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A strategy for high-throughput analysis of levosimendan and its metabolites in human plasma samples using sequential negative and positive ionization liquid chromatography/tandem mass spectrometric detection

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Levosimendan (Simdax®) is an approved drug in approximately 40 countries and currently in phase III clinical studies in the USA and Europe. An accurate, high-throughput and rugged assay is critical to support these clinical trials. Due to the mechanism of drug metabolism, the drug and its active metabolites often have significant differences in their chemical properties. In order to achieve high assay throughput and low sample volumes, a single bioanalytical assay for the drug and its metabolites is preferred. However, this need may prevent the optimization of both high-performance liquid chromatography (HPLC) and mass spectrometric ionization conditions. The chemical properties of levosimendan are significantly different from those of its two active metabolites, OR-1855 and OR-1896. Here, we present a novel strategy for high-throughput analysis of levosimendan and its metabolites. A 96-well liquid/liquid extraction procedure was developed for sample preparation. A single liquid chromatography/tandem mass spectrometry (LC/MS/MS) system with two separate mobile phases, shared backwash solvent and conditioning solvent, was developed to perform sequential LC separation for levosimendan and the metabolites. Levosimendan was eluted by 5 mM ammonium acetate in 33.3% acetonitrile and detected using negative ionization mode MS/MS monitoring. The metabolites were eluted by 5 mM ammonium acetate and 0.2% acetic acid in 20% acetonitrile and detected with positive ionization mode MS/MS monitoring. The method has been demonstrated to have excellent precision and accuracy, with high assay ruggedness during method validation and clinical sample analysis. The linear dynamic ranges were approximately 200–50 000 pg/mL for levosimendan and approximately 500–130 000 pg/mL for both metabolites. The coefficient of determination (r^2) for all analytes was greater than 0.9985. The intra-assay %CVs for QC samples were from 0.9% to 2.0% for levosimendan, 0.9% to 3.2% for OR-1855, and 0.4% to 4.9% for OR-1896. The inter-assay %CVs for QC samples were from 1.2% to 1.8% for levosimendan, 1.3% to 2.7% for OR-1855, and 1.4% to 3.4% for OR-1896. The mean % biases for QC samples were from 1.5% to 5.5% for levosimendan, −1.4% to 2.6% for OR-1855, and −0.3% to 4.5% for OR-1896. By using a single extraction approach coupled with sequential LC/MS/MS analysis for levosimendan and its metabolites, the assay maintained high throughput and low sample volume usage.

Levosimendan (Simdax®) is a novel intravenous positive inotropic agent that has been approved for the therapy of management for severe heart failure in approximately 40 countries, and is currently in phase III clinical studies in the USA and Europe.[1–3] An accurate, high-throughput, and robust assay is critical to support these clinical trials. However, to our knowledge there are only a select few publications that describe assays for the measurement of levosimendan in human plasma samples, although individual labs may have validated assays. Li *et al*. have also described a chiral separation capillary electrophoresis method for the quantitative analysis of levosimendan with an assay range from 25–500 μg/mL.[4] Karlsson *et al*. have described an automated online dialysis chromatographic assay using a Gilson ASTED system, with a lower limit of quantitation (LLOQ) of 5 ng/mL.[5]

Liquid chromatography/tandem mass spectrometry (LC/MS/MS) has been routinely used for the quantitative analysis of drugs and their metabolites for preclinical and clinical studies.[6,7] Liquid/liquid extraction (LLE) is one of the primary sample extraction procedures prior to LC/MS/

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MS detection.[8–24] LLE generally provides cleaner extracts than solid-phase extraction (SPE) and protein precipitation processes. LLE is also more cost effective than SPE, particularly if non-halogenated extraction solvents such as hexane and ethyl acetate are used. In our laboratory, we have developed reliable, rugged, 96-well format, semi-automatic LLE procedures for other drug samples.[25,26] Here we report a 96-well format LLE MS/MS assay for the quantitative analysis of levosimendan and its two active metabolites, OR-1855 and OR-1896, the chemical structures of which are presented in Fig. 1. A novel strategy utilizing a single extraction and two LC injections for negative and positive ionization, respectively, was also implemented during assay development and validation.

Due to varying mechanisms of drug metabolism, a given drug and its active metabolites often have significant differences in their chemical properties. In order to achieve high assay throughput and low sample volume usage, a single bioanalytical assay for the drug and metabolites is desired. However, this need may prevent the optimization of both high-performance liquid chromatography (HPLC) and mass spectrometric conditions. The properties of levosimendan are significantly different from those of its two active metabolites, OR-1855 and OR-1896. While negative electrospray ionization (ESI) gives excellent sensitivity for levosimendan, positive ionization gives increased sensitivity for OR-1855 and OR-1896 relative to negative mode. The optimized mobile phase conditions for chromatography and ESI are also dissimilar for levosimendan and the metabolites. The 96-well LLE method was developed to extract levosimendan and the metabolites simultaneously.

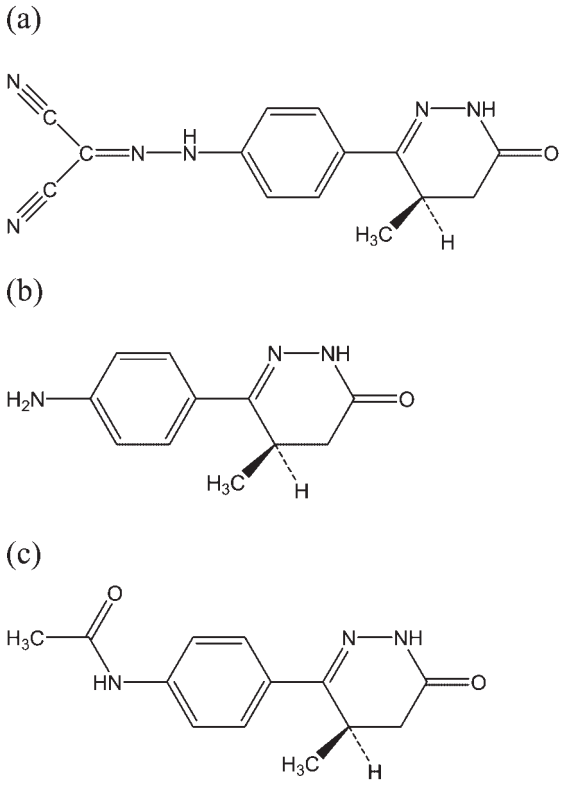

Figure 1. Chemical structures of (a) levosimendan, (b) OR-1855, and (c) OR-1896.

An HPLC system was configured to have separate injections and to deliver the corresponding mobile phases for the analysis of levosimendan and two metabolites. The backwash and hardware configurations are shared by these injections. The injection volumes are also tailored to provide sufficient signal-to-noise ratio for both LC runs. Levosimendan and metabolite analysis can be completed sequentially from the same reconstituted extracts without manual intervention.

As demonstrated during assay validation and sample analysis for clinical studies, this strategy provides optimized usage of sample volume and sample extraction time, along with rugged, precise, and reproducible assay performance. This strategy could also be used as an example for other drugs that have significantly different properties from their metabolites.

EXPERIMENTAL

Chemicals and reagents

Methanol, acetonitrile, hexane, and ethyl acetate, all HPLC grade, were purchased from EMD (Gibbstown, NJ, USA). Glacial acetic acid was also supplied by EMD. Ammonium acetate, A.C.S. grade, was supplied by J.T. Baker (Phillipsburg, NJ, USA). Normal human plasma with potassium (K) EDTA as anticoagulant (NHP-K EDTA) was supplied by Biological Specialty Corporation (Colmar, PA, USA). Reference materials for levosimendan, OR-1855, OR-1896, and the internal standards d_3-OR-1259, d_3-OR-1539, and d_3-OR-1896 were supplied by Orion Corporation (Espoo, Finland).

Instrumentation

Plasma samples were transferred using a Hamilton (Reno, NV, USA) Microlab AT2 Plus automated liquid handler, which was also used for addition of internal standards, ammonium acetate buffer, and extraction solvent. The Shimadzu (Kyoto, Japan) HPLC system included two Shimadzu LC-10 AD VP HPLC pumps, and a Shimadzu SIL-HTc autosampler with integrated system controller. An 1100 series HPLC pump and degasser system from Agilent Technologies (Palo Alto, CA, USA) was used to deliver backwash solvent and conditioning solvent for the pre-column regeneration. The EHMA model valves used to control LC flow between the mass spectrometer inlet and waste line were from Valco Instruments (Houston, TX, USA). An API-4000 mass spectrometer, ESI probe, and computer control system were from MDS Sciex (Toronto, ON, Canada). A YMC (Milford, MA, USA) AQ C18 2.0×150 mm 5 μm column was used for LC separation, along with a pre-column consisting of an Opti-Lynx Reliasil C18 2.1×10 mm guard cartridge from Optimize Technologies (Oregon City, OR, USA), and an Upchurch (Oak Harbor, WA, USA) SS Blu $0.188 \times 0.024 \times 0.254''$ frit. Additional items used included extraction plates from Marsh Biomedical (Rochester, NY, USA), AB-0813 polypropylene-aluminum film and an AB-0384 heat sealer from ABgene (Epsom, UK), and a VX-2500 multi-tube vortexer from VWR (West Chester, PA, USA). Analyst version 1.3.2 was used for data acquisition and for integration of chromatograms.

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Preparation of standard and quality control samples (QCs)

Separate stock solutions were used in preparations of standards and QCs. Working solutions were prepared by diluting stock solutions with 1:1 acetonitrile/water. Standard levels 1 to 10 were prepared by adding the appropriate volume of working solution into a class A volumetric flask and diluting to the mark with pooled NHP-K EDTA. QC levels 1 to 5 were prepared in the same manner. Standards and QCs were then aliquoted into polypropylene tubes and stored in freezers maintained at approximately −70°C. Additional QCs were stored at approximately −20°C for the purpose of stability evaluation. The concentration ranges of the prepared standards were from 200.13–50 032.40 pg/mL for levosimendan, from 504.04–126 009.60 pg/mL for OR-1855, and from 533.75–133 440.00 pg/mL for OR-1896.

Sample extraction

Samples were thawed at room temperature, followed by mixing to ensure homogeneity. Volumes of 300 μL of each plasma sample were loaded into the appropriate wells of a 96-well plate using the Hamilton automated liquid handler. The Hamilton was then used for the addition of 25 μL of 25 mM ammonium acetate, followed by 1200 μL of 80:20 ethyl acetate/hexane as extraction solvent. The plate was then sealed with polypropylene/aluminum film and shaken for 5 min using a multi-tube vortexer. After centrifugation at 4000 rpm for 5 min, the heat seal was punctured and 900 μL of the organic layer was transferred using the Hamilton to a clean 96-well plate. The organic extract was then dried under nitrogen at room temperature, reconstituted with 200 μL of a 15% acetonitrile solution containing 5 mM ammonium acetate, sealed with a 96-well plate cap map, and shaken using the multi-tube vortexer for approximately 3 min. Samples were then injected onto the LC/MS/MS system, using 10 μL for levosimendan and 60 μL for the metabolites.

Chromatographic conditions

Isocratic HPLC methods were employed for separation of both levosimendan and the metabolites. The mobile phase for levosimendan consisted of 5 mM ammonium acetate in 33.3% acetonitrile/water, and the mobile phase for the metabolites consisted of 5 mM ammonium acetate and 0.2% (by volume) of acetic acid in a 20% acetonitrile/water solution. The flow rates for both acquisitions were set at 0.35 mL/min.

Two LC pumps were configured to deliver the two separate mobile phases for levosimendan and the metabolites. They were controlled by one Shimadzu controller, and connected via a 'T' mixer connection prior to the inlet of the autosampler. The remaining autosampler, pre-column, and column configurations are identical to those described in our previous publication.[21] During sample analysis, the LC controller is programmed to utilize the appropriate pump and mobile phase for injection of either levosimendan or metabolites, and the two injections can be done sequentially without additional intervention.

Timing for levosimendan acquisition was such that at 0.5 min the guard column was switched offline and washed at 2.0 mL/min with backwash solvent (acetonitrile/water, 95:5), followed by conditioning solvent. At 1.6 min, flow was diverted from waste to the mass spectrometer, followed by initiation of data acquisition at 1.8 min for a 1.5-min period. At 2.0 min, the backwash pump was switched from backwash solvent A to conditioning solvent for re-equilibration, which contained 5 mM ammonium acetate in a 20% acetonitrile/water solution. At 3.8 min, the guard column was switched back online, followed by diversion from the mass spectrometer to waste at 4.3 min.

Timing for the metabolite acquisition was designed similarly to that for levosimendan, but is shifted slightly later. At 0.8 min the guard column was switched offline and washed at 2.0 mL/min with backwash solvent and then conditioning solvent. At 2.2 min, flow was diverted from waste to the mass spectrometer. At 2.3 min, the backwash pump was switched from backwash solvent to conditioning solvent, followed by initiation of data acquisition at 2.9 min for a 1.8-min period. At 4.2 min, the guard column was switched back online, followed by diversion from the mass spectrometer to waste at 4.6 min.

MS/MS detection

By taking advantage of different ionization modes, LC/MS/MS detection was performed using a MDS Sciex API 4000 triple quadrupole mass spectrometer with an ESI Turboionspray ionization probe, operated in negative mode for levosimendan and positive mode for the metabolites. The computer control system was Analyst version 1.3.2. For levosimendan, the multiple reaction monitoring (MRM) detection channel was m/z 279.1 → 227.2. Typical source settings were 10 for the CUR gas, 35 for GS1, 75 for GS2, 12 for CAD, and 500°C for the source temperature. Additional MS parameters were −95 for DP, −3 for EP, −30.6 for CE, and −10.2 for CXP. For the metabolites, MRM mass channels were m/z 204.2 → 159.1 for OR-1855 and m/z 246.3 → 204.2 for OR-1896. Source settings for the metabolites were 10 for the CUR gas, 60 for GS1, 80 for GS2, 9 for CAD, and 500°C for the source temperature. Additional MS parameters for OR-1855 were 55 for DP, 10 for EP, 31 for CE, and 8.9 for CXP. Additional MS parameters for OR-1896 were 70 for DP, 10.7 for EP, 31.6 for CE, and 12.2 for CXP. Because internal standards are d_3-deuterated analogs, mass channels monitored for the internal standards are set 3 units higher than their respective analytes, and use the same spectrometric conditions.

Quantitation

D_3-OR-1259, d_3-OR-1539, and d_3-OR-1896 were used as internal standards for levosimendan, OR-1855, and OR-1896, respectively. Peak areas for the analytes and internal standards were determined using Analyst 1.3.2 and loaded into the Watson LIMS system. For each analytical batch, a calibration curve was derived from the peak area ratios (analyte/internal standard) using weighted linear least-squares regression of the area ratio versus the concentration of the standards. A weighting of $1/x^2$ (where x is the concentration of a given standard) was used for curve fit. The regression equation for the calibration curve was used to back-calculate the measured concentration for each standard and QC and the results were compared to the theoretical

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concentration to obtain the accuracy, expressed as a % bias from the theoretical value, for each standard and QC measured.

RESULTS AND DISCUSSION

Liquid/liquid sample extraction

The results of our investigation into the extraction efficiency of levosimendan and its metabolites are shown in Fig. 2. Hexane and ethyl acetate were used as extraction solvents. While hexane generally provides better extraction efficiency for non-polar compounds, ethyl acetate generally gives better efficiency for polar compounds. The pH of the extraction mixture was varied by adding 50 μL of either 0.01% acetic acid in 25 mM ammonium acetate buffer, 0.05% acetic acid in 25 mM ammonium acetate buffer, 0.1% ammonium hydroxide in 25 mM ammonium acetate buffer, or 25 mM ammonium acetate by itself. As shown in Fig. 2, the extraction efficiencies for all three analytes are unchanged regardless of the buffer used. Additionally, 100% ethyl acetate as the extraction solvent provided the highest recovery for all three analytes. However, when 100% ethyl acetate was used as extraction solvent, an opaque layer between the aqueous and organic phases was formed which prevented a clean transfer of the organic layer into the 96-well plate used for dry-down. Increasing the percentage of hexane reduces the thickness of this opaque layer. As a result, 80:20 ethyl acetate/hexane was selected as the extraction solvent without significant loss of extraction efficiency. Using these extraction conditions, all three analytes could be extracted simultaneously.

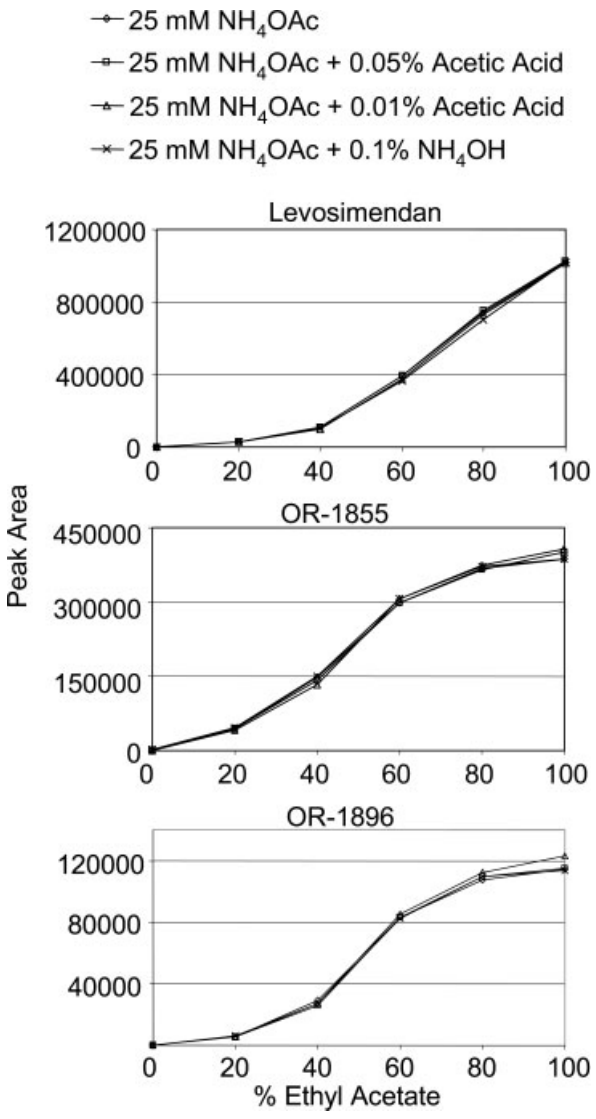

Figure 2. . Extraction efficiency test for levosimendan and metabolites.

LC/MS/MS detection

Because levosimendan and the metabolites are analyzed in two separate injections, the LC conditions could be tailored to give optimum chromatographic peak shape and proper compound retention so that the analytes were adequately separated from both the solvent front and endogenous matrix components. The separate mobile phases for levosimendan and the metabolites were also selected to give the best sensitivity for their respective compounds. It was found that high concentrations (i.e. 20 mM or higher) of ammonium acetate in the mobile phase greatly reduced signal intensity for both levosimendan and the metabolites. The retention time for levosimendan is approximately 2.3 min (data acquisition starts at 1.8 min). For the metabolites, retention times for OR-1855 and OR-1896 are approximately 3.3 and 3.8 min, respectively (data collection begins at 2.9 min).

The setup of autosampler, LC pumps and controller is critical to achieve sequential analysis of both levosimendan and the metabolites without manual intervention. A mixer was selected as a 'T' connection from both LC pumps that only allowed one mobile phase to flow through the autosampler and into the column without the use of an additional switching valve. The guard column was reconditioned after backwash and prior to being switched back inline with the analytical column for the next sample injection. The conditioning solvent was selected to be common for the analysis of both levosimendan and the metabolites.

Mass spectra and tandem mass spectra were obtained by infusion of levosimendan and the metabolites individually via a 'T' connection between the LC column and the mass spectrometer inlet. The tandem mass spectra are shown in Fig. 3. While the deprotonated peak is the predominant form of the molecular ion for levosimendan, the protonated peaks are the primary forms of the molecular ion for the two metabolites. Increasing the declustering potential (DP) will generally create source fragmentations for all three analytes (results not presented here). MS/MS spectra give a major product ion at *m/z* 227 for levosimendan. In addition to the primary MS/MS fragment ions at *m/z* 159 for OR-1855 and at *m/z* 204 for OR-1896, several other intense fragment ions are generated for both OR-1855 and OR-1896. The statistical distribution of the precursor charges to the fragments is one of the reasons for the relatively low sensitivity of both metabolites. The sensitivity of levosimendan, however, greatly benefits from the negative ionization mode that provides a high ionization efficiency, low background noise level, and a preferentially formed product ion. The d_3

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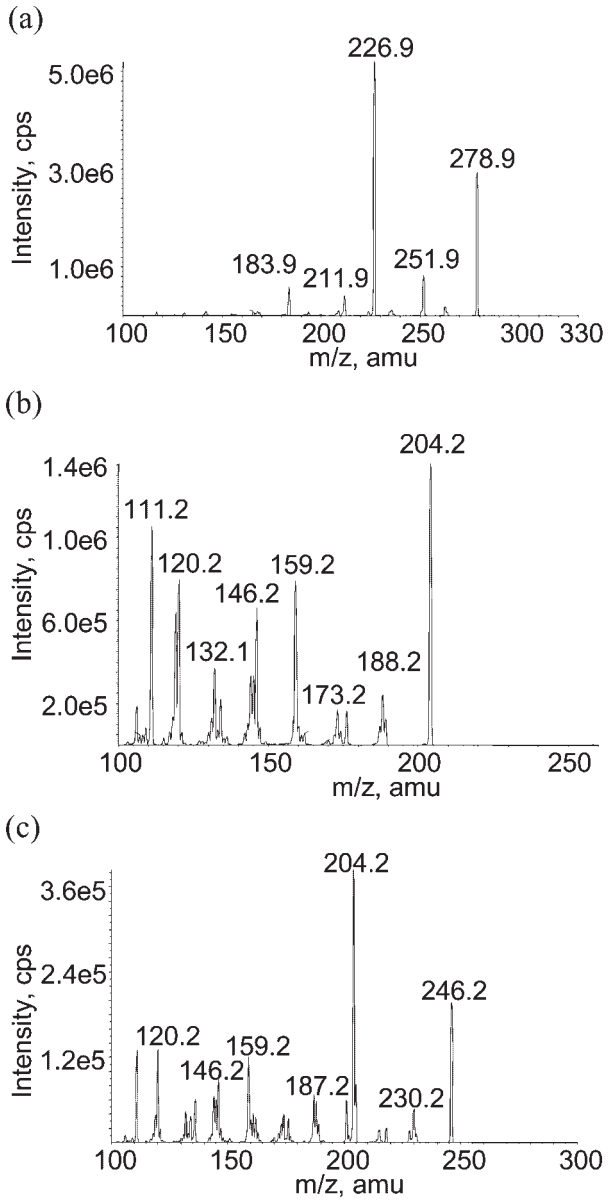

Figure 3. MS product ion spectra of (a) levosimendan, (b) OR-1855, and (c) OR-1896.

isotopically labeled internal standards for levosimendan and the metabolites give molecular and product ions at 3 *m/z* units above their respective analytes (spectra not shown).

Assay validation

The validation experiments were designed with reference to '*Guidance for Industry – Bioanalytical Method Validation*' recommended by the Food and Drug Administration of the United States.[27] The experimental design and results of some important criteria of method validation are presented in the following sections.

Linearity

The evaluation of the linearity of the calibration curve was obtained from three consecutively prepared batches. The linear dynamic range evaluated was from 200.13–50 032.40 pg/mL for levosimendan, from 504.04–126 009.60 pg/mL for OR-1855, and from 533.75–133 440.00 pg/mL for OR-1896. Minimum and maximum correlation coefficients (r^2), along with the mean back-calculated concentrations and mean biases of the standards, are presented in Table 1.

Accuracy and precision of QCs, LLOQ, and ULOQ samples

Eighteen replicates of QCs, LLOQ (lower limit of quantitation), and ULOQ (upper limit of quantitation) samples from three consecutive runs were used to evaluate the precision and accuracy at each concentration level. LLOQ and ULOQ samples are replicates of standard 1 and 10, respectively, but are not used in generation of the standard curve. Results indicate a high degree of accuracy and precision, with 270 QC results from 90 QCs within ±14.3% of theoretical for all three analytes, 54 results from 18 LLOQ samples within ±11.1% of theoretical for all three analytes, and 54 results from 18 ULOQ samples being within ±4.5% of theoretical for all three analytes. Additionally, inter-run % coefficient of variance (CV) values of a given concentration for all replicates of all analytes were less than or equal to 4.0%. Inter-run mean back-calculated concentrations, mean biases, and %CV values of QCs are presented in Table 2. Corresponding data for LLOQ and ULOQ samples are given in Table 3.

Selectivity

Selectivity was evaluated by extracting blank plasma samples from six different lots of matrix and comparing the response at the retention times of the analytes to the response at the LLOQ. Selectivity was evaluated for blank plasma samples both with and without deuterated internal standard. Additionally, due to the molecular weight of ibuprofen (MW = 206.29) being near to the *m/z* values monitored for OR-1855, OR-1896, and their internal standards, selectivity was also evaluated for plasma samples spiked with 30 μg/mL of ibuprofen, approximately equal to plasma concentrations of a standard 400 mg over-the-counter ibuprofen dose. No peaks were observed for all blank plasma samples.

Extraction recovery

In order to determine extraction recovery, three recovery control solutions were prepared in 1:1 acetonitrile/water at the concentrations of QCs 1, 3, and 5. Volumes of 300 μL of recovery control solution were added to extracted NHP-K EDTA with internal standard prior to dry-down. The area ratios (analyte/internal standard) for the recovery controls were then determined at each level, and compared to the area ratios obtained from extracted QCs at the corresponding level. Extraction recovery was calculated by dividing the area ratios of individual QCs by the mean area ratio of the recovery control solutions. Overall mean extraction recoveries ranged from 19.2–36.0% for levosimendan, from 33.3–62.0% for OR-1855, and from 31.9–55.5% for OR-1896. Although extraction recoveries are considerably less than the theoretical maximum of 75%, as determined from volumes of extraction solvent added and recovered, extraction recovery is still sufficient for accurate and reproducible results throughout the assay range due to a use of stable isotope labeled internal standards.

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Table 1. Mean concentrations, mean % biases, and r^2 ranges of calibration curves (n = 3)

	Std 1		Std 2		Std 3		Std 4	
Analyte	Mean conc.	Mean % bias	Mean conc.	Mean % bias	Mean conc.	Mean % bias	Mean conc.	Mean % bias
Levosimendan	195.61	−0.2	413.22	3.2	614.40	2.3	1181.60	−1.6
OR-1855	493.74	−2.0	1038.01	3.0	1526.34	0.9	3036.47	0.4
OR-1896	522.25	−2.2	1076.60	1.1	1658.35	3.6	3241.85	1.2

	Std 5		Std 6		Std 7		Std 8	
Analyte	Mean conc.	Mean % bias	Mean conc.	Mean % bias	Mean conc.	Mean % bias	Mean conc.	Mean % bias
Levosimendan	2414.40	0.5	6471.19	3.5	12482.46	−0.2	24774.47	−1.0
OR-1855	6149.16	1.7	16174.65	2.7	31227.86	−0.9	61774.76	−2.0
OR-1896	6577.73	2.7	16900.09	1.3	32860.69	−1.5	65951.03	−1.2

	Std 9		Std 10		r^2	
Analyte	Mean conc.	Mean % bias	Mean conc.	Mean % bias	Minimum	Maximum
Levosimendan	39401.60	−1.6	48533.41	−3.0	0.998814	0.999355
OR-1855	98833.27	−2.0	123688.58	−1.8	0.998514	0.999487
OR-1896	104433.49	−2.2	130079.08	−4.5	0.998793	0.999612

Matrix effect

The effect of the plasma-anticoagulant matrix on the concentration determination of the analytes was investigated by preparing samples at the concentrations of QC 1 in multiple lots of unpooled matrix. The absence of any significant matrix effect was confirmed by 15 different lots of matrix having mean biases less than 15%.

Dilution

The suitability of study samples being diluted with drug-free plasma on the day of assay without undergoing an additional freeze/thaw cycle was evaluated as part of the validation. A QC level used specifically for dilutions was prepared with the concentrations of the three analytes being above the ULOQs during validation, at 63 245.00 pg/mL for levosimendan, 147 812.00 pg/mL for OR-1855, and 147 480.00 pg/mL for OR-1896. Prior to loading into the 96-well plate, 60 μL of dilution QC were mixed with 540 μL of blank matrix to achieve a 10× dilution. The diluted QC was then loaded into the plate and extracted as normal. Results for dilution QCs gave mean % biases of −6.8%, −10.6%, and −7.8% for levosimendan, OR-1855, and OR-1896, respectively. Corresponding CVs were 3.5%, 2.5%, and 3.5%.

Table 2. Inter-run mean concentrations, mean % biases, and % CVs for QC samples (n = 18)

	QC 1			QC 2			QC 3		
Analyte	Mean conc.	Mean % bias	% CV	Mean conc.	Mean % bias	% CV	Mean conc.	Mean % bias	% CV
Levosimendan	426.91	5.5	1.8	512.32	5.5	1.5	5232.05	3.4	1.2
OR-1855	970.90	2.6	2.6	1160.89	2.3	2.7	11829.20	0.0	1.3
OR-1896	986.59	4.5	2.7	1175.60	3.8	3.4	11893.70	0.8	1.9

	QC 4			QC 5		
Analyte	Mean conc.	Mean % bias	% CV	Mean conc.	Mean % bias	% CV
Levosimendan	20507.40	1.3	1.7	39866.47	−1.5	1.5
OR-1855	47522.78	0.5	1.8	93309.63	−1.4	1.4
OR-1896	47656.90	1.0	1.3	94070.82	−0.3	1.8

Table 3. Inter-run mean concentrations, mean % biases, and % CVs for LLOQ and ULOQ samples (n = 18)

	LLOQ			ULOQ		
Analyte	Mean conc.	Mean % bias	% CV	Mean conc.	Mean % bias	% CV
Levosimendan	193.05	−3.5	2.8	48978.09	−2.1	1.6
OR-1855	506.61	−0.1	2.5	124302.36	−1.4	2.1
OR-1896	520.82	−2.4	4.0	131223.76	−1.7	1.8

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Stability

To demonstrate that accurate measurement of analyte concentrations will not be compromised by degradation of the analytes, samples were tested under various conditions to establish stability throughout the sample collection, extraction, and analysis process.

Frozen storage stability of NHP-K EDTA samples was evaluated as follows. Multiple sets of stability QCs were prepared and stored at both −20°C and −70°C. For initial testing, one set of QCs was assayed in triplicate and quantitated using a set of calibration standards prepared on the same date as the QCs. After a documented period of time in frozen storage, the stability of QCs was retested using newly prepared calibration standards. The mean concentrations of each QC level were then compared to the mean concentrations determined from the initial testing. The time difference between the initial testing of stability QCs and the preparation date of the calibration standards used for retesting was the established frozen storage stability period. Stability has been established for 143 days at −20°C, with mean % differences for stability QCs being less than or equal to ±9.7% for levosimendan, ±2.2% for OR-1855, and ±2.5% for OR-1896. Stability has also been established for 143 days at −70°C, with mean % differences for stability QCs being less than or equal to ±1.4% for levosimendan, ±2.6% for OR-1855, and ±3.8% for OR-1896.

In order to demonstrate suitability for samples undergoing multiple assays, the stability of samples subjected to multiple freeze/thaw cycles with a corresponding period at room temperature was evaluated as follows. Freeze/thaw stability QCs were subjected to multiple freeze/thaw cycles to simulate conditions that would occur during sample analysis. The freeze/thaw QCs were completely thawed at room temperature, allowed to remain at room temperature for a documented period of time, and then placed back in storage. The samples remained in the freezer for at least 12 h before being removed for another thaw. The freeze/thaw stability QCs were then assayed in a single batch along with standards and QCs that had undergone only one freeze/thaw cycle, with concentrations of all QCs being calculated from the same calibration curve. The mean concentrations of the freeze/thaw stability QCs were then compared to the mean concentrations of QCs that underwent only one freeze/thaw cycle. Freeze/thaw stability was investigated for samples stored at both −20°C and −70°C, with a total time at room temperature of 28 h. For samples stored at −20°C, % differences were within ±2.1% for levosimendan, ±1.8% for OR-1855, and ±2.4% for OR-1896. For samples stored at −70°C, % differences were within ±1.6% for levosimendan, ±1.8% for OR-1855, and ±1.2% for OR-1896.

Autosampler stability, or the stability of the analytes in reconstitution solution during injection, was tested in the following manner. A batch consisting of standards and QCs was injected onto the LC/MS/MS system. After a period of storage in the autosampler, the batch was reinjected, and the reinjected QCs quantitated using the originally injected standard curve. The time difference between the first injection of the final QC and the reinjection of the final QC was the demonstrated autosampler stability period, and was successfully established for approximately 120 h in an autosampler set at 10°C. Mean % biases were less than or equal to ±0.5% for levosimendan, ±3.1% for OR-1855, and ±2.9% for OR-1896.

The suitability of prepared samples to be stored before injection or reinjection, known as batch storage stability, was established by preparing a batch and storing it in the autosampler for a documented period of time prior to analysis. The time difference between the completion of sample preparation and injection of the batch was the demonstrated batch storage stability period. Injection of a stored batch after 126 h at 10°C in the autosampler gave mean % biases of less than or equal to ±6.2% for levosimendan, ±3.5% for OR-1855, and ±1.6% for OR-1896.

Application to clinical samples

This validated assay has been applied in the concentration determination of levosimendan, OR-1855, and OR-1896 in human plasma for clinical studies. The absence of quantifiable concentrations in all pre-dose samples further proved the selectivity of the assay. Sample LC/MS/MS chromatograms from a patient sample after infusion are shown in Fig. 4. Inter-run QC performance during sample analysis is

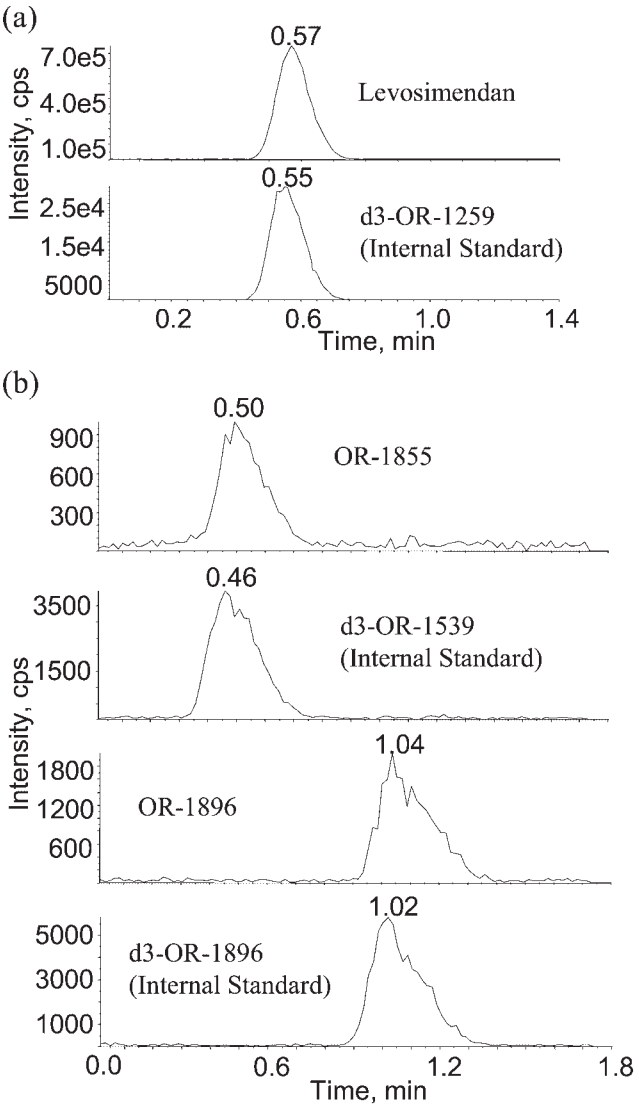

Figure 4. LC/MS/MS chromatograms of (a) levosimendan and (b) metabolites from a clinical subject after a 1-h infusion.

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Table 4. QC performance during sample analysis (n = 28 for levosimendan, n = 20 for OR-1855 and OR-1896)

	QC 1			QC 2			QC 3		
Analyte	Mean conc.	Mean % bias	% CV	Mean conc.	Mean % bias	% CV	Mean conc.	Mean % bias	% CV
Levosimendan	485.27	3.4	1.8	1951.33	3.9	1.4	6039.76	2.9	1.4
OR-1855	1224.10	1.1	2.7	4939.38	2.0	3.7	15262.50	0.9	2.6
OR-1896	1177.42	1.4	3.7	4721.87	1.6	2.3	14547.92	0.2	1.6

	QC 4			QC 5		
Analyte	Mean conc.	Mean % bias	% CV	Mean conc.	Mean % bias	% CV
Levosimendan	23785.98	0.2	2.0	42109.71	−0.3	1.6
OR-1855	61236.58	1.2	3.2	108682.38	−0.3	2.7
OR-1896	57712.91	−0.6	1.7	104265.81	−0.3	2.4

presented in Table 4. The ruggedness of the assay was further demonstrated by having 170 results from 70 standard samples within ±10.9 of their theoretical values for all three analytes, and 340 QC results from 140 QC samples within ±9.5% of their theoretical values for all three analytes. Mean % biases for QC samples were less than or equal to ±3.9% for levosimendan, ±2.0% for OR-1855, and ±1.6% for OR-1896. Additionally, this method has been successfully transferred for sample analysis of clinical samples and plasma protein binding assays at a Contract Research Organization (CRO).

CONCLUSIONS

Here we have reported a rugged LC/MS/MS method and its application for the precise and accurate analysis of levosimendan and its metabolites for clinical studies. Due to their disparate chemical properties, separate LC conditions and mass spectrometric ionization modes were employed for the analysis of levosimendan and the metabolites. A semi-automated 96-well format liquid/liquid extraction method was developed for sample preparation of all three analytes. A strategy to combine these analyses into a single assay setup was fully implemented with the necessary configurations of autosampler, LC controller, LC pumps, and mobiles phases. This approach could also be used as a model to develop other methods utilizing sequential LC/MS/MS analysis of drug compounds and metabolites that may require separate LC and mass spectrometric conditions.

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