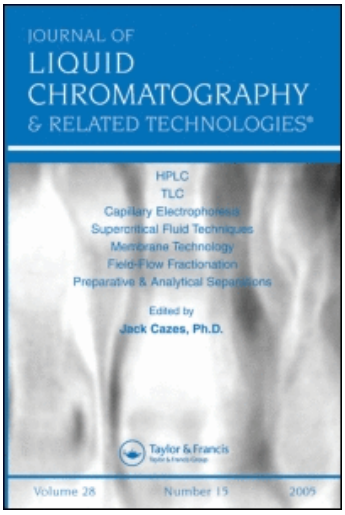

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Quantitative Determination of Cannabinoid Receptor Antagonist Surinabant in Human Plasma by LC-UV and LC-ESI-MS/MS Methods

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Abstract: Surinabant [5-(4-bromophenyl)-1-(2,4-dichlorophenyl)-4-ethyl-*N*-(1-piperidinyl)-1*H*-pyrazole-3-carboxamide] is a cannabinoid receptor type 1 antagonist which is believed to indirectly inhibit the dopamine-mediated reward system for food, alcohol and nicotine addiction. Currently, there is no analytical method reported for the determination of surinabant in biological matrices. In this work, a liquid chromatographic (LC) method with both ultraviolet (UV) and electrospray ionization tandem mass spectrometric (ESI-MS/MS) detection has been developed and validated for the quantitative measurement of surinabant in human plasma to support the clinical investigation of this new drug. The compound AM251 was used for internal calibration. A protein precipitation procedure was employed for plasma sample preparation. Chromatographic separation of surinabant and internal standard was carried out on a Waters YMC^{TM} Pro C4 cartridge column using a mobile phase containing 99.9% CH_3CN/H_2O (50:50, v/v) and 0.1% HCOOH. The LC-UV detection was accomplished by monitoring the absorption at 258 nm, which had an LLOQ of 100 ng/mL and a calibration range of 100–1500 ng/mL for surinabant. The LC-ESI-MS/MS detection was achieved using positive multiple-reaction-monitoring (MRM) mode for surinabant (m/z $523 \rightarrow 423$) and the internal standard (m/z $555 \rightarrow 455$), which had an LLOQ of 5.00 ng/mL and a calibration curve of 5.00–1000 ng/mL.

Keywords: Cannabinoid-receptor antagonist, Human plasma, LC-ESI-MS/MS, LC-UV, Surinabant

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INTRODUCTION

Surinabant [5-(4-bromophenyl)-1-(2,4-dichlorophenyl)-4-ethyl-*N*-(1-piperidinyl)-1*H*-pyrazole-3-carboxamide] (Fig. 1) is an antagonist of cannabinoid receptor type 1 (CB_1), which was developed by Sanofi Aventis.[1] Cannabinoid (CB) receptors are a class of G protein-coupled receptors which are divided into two well-defined subtypes, CB_1 and CB_2. The CB_1 receptor is widely expressed in the brain, but also found in the lungs, liver and kidneys, and the CB_2 receptor is mainly expressed in the immune system and in hematopoietic cells.[2] CB receptors can be activated by a group of compounds known as endocannabinoids (endogenous) or cannabinoids (exogenous), which produce a cascade of biological responses, including the inhibition of adenylate cyclase and thereby the production of cAMP,[3] the enhancement of inwardly rectifying and A-type potassium channels,[4] inhibition of presynaptic N- and P/Q-type calcium channels[4] and activation of MAP kinase.[5] These biochemical processes are involved in cognition, memory, anxiety, control of appetite, emesis, motor behavior, sensory, and autonomic and neuroendocrine responses, as well as in immune and inflammatory responses.[6] CB-receptor antagonists bind to the receptor and decrease the CB receptor-mediated responses.[7]

Surinabant is a second-generation CB_1 receptor antagonist, which has longer duration of action than the first-generation CB_1 receptor antagonist rimonabant, and enhanced oral activity.[8–10] CB1 receptors are widely expressed in hypothalamus and nucleus accumbens which are involved in food intake control and feeding behavior. These areas of the central nervous system are associated with the mesolimbic dopamine pathway which regulates the reward strength circuitry.[7,11]

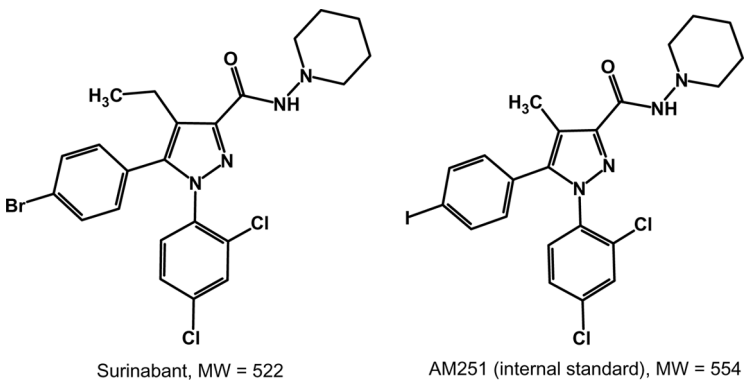

Figure 1. The chemical structures of surinabant and internal standard.

Therefore, surinabant is believed to indirectly inhibit the dopamine-mediated reward system for food, alcohol and nicotine addiction. Surinabant has been in clinical trials in Europe for treatment of obesity[12] and nicotine addition.[13]

Currently, there is no analytical method publicly available for quantitative measurement of surinabant in biological media. It is necessary to develop an analytical method to study the pharmacological and toxicological properties of this experimental new drug. Further, the analytical method developed will arm researchers with a quantitative tool to investigate endocannabinoids and their signaling system.

This work describes the development and validation of a liquid chromatographic method with either ultraviolet (UV) or electrospray ionization tandem mass spectrometric (ESI-MS/MS) detection for the quantitative measurement of surinabant in human plasma. While UV spectrometers are widely available, relatively inexpensive and easy to use, the limit of detection afforded by a UV spectrometer may not be low enough for certain investigations. To achieve a lower limit of detection, an electrospray ionization tandem mass spectrometer has been used. For surinabant quantitation, the compound AM251 was used as internal standard and plasma samples were prepared by acetonitrile protein precipitation before subjected to chromatographic separation. The separation of surinabant and internal standard was done on a C4 column, and the detection was carried out by either UV detector at 258 nm or ESI-MS/MS in positive MRM mode. The methods developed provide quantitative measures for surinabant in human plasma. These methods can be used for the pharmacokinetic study of surinabant in clinical trials, as well as, to study the modulation of CB receptors.

EXPERIMENTAL

Chemicals and Solutions

Acetic acid, HPLC-grade acetonitrile, dimethyl sulfoxide (DMSO), formic acid, and 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1H-pyrazole-3-carboxamide trifluoroacetate salt (AM251) were from Sigma-Aldrich (St. Louis, MO, USA). 5-(4-bromophenyl)-1-(2,4-dichloro-phenyl)-4-ethyl-*N*-1-piperidinyl-1H-pyrazole-3-carboxamide (surinabant) was obtained from the drug repository (NIMH code S-908) of National Institute of Mental Health (Bethesda, MD, USA). Pooled human plasma containing no detectable surinabant was from Haemtech, Inc (Essex Junction, Vermont, USA).

A stock solution (1.00 mg/mL) of surinabant ($C_{23}H_{24}Cl_2N_4BrO$) was prepared by weighing 1.32 mg of the compound into a 1.5 mL Eppendorf

tube (Eppendorf, Westbury, NY, USA) and dissolving it with 1.32 mL of DMSO. A stock solution (1.00 mg/mL) of AM251 trifluoroacetate salt ($C_{22}H_{21}N_4OCl_2I \cdot C_2HF_3O_2$) was prepared by weighing 1.18 mg of the compound into a 1.5 mL Eppendorf tube and dissolving it with 1.18 mL of DMSO. After the compounds had dissolved in DMSO, both surinabant and AM251 stock solutions were transferred to 1.5 mL amber glass vials and stored at $-20°C$ when not in use.

A working solution (100 μg/mL) of surinabant was prepared by a dilution (1/10) of the surinabant stock solution with DMSO. A working solution (5.00 μg/mL) of AM251 was prepared by a three-step dilution (1/8.3, 1/10, 1/2) of the AM251 trifluoroacetate salt stock solution with DMSO. A mobile phase for liquid chromatographic separation was prepared by mixing 99.9% CH_3OH/H_2O (50:50, v/v) and 0.1% HCOOH. The above solutions were stored at 4°C when not in use.

Plasma Calibrators and Controls

Pooled human plasma containing no detectable surinabant was used as the blank plasma to prepare human plasma calibrators and controls for this study. Surinabant standard solutions (0.100, 0.1500, 0.200, 1.00, 1.50, 2.00, 5.00, 10.0, 15.0, 20.0 and 30.0 μg/mL) were prepared by a serial dilution of the working solution (100 μg/mL) with DMSO. Surinabant plasma calibrators (5.00, 10.0, 50.0, 100, 250, 500, 750, 1.00×10^3 and 1.50×10^3 ng/mL) were prepared by a 1:20 dilution of the corresponding surinabant standard solutions with the blank plasma. Surinabant plasma controls (7.50, 75.0, 150, 600, 750 and 1.20×10^3 ng/mL) were prepared by a 1:20 dilution of the corresponding surinabant standard solutions with the blank plasma. All plasma samples were stored at $-20°C$ until use.

Plasma Sample Preparation

Plasma samples (e.g., surinabant plasma calibrators, surinabant plasma controls) were prepared using the following protocol. First, 200 μL of plasma sample was pipetted into a 1.5 mL Eppendorf tube, then followed by 10.0 μL of the AM251 working solution (5.00 μg/mL) and 50.0 μL of acetic acid. After vortex mixing, 1.00 mL of acetonitrile was added. The solution was vortexed, then centrifuged at 13,000 g for 15 min. After centrifugation, the supernatant was transferred to a 1.5 mL Eppendorf tube, and evaporated to dryness in a TurboVap LV Evaporator (Caliper Life Sciences, Hopinkton, MA, USA) at 55°C with N_2 pressure of 20 psi for 2 h. Prior to analysis, the sample was reconstituted in 200 μL of the mobile phase.

Recovery of Plasma Surinabant

The recovery of plasma surinabant by sample preparation procedure was assessed using surinabant plasma controls and surinabant reference solutions at three different concentrations, which were 7.50, 75.0, 750 ng/mL for LC-ESI-MS/MS and 150, 600 and 1.20×10^3 ng/mL for LC-UV. The reference solutions contained the same sample matrix as that of plasma controls, which were prepared using blank plasma as samples by the same sample preparation procedure as that of plasma controls, and reconstituted in the mobile phase with corresponding surinabant concentrations.

LC-UV Method

Instrumentation. A System Gold® HPLC BioEssential 126/128 from Beckman Coulter (Fullerton, CA, USA) was used in this work, which consisted of a 126-gradient pump, a 508-autosampler and cooler that was set at ambient temperature, a stainless steel in-line filter (0.5 μm pore, 0.23 μL dead volume) from Upchurch Scientific (Oak Harbor, WA, USA), a YMC™ Pro C4 cartridge column (3 μm, 120 Å, 2.0 mm × 50 mm) from Waters (Milford, MA, USA), a 168-photo diode array detector the was set at 258 nm, and a 32-karat workstation. The fluidic connection of the system was made using stainless steel tubing (0.0625 in. o.d., 0.0100 in. i.d.). Data acquisition and peak integration was done by 32-karat software (version 8.0). The peak area ratios of surinabant to the internal standard were plotted against plasma surinabant concentrations for a calibration curve.

Chromatographic Separation. Analytical separation of surinabant and AM251 was performed at ambient temperatures on the Waters YMC™ Pro C4 cartridge column. Prior to the analysis, the cartridge column was first equilibrated with the mobile phase containing 99.9% CH_3OH/H_2O (50:50, v/v) and 0.1% HCOOH at a flow rate of 0.200 mL/min for about 30 min. For the subsequent analysis, 20 μL of sample was injected with the autosampler, and the analytes were separated on the column by isocratic elution at a flow rate of 0.200 mL/min.

LC-ESI-MS/MS Methods

Instrumentation. The LC-ESI-MS/MS system used in this work consisted of an HP1100 pump by Hewlett Packard (Palo Alto, CA, USA), an HP1100 autosampler, a stainless steel in-line filter (0.5 μm pore, 0.23 μL dead volume) by Upchurch Scientific (Oak Harbor, WA, USA),

a YMC^{TM} Pro C4 cartridge column (3 μm, 120 Å, 2.0 mm × 50 mm) by Waters (Milford, MA, USA), a stainless steel splitting tee (1/16″ × 0.25 mm) by Valco (Houston, TX, USA), and a Quattro II electrospray ionization triple quadrupole mass spectrometer by Micromass (Manchester, UK). The fluidic connection of the system was made using high-pressure polyether ether ketone (PEEK) tubing (0.0625 in. o.d., 0.0100 in. i.d.). The post-column split ratio was 1:2 with a smaller flow (ca. 63 μL/min) to the MS detector and the larger one to the waste. Data acquisition and peak integration were accomplished using the Micromass Masslynx software (version 3.4). The peak area ratios of surinabant to the internal standard were plotted against plasma surinabant concentrations for a calibration curve.

Chromatographic Separation. The separation conditions were the same as those described in the LC-UV method section

ESI-MS/MS Detection. The Micromass Quattro II triple quadrupole mass spectrometer was operated under the positive electrospray ionization mode (ESI^+). The mass spectrometer was tuned by infusion of a mixture of surinabant (2.0 μg/mL) and AM251 (2.0 μg/mL) in the mobile phase at a flow rate of 3 μL/min with a syringe pump (Harvard Apparatus, South Natick, MA, USA). Ionization conditions were optimized as follows: nitrogen sheath and desolvation gas at 10 and 350 L/h, capillary at 3.5 kV, HV lens at 0.5 kV, cone at 45 V, skimmer at 1.5 V, RF lens at 0.2 V, ion source temperature at 95°C, ion energy at 0.1 V, low- and high-mass resolution at 15, and multiplier at 700 V. Full-scan spectra were acquired in the continuum mode at a scan rate 400 u/s. Multiple reaction monitoring (MRM) mode was used for quantitation, which was set at the following conditions: m/z 523 → 423 for surinabant, m/z 555 → 455 for AM251, argon collision gas at 2.0–2.5 μbar, cone at 45 V, collision energy at 20 V for both analytes, low- and high-mass resolution at 10 for quadrupole 1 and 15 for quadrupole 3, dwell time at 0.4 s, and the inter-scan delay at 0.01 s. The ionization parameters were the same as those described previously.

RESULTS AND DISCUSSION

Preparation of Surinabant Standards and Plasma Calibrators

When selecting a solvent for dissolution of analytes into a solution, there are two key considerations; first, the solvent chosen must dissolve the compound completely to produce a homogenous solution; secondly, the solvent should not cause matrix precipitation upon its addition to

biological samples. As shown in Fig. 1, both surinabant and AM251 are hydrophobic compounds which have low solubilities in aqueous solution. Therefore, it is difficult to dissolve either compound directly in biological samples.

Our experiments showed that protonation of amine groups on surinabant and AM251 with 0.1 M HCl had little effect on dissolving the compounds in either water or plasma. Since surinabant has a similar structure with rimonabant which is completely soluble in DMSO and the addition of DMSO at 25% or lower does not cause protein precipitation in plasma,[14] DMSO has been chosen as the solvent for the preparations of surinabant and AM251 stock and working solutions, as well as surinabant standard solutions. The percent composition of DMSO in surinabant plasma calibrators and controls in this work was 10% (v/v) in which there was no protein precipitation observed.

Plasma Sample Preparation

Plasma samples were prepared by a simple protein precipitation procedure using 100% CH_3CN as solvent. Prior to the addition of acetonitrile, acetic acid was added to plasma samples to reduce the binding between the analytes and plasma proteins due to protonation. The use of acetic acid improved the recovery of surinabant and AM251 and enhanced the mass sensitivity in the consequent ESI-MS-MS detection.

After protein precipitation, the supernatant containing surinabant and AM251 was evaporated to dryness in a TurboVap LV Evaporator. The drying time may be shortened by increasing either the temperature of the water bath or the pressure of nitrogen flow.

The sample preparation procedure used has been proven by the recovery data (Table 1) to be adequate for the analysis of surinabant by LC-UV and LC-ESI-MS/MS methods.

LC-UV Method

In this work, a Waters YMC^{TM} Pro C4 column was used instead of C8 or C18 column because its use reduced organic solvent consumption while producing optimal analyte resolution and retentions. Due to the presence of an ethyl group at the 4-position of its pyrazole ring, surinabant is more hydrophobic than AM251 (Figure 1) despite its smaller molecular mass. The elution sequence from a C4 column was AM251 (7.4 min) first, then surinabant (9.4 min), and the total run time for each sample was about 11.0 min (Fig. 2). UV detection was carried at 258 nm because surinabant and AM251 displayed maxima absorptivities and the blank human

Table 1. Recovery, precision and accuracy data for surinabant

Surinabant (ng/mL)	Recovery (%)	Mean peak area ratio	Standard deviation	CV (%)	Accuracy (%)
HPLC-UV Method					
150	103	0.330	0.0099	3	86.9
600	95.0	1.37	0.054	4	96.5
1.20×10^3	97.0	2.81	0.086	3	92.7
LC-ESI-MS/MS Method					
7.50	101	0.0344	0.0026	8	92.3
75.0	96.0	0.321	0.0093	3	87.2
750	100	2.56	0.15	6	92.6

All determinations were based on the mean value obtained from five separate samples by duplicate measurements at each concentration. The concentration of internal standard was fixed (250 ng/mL).

plasma showed no detectable interferences within the retention range of the analytes.

The recovery studies were conducted using the protein precipitation procedure and LC-UV method with surinabant plasma controls and surinabant reference solutions at three different concentrations (150, 600 and 1.2×10^3 ng/mL). The relative recoveries of surinabant were determined by comparing the mean-peak-area ratios of surinabant to

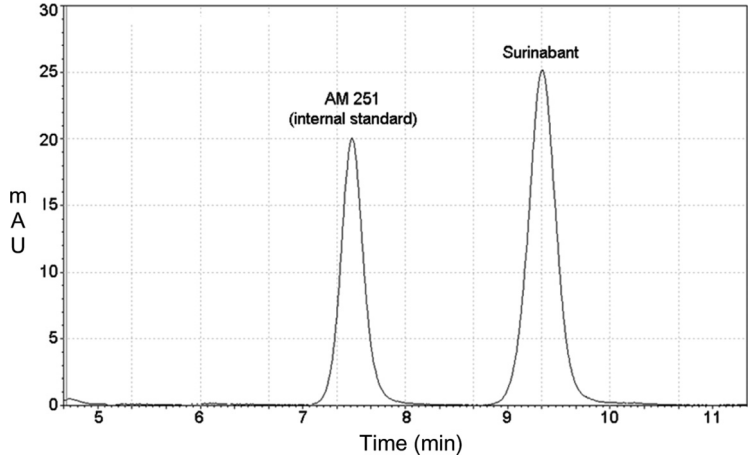

Figure 2. The representative UV chromatogram of human plasma spiked with 250 ng/mL AM251 and 500 ng/mL surinabant. The experimental conditions described in the LC-UV method section.

the internal standard in the plasma controls to those of surinabant to the internal standard in the reference solutions. As shown in Table 1, the mean relative recoveries of surinabant ranged 95.0–103%.

The precision of the LC-UV method was determined by analyzing surinabant plasma controls at three different concentrations (150, 600 and 1.2×10^3 ng/mL) and was determined by the duplicate measurements of the peak-area ratio of surinabant to the internal standard from five separate samples at each concentration. As shown in Table 1, the precision expressed in terms of percent coefficient of variation (%CV) was $\leq$4%.

The linear dynamic response of the LC-UV method was determined by the peak-area ratios of surinabant to internal standard versus the concentration of surinabant in plasma. A linear calibration range of 100–1500 ng/mL with a correlation coefficient of 0.999 was achieved. A calibration equation, $y = 0.00232x - 0.0619$, was derived from the average peak area ratio of two separate injections from a single sample at each of the six concentrations (100, 250, 500, 750, 1000 and 1500 ng/mL) over the calibration range. Accuracies were 86.9, 96.5 and 92.7% at the concentrations of 150, 600 and 1.20×10^3 ng/mL by duplicate measurements. The lower limit of quantitation (LLOQ) of the LC-UV method was determined by the lowest plasma surinabant calibrator on the calibration curve to be 100 ng/mL with a CV and an accuracy of 8 and 95.0%, respectively.

LC-ESI-MS/MS Method

In the LC-ESI-MS/MS method, the chromatographic separation conditions were the same as those described in the LC-UV method. However, the elution times of AM251 and surinabant were slightly longer than (ca. 0.9 min) those of the LC-UV method due to a longer fluidic connection in the LC-ESI-MS/MS system, which were 8.3 and 10.3 min, respectively (Fig. 3). The total run time for each sample was approximately 12.0 min.

The full-scan mass spectra by positive electrospray ionization (Figs. 4a and 4c) revealed that surinabant and AM251 produced predominant protonated molecular ions at m/z 523 and 555, respectively. These parent ions were fragmented to produce daughter mass spectra which showed that the predominant product ions were m/z 423 for surinabant and m/z 455 for AM251 (Figs. 4b and 4d). These product ions were measured by the multiple-reaction-monitoring (MRM) mode for internal calibration and quantitation of surinabant. The selectivity and specificity of the LC-ESI-MS/MS method by MRM mode for the measurement of surinabant and AM251 is illustrated by the mass chromatograms (Fig. 3) which show no interference from the plasma matrices.

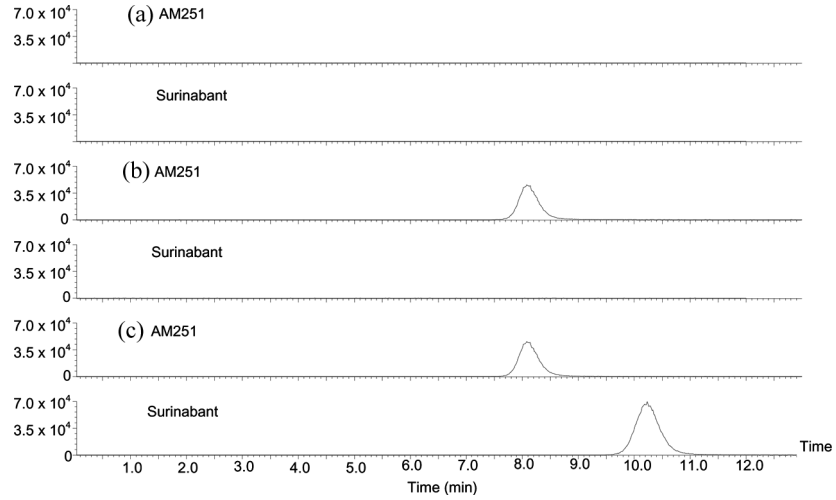

Figure 3. The representative mass chromatograms of human plasma samples. (a) The double blank; (b) the pooled human plasma spiked with AM251 at 250 ng/ml; and (c) the pooled human plasma spiked with surinabant at 500 ng/mL and AM251 at 250 ng/mL. The experimental conditions were described in the LC-ESI-MS/MS methods section.

The fragmentation of surinabant produced a predominant product ion of 100 amu less than its parent ion (Fig. 4b), which displayed an identical pattern as those of rimonabant and AM251 in our previous work.[14] Therefore, the fragmentation reaction of surinabant is proposed in Fig. 5.

The recovery studies were conducted using the protein precipitation procedure and LC-ESI-MS/MS method with surinabant plasma controls and surinabant reference solutions at three different concentrations (7.50, 75.0 and 750 ng/mL). As shown in Table 1, the mean relative recoveries of surinabant ranged 96.0–101%.

The precision (%CV) determined by the triplicate measurements of the peak area ratio of surinabant to the internal standard from five separate samples at each of the three concentrations was $\leq$8% (Table 1).

The linear calibration range of the LC-ESI-MS/MS method was 5.00 to 1000 ng/mL with a correlation coefficient of 0.999. A calibration equation, $y = 0.00373x - 0.00941$, was derived from the average peak area ratio of three separate injections from a single sample at each of the six concentrations (5.00, 10.0, 50.0, 100, 500 and 1000 ng/mL) over the calibration range. Accuracies were 92.3, 87.2 and 92.6% at the concentrations of 7.50, 75.0 and 750 ng/mL by triplicate measurements. The LLOQ for the LC-ESI-MS/MS method was determined to be 5.00 ng/mL with a CV and an accuracy of 6 and 90.9%, respectively.

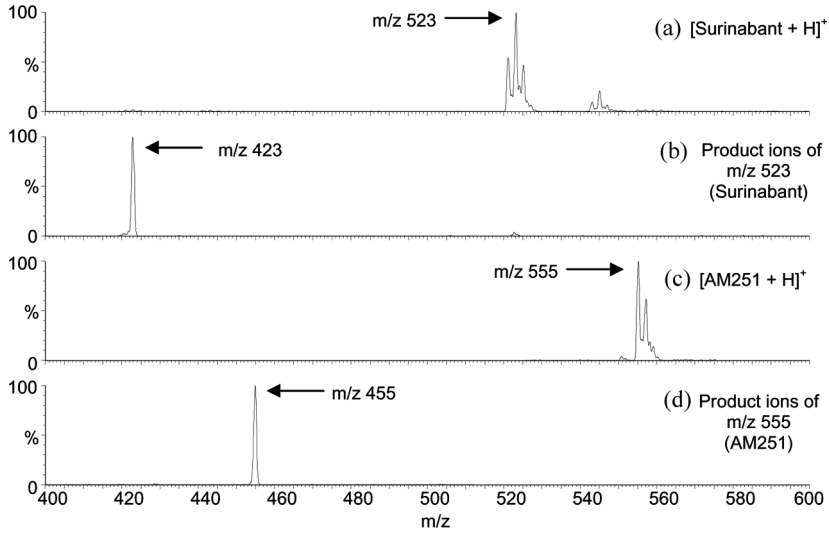

Figure 4. The mass spectra of surinabant and AM251 by the positive electrospray ionization. (a) The mass spectrum of surinabant; (b) the daughter spectrum of m/z 523 (surinabant); (c) the mass spectrum of AM251; and (d) the daughter spectrum of m/z 555 (AM251). The experimental conditions were described in the LC-ESI-MS/MS methods section and the concentrations of surinabant and AM251 were 2.00 μg/mL.

In comparison to the LC-UV method, the LC-ESI-MS/MS method has a lower detection limit, higher selectivity and specificity. It is well suited for the measurement of surinabant in complex biological matrices.

Stability

The stability of surinabant in plasma was determined by three aliquots at each low and high controls after three freeze (−20°C) and thaw (room

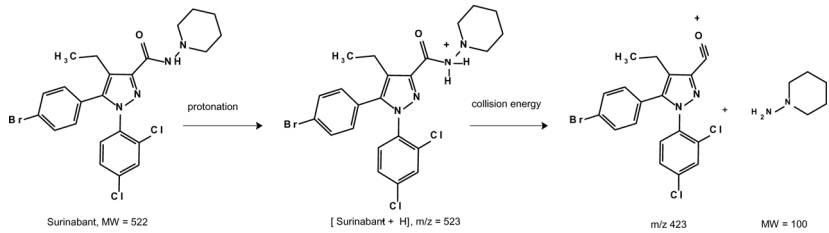

Figure 5. The proposed fragmentation scheme of surinabant.

temperature) cycles. The results showed no significant degradation with relative errors 4 and 6% for the low and high controls, respectively. The short-term temperature stability of surinabant in plasma was assessed by storing the surinabant plasma controls at room temperature for 24 h. For this experiment, the maximum relative error observed was 4% which indicated that surinabant plasma samples were stable at room temperature for at least 24 h. A study was conducted to ascertain the necessity of storing surinabant samples in amber vials to protect the compound from light degradation. Surinabant plasma controls stored in amber versus clear vials for six weeks at −20°C gave a maximum relative error of 7% which showed that surinabant could be stored safely under these conditions without protection from light.

CONCLUSIONS

Two methods, an LC-UV and an LC-ESI^+-MS/MS method have been developed and validated for the quantitative measurement of surinabant in human plasma using AM251 as the internal standard. Plasma samples were prepared by a protein precipitation procedure with acetonitrile, which resulted in recoveries of surinabant (95.0–103%). Chromatographic separation of surinabant and internal standard was carried out on a Waters YMC^{TM} Pro C4 cartridge column with a mobile phase containing 99.9% CH_3CN/H_2O (50:50, v/v) and 0.1% HCOOH. The LC-UV detection was accomplished at 258 nm for both surinabant and the internal standard, which had an LLOQ of 100 ng/mL and a calibration range of 100–1500 ng/mL for surinabant. The LC-ESI-MS/MS detection was achieved by positive MRM mode at m/z $523 \rightarrow 423$ for surinabant and m/z $555 \rightarrow 455$ for the internal standard, which had an LLOQ of 5.00 ng/mL and a calibration curve of 5.00–1000 ng/mL for surinabant. The low limits of detection achieved with these methods allow for their applications to a variety of studies in pharmacology, toxicology and biochemical mechanisms of surinabant.

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