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Determination of SR 49059 in human plasma and urine by LC-APCI/MS/MS

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Abstract

SR 49059 ((2S 1-[(2R 3S)-5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzene-sulfonyl)-3-hydroxy-2,3-dihydro-1H-indole-2-carbonyl]-pyrrolidine-2-carboxamide) is an orally active non-peptide vasopressin V_{1a} antagonist. A sensitive, selective, and robust LC-MS/MS method was developed to determine the plasma and urine concentrations of SR 49059 in support of clinical studies. Plasma samples were prepared based on a rapid extraction procedure using Chem Elut™ cartridges. The extracted samples were analyzed on a C_{18} HPLC column interfaced with a Finnigan TSQ 700 mass spectrometer. Positive atmospheric chemical ionization (APCI) was employed as the ionization source. The analyte and its internal standard ($^2H_6$-SR 49059) were detected by use of multiple reaction monitoring (MRM) mode. The plasma matrix had a calibration range 0.2–20 ng ml^{-1}, with within and between run accuracy and precision both less than 10%. The chromatographic run time was approximately 3 min. Urine samples were prepared based on a simple dilution with water, followed by analysis under the same conditions as plasma. The calibration range for urine matrix was 20–5000 ng ml^{-1}, with within and between run accuracy and precision less than 11%. The method has been successfully applied to the clinical sample analysis. The plasma assay was also evaluated on a Finnigan TSQ 7000 mass spectrometer. The performance based on precision and accuracy was virtually identical to that on the TSQ 700, with the exception of linearity in calibration curve (the TSQ 700 was linear, the TSQ 7000 was quadratic). © 1997 Elsevier Science B.V.

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1. Introduction

Vasopressin (AVP) is released from the posterior pituitary gland either in response to increased plasma osmolality detected by brain osmoreceptors or to decreased blood volume and blood pressure sensed by the low-pressure volume receptors and the atrial baroreceptors. Vasopressin-induced antidiuresis, mediated by renal epithelial V_2 receptors and vasoconstriction, mediated by vascular V_{1a} receptors, help maintain normal plasma osmolality, blood volume and blood pressure. Various effects of vasopressin are mediated via different vasopressinergic sub type receptors [1,2]. V_{1a} receptors are mainly located on vascular

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smooth muscle cells, hepatocytes, plateletes, cortico adrenal and uterus cells. Stimulation of V_{1a} receptors results in an increase in intracellular calcium and increased turnover of phosphatidylinositol. SR 49059 (Fig. 1), a new molecular entity, is a potent and selective orally-effective non-peptide vasopressin V_{1a} antagonist, with marked affinity, and selectivity for both animal and human vasopressin V_{1a} receptors. The compound was discovered and is developed by Sanofi Recherche [3]. Antagonism of V_{1a} receptors may induce a variety of biological effects in mammals. Due to different localisation of the V_{1a} receptor, the compound is currently undergoing exploratory clinical investigations to define its indications [4–6]. Due to the marked potency of SR 49059, a highly sensitive bioanalytical method for the drug quantitation in plasma is essential to support pharmacokinetic analysis in the clinical trials.

LC-MS/MS, as a quantitative bioanalytical technique with high sensitivity and selectivity, has been extensively used in the pharmaceutical industry. Representative applications can be found in the literature [7–13].

In this study, a LC-MS/MS method based on atmospheric pressure chemical ionization (APCI) was developed and validated for SR 49059 in human plasma and urine. The deuterium-labeled analog ($[^2H]_6$-SR 49059, Fig. 1) was used as the internal standard. The performance of the method including accuracy, precision, robustness, throughput, etc. is reported. The utility of the method is demonstrated using the pharmacokinetic data from a clinical study as an example.

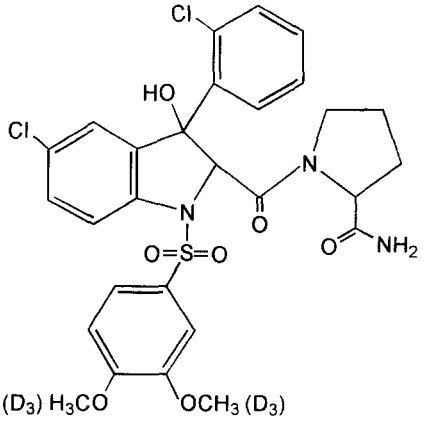

Fig. 1. The structure of SR 49059. The deuterium labels are indicated by D_3 in the parenthesis.

2. Experimental

2.1. Materials

SR 49059 and $[^2H]_6$-SR 49059 (isotopic purity > 99.5%) were synthesized at Sanofi Research (Montpelier, France and Alnwick, UK, respectively). Ammonium acetate, HPLC grade, was obtained from EM Science (Gibbstown, NJ). Acetonitrile, water, dichloromethane, hexane and methanol, all HPLC grade, were obtained from J.T. Baker (Phillipsburg, NJ). Helium, argon and nitrogen, all research grade, were from Air Products and Chemicals (Allentown, PA). Sterile human plasma was obtained from Rockland (Gilbertsville, PA). Control urine was obtained from healthy volunteers in house. Chem Elut™ (6 cc barrel volume, 1 ml sample size) and solid phase extraction cartridges (3 cc/50 mg, 6cc/250 mg and 6 cc/500 mg) were from Varian (Harbor City, CA).

2.2. Sample preparation

(a) Chem Elut™ for plasma samples. Plasma samples (1 ml) were loaded to Chem Elut™ cartridges, followed by waiting for about 5 min to allow the aqueous sample to be absorbed to the silica surface. The drug was eluted twice (4 ml each) with dichloromethane/hexane (1:1) under gravity. The organic fractions were collected and combined, followed by evaporation on a Turbovap (Zymark, Hopkinton, MA) at 50°C and 5–20 psi N_2. Residue was reconstituted in 200 μl of water/methanol (1:1) and vortexed. After centrifugation at 2500 rpm for 5 min, 75 μl of the supernatant was injected onto LC-MS/MS. The extraction recovery was estimated to be approximately 90% (see Section 5 for the determination of extraction recovery).

(b) Solid phase extraction (SPE) for plasma samples. SPE was evaluated in the method devel-

opment as an option for plasma sample preparation. The cartridges used include C_{18} and C_1 with varying sizes from 3 cc/50 mg to 6 cc/500 mg. Plasma sample (1 ml) was loaded to the cartridge (already conditioned with methanol and water), followed by washing with 2 ml of water/acetonitrile. Three ratios of water/acetonitrile were tested including 50/50, 70/30, and 80/20. The pH was also evaluated by replacing water with appropriate 50 mM buffer (pH 1.8, 5.0 and 7.0 with formic acetate, ammonium acetate, and phosphate, respectively). Elution was carried out by 2 ml of acetonitrile or methanol. The organic eluent was collected and evaporated under N_2 at room temperature. The residue was reconstituted into 200 μl of water/methanol (1:1) prior to injection on to the LC-MS/MS system.

(c) Urine sample preparation. Urine samples (0.1 ml) were diluted 10 fold with water/MeOH (1:1). After transferring 200 μl of the diluted sample into autosampler vials, 125 μl was injected onto LC-MS/MS.

3. LC/MS/MS analysis

3.1. Plasma

An HPLC system consisted of two Schimadzu LC-10AD pumps, an SCL-10A controller, an SIL-10A autosampler with a 100-μl loop and a 33 × 4.6 mm, 3 μm Supelcosil LC-18-DB column. The mobile phase was 35 mM ammonium acetate in water (A) or methanol (B) at a flow rate of 1 ml min^{-1} with the following gradient: 0 min, 50% B; 2 min, 95% B; 3 min, 95% B; 3.1 min, 50% B.

The effluent from the HPLC was directed through a Finnigan atmospheric pressure chemical ionization (APCI) interface into a Finnigan TSQ 700 system. The vaporizer and capillary heater temperatures were set at 500 and 200°C, respectively. The current on the Corona discharge needle was fixed at 5 μA. The collision gas (argon) pressure was established at 2.5 mTorr; the collision energy (voltage in the collision cell) was −16.7 V. A gain of 8 and an electron multiplier voltage of 1800 V were used. The sheath and auxiliary gas settings were 40 psi and 2 cc min^{-1}, respectively. The dwell time was 300 ms. The instrument was operated in the positive ion mode. An ammonium adduct ion of the drug $[M + NH_4]^+$ at m/z 637 (643 for internal standard) was admitted to the first quadrupole (Q1). After the collision induced fragmentation in Q2, the product ion at m/z 603 (609 for internal standard) was monitored in Q3. Unit resolution (at half peak height) was used for both Q1 and Q3. Data processing was carried out using a Finnigan QUAN (Version 1.00) data analysis program. Peak area ratios based on multiple reaction monitoring (MRM) of the drug (m/z 637 → 603) and the internal standard (m/z 643 → 609) were utilized for construction of calibration curve and quantitation.

For the comparative study on the TSQ 7000 mass spectrometer, essentially the same conditions were used as that on the TSQ 700 except that an electron multiplier voltage of 1700 V was used. The TSQ 7000 was interfaced with a HP 1090 LC system consisting of an autosampler, a binary solvent delivery system and an injection loop of 125 ul. All LC conditions were the same as that on the Schimadzu.

3.2. Urine

A TSQ 7000 mass spectrometer and an HP 1090 HPLC system were used. All conditions were the same as that in the plasma comparative study.

4. Results

SR 49059 under the experimental conditions employed was detected in the Q1 scan predominantly as a positive ammonium adduct ion $[M + NH_4]^+$ at m/z 637 (Fig. 2A). The product ion spectrum for SR 49 059 was obtained by collision induced dissociation and is shown in Fig. 2B. The fragmentation mechanisms were not entirely elucidated but appeared to involve dehydration of the ammonium adduct (m/z 619, $[M + NH_4 - H_2O]^+$), dehydroxylation of molecular ion (m/z 603, $[M + H - OH]^+$) and cleavage of formamide (m/z 575, $[M\text{-}CONH_2]^+$). The fragment at m/z 603 ($[M + H - OH]^+$) was the principle ion in the

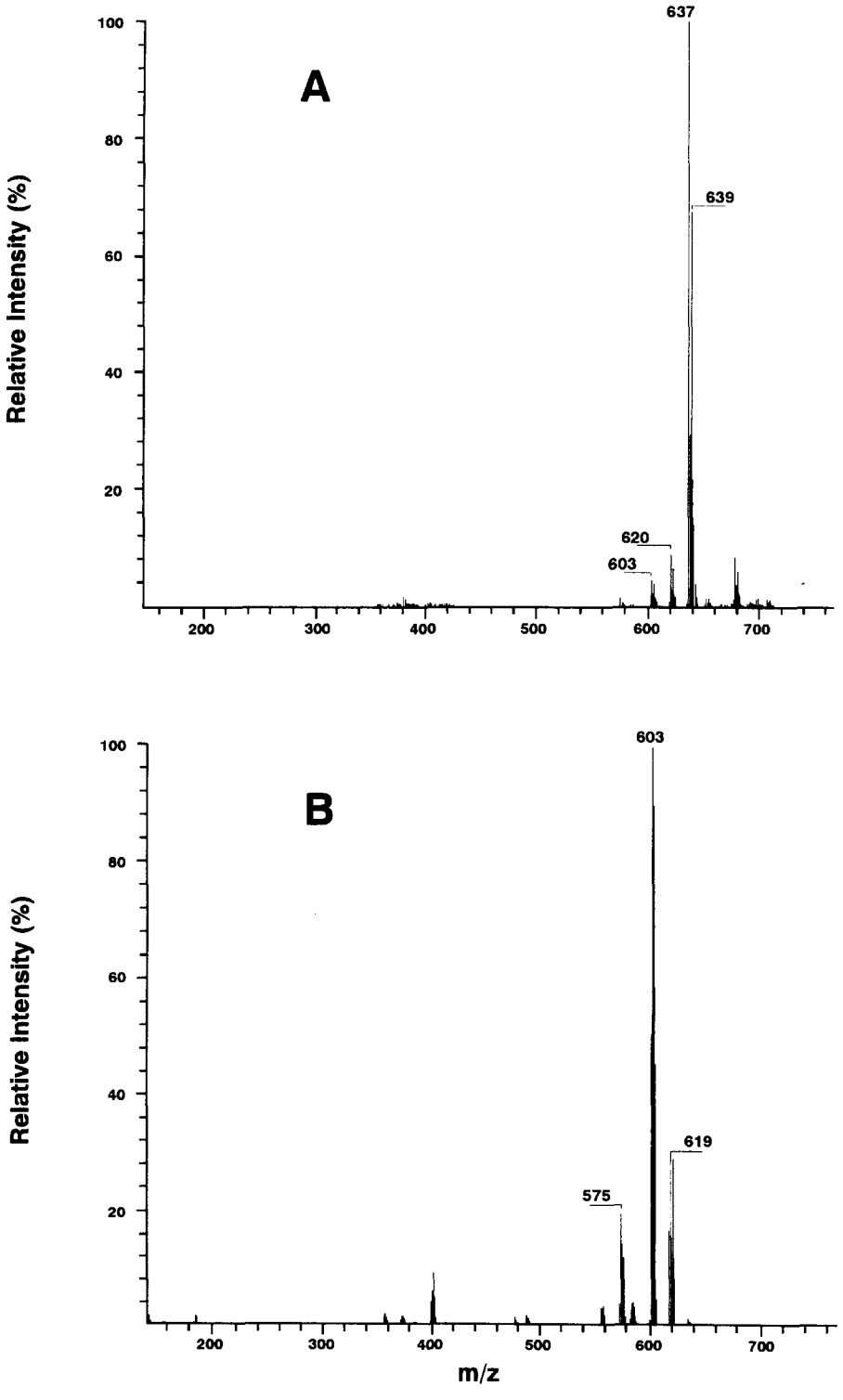

Fig. 2. Full scan mass spectrum of SR 49059 (A) under positive APCI and the product ion mass spectrum of the ammonium adduct ion of SR 49059 (m/z 637) (B).

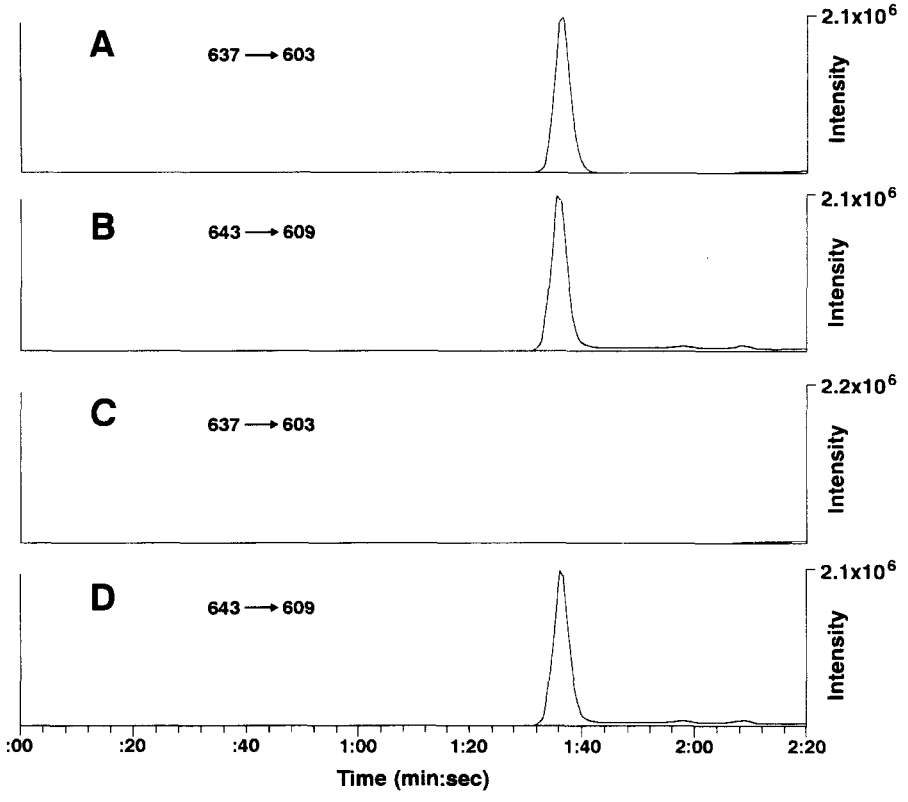

Fig. 3. Chromatograms obtained through SRM (SR 49 059, 637 = > 603; internal standard, 643 = > 609) from the extracted samples of a subject. The plasma was collected prior to (C and D), and 3 h after (A and B), an oral administration of 100 mg. The drug level measured was 3.00 ng ml^{-1} (A).

product spectrum, and was utilized to monitor SR 49 059. The spectra obtained from the TSQ 700 and the TSQ 7000 were virtually identical. MRM chromatograms obtained from the extracted plasma samples of a subject following an oral administration of 100 mg SR 49 059 are shown in Fig. 3. Chromatograms were identical for the TSQ 700 (plasma) and the TSQ 7000 (plasma and urine). Retention time and chromatographic profile were reproducible throughout the entire study, in spite of the periodical column replacement.

4.1. Calibration

Plasma calibration curves consisted of SR 49 059 concentration levels of 0.2, 0.4, 0.7, 1, 2, 5, 10 and 20 ng ml^{-1}, with triplicates at both ends and singlets in between (Fig. 4). The signal response of SR 49 059 to the plasma concentration was linear over the entire calibration range on the TSQ 700, while the best fit on the TSQ 7000 was quadratic (Fig. 4). A weighting of $1/Y^2$ was used for both linear and quadratic curves.

Urine calibration curves contained SR 49 059 concentration levels of 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng ml^{-1}, also with triplicates at both ends and singlets in between. A quadratic curve (Fig. 4) with $1/Y^2$ weighting was used.

4.2. Precision and accuracy

Precision and accuracy were assessed based on both within and between run analysis (TSQ 700). In the within run analysis, 6 replicates were analyzed at each concentration level; while in the

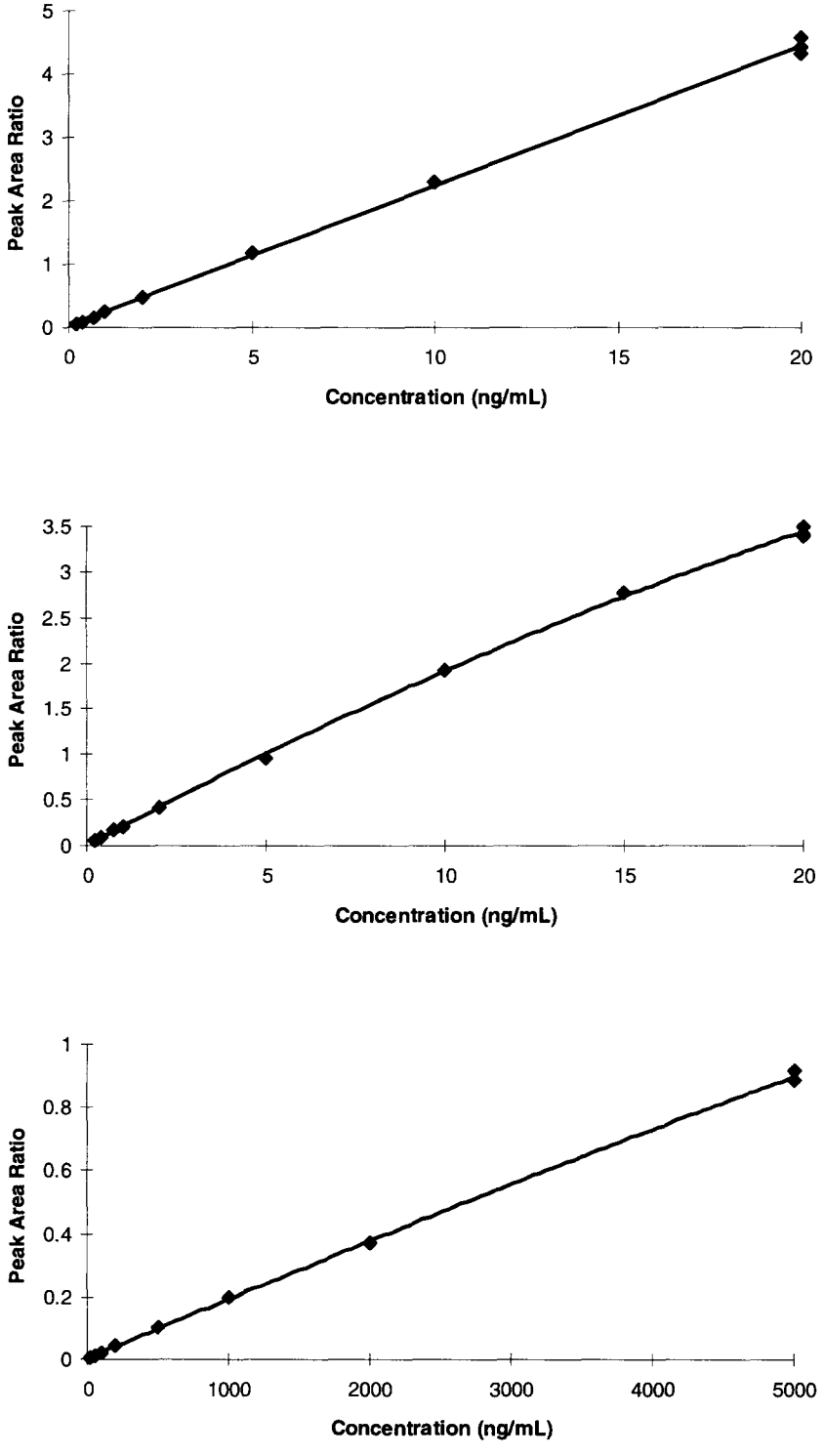

Fig. 4. Representative calibration curves for the quantitation of SR 49059 in human plasma (top panel, TSQ 700; middle panel, TSQ7000) and human urine (bottom panel, TSQ 7000).

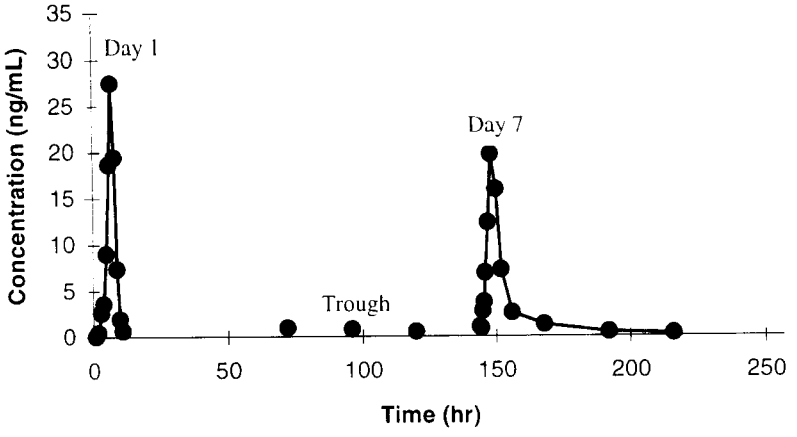

Fig. 5. Plasma concentrations of SR 49 059 in a subject following daily (uid) oral administration of 300 mg for 7 days.

between run analysis, duplicates were used at each concentration level. The results from both plasma and urine samples are given in Table 1. The coefficients of variance (CV%) and mean percentage differences from nominal (M%D) for all the analysis were below 11%.

Precision and accuracy were also assessed on three TSQ 7000 instruments. The data are given in Table 2. The precision and accuracy were similar between the TSQ 700 and all of the TSQ 7000 instruments.

4.3. Stability of SR 49 059 during the sample preparation

The stability of SR 49 059 in the sample preparation was assessed based on several aspects including (1) freeze/thaw, in which control plasma and urine fortified with SR 49 059 was subject to three cycles of freeze (− 20°C) and thaw, followed by the measurement of drug levels, (2) post-sample process stability, in which the dry samples, obtained after Chem Elut™ process and evapora-

Table 1
Precision and accuracy of within and between run analysis

	Nominal (ng ml^{-1})	Within day[a]		Between day[b]	
		Precision	Accuracy[c]	Precision	Accuracy[d]
Plasma	0.2	5.9	−8.0	9.0	−4.5
	0.4	5.2	−1.0	8.7	0.75
	2.0	1.7	3.5	2.9	3.0
	15	1.6	−2.0	3.0	0.67
	20	3.0	−3.0	2.6	−0.5
Urine	20	5.7	10.5	5.4	8.5
	50	2.4	2.4	2.9	3.0
	500	1.4	−2.0	3.0	1.4
	5000	0.6	0.2	1.4	−2.2

[a]Six replicates at each level.
[b]Based on 6 runs (the first between run analysis used the first two samples in the within day run analysis), duplicates at each level in each run.
[c]Coefficient of variance in percentage (CV%).
[d]Mean percentage difference from nominal (M%D).

Table 2
Within run analysis data for SR 49 059 in human plasma from a TSQ 700 and three TSQ 7000 mass spectrometers

Nominal concentration (ng ml^{-1})	TSQ 700		TSQ 7000-A[a]		TSQ 7000-B[a]		TSQ 7000-C[a]	
	Precision[b]	Accuracy[c]	Precision[b]	Accuracy[c]	Precision[b]	Accuracy[c]	Precision[b]	Accuracy[c]
0.2	5.9	−8.0	12	1.9	4.4	−13	5.0	2.7
0.4	5.2	−1.0	4.7	−6.1	8.5	−1.3	13	−7.8
2.0	1.7	3.5	1.6	−0.17	1.5	2.8	4.0	0.33
15	1.6	−2.0	2.5	2.4	1.3	2.9	3.1	4.3
20	3.0	−3.0	6.2	7.7	0.85	7.5	3.1	6.2

[a]Three TSQ 7000 uniquely identified as A, B and C.
[b]Coefficient of variance in percentage (CV%).
[c]Mean percentage difference from nominal (M%D).

tion, were stored at room temperature (RT) for 4 days followed by 2 days in reconstitution solution at room temperature prior to analysis by LC/MS, and (3) room temperature stability, in which the SR 49 059 fortified plasma and urine samples were kept at room temperature for 24 h prior to sample preparation. Two concentration levels (0.4 and 15 ng ml^{-1} for plasma, 50 and 5000 ng ml^{-1} for urine) and 6 replicates at each level were used for all the stability experiments. SR 49 059 was found to be stable in any of the sample preparation aspects examined (Table 3).

4.4. Application to clinical sample analysis

This method has been successfully applied to the determination of plasma and urine concentration levels of SR 49 059 in support of pharmacokinetic analysis in a clinical study. The plot of plasma concentration vs. sampling time obtained from a subject following daily oral administration (uid) of 300 mg for 7 days is shown in Fig. 5. The urine concentrations from the same subject are given in Table 4.

Table 3
Stability of SR 49059 in the sample preparation

	Experiment	Precision[a]		Accuracy[b]	
		0.4 (ng ml^{-1})	15 (ng ml^{-1})	0.4 (ng ml^{-1})	15 (ng ml^{-1})
Plasma	Free/thaw 3 cycles	5.2	0.65	5.0	5.3
	Processed sample	6.7	0.96	1.0	2.0
	Room temperature	2.6	1.7	−8.8	1.3
	Experiment	50 (ng ml^{-1})	5000 (ng ml^{-1})	50 (ng ml^{-1})	5000 (ng ml^{-1})
Urine	Free/thaw 3 cycles	1.9	1.1	−2.2	−8.2
	Processed sample	3.6	1.7	2.0	−2.2
	Room temperature	1.5	1.1	0.4	0

[a]Coefficient of variance in percentage (CV%).
[b]Mean percentage difference from nominal (M%D).

5. Discussion

To monitor the sample preparation in the method development, signal suppression (e.g. ion suppression by matrix material) and extraction recovery were evaluated among many parameters. Signal suppression was measured based on the difference in signal intensity between the standards (stock solution direct injection) and the processed control plasma residues spiked with the standards post sample preparation. Extraction recovery was determined based on the difference in signal intensity between the spiking of standards pre and post sample preparation (into the control plasma and the extracted residue, respectively). SPE was evaluated based on cartridge size (amount of packing material), percentage of organic solvent in the washing solution, pH of aqueous portion of the washing solution, organic solvent in the elution and the surface chemistry of the packing material (see Section 2 for more details). Chem Elut™ was evaluated based on the organic solvents used for elution. It was found that C_{18}, 6 cc/500 mg, 80/20 water/acetonitrile washing, acetonitrile elution in the SPE and hexane/DCM (1:1) elution in the Chem Elut™ represented the best conditions. Once optimized, the SPE and Chem Elut™ were compared for signal suppression and extraction recovery. The results suggested that these two approaches were equally effective (high extraction recovery, 90% or greater and virtually no signal suppression). Chem Elut™ was selected for use in this study because of the low cost (typically less than half of the cost compared to SPE cartridges), the ease of operation (no equilibration, no washing, no vacuum) and most importantly the high through put in the sample preparation. In contrast to typical LC/MS assays where the operation speed is limited by the sample clean up procedure [5], the turn-around was so fast in the sample preparation using Chem Elut™ that the rate limiting step in this assay was the LC/MS instrument analysis.

Table 4
Urine concentrations of SR 49 059 from a subject following daily oral administration (uid) of 300 mg for 7 days

Day	Hours	Concentration (ng ml^{-1})
1	0	<MQL[a]
	0–4	1.54×10^3
	4–8	2.23×10^3
	8–12	167
	12–16	442
	16–24	185
7	0–4	827
	4–8	2.04×10^3
	8–12	104
	12–16	389
	16–24	357

[a]Less than minimum quantifiable level; MQL = 20 ng ml^{-1}.

APCI was observed to be significantly more sensitive than electrospray ionization (ESI) for SR 49059.

Ammonium acetate as mobile phase additive was found in this study to be necessary for the signal intensity in the APCI mode, using methanol as mobile phase organic modifier. The signal of ammonium adduct $[M + NH_4]^+$ formed in the presence of ammonium acetate was approximate 50% more intense than that of the molecular ion $[M + H]^+$ monitored in the absence of ammonium acetate. More importantly, the signal level of $[M + MH_4]^+$ was highly stable from day to day. When acetonitrile (ACN) was evaluated as the organic modifier in the mobile phase, an adduct $[M + NH_4 + ACN]^+$ was formed as the predominant ion. This adduct ion was found to co-exist with and decrease proportionally to $[M + NH_4]^+$ ion as ammonium acetate concentration increased, suggesting that ammonium has much higher affinity for SR 49 059 than does ACN in the adduct formation especially considering that acetonitrile was in large excess. It appeared that $[M + NH_4 + ACN]^+$ and $[M + NH_4]^+$ were at equilibrium, as indicated by the reversibility between these ions when ammonium acetate concentration varied. The $[M + NH_4 + ACN]^+$ ion showed poor signal stability probably due to the weak association of ACN with the drug.

Gradient elution was used in this study because of significant band broadening observed under isocratic conditions. In addition, column life span was prolonged by gradient elution probably due to the washing effects of gradient which minimizes the accumulation of matrix materials on the column.

The method was found to be (1) rugged (total over 50 runs by 6 analysts on 4 instruments without failure), (2) specific, as demonstrated by large number of pre-dose and control plasma samples, and (3) highly efficient, due to the simplicity of the sample preparation procedure. The typical sample clean up time for 100 samples on Chem Elut™ was approximately 1 h, with only two steps involved in the sample clean up—loading and elution (compared to condition, loading, washing and elution in SPE). In addition, the Chem Elut™ operation did not require vacuum for solvent flow. The gravity elution eliminated the time and attention required for vacuum control, significantly improving the efficiency.

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