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Short communication

Development and validation of a sensitive LC/MS/MS method for the simultaneous determination of naloxone and its metabolites in mouse plasma

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ABSTRACT

A rapid, specific, and reliable LC–MS/MS based bioanalytical method was developed and validated for the simultaneous determination of naloxone (NLX) and its two metabolites, 6β-naloxol (NLL) and naloxone-3β-D-glucuronide (NLG) in mouse plasma. The optimal chromatographic behavior of these analytes was achieved on an Aquasil C18 column (50 mm × 2.1 mm, 5 μm) using reversed phase chromatography. The total LC analysis time per injection was 2.5 min with a flow rate of 1.0 mL/min with gradient elution. Sample preparation via protein precipitation with acetonitrile in a 96-well format was applied for analyses of these analytes. The analytes were monitored by electrospray ionization in positive ion multiple reaction monitoring (MRM) mode. Modification of collision energy besides chromatographic separation was applied to further eliminate interference peaks for NLL and NLG. The method validation was conducted over the curve range of 0.200/0.400/0.500 to 100/200/250 ng/mL for NLX/NLL/NLG, respectively, using 0.0250 mL of plasma sample. The intra- and inter-day precision and accuracy of the quality control samples at low, medium, and high concentration levels showed ≤6.5% relative standard deviation (RSD) and −8.3 to −2.5% relative error (RE). The method was successfully applied to determine the concentrations of NLX, NLL, and NLG in incurred mouse plasma samples.

1. Introduction

Naloxone (NLX), as shown in Fig. 1, is mainly used as a specific mu-opioid receptor antagonist for the treatment of opioid overdose and to reverse opioid-induced depression following surgery [1–3]. As shown in Fig. 1, 6α/β-naloxol (NLL) and naloxone-3β-D-glucuronide (NLG) are the metabolites of NLX. The potency of NLL to precipitate opioid withdrawal from acute morphine dependence has been reported [4]. It has also been demonstrated that NLG can antagonize the mobility-lowering effect of morphine in the rat colon [5,6]. Therefore, development of a bioanalytical method for the quantification of NLX, NLL, and NLG in plasma samples is needed to better understand their toxicokinetic and pharmacokinetic behaviors.

There are several high-performance liquid chromatography (HPLC)-based bioanalytical methods published on the quantification of NLX [7–11]. Most of these published methods showed long HPLC run times (over 10 min) and higher detection limits (greater than 2.00 ng/mL). In recent years, liquid chromatography–tandem mass spectrometry (LC–MS/MS)-based bioanalytical methods have also been used for the determination of NLX in biological fluids [12–14]. With MS detection, the detection limits were decreased to as low as 0.0250 ng/mL. However, long HPLC run times of greater than 10 min were needed in these published LC–MS/MS methods. Among these published LC–MS/MS methods, one has reported the quantification of both NLX and NLG [12]. No published reports are available yet with regard to the determination of NLL in biological samples.

The present study was undertaken to develop and validate a simple, high throughput, sensitive, and convenient bioanalytical assay for the simultaneous determination of NLX, NLL, and NLG in mouse plasma by LC–MS/MS. Subsequently, this validated LC–MS/MS method was applied to simultaneously quantify NLX, NLL, and NLG in incurred mouse plasma samples.

2. Experimental

2.1. Chemical, reagents, materials, and apparatus

NLX, NLL, NLG and their corresponding deuterated internal standards, including NLX-d_5, NLL-d_5, and NLG-d_5, with purities of 100%

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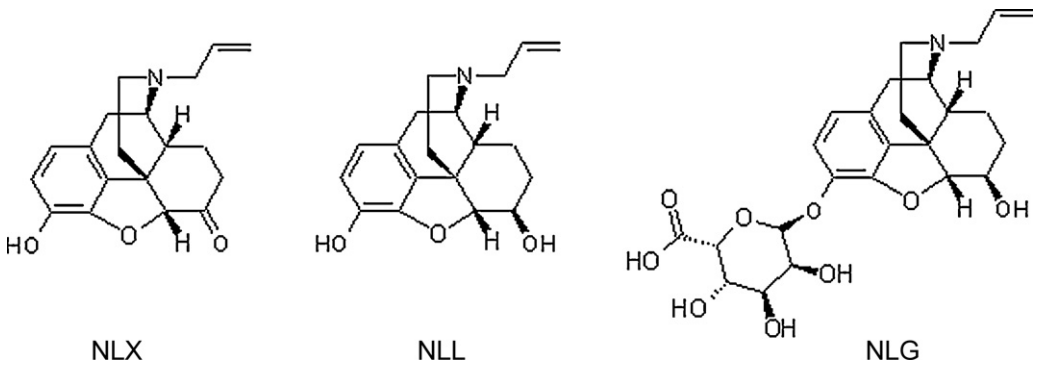

Fig. 1. Chemical structures of Naloxone (NLX), 6β-Naloxol (NLL), and Naloxone-3β-D-glucuronide (NLG).

were purchased from Cerilliant Corporation (Round Rock, TX, USA). HPLC grade acetonitrile and formic acid (≥96%) were obtained from Sigma–Aldrich (St. Louis, MO, USA). A PURELAB Ultra system from ELGA (Marlow, UK) was used in the laboratory to produce deionized water. Mouse plasma with K_2-EDTA as the anticoagulant was obtained from Biochemed (Winchester, VA, USA).

An automated SPE system (Quadra 96, model 96-320) for adding organic solvents (ethyl acetate) and transferring samples during sample preparation was obtained from Tomtec (Hamden, CT, USA). A 96-well sample concentrator (SPE DRY-96) with a temperature control from Jones Chromatography (Lakewood, CO, USA) was used for evaporating samples.

2.2. Chromatographic conditions

The HPLC system consisted of solvent delivery system LC-20AD, autosampler SIL-20AC, column oven CTO-20AC, degasser DGU-20A_3, and controller CBM-20A from Shimadzu (Kyoto, Japan). Chromatographic separation of NLX, NLL, NLG, NLX-d_5, NLL-d_5, and NLG-d_5 was evaluated on an Aquasil C18 column (50 mm × 2.1 mm, 5 μm) from Thermo Electron (Bellefonte, PA, USA) and several other columns including a Gemini C18 column (50 mm × 2.0 mm, 5 μm), a Synergi Polar-RP column (50 mm × 2.0 mm, 4 μm), a Synergi Max-RP column (50 mm × 2.0 mm, 4 μm) from Phenomenex (Torrance, CA, USA) for reversed phase chromatography and on a Kromasil silica column (50 mm × 3.0 mm, 5 μm) from Thermo Electron (Bellefonte, PA, USA) for hydrophilic interaction liquid chromatography during method development. Formic acid (0.1% in water) was used as mobile phase A (MA) and 0.1% formic acid in acetonitrile was used as mobile phase B (MB). For method validation and sample analysis, the chromatographic analysis was conducted using gradient elution on an Aquasil C18 column. The HPLC program for gradient elution was as follows: 2% of MB (0–0.1 min), from 2% to 75% of MB (0.1–1.2 min), 75% of MA (1.2–1.9 min), from 75% to 2% of MB (1.9–2.0 min), and 2% of MB (2.0–2.50 min). Separation was performed at a flow rate of 1.0 mL/min. Sample injection volume was 15 μL. Column temperature was set at 25 °C. The cycle time between two consecutive injections was approximately 3.0 min.

2.3. Mass spectrometric conditions

An API 5000 triple quadrupole mass spectrometer (MDS-Sciex, Concord, Canada) with Turboionspray™ (TIS) interface was operated in positive ionization mode with multiple reaction monitoring (MRM) for LC–MS/MS analyses. The mass spectrometric parameters were optimized to improve the MRM sensitivity. However, optimal collision energy was not applied to NLL and NLG to minimize some interference peaks near the analyte peaks of interest. The instrument parameters for monitoring NLX, NLL, NLG, NLX-d_5, NLL-d_5, and NLG-d_5 during method validation and sample analysis were as follows: TIS temperature, 600 °C; TIS voltage, 3500 V; curtain gas (CUR), nitrogen, 25; nebulizing gas (GS1), 80; TIS gas (GS2), 60; collision gas, 10; declustering potential (DP), 80 V; entrance potential (EP), 10 V; collision energy (CE), 52 eV for NLX, NLG, NLX-d_5, and NLG-d_5 and 45 eV for NLL and NLL-d_5; collision cell exit potential (CXP), 22 V. The following precursor to product ion transitions were used for the MRM of NLX, NLL, NLG, NLX-d_5, NLL-d_5, and NLG-d_5, respectively, m/z 328.2 → 212.1, m/z 330.2 → 294.1, m/z 504.2 → 310.1, m/z 333.1 → 212.1, m/z 335.2 → 299.1, and m/z 509.2 → 315.1 with dwell times of 20 ms. The mass spectrometer was operated at unit mass resolution for both the first and third quadrupoles.

2.4. Preparation of standard solutions

Stock solutions of NLX at 1.00 mg/mL, NLL at 1.00 mg/mL, and NLG at 0.100 mg/mL in methanol were prepared by the manufacturer. Similarly, NLX-d_5, NLL-d_5, and NLG-d_5 at 0.100 mg/mL in methanol were directly obtained from the manufacturers. Intermediate standard solutions at the desired concentration for the preparation of calibration curve and QC samples were made by serial dilution with acetonitrile/water (50/50, v/v) starting from their respective concentrated stock solutions. The intermediate internal standard solution (10.0/20.0/25.0 ng/mL) was diluted from the stock internal standard solution of NLX-d_5, NLL-d_5, and NLG-d_5 at 0.100 mg/mL with acetonitrile/water (50:50, v/v). These standard solutions were stored in glass vials and kept refrigerated (2–8 °C).

2.5. Preparation of calibration standards and quality control samples

Calibration standards were prepared daily by spiking an appropriate quantity of the intermediate standard solutions into mouse plasma. Eight calibration standards for NLX, NLL, and NLG, respectively, were prepared at 0.200/0.400/0.500, 0.400/0.800/1.00, 2.00/4.00/5.00, 5.00/10.0/12.5, 20.0/40.0/50.0, 50.0/100/125, 90.0/180/225, and 100/200/250 ng/mL. Quality control samples were prepared by spiking an appropriate amount of intermediate standard solutions into mouse plasma to reach the desired concentration with non-matrix composition less than 5% of the final volume. Lower limit of quantitation QC (LLOQ), low QC (LQC), medium QC (MQC), high QC (HQC), and dilution QC (DQC) were prepared at 0.200/0.400/0.500 ng/mL, 0.600/1.20/1.50 ng/mL, 10.0/20.0/25.0 ng/mL, 80.0/160/200 ng/mL, and 500/1000/1250 ng/mL, respectively, for NLX, NLL, and NLG. All QC samples were aliquoted into 1.4 mL polypropylene vials and stored at −20 °C.

2.6. Sample preparation

A volume of 25.0 μL of each calibration standard, QC sample, incurred sample, and blank matrix control sample was aliquoted into individual wells in a 96-well plate. Next, 25.0 μL of the intermediate internal standard solution of NLX-d_5, NLL-d_5, and NLG-d_5 at 10.0/20.0/25.0 ng/mL were added to individual wells containing samples with the exception of the blank control samples, to which 25.0 μL of acetonitrile/water (50/50, v/v) were added. Then 300 μL of acetonitrile were added to each sample. Vortexing at high speed for approximately 5 min was applied to mix the sample and precipitate proteins. The 96-well plate with samples was centrifuged at 3000 rpm for approximately 5 min. After centrifugation, 300 μL of the supernatant were transferred to a clean 96-well plate using an automated SPE system. The extract was evaporated to dryness using a 96-well sample concentrator (SPE DRY-96) set at 50 °C for about 15 min. The resulting dry residues were reconstituted in 100 μL of acetonitrile/water (2/98, v/v) for LC–MS/MS analysis.

2.7. Data analysis

Sciex Analyst software (version 1.5.1) was used for the data acquisition and analysis of NLX, NLL, and NLG. The calibration curves (analyte peak area/IS peak area versus analyte concentration) of NLX, NLL, and NLG were obtained based upon the least square linear regression fit ($y = mx + b$) with a weighting factor of $1/x^2$. The coefficient of determination (r^2) was set as >0.98 for the acceptance criteria of the calibration curves. The accuracy and precision were required to be within 100 ± 15% of the nominal concentration and ≤15% RSD, respectively, for LQC, MQC, HQC, and DQC samples while the accuracy and precision were required to be within 100 ± 20% of the nominal concentration and ≤20% RSD for LLOQ samples in the intra-batch and inter-batch assay.

2.8. Method validation

This study was performed in compliance with the principles of Good Laboratory Practice (GLP). NLX, NLL, and NLG were validated over the range of 0.200/0.400/0.500 ng/mL to 100/200/250 ng/mL for the LC–MS/MS assay. Matrix effect, specificity, sensitivity, carryover, linearity, precision, accuracy, dilution integrity, and stability were evaluated during method validation. The matrix effect was determined by calculating Matrix Factor (MF), which was obtained as a ratio of the analyte peak response in the presence of matrix ions to the analyte peak response in the absence of matrix ions by post-extraction spiking analyte at the MQC level into blank plasma extracts and blank water extracts. The specificity was assessed by testing 6 lots of blank plasma extract for the presence or absence of interference as. Sensitivity of analytes was determined by calculating the signal to noise ratios of LLOQ samples. Carryover of analytes was also evaluated by analyzing blank plasma extract samples immediately after an upper limit of quantification (ULOQ) sample or HQC sample. The linearity of the calibration curve was evaluated as described in Section 2.7.

The precision and accuracy of the method were assessed by the analyses of three separate batches of mouse plasma samples. Each batch consisted of one set of calibration standards (eight concentration levels) and six replicates of QC samples at each of LLOQ, LQC, MQC, and HQC levels. Dilution integrity was evaluated by a 10-fold dilution of the DQC sample with blank plasma prior to extraction in one of the three batches. The short-term matrix stability was evaluated in one of the three validation batches, in which the LQC and HQC samples were subjected to three freeze–thaw cycles (freeze–thaw stability) or exposed to room temperature (~22 °C) for approximately 26 h (room temperature stability) prior to extraction. To determine the storage and re-injection reproducibility of the processed samples, one of the three batches of extracted samples was stored in the autosampler (5 °C) for approximately 92 h before re-injection for LC–MS/MS analysis. The long-term stability was evaluated in an additional batch in which LQC and HQC samples were stored at approximately −20 °C for 40 days. Freshly prepared calibration standards were utilized for each of the stability evaluations.

3. Results and discussion

3.1. LC–MS/MS analysis

Several challenges resulting from the very different polarities of NLX, NLL, and NLG in the simultaneous determination of their concentrations were encountered during method development. The first one was to optimize chromatographic conditions to achieve sufficient retention for NLG, a very polar analyte. Different reversed phase columns including Aquasil C18, Gemini C18, Synergi Polar-RP, and Synergi Max-RP were evaluated in terms of retention time, peak shape, and sensitivity. For all these columns, the initial mobile phase conditions for gradient elution require low percentage of MB (lower than 3%MB) to achieve sufficient retention for NLG. Among these columns, Aquasil C18 and Synergi Polar-RP showed slightly better retention than that of Gemini C18 and Synergi Max-RP. Similar peak shape for NLG was observed on both Aquasil C18 and Syngergi Polar-RP column. However, slightly better sensitivity for NLG was observed on Aquasil C18. In addition, good peak shape was not observed from a Kromasil silica column under hydrophilic interaction chromatography. Therefore, an Aquasil C18 column was chosen for further evaluation. The second challenge was to find an appropriate gradient elution condition for separating interference peaks in extracted samples from the analytes of interest. Interference peaks for all three analytes were observed in extracted samples under initial chromatographic conditions during method development. For the MRM transition channel of NLL (see Fig. 2), the use of shallow gradient elution can better separate interference peaks from NLL. Although the interference peaks were separated from NLL in extracted control samples, they might present potential problems during incurred sample analysis. In addition, if the gradient is too shallow it can deteriorate the peak shape of NLG and lower the sensitivity of NLX. Therefore, further minimization of interference via optimization of MS detection or sample preparation is needed.

For MRM mass spectrometric detection, the most prominent product ion of NLX, NLL, and NLG was selected as described in Section 2.3. The use of alternative collision energy for these compounds does not significantly decrease their MS response so that it is suitable for eliminating interference peaks. In this case, the interference peaks for NLL were minimized by increasing the CE from 37 to 52, which almost completely eliminated the interference peaks without significant compromise of the MS response for NLL (see Fig. 2). The full method validation and sample analysis were conducted under the new mass spectrometric conditions. However, better sample clean-up approaches are needed if interference peaks cannot be eliminated by differentiating CE or optimizing chromatographic separation. Therefore, adjusting CE can be used as an alternative tool to eliminate or minimize interference peaks if analytes of interest and interference peaks/analytes have very different CE. Combination of chromatographic separation and fine-tuning of mass spectrometric parameters can sometimes provide an efficient way to eliminate interference peaks.

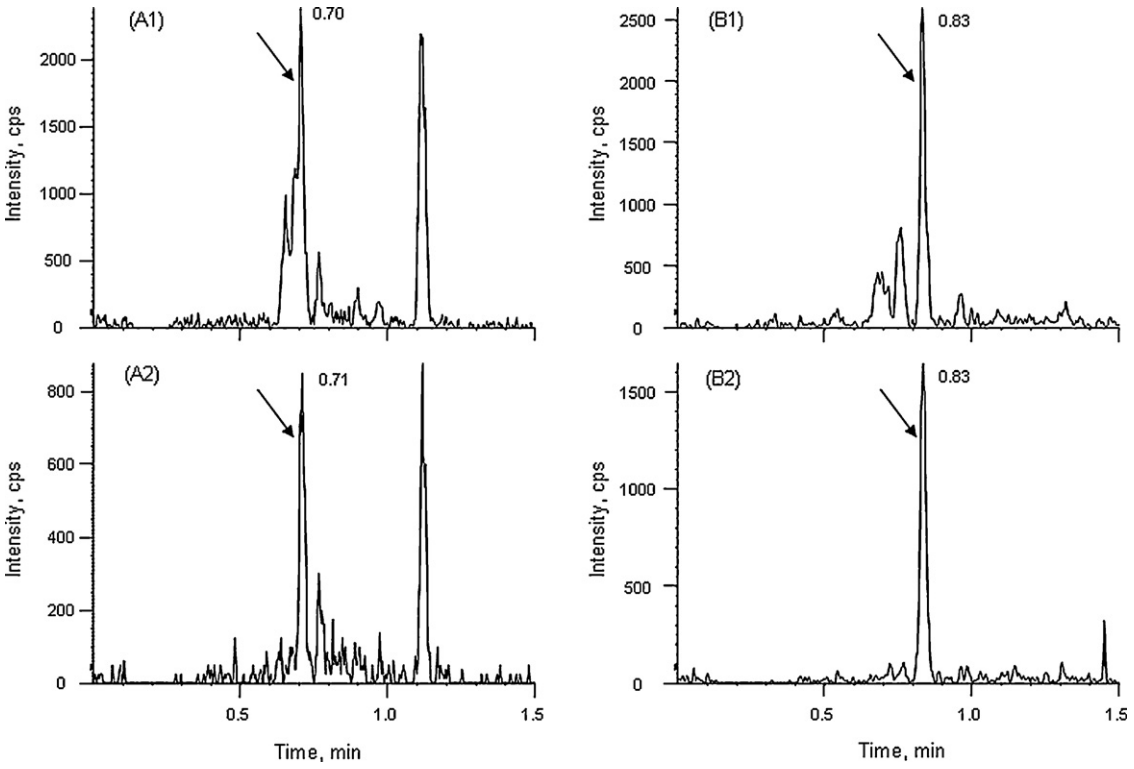

Fig. 2. Representative LC–MS/MS chromatograms of an extract from a plasma sample at the LLOQ monitoring NLL at m/z 330.2 → 299.1; the left panel of chromatograms was obtained from a sharper HPLC gradient chromatographic condition in contrast to that for method validation; the right panel of chromatograms was obtained from chromatographic conditions for method validation with a shallow HPLC gradient elution; the top row of chromatograms was detected at optimal collision energy (CE) of 37; the bottom row of chromatograms was detected at deoptimized CE of 52.

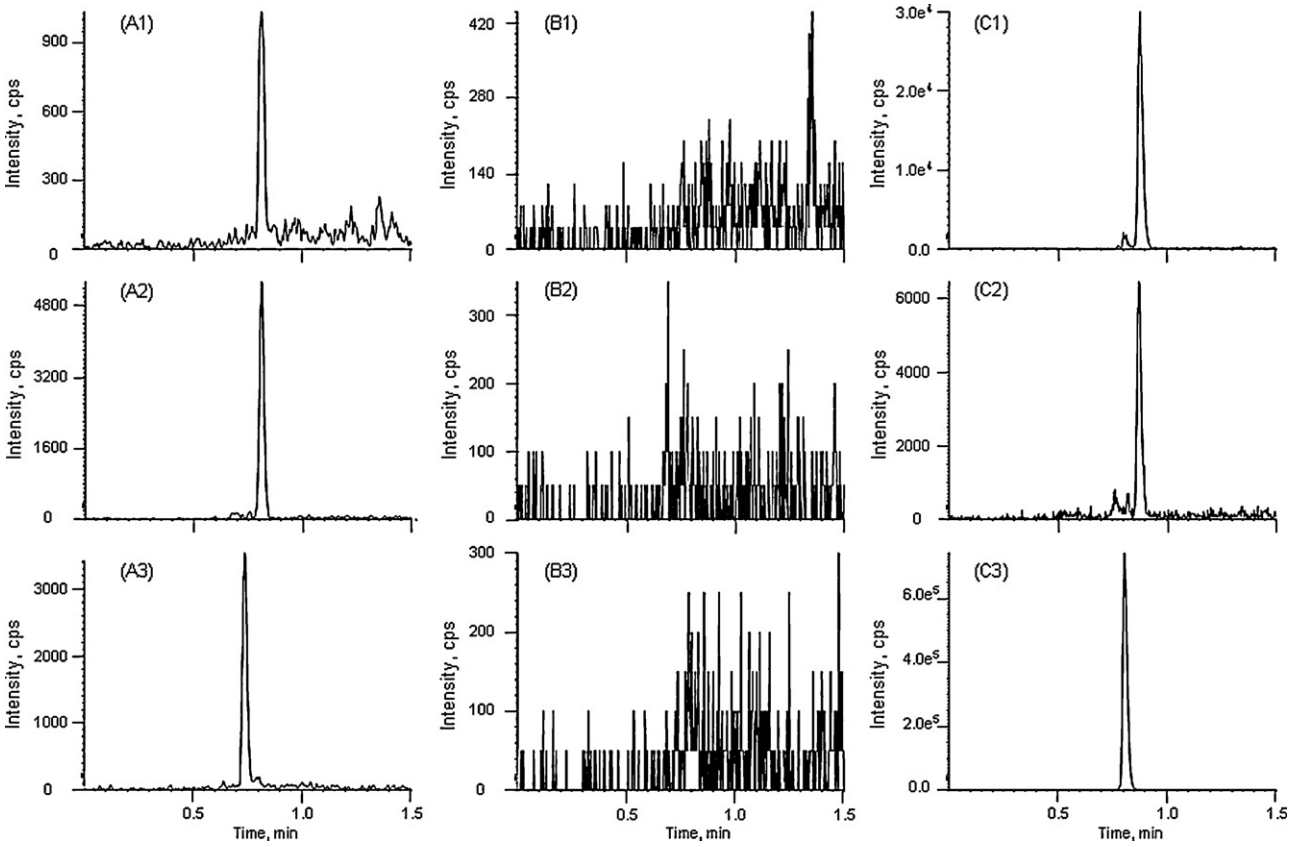

Fig. 3. Representative LC–MS/MS chromatograms of extracts from (A) a plasma sample at the LLOQ level (left panel); (B) a blank plasma (middle panel); (C) an incurred plasma sample from Day27, 0.25 h (right panel). The top row of chromatograms monitors NLX at m/z 328.2 → 212.1; the middle row of chromatograms monitors NLL at m/z 330.2 → 299.1; and the bottom chromatograms monitors NLG at m/z 504.2 → 310.0.

Table 1
Precision and accuracy of quality control samples of NLX, NLL, and NLG.

	LLQC (0.200/0.400/0.500 ng/mL)			LQC (0.600/1.20/1.50 ng/mL)			MQC (10.0/20.0/25.0 ng/mL)			HQC (80.0/160/200 ng/mL)			DQC (0.200/0.400/0.500 ng/mL)		
	NLX	NLL	NLG	NLX	NLL	NLG	NLX	NLL	NLG	NLX	NLL	NLG	NLX	NLL	NLG
Day 1															
Mean ($n=6$)	0.208	0.394	0.467	0.599	1.26	1.53	1.22	4162	26.7	79.4	162	209	465	905	1120.00
RSD (%)	7.6	5.1	4.8	4.4	6.4	2.0	2.1	3.0	1.4	2.9	1.9	2.3	2.9	2.2	1.8
RE (%)	4.0	−1.5	−6.6	−0.2	7.0	25.0	25.0	6.0	6.8	−0.7	1.3	4.5	−7.0	−9.5	−10.4
Day 2															
Mean ($n=6$)	0.202	0.404	0.458	0.638	1.29	1.57	10.2	21.4	26.9	78.9	160	207			
RSD (%)	6.5	4.7	3.4	5.1	2.2	2.5	2.2	2.3	2.2	2.2	2.2	2.0			
RE (%)	1.0	1.0	−8.4	6.3	7.5	4.7	2.0	7.0	7.6	−1.4	0.0	3.5			
Day 3															
Mean ($n=6$)	0.207	0.406	0.524	0.585	1.19	1.48	10.5	21.0	27.0	83.9	165	207			
RSD (%)	9.9	3.1	4.4	6.2	3.5	5.2	2.5	3.0	3.4	2.6	3.8	6.5			
RE (%)	3.5	1.5	4.8	−2.5	−0.8	−1.3	5.0	5.0	8.3	4.9	3.1	3.5			
Inter-day															
Mean ($n=18$)	0.206	0.401	0.483	0.607	1.25	1.53	10.3	21.2	26.9	80.7	163	207			
RSD (%)	7.8	4.3	7.5	6.2	5.4	4.2	2.7	2.7	2.4	3.8	2.9	3.9			
RE (%)	3.0	0.3	−3.4	1.2	4.2	2.0	3.0	6.0	7.6	0.9	1.9	3.5			

Note: A 10-fold dilution of the DQC with blank matrix prior to extraction was applied.

3.2. Matrix effect and specificity

The MF of 0.75, 0.75, and 1.04 were obtained for NLX, NLL, and NLG, respectively. A MF value of less than one indicates ionization suppression for NLX and NLL. However, the I.S. normalized MF of 1.06, 0.98, and 1.00 respectively, which are close to one, were obtained as a ratio of the MF of NLX, NLL, and NLG to the MF of their corresponding deuterated I.S. This suggested that matrix effect on these analytes can be compensated by their isotope labeled I.S. Therefore, the matrix effect on analysis of NLX, NLL, and NLG can be minimized.

Under the current LC–MS/MS and sample preparation conditions, interference peaks were eliminated in the chromatographic region of NLX, NLL, NLG and their internal standards (Figs. 2 and 3), suggesting specificity of this assay.

3.3. Linearity, sensitivity, and carryover

Linearity was assessed based on the average of eight calibrators analyzed in three separate batches. Acceptable linearity was achieved in the range of 0.200/0.400/0.500 to 100/200/250 ng/mL. For all three analytes, the coefficient of determination (r^2) was greater than 0.992 in all validation batches. The back-calculated results for all calibration standards showed ≤7.2% RSD and −8.0 to 4.0% RE for NLX, ≤5.6% RSD and −8.0 to 4.0% RE for NLL, ≤7.4% RSD and −7.0 to 2.2% RE for NLG.

The assay sensitivity was determined by the analysis of LLOQ samples ($n=6$) in three separate validation batches. A signal-to-noise ratio (S/N) of approximately 20, 100, and 60 was obtained at the LLOQ of 0.200/0.400/0.500 ng/mL level for NLX, NLL, and NLG, respectively (shown in Fig. 3). Acceptable precisions of 6.4% RSD for NLX, 5.1% RSD for NLL, 7.5% RSD for NLG and accuracies of 3.0% RE for NLX, 0.3% RE for NLL, −3.4% RE for NLG were obtained for inter-day assay (Table 1).

In addition, carryover from previous concentrated samples was determined to be negligible.

3.4. Precision and accuracy

The precision and accuracy of the method were determined by analyzing QC samples at the low (0.600/1.20/1.50 ng/mL, LQC), medium (10.0/20.0/25.0 ng/mL, MQC), and high

Table 2
Freeze/thaw stability, room temperature stability, re-injection reproducibility, and long-term stability of NLX, NLL, and NLG.

	LQC (0.600/1.20/1.50 ng/mL)			HQC (80.0/160/200 ng/mL)		
	NLX	NLL	NLG	NLX	NLL	NLG
Stability after five freeze–thaw cycles						
Mean ($n=6$)	0.627	1.24	1.62	79.4	157	182
RSD (%)	4.4	1.4	6.0	6.2	4.7	5.6
RE (%)	4.5	3.3	8.0	−0.7	−1.9	−9.0
Room temperature stability ~26 h						
Mean ($n=6$)	0.608	1.22	1.53	82.2	166	199
RSD (%)	6.2	6.8	8.4	4.5	2.2	6.0
RE (%)	1.3	1.7	2.0	2.8	3.8	−0.5
Re-injection reproducibility ~92 h						
Mean ($n=6$)	0.614	1.21	1.6	81.3	157	200
RSD (%)	5.7	3.8	3.3	2.8	2.1	0.3
RE (%)	2.3	0.8	6.7	1.6	−1.9	0.0
Storage at −20 °C for 40 days						
Mean ($n=6$)	0.604	1.36	1.43	80.3	168	189
RSD (%)	9.0	2.3	5.7	2.0	2.5	2.4
RE (%)	0.7	13.0	−4.7	0.4	5.0	−5.5

(80.0/160/200 ng/mL, HQC) levels. The intra-batch precision was ≤6.5% RSD and the intra-batch accuracy was in the range of −1.4 to −8.3% RE over the three concentration levels evaluated for all three analytes (Table 1). The inter-batch precision and accuracy of QC samples at different levels are also shown in Table 1. These results indicated that excellent precision and accuracy can be achieved for this assay under the current method validation conditions.

3.5. Dilution integrity

As shown in Table 1, the results demonstrated that samples with a concentration greater than the upper limit of the standard curve could be quantified with reliable precision and accuracy after being appropriately diluted with blank matrix.

3.6. Stability

The short-term stability under various experimental conditions and long-term stability experiment as described in Section 2.8 were evaluated using LQC and HQC samples. As a result, no stability issue was observed from any of these experiments (Table 2).

3.7. Application of the method and incurred sample reanalysis

A representative chromatogram of an incurred sample (male, day 27, hour 25) is shown in Fig. 3, which indicates similar chromatographic behavior to QCs. More than 250 incurred samples were analyzed and exhibited no method related problems during quantification of NLX, NLL, and NLG.

Incurred sample reanalysis (ISR) was performed using 25 incurred samples (10%) from the study randomly selected and re-analyzed in a separate batch run. The differences in concentrations between the ISR and the initial values were calculated (data not shown). 85% of reanalyzed samples showed less than 20% differences for NLX and NLL. 95% of reanalyzed samples showed less than 20% differences for NLG. The incurred sample reanalysis results meet our acceptance criteria, which require two thirds of reanalyzed sample show less than 20% differences.

4. Conclusion

A rapid, specific, and reliable LC–MS/MS based bioanalytical method has been successfully developed and validated to simultaneously quantify NLX, NLL, and NLG in mouse plasma. The current chromatographic conditions provide both good retention and peak shape for the analysis of all three analytes. The use of different collision energies can sometimes significantly reduce background noise and interference peaks without a sacrifice of sensitivity. Therefore, the combination of chromatographic separation and mass spectrometric parameters modification can efficiently shorten the HPLC run time. The relatively short sample preparation time together with the short LC run time make the present method practical for high throughput sample analysis in a cost-effective manner. The present assay demonstrates highly reproducible chromatographic and statistical results in terms of precision and accuracy during method validation. The successful application of this method to a toxicokinetic study supports its applications in future pharmacokinetic study of clinical samples.

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