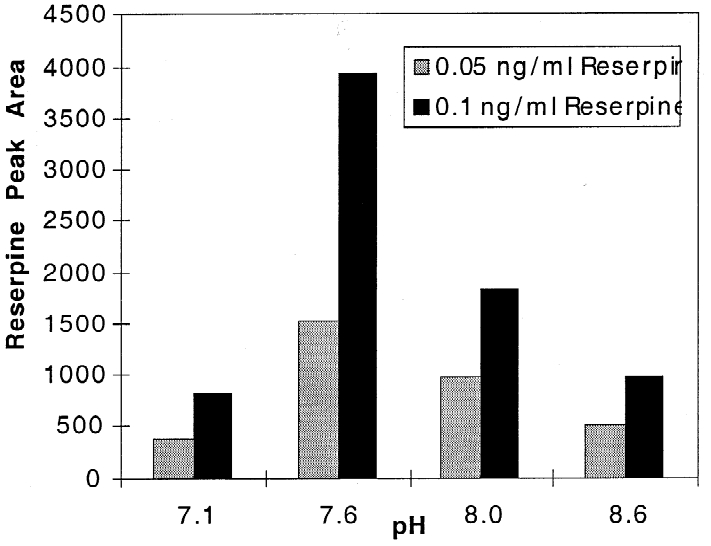

Fig. 2. Effects of pH in LC mobile phase on the analyte response for reserpine via turbo ionspray LC–MS–MS (expressed as peak area of reserpine).

grams and sensitivities had been achieved without further experiments.

Reserpine is a weakly basic compound and its reported pK_a is 7.4 [16]. Theoretically, the maximal sensitivity of electrospray ionization in the positive ion mode could be achieved when the pH of the LC mobile phase is adjusted to two units below pK_a of the analytes [10,11]. But in these experiments, the pH of the LC mobile phase was maintained slightly above the pK_a for reserpine and the SRM LC–MS detection limits improved. The highest sensitivity achieved was with pH 7.6, which is close to the pK_a of reserpine (Fig. 2). These results are somewhat counter to the conventional theory of electrospray ionization [10–12]. Mansoori et al. described observation of intense $[M+H]^+$ ions electrosprayed from strongly basic solutions and of $[M-H]^-$ ions from strongly acidic solutions as ''wrong-way-round'' electrospray ionization (ESI) [12]. The mechanism for ''wrong-way-round'' ESI is still unclear. Wholesale electrochemical acidification [12] of basic solutions is not supported by pH measurements on collected spray, and in any case the electrochemical currents are far too small. Furthermore, their work showed the insensitivity of ESI abundance of analyte ions to pH of the prespray bulk solution [12]. Temesi and Law reported some controversial results for effects of electrolyte concentrations on the responses of some tested compounds [17]. The effects of buffers were very compound dependent. The reason for their results was unclear, although it may be related to changes in the ionization status of the analytes as the eluent pH changed [17]. It is apparent that all factors influencing analyte sensitivities are not well understood even though electrospray ionization has been extensively used to-date. We speculate that pH of the sample solution or LC mobile phase is not the single factor influencing analyte responses in LC–MS experiments. The sensitivity of electrospray LC–MS technologies is influenced by the combined effects of all buffers, additives, solvents and matrix effects in the LC–MS system. This speculation is supported by the results in another study in our laboratory in which the optimal pH of the LC mobile phase for optimal response was also close to the pK_a of an acidic analyte detected in the negative ion mode with turbo ionspray (unpublished results). The effects of pH on the sensitivity of reserpine in positive ion electrospray warrant further investigation and may shed some light onto our general understanding of the ionization processes. In conclusion, however, it is clear from the studies described in this report that the electrospray detection limits for both reserpine and its I.S. are improved by maintaining the mobile phase at pH 7.6 in this analysis (Fig. 2).

3.2. 96-Well LLE

The calibration curves were fit by a weighted $(1/x^2)$ linear regression. Coefficients of determination (R^2) were $\geq$0.9942 for reserpine in mouse plasma. The assay was highly specific for reserpine and rescinnamine. No chromatographic interferences were observed in any of the control plasma samples analyzed (Figs. 3 and 4). The plasma LLQ experiment demonstrated that 0.02 ng/ml is an achievable LLQ for reserpine. The precision (RSD) was 12.5% for reserpine at this level. The mean percent deviation (% Dev) from the nominal value was 7.09% (Table 1).

Table 2 summarizes the accuracy and precision data for the reserpine QC samples. The intra- and inter-assay accuracy (expressed as % Dev, % deviation from nominal) ranged from −8.17 to 8.61% for reserpine for QC1, QC2 and QC3 concentration levels. The intra- and inter-assay precision data (expressed as RSD, relative standard deviation) ranged from 1.75 to 10.9% for reserpine at QC1, QC2 and QC3. Table 3 presents the accuracy and precision data for reserpine standards in mouse plasma. The accuracy ranged from −3.52 to 6.37% for standards 1–8. The precision data ranged from 2.21 to 8.91% for standards 1–8. These data indicate acceptable inter- and intra-assay accuracy and precision for the determination of reserpine in mouse plasma.

3.3. Disodium EDTA as a ''protein-denaturing'' reagent

During method development and validation, different lots of control mouse plasma with dipotassium EDTA as anticoagulant were obtained from Lampire Biological Labs. used to prepare standards and QC samples. Reserpine and the I.S. were not found to

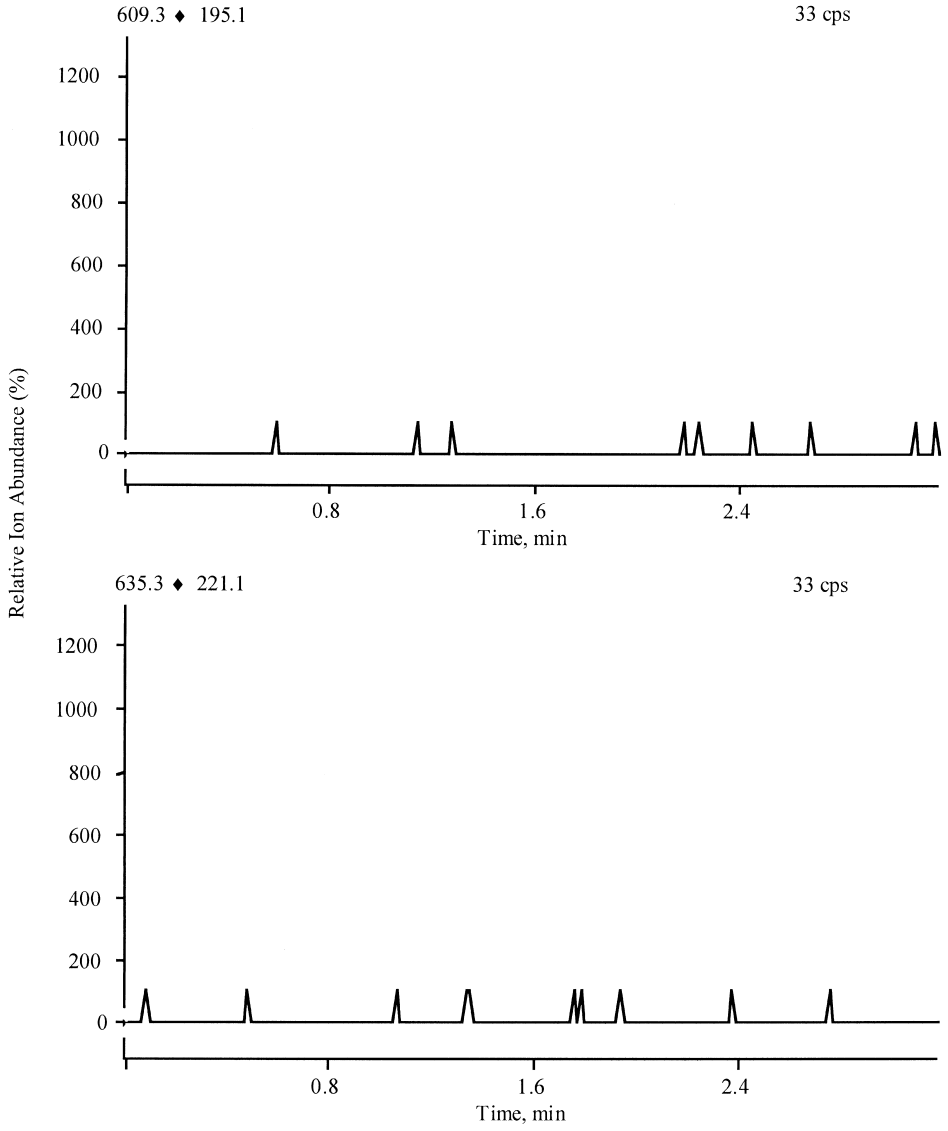

Fig. 3. SRM LC–MS chromatograms of reserpine (upper panel) and its I.S. (lower panel) in control blank mouse plasma extract analyzed by an API III^{Plus}. Reserpine m/z 609.3→m/z 195.1, retention time 2.55 min. I.S. m/z 635.3→m/z 221.1, retention time 2.55. Chromatogram normalized to the same scale as in Fig. 4.

bind significantly to proteins in the different lots of control mouse plasma. However, the behavior of reserpine and the I.S. fortified into the FVB/N mouse plasma samples (dipotassium EDTA as anti-coagulant) from the preclinical study of Pharmacia & Upjohn behaved very differently. In 30% of the preclinical study samples, the I.S. (50 μl of 5 ng/ml added to each sample) was not detected, and in about 20% of the preclinical study samples, the response of the I.S. was only 10–20% of those in control plasma. The results of these experiments suggested that protein-binding of the analytes in the FVB/N mouse plasma of the preclinical study samples was a major cause of the reduced recoveries. The reasons why protein-binding only occurred in some of the pre-clinical study samples, but not in the control mouse

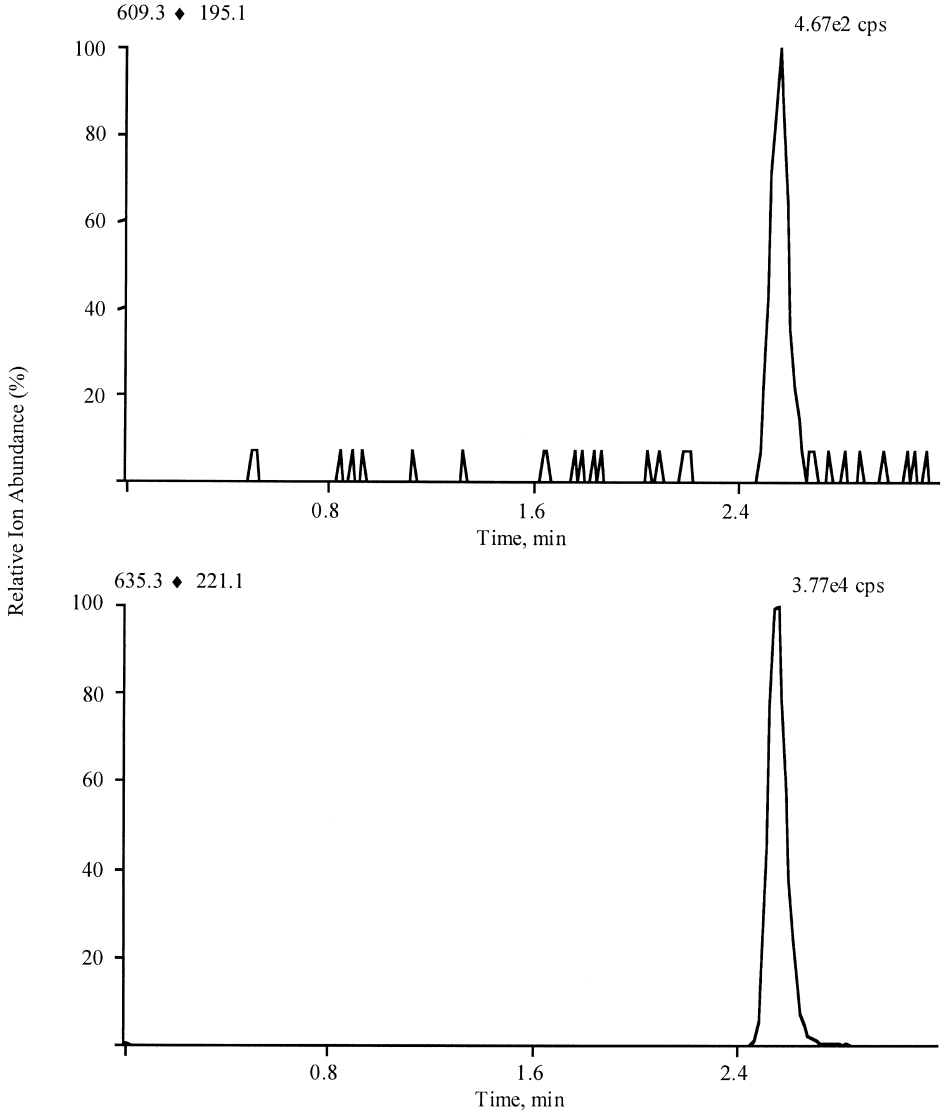

Fig. 4. SRM LC–MS chromatograms of reserpine (upper panel) and its I.S. (lower panel) in calibration standard 1 in mouse plasma extract containing reserpine (0.02 ng/ml) and I.S. (5 ng/ml) analyzed by an API III^{Plus}.

plasma samples, have not been fully characterized. However, these experiments suggest that proteins and perhaps other components in plasma were very different among individual FVB/N mice and that the analyte and I.S. were present in an unbound form at very low concentrations (I.S. concentration was 5 ng/ml and 50 μl was used in each sample). Furthermore, it has been suggested that the blood sampling vehicles (tubes and contents) and the actual amount of blood drawn might partially contribute to the variations (unpublished communications).

Fig. 5 shows the effects of disodium EDTA and five commonly used protein-denaturing reagents on the I.S. responses (expressed as peak area) in the FVB/N mouse plasma of the preclinical study samples. The reserpine responses were not compared because the reserpine concentrations in these preclinical study samples were unknown. The results

Table 1
Lower limit of quantitation of reserpine in mouse plasma

Plasma lot No.	Reserpine concentration (0.02 ng/ml)	% Dev[a] from nominal
618	0.0246	22.8
619	0.0228	14.2
620	0.0170	−15.0
621	0.0213	6.45
622	0.0229	14.7
626	0.0199	−0.550
Mean	0.0214	7.09
SD	0.00268	
Precision (RSD, %)[b]	12.5	

[a] % Dev=[(calculated concentration−nominal)/nominal]×100.
[b] RSD, %=(standard deviation/mean)×100.

from the method validation experiments showed that reserpine behaved like the I.S. Without the addition of protein-denaturing reagents to these preclinical study samples, no signal was observed for the I.S. By adding any of the protein-denaturing reagents (listed in Fig. 5) to the same preclinical study samples which showed no I.S. response in the absence of a protein-denaturing reagent, all these preclinical study samples showed an increase in the I.S. response. The response observed for the I.S. following treatment with acetonitrile as a protein-denaturing reagent was the lowest. Protein precipitation by acetonitrile may cause the analyte to co-precipitate and carry the analyte out of solution. It was observed that after adding the protein-denaturing reagents followed by vortex mixing, the samples

Table 2
Summary of accuracy and precision for the determination of reserpine in mouse plasma for QC1, QC2 and QC3 samples from three validation runs

Run No.		Nominal concentration (ng/ml)		
		QC1, 0.06	QC2, 2.0	QC3, 8.0
1	Mean (ng/ml)	0.0618	2.01	8.69
	n	6	6	6
	Within-run precision (RSD, %)	5.09	3.63	2.95
	Within-run accuracy (% Dev)	3.07	0.330	8.61
2	Mean (ng/ml)	0.0585	2.06	8.69
	n	5	6	6
	Within-run precision (RSD, %)	3.62	2.71	1.75
	Within-run accuracy (% Dev)	−2.53	3.00	8.61
3	Mean (ng/ml)	0.0551	1.95	8.39
	n	6	6	6
	Within-run precision (RSD, %)	10.9	2.26	2.30
	Within-run accuracy (% Dev)	−8.17	−2.41	4.91
	Grand mean (ng/ml)	0.0585	2.01	8.59
	n	17	18	18
	Between-run precision (RSD, %)	8.36	3.57	2.80
	Between-run accuracy (% Dev)	−2.54	0.308	7.38

Table 3
Accuracy and precision for the SRM LC–MS determination of reserpine in mouse plasma for calibration standards from three validation runs

	Standard (ng/ml)							
	STD 1, 0.02	STD 2, 0.04	STD 3, 0.1	STD 4, 0.4	STD 5, 2	STD 6, 5	STD 7, 7.5	STD 8, 10
Mean (ng/ml)	0.0199	0.0413	0.0980	0.386	1.97	4.92	7.53	10.6
Precision (RSD, %)	6.10	4.32	8.91	2.35	2.21	2.72	2.85	2.38
Accuracy (% Dev)	−0.533	3.32	−1.96	−3.52	−1.33	−1.58	0.352	6.37

with acetonitrile, TCA, TFA and acetic acid appeared more cloudy than those with disodium EDTA and formic acid. The samples with added disodium EDTA were clearer after vortex-mixing and had the highest response. In these instances, the I.S. which was more than four-fold higher than the samples treated with formic acid. The latter produced the best results among conventional protein-denaturing reagents used in this study. Although acetonitrile, TCA, and formic acid are commonly used as protein-denaturing reagents to precipitate proteins and release protein-bound drugs in biological samples [18–21], these reagents were not adequate to release reserpine and rescinnamine in the FVB/N mouse plasma studied in this work. In our study, only excess disodium EDTA was able to effectively release these analytes in the FVB/N mouse plasma. Most interestingly, we found that EDTA released the highly-bound reserpine to about the same degree as normal denaturants (LLE) did with conventional mouse plasma.

We do not have experimental evidence which suggests why excess EDTA treatment is required for maximal release of analytes in some of the FVB/N mouse plasma samples described in this work. But since EDTA is a well-known divalent cation chelating reagent and many of major serum proteins are metalloproteins, we propose that the plasma pro-

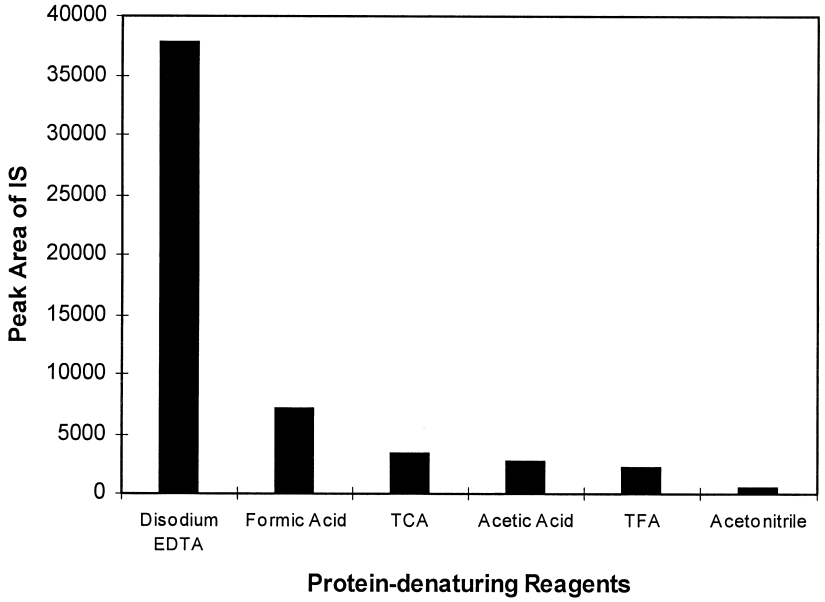

Fig. 5. Effects of protein-denaturing reagents on the responses (expressed as peak area) of I.S. in FVB/N mouse plasma (analyzed by an API III^{Plus}), for which the I.S. was not detectable without adding protein-denaturing reagents.

tein(s) which bind the analytes and cause undetectable response in some of FVB/N mouse plasma samples are most likely to be metalloproteins. Previous studies on many metalloproteins, e.g., α-lactalbumin and interstitial collagenase showed that removal of divalent cations resulted in a significant tertiary structure change characterized by exposure of some tryptophan residues to a polar environment [13–15]. Thus it is reasonably speculated that the treatment with excess EDTA could alter the conformation of the analyte-bound protein and weaken secondary analyte binding affinity. The ''denatured'' protein then could shift the binding equilibrium to release the free form of the analytes. It should be noted that a minor disadvantage in using excess disodium EDTA is that it tends to reduce the electrospray ion current responses of both analytes when used with control mouse plasma. The cause of reduced responses has not been fully studied. Without treatment of the protein-denaturing reagent, the recovery of reserpine in the control mouse plasma obtained from Lampire Biological Labs. was estimated to be 90%. With excess disodium EDTA as a ''protein-denaturing reagent'', the recovery of reserpine in the control mouse plasma was only 43.5% and the API 3000 was needed to achieve the required LLQ at 0.02 ng/ml in the FVB/N mouse plasma samples.

4. Conclusions

The turbo ionspray LC–MS–MS assay procedure using 96-well LLE for reserpine in FVB/N mouse plasma has proven to be sensitive, specific, accurate and reproducible. The single-step 96-well LLE procedure reduced the sample preparation time from approximately 2 days by the previously published method [5] to less than 3 h for 96 samples. The relatively low quantitation detection limits reported here allows reliable and reproducible quantitation of reserpine down to a level of 0.02 ng/ml in mouse plasma based on 0.1-ml samples. The sensitivity of LC–MS–MS in the positive ion mode was increased significantly by optimizing the pH of the LC mobile phase compared to previously reported work [5]. The optimal mobile phase pH for sensitive electrospray detection was determined to be 7.6, which is close to the pK_a of reserpine and counter to the commonly accepted conventional theory of pH effects on electrospray ionization [10–12]. We speculate that the pH of the sample solution or mobile phase is not the single factor to influence responses in electrospray LC–MS experiments, and these responses could be influenced by the combined effects of all buffers, additives and solvents in the LC–MS system.

Reserpine and rescinnamine were found to be highly bound in certain FVB/N mouse plasma samples from the preclinical study. Disodium EDTA was the most effective ''protein-denaturing'' reagent among the six reagents selected in this work to release reserpine and rescinnamine in the FVB/N mouse plasma. The mechanism of excess disodium EDTA as a special ''protein-denaturing'' reagent to facilitate the release of reserpine and rescinnamine in the FVB/N mouse plasma was speculated as the removal of the protein-bound divalent cations from metalloproteins to induce a conformational change of the protein causing the weaker binding affinity for the analytes. Moreover, there were significant adsorptive losses of reserpine at low concentrations when stored in polypropylene vials in freezers (−70°C) for more than 3 days. This phenomenon needs to be further investigated in future studies.

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