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Simultaneous Determination of the Novel Neuroprotective Agent KR-31378 and its Metabolite KR-31612 Using High Performance Liquid Chromatography with Tandem Mass Spectrometry in Human Plasma

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An LC/MS/MS method for the simultaneous determination of a neuroprotective agent for ischemia-reperfusion damage, KR-31378 and its N-acetyl metabolite KR-31612 in human plasma was developed. KR-31378, KR-31612 and the internal standard, KR-31543 were extracted from human plasma by liquid-liquid extraction. A reverse-phase HPLC separation was performed on Luna phenylhexyl column with the mixture of acetonitrile-5 mM ammonium formate (55:45, v/v) as mobile phase. The detection of analytes was performed using an electrospray ionization tandem mass spectrometry in the multiple reaction monitoring mode. The lower limits of quantification for KR-31378 and KR-31612 were 2.0 ng/ml. The method showed a satisfactory sensitivity, precision, accuracy, recovery and selectivity.

Key words: KR-31378, KR-31612, LC/MS/MS, Neuroprotective, Ischemia

INTRODUCTION

KR-31378[(2S,3S,4R)-N-(6-amino-3,4-dihydro-2-dimethoxymethyl-3-hydroxy-2-methyl-2H-benzopyranyl)-N'-benzyl-N"-cyanoguanidine] (Fig. 1) was synthesized by Korea Research Institute of Chemical Technology (Taejeon, Korea) as a new therapeutic agent for neuroprotection (Yoo *et al.*, 2001). KR-31378 possesses both anti-oxidant and potassium channel modulating activities. KR-31378 has shown to protect cultured rat cortex neurons against iron-induced oxidative injury *in vitro* (Hong *et al.*, 2002). A significant reduction in infarct volume at 24 h following occlusion was also observed in the rat transient cerebral-ischemia model (Kim *et al.*, 2002). KR-31378 is currently being evaluated in a preclinical study as a new neuroprotective agent for ischemia and reperfusion damage. N-acetyl-KR-31378 (KR-31612), a metabolite of KR-31378 was observed *in vivo* after intravenous or oral administration of KR-31378 to the rats (Kim *et al.*, 2000).

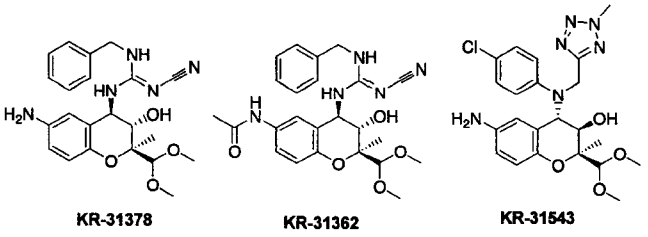

Fig. 1. Chemical structures of KR-31378, KR-31612 and KR-31543 (internal standard)

HPLC method using a UV detector has been reported for the analysis of KR-31378 in human plasma and urine (Lee *et al.*, in press) with the detection limits of 0.2 and 0.5 μg/ml, respectively. However, the concentration of its metabolite, KR-31612 (Fig. 1), could not be analyzed. The

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objective of this study was to develop and validate an LC/MS/MS method for the rapid analysis of KR-31378 and KR-31612 in human plasma. This method offers the advantages of a shorter run time with excellent specificity and sensitivity to support the further clinical development of KR-31378.

MATERIALS AND METHODS

Materials

KR-31378, KR-31612 and KR-31543 (internal standard, Fig. 1) were synthesized by Dongbu Hannong Chemical Co. (Taejeon, Korea). Acetonitrile and methylene chloride (HPLC grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and the other chemicals were of HPLC grade or the highest quality available. Drug-free human plasma containing heparin as the anticoagulant was obtained from healthy volunteers.

Preparation of calibration standards and quality control samples

Primary stock solutions of KR-31378, KR-31612 and KR-31543 (1 mg/mL) were prepared in acetonitrile. Working standard solutions of KR-31378 and KR-31612 were prepared by diluting each primary solution with 50% acetonitrile. The working solution for internal standard (1 μg/mL) was prepared by diluting an aliquot of stock solution with 50% acetonitrile.

Human plasma calibration standards of KR-31378 and KR-31612 (2, 5, 10, 40, 100, 250, 500 and 1000 ng/mL) were prepared by spiking appropriate amount of the working standard solutions into drug-free human plasma. Quality control (QC) samples at 5, 25 and 400 ng/mL were prepared in bulk by adding the appropriate working standard solutions to drug-free human plasma. The QC samples were aliquoted (100 μL) into polypropylene tubes and stored -20°C until analysis.

Sample preparation

To 100 μL blank plasma, calibration standards and QC samples, 10 μL of internal standard working solution and 100 μL of 0.1 M sodium hydroxide were added to 2-mL Eppendorf tubes. The samples were extracted with 700 μL of dichloromethane by vortex-mixing for 5 min and centrifuged at 5000 × g for 5 min. The organic layer was separated and evaporated to the dryness under nitrogen at 35°C. The residues were dissolved in 50 μL of 50% acetonitrile and 10 μL were injected onto LC/MS/MS system.

LC/MS/MS analysis

For LC/MS/MS analysis, the chromatographic system consisted of a Nanospace SI-2 pump, a SI-2 autosampler and a S-MC system controller (Shiseido, Tokyo, Japan). The separation was performed on a Luna phenylhexyl column (3 μm, 2 mm i.d. × 100 mm, Phenomenex, Torrance, CA, USA) using a mixture of acetonitrile-ammonium formate (5 mM, pH 7.0) (55:45, v/v) at a flow rate of 0.2 mL/min. The column temperature was 30°C. The effluent was introduced directly into the tandem quadrupole mass spectrometer (Quattro LC, Micromass UK Ltd, UK) through the positive ionization electrospray interface. The ion source and desolvation temperature were held at 120°C and 250°C, respectively. The nebulizing and collision gases were nitrogen and argon, respectively. The cone voltage was 25 eV and the optimum collision energies for KR-31378, KR-31612 and KR-31543 were 30, 30 and 25 eV, respectively. Multiple reaction monitoring (MRM) mode using specific precursor/product ion transitions was employed for the quantification. Detection of the ions was performed by monitoring the decay of *m/z* 426.6 parent ion to *m/z* 187.3 daughter ion for KR-31378, the decay of *m/z* 468.3 parent ion to *m/z* 362.1 daughter ion for KR-31612 and the decay of *m/z* 475.1 parent ion to *m/z* 219.9 daughter ion for KR-31543. Peak areas for all components were automatically integrated using MassLynx software.

Method validation

Batches, consisting of three calibration standards at each concentration, were analyzed on three different days to complete the method validation. In each batch, QC samples at 5, 25 and 400 ng/mL were assayed in sets of five replicates to evaluate the intra- and inter-day precision and accuracy.

RESULTS AND DISCUSSION

LC/MS/MS

Luna phenylhexyl column with the mobile phase of acetonitrile and 5 mM ammonium formate (pH 7.0) resulted in the short chromatographic run time (4.5 min) with satisfactory separation of KR-31378, KR-31612 and KR-31543 (internal standard). The retention times for KR-31378, KR-31612 and KR-31543 were 2.0, 1.9 and 3.8 min, respectively.

The electrospray ionization method gave the optimum sensitivity for KR-31378 and KR-31612 in positive ion mode. The quantification was performed by MRM detection mode using the specific precursor/product ion transitions. The mass spectra of KR-31378, KR-31612 and KR-31543 indicated the protonated molecule ([MH^+]) at *m/z* 426, 469 and 475, respectively, to be the most abundant ion. Therefore, [MH^+] ion of each compound was selected as the precursor ion. The collision-induced dissociation product ion mass spectra for KR-31378, KR-31612 and

KR-31543 yielded several predominant product ions (Fig. 2). The product ions at *m/z* 187, 362 and 220 were selected based on the specificity and sensitivity for KR-31378, KR-31362 and KR-31543, respectively. The precursor/product ion transitions were *m/z* 426 → 187 for KR-31378, *m/z* 468 → 362 for KR-31612 and *m/z* 475 → 220 for KR-31543 (internal standard).

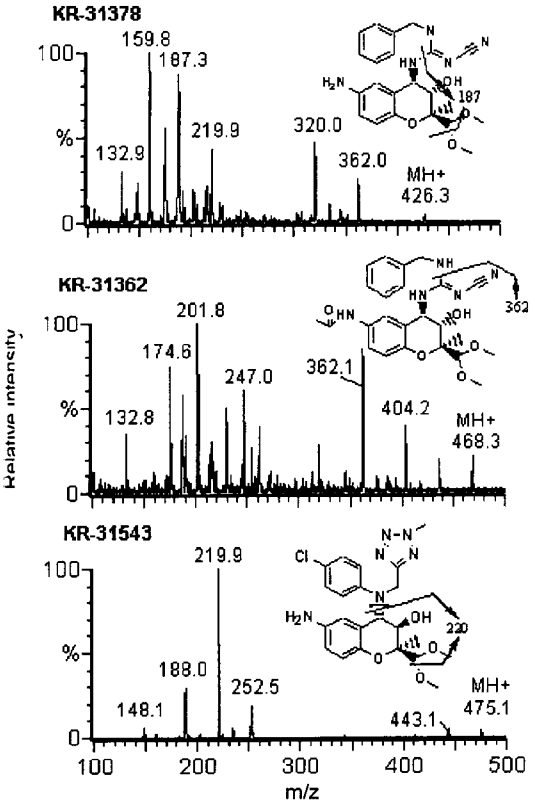

Fig. 2. Product ion mass spectra of KR-31378, KR-31612 and KR-31543 (internal standard).

Fig. 3 shows representative chromatograms of the extracts obtained with blank human plasma and human plasma spiked with KR-31378 and KR-31612 (2.0 and 50.0 ng/mL). There were no peaks of interfering with KR-31378, KR-31612 and KR-31543 at their retention times in the blank human plasma (Fig. 3a). No significant change in the peak areas of KR-31378, KR-31612 and KR-31543 in the extracts from human plasma was

Table I. Calculated concentrations of KR-31378 and KR-31612 in calibration standards prepared in human plasma. Values are mean ± SD (n = 9)

Prepared concentration (ng/ml)	Calculated concentration (ng/ml)	
	KR-31378	KR-31612
2.0	2.0 ± 0.1	2.0 ± 0.2
5.0	4.9 ± 0.4	5.2 ± 0.4
10.0	9.3 ± 0.7	9.7 ± 1.2
40.0	37.0 ± 2.9	40.2 ± 2.4
100.0	100.2 ± 3.4	102.5 ± 4.5
250.0	241.4 ± 11.7	246.5 ± 13.4
500.0	523.6 ± 22.9	500.6 ± 17.7
1000.0	987.0 ± 18.7	989.0 ± 37.6

Table. II. Absolute recoveries of KR-31378, KR-31612 and KR-31543 (internal standard) from spiked human plasma

Concentration (ng/mL)	Recovery (%, mean ± SD, n=5)		
	KR-31378	KR-31362	KR-31543
5	90.5 ± 3.6	97.9 ± 9.0	-
25	90.5 ± 7.2	101.7 ± 8.2	-
400	96.4 ± 3.9	97.8 ± 4.1	-
1000	93.0 ± 4.0	89.4 ± 5.7	-
100	-	-	85.1 ± 3.8

- : Not assayed

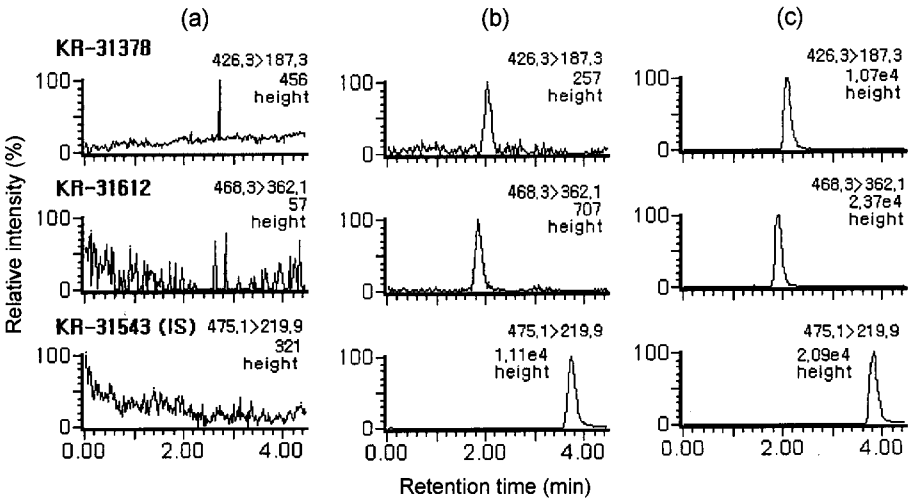

Fig. 3. Representative LC-MS/MS chromatograms of (a) a blank human plasma and human plasma samples spiked with (b) 2.0 ng/ml and (c) 50.0 ng/ml of KR-31378 and KR-31612.

Table. III. Precision and accuracy of KR-31378 and KR-31612 in human plasma quality control samples

Analytes	Statistical variable	Intra-batch (n=5)			Inter-batch (n=15)		
		5 ng/mL	25 ng/ml	400 ng/ml	5 ng/ml	25 ng/ml	400 ng/ml
KR-31378	Mean	4.9	23.1	426.0	5.0	24.3	410.7
	CV (%)	6.6	2.8	6.3	5.6	5.9	8.7
	Accuracy (%)	98.6	92.4	106.5	100.2	97.2	102.7
KR-31362	Mean	5.0	26.5	383.4	4.9	25.2	376.7
	CV (%)	8.8	5.1	3.8	7.4	7.9	4.1
	Accuracy (%)	100.6	106.0	95.8	97.5	100.7	94.2

observed compared to the matching reference standard solutions without plasma matrix component. Sample carryover effect was not observed.

Linearity

The calibration curves for KR-31378 and KR-31612 in plasma were linear over the concentration range 2.0-1000.0 ng/mL and reproducible with mean ± standard deviation for the constants in the regression equation of $y = (0.00557 \pm 0.00025)x - (0.00044 \pm 0.00015)$ and $y = (0.0109 \pm 0.0011)x - (0.0011 \pm 0.0011)$, respectively. The mean cor-relation coefficients (r^2) were 0.998 and 0.996 for KR-31378 and KR-31612, respectively. Acceptable accuracy and precision were obtained over the concentration range examined for KR-31378 and KR-31612 (Table 1).

Recovery

The extraction recoveries of KR-31378 and KR-31612 from spiked human plasma were determined at the concentrations of 5.0, 25.0, 400.0 and 1000.0 ng/mL in five replicates. The recoveries of KR-31378 ranged from 90.5 to 96.4%. The recoveries of KR-31362 ranged from 89.4 to 101.7%, with that of KR-31543 (internal standard) being 85.1 ± 3.8% (Table 2). The simple liquid-liquid extraction with dichloromethane has been successfully applied to the extraction of KR-31378 and KR-31612 from human plasma.

Sensitivity

The lower limit of quantitation (LLOQ) was set at 2.0 ng/ml for KR-31378 and KR-31362 using 100 μL of human plasma. As shown in Fig. 3b, the peak heights for two analytes are at least three times the background noise. The mean percent accuracy values 97.4% for KR-31378 and 101.0% for KR-31612 with coefficients of variation (CV) of 6.0 and 5.1%, respectively, at the LLOQ (Table 1).

Intra-batch and inter-batch accuracy and precision

QC samples containing KR-31378 and KR-31612 were prepared and analyzed in three assay batches. Table 3 shows a summary of intra- and inter-batch precision and accuracy. The intra-batch accuracy for KR-31378 and KR-31612 ranged from 92.4 to 108.3% at three different concentrations with the precision (CV) between 2.6 and 8.8%. The inter-batch accuracy for KR-31378 and KR-31612 ranged from 94.2 to 102.7% at three different concentrations with the precision (CV) between 4.1 and 8.7%. These results indicated that the present method has a satisfactory accuracy, precision and reproducibility.

Stability

KR-31378, KR-31362 and KR-31543 were stable throughout the extraction procedure and in the extracts. The re-analysis of the reconstituted extracts stored for 24 hours at 4°C showed acceptable accuracy for QC samples.

CONCLUSION

A rapid, sensitive and selective LC/MS/MS method was developed for the simultaneous determination of KR-31378 and KR-31362 in human plasma. The analytes and the internal standard were extracted from plasma using a simple liquid-liquid extraction, and chromatographed on a phenylhexyl column. The method was proved to be suitable for the high-throughput pharmacokinetic study.

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