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A sensitive LC/MS/MS method using silica column and aqueous–organic mobile phase for the analysis of loratadine and descarboethoxy-loratadine in human plasma

Weng Naidong *, T. Addison, T. Schneider, X. Jiang, T.D.J. Halls

Covance Laboratories Inc., Department of Bioanalytical Chemistry, 3301 Kinsman Boulevard, Madison, WI 53704, USA

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Abstract

A sensitive method using liquid chromatography with tandem mass spectrometric detection (LC/MS/MS) was developed and validated for the simultaneous analysis of antihistamine drug loratadine (LOR) and its active metabolite descarboethoxy-loratadine (DCL) in human plasma. Deuterated analytes, i.e. LOR-d_3 and DCL-d_3 were used as the internal standards (I.S.). Analytes were extracted from alkalized human plasma by liquid/liquid extraction using hexane. The extract was evaporated to dryness under nitrogen, reconstituted with 0.1% (v/v) of trifluoroacetic acid (TFA) in acetonitrile, and injected onto a 50 × 3.0 mm I.D. 5 μm, silica column with an aqueous–organic mobile phase consisted of acetonitrile, water, and TFA (90:10:0.1, v/v/v). The chromatographic run time was 3.0 min per injection and flow rate was 0.5 ml/min. The retention time was 1.2 and 2.0 min for LOR and DCL, respectively. The tandem mass spectrometric detection was by monitoring singly charged precursor → product ion transitions: 383 → 337 (m/z) for LOR, 311 → 259 (m/z) for DCL, 388 → 342 (m/z) for LOR-d_3, and 316 → 262 (m/z) for DCL-d_3. The low limit of quantitation (LLOQ) was 10 pg/ml for LOR and 25 pg/ml for DCL. The inter-day precision of the quality control (QC) samples was 3.5–9.4% relative standard deviation (R.S.D.). The inter-day accuracy of the QC samples was 99.0–107.9% of the nominal values.
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1. Introduction

Loratadine (LOR), trade-name Claritin®, is a second-generation long-acting tricyclic antihistamine with selective peripheral histamine H_1-antagonistic activity [1]. The relatively less sedating characteristic of the second-generation H_1 blockers is due mainly to their less complete distribution into the central nervous system. LOR is used for relieve of nasal and non-nasal symptoms of seasonal allergies and skin rash. LOR also presents weak platelet-activating factor (PAF) antagonistic properties. Taking into account the physiological importance of PAF in asthma, it was of therapeu-

* Corresponding author. Tel.: +1-608-242-2652; fax: +1-608-242-2735.
E-mail address: naidong.weng@covance.com (W. Naidong).

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tic interest to antagonize by a single molecule the action of both H_1 and PAF. Like several other second-generation H_1 antagonists, LOR is metabolized by the CYP 3A4 system and thus is subject to important interactions when other drugs inhibit this subtype of P450 enzymes. Descarboethoxyloratadine (DCL) is the major active metabolite of LOR. To support a drug–drug interaction study, measurements of as low as 10 pg/ml of LOR and 25 pg/ml of DCL in human plasma were required.

Only a few analytical methods for assaying LOR and/or DCL in biological fluids have appeared in literature, which included a cathodic adsorptive stripping voltammetric method [2], liquid chromatographic (LC) methods [3,4], a gas chromatographic (GC) method [5], GC with mass spectrometry (GC/MS) methods [6,7], and LC with tandem mass spectrometry (LC/MS/MS) methods [8,9]. Only four methods mentioned the simultaneous analysis of LOR and DCL [3,5,8,9]. The LC method described by Zhong and Blume [3] had a low limit of quantitation (LLOQ) of 0.5 ng/ml. LLOQ of 0.1 ng/ml was achieved with the GC method by Johnson et al. [5]. The LC/MS/MS method described by Sutherland et al. [8] also had a LLOQ of 0.1 ng/ml. The LC/MS/MS method described by Ramanathan et al. [9] had a long run time (50 min) and was more suitable for qualitative work. Sensitive bioanalytical methods capable of measuring low pg/ml of LOR and DCL have not been reported.

Therefore, a sensitive and high-throughput LC/MS/MS method for assaying LOR and DCL in human plasma was developed. In order to improve the method ruggedness, deuterated LOR and DCL were used as the internal standards (I.S.). The chemical structures of LOR, DCL and their I.S., i.e. LOR-d_3 and DCL-d_3 are shown in Fig. 1. The novel use of silica column and aqueous–organic mobile phase provided improved sensitivity for an LC/MS/MS method. As low as 10 pg/ml LOR and 25 pg/ml DCL in human plasma can be reliably quantified.

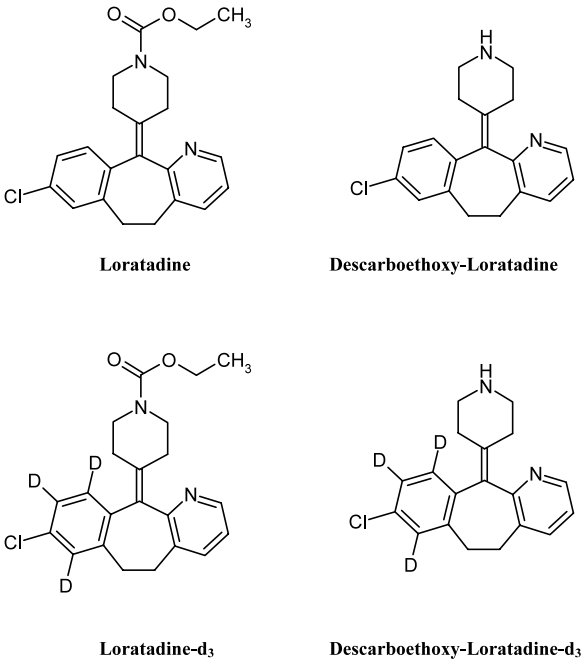

Fig. 1. Chemical structures of loratadine (LOR), descarboethoxy-loratadine (DCL), loratadine-d_3 (LOR-d_3), and descarboethoxy-loratadine-d_3 (DCL-d_3).

2. Experimental

2.1. Chemicals and reagents

LOR (purity 100%), DCL (purity 99.4%), LOR-d_3 (purity 91%, isotopic purity 100%), and DCL-d_3 (purity 99%, isotopic purity 100%) were supplied by Custom Synthesis Services (Madison, WI, USA). Hexane and ammonium hydroxide of ACS reagent grade were from Fisher Scientific (St. Louis, MO, USA). Acetonitrile, methanol, TFA, and water of HPLC grade were also from Fisher Scientific. Control human plasma, sodium heparin as an anticoagulant, was from Biochemed (Winchester, VA, USA) and was stored in a freezer at $-20\,^{\circ}C$.

2.2. Calibration standards, quality control (QC) samples and sample extraction

Standards and quality control (QC) samples were made from separate stock solutions (0.1 mg/ml in methanol) of LOR and DCL. Working spiking solutions were prepared in methanol at concentrations of 100/250, 200/500, 500/1250, 2000/5000, 5000/12 500, 8000/20 000, and 10 000/

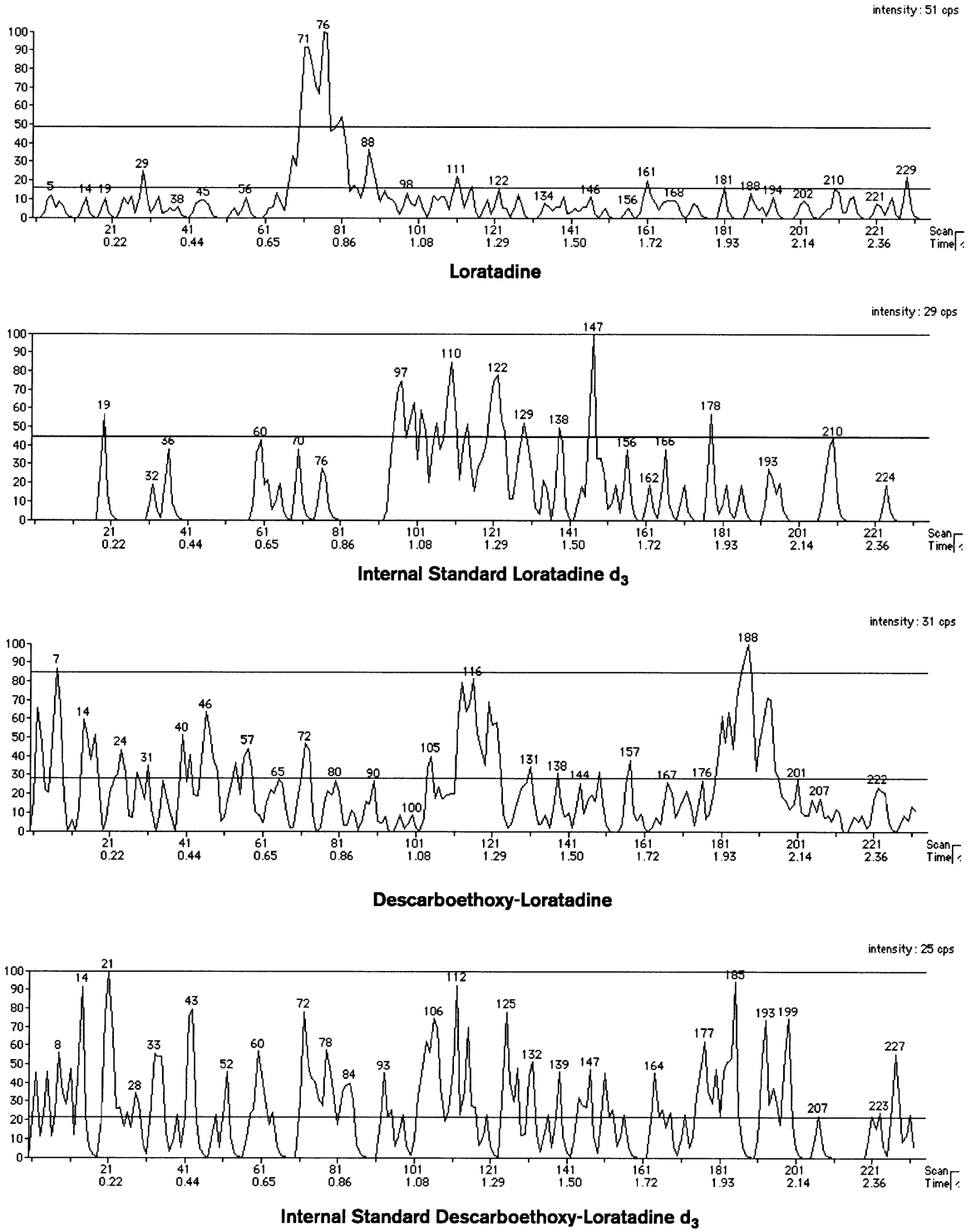

Fig. 2. Chromatogram of a blank plasma sample.

25 000 pg/ml for LOR/DCL. These solutions were stable for at least 25 days when stored at 2–8 °C and foil-covered. QC samples at levels of 30.0/75.0, 120/300, 720/1800, and 2000/5000 pg/ml for LOR/DCL were prepared by adding appropriate amount of LOR and DCL stock solutions to blank human plasma. The amount of methanol in QC samples was always less than 0.2%. All QC samples were aliquoated and stored frozen at −20 °C.

Calibration standards were prepared by aliquoting 100 μl of each working calibration solution into 16 × 125-mm glass tubes with screw caps and containing 1.0 ml of blank plasma. Aliquots (1.0 ml) of QC and blank plasma samples were transferred into the glass tubes and 100 μl of methanol was added. Fifty (50) μl of I.S. solution (20/100 ng/ml of LOR-d_3/DCL-d_3 in methanol) was added to all tubes except blank plasma samples. Fifty (50) μl of 0.1% ammonium hydroxide solution was added to each tube and samples were vortex-mixed for 30 s. Five (5) ml of hexane was added to each tube and samples were vortex-mixed for 2 min. The tubes were then centrifuged at 1200 × *g* at room temperature for 5 min using a Beckmann Centrifuge Model J6-MC (Fullerton, CA, USA). The lower aqueous layer was frozen in a dry ice/acetone bath and the upper organic layer was decanted into a 12 × 125-mm polypropylene culture tube. The organic solvent was evaporated to dryness under nitrogen in a Zymark TurboVap LV Evaporator (Hopkinton, MA, USA) set at 40 °C and approximately 10-psi nitrogen pressure. The samples were reconstituted with 200 μl of 0.1% (v/v) TFA in acetonitrile by vortex mixing for 2 min. The samples were transferred to limited-volume polypropylene vials.

2.3. LC/MS/MS

The LC/MS/MS system consisted of a Shimadzu HPLC system (Kyoto, Japan) and a PE Sciex API 3000 tandem mass spectrometer (Concord, Ontario, Canada) with a turbo ion-spray ionization source operated in a positive ion mode. The analytical column, Betasil silica of 50 × 3.0 mm I.D., 5 μm, was from Keystone Scientific (Bellefonte, PA, USA). The mobile phase was acetonitrile–water–TFA (90:10:0.1, v/v/v). The injection volume was 35 μl. The flow rate was 0.5 ml/min. Autosampler carry-over was determined by injecting first the highest calibration standard then an extracted blank sample. No carry-over was observed. Without any column-regeneration, one column was used for at least 500 injections of the extracted samples.

Sensitivity of the multiple reaction mode (MRM) was optimized by infusing 0.1 μg/ml LOR, DCL, LOR-d_3, and DCL-d_3 in a mixture of acetonitrile, water, and TFA (50:50:0.05, v/v/v) at 10 μl/min, using a Harvard '22' syringe pump (Harvard Apparatus, South Natick, MA, USA). The Ionspray needle was maintained at 5 kV. The turbo gas temperature was 300 °C and the auxiliary gas flow was 8.0 l/min. Nebulizing gas, curtain gas, and collision gas flows were at instrument settings of 10, 10, and 5, respectively. Singly charged precursor to product ion transitions were monitored at 383 → 337 (*m*/*z*) for LOR, 311 → 259 (*m*/*z*) for DCL, 388 → 342 (*m*/*z*) for LOR-d_3, and 316 → 262 (*m*/*z*) for DCL-d_3. The collision energy was 33 eV for both LOR and DCL. The dwell time was 200 ms for LOR and DCL, and 100 ms for LOR-d_3 and DCL-d_3. Both quadrupoles were maintained at unit resolution. A Power Mac G3 workstation running Sample Control and MacQuan was used for data acquisition and processing. A weighted 1/concentration^2 linear regression was used to generate calibration curves from standards and calculate the concentrations of QC samples.

3. Results and discussion

3.1. Development of the LC/MS/MS method

In this paper, a LC/MS/MS method using silica column and aqueous–organic mobile phase to analyze LOR and DCL in human plasma was presented. The selection of silica column and aqueous–organic mobile phase was based on our previous experiences with other polar compounds [10–14]. LC/MS/MS methods using silica columns operated with aqueous–organic mobile phases are viable means of analyzing polar compounds in

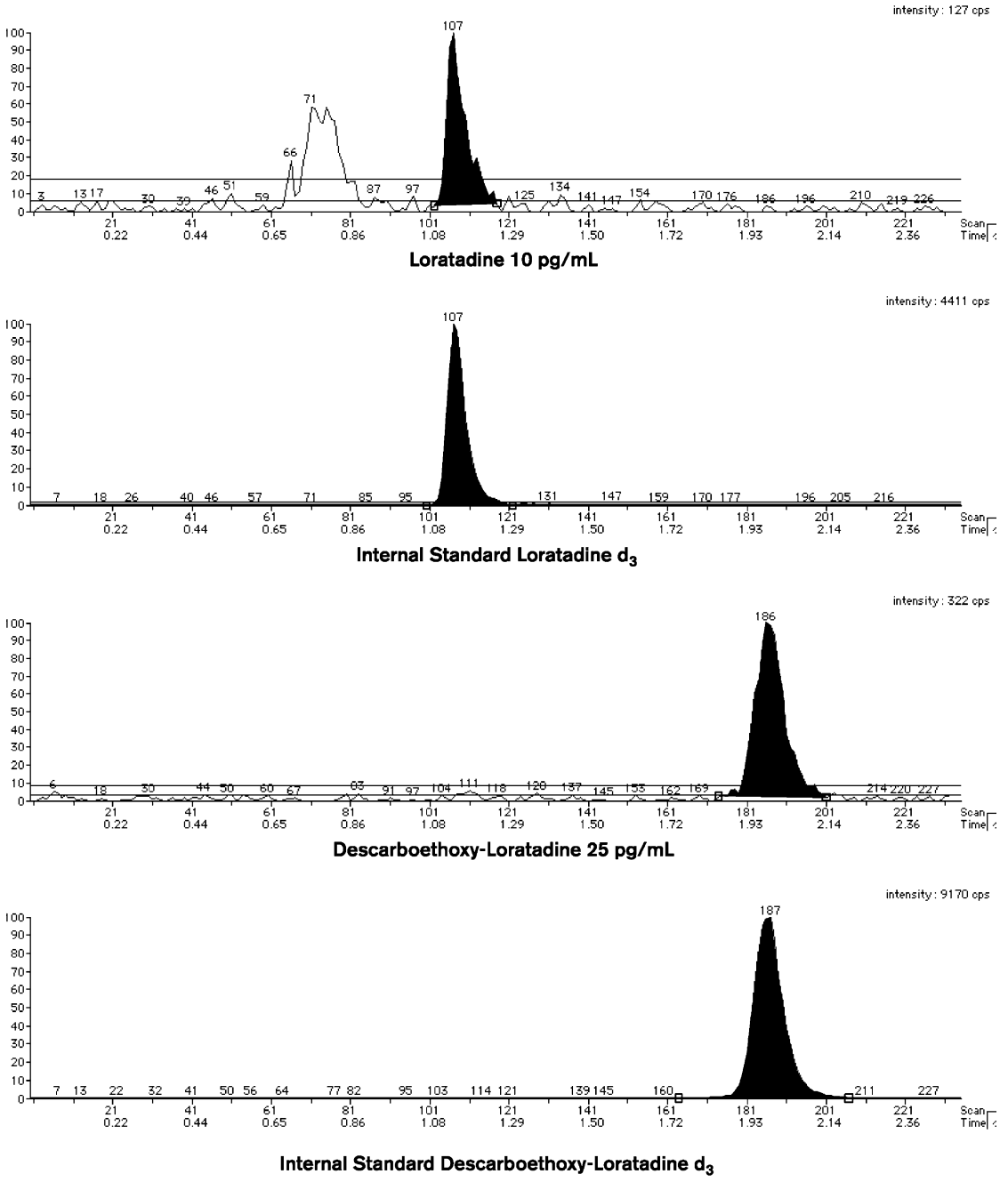

Fig. 3. Chromatogram of a blank plasma sample fortified with LLOQ (10 pg/ml) of LOR, LLOQ (25 pg/ml) of DCL, and I.S.

biological fluid. Traditionally, reversed-phase LC/MS/MS methods were used for analysis of analytes in biological fluids. However, reversed-phase LC/MS/MS for analysis of polar analytes could be a challenge. Highly aqueous mobile phase is used to retain polar analytes on the reversed-phase columns. In order to achieve spraying conditions at LC/MS interface necessary for adequate sensitiv-

ity, mobile phase of highly aqueous solutions should be avoided [15].

Even though TFA has been reported to suppress electrospray signals due to its ion-pairing activity in the gas phase with the analyte ions [16], the gain in sensitivity by going to higher organic content (90% acetonitrile) was so large that the aqueous–organic mobile phase still gave much better signal-to-noise ratio (and peak shape) than a typical reversed-phase LC/MS/MS. If 1% formic acid instead of 0.1% TFA was used, 75% acetonitrile and 25% water were required to obtain approximately the same retention as those from the TFA mobile phase. The sensitivity of both analytes thus obtained was about the same as that with TFA mobile phase but the peaks for both LOR and DCL were slightly broader. In this study mobile phases with methanol was not tried. Unlike classic normal-phase LC where the trace amount (in the ppm range) of water in the mobile phase has to be strictly controlled, the aqueous–organic mobile phase can be easily prepared and the LC/MS/MS condition is compatible with the biological sample extraction techniques. The acidic mobile phase nature enhances the protonation and in turn improved sensitivity of the basic analyte like LOR and DCL. Water is the stronger eluting solvent on the silica column than acetonitrile. The advantage of using a reconstitution solution, acetonitrile, with elution strength weaker than the mobile phase has been discussed [17].

Figs. 2 and 3 show the chromatograms of plasma blank, and 10/25 pg/ml (LLOQ) of LOR/DCL in plasma, respectively. To avoid detrimental matrix effects, analytes should be resolved from the solvent front. The capacity factors (k′) for LOR and DCL were 2.0 and 4.0, respectively, indicating they were well resolved from the solvent front. Known amounts of analytes at 10/25 pg/ml of LOR/DCL were fortified into each of the six lots of plasma. These samples, together with blank samples, were run together with one set of calibration standards extracted from one lot of plasma. All six lots of human plasma were shown to be free of interference for LOR and DCL as well as their corresponding I.S. Liquid/liquid extraction using hexane was relatively selective. When the blank plasma was fortified with compounds of diverse polarity, including clonidine, albuterol, fentanyl, ritonavir, and naltrexone, only fentanyl could be extracted by using hexane. Fentanyl did not cause any interference to LOR and DCL because of MS selectivity. Other common medications such as aspirin, caffeine, acetaminophen and ibuprofen were also too polar to be extracted by hexane. For the fortified samples, the relative standard deviation (R.S.D.) and relative error (R.E.) for LOR at 10 pg/ml is 11.9 and +11.7%, respectively. The R.S.D. and R.E. for DCL at 25 pg/ml is 4.0 and −0.7%, respectively. These results indicated the lack of matrix effect difference among the tested plasma lots.

I.S. should mirror the analytes during extraction, chromatography and MS detection. I.S. should have similar extraction recovery as the analytes. To compensate for any potential inconsistent response due to matrix effects, I.S. should elute close to the analytes on the column. Whenever possible, stable isotopes of the analytes should be used as I.S. to compensate for the potential matrix effects caused by co-eluting endogenous components in biological fluids. The detrimental matrix effects have been well documented in the literature and have been identified as the primary causes for the failure of the quantitative bioanalytical LC/MS/MS methods [18–20]. In this report, deuterated analytes were used as the I.S. The mass transition $383 \rightarrow 337$ (m/z) was monitored for LOR and the LOR-d_3 mass transition monitored would normally be 386 (Cl_{35}) $\rightarrow$ 340 (m/z). However, an interference peak from isotopic effect from LOR using that transition was observed at the LOR-d_3 channel using $386 \rightarrow 340$ (m/z) transition. Therefore, transition 388 (Cl_{37}) $\rightarrow$ 342 (m/z) was chosen as the IS channel and no isotopic effects from LOR were observed. Since there are 5 amu difference between the product ions of LOR and LOR-d_3, both chlorine and deuterium groups are retained during the fragmentation. The product ion arose from the loss of CH_3CH_2OH of the protonated molecule. For the same reason, m/z 316 (Cl_{37}) instead of 314 (Cl_{35}) was chosen as the precursor ion for DCL-d_3 transition. Either the chlorine or two deuterium groups were lost during the fragmentation because

Table 1
Precision and accuracy of calibration standards ($n = 4$)

	LOR (pg/ml)								Slope	r^2	Y-intercept
	10.0	20.0	50.0	100	200	500	800	1000			
Mean	10.3	19.7	50.4	106	203	493	808	967	3.17E-03	0.9975	−1.30E-03
R.S.D. (%)	4.7	4.7	7.2	1.8	3.5	2.7	5.6	4.2	5.2	0.2	−203
R.E. (%)	+2.8	−1.6	+0.8	+5.9	+1.3	−1.4	+1.0	−3.3			
	DCL (pg/ml)										
	25.0	50.0	125	250	500	1250	2000	2500			
Mean	24.7	50.3	128	255	500	1223	1973	2495	1.14E-03	0.9995	4.54E-03
R.S.D. (%)	0.6	1.5	1.6	2.9	4.2	1.7	4.0	1.1	2.2	0.0	52
R.E. (%)	−1.1	+0.5	+2.1	+1.8	+0.1	−2.2	−1.4	−0.2			

there were only 3 amu difference between the product ions of DCL and DCL-d_3.

Since LOR and DCL have basic functional groups, they could be extracted from alkalized human plasma using liquid/liquid extraction method. A simple one-step liquid/liquid extraction of LOR and DCL from alkaline plasma was developed. Both LOR and DCL were extracted into hexane. The hexane phase was evaporated to dryness and reconstituted with acetonitrile containing 0.1% TFA. Sutherland et al. [8] also described a liquid/liquid extraction procedure to extract LOR and DCL. LOR and DCL were initially extracted into toluene. DCL was then back extracted into 2% formic acid. The organic phase containing LOR was evaporated to dryness and reconstituted with the 2% formic acid containing back-extracted DCL. This extraction procedure was time-consuming and was less suitable for high throughput sample analysis. For the method described here, the mean recoveries for LOR and DCL were 53 and 58%, respectively. The recov-

Table 2
Precision and accuracy of quality control samples

	LOR (pg/ml)							
	Intraday ($n = 6$)					Interday ($n = 18$)		
	30.0	120	720	720[a]	2000[a]	30.0	120	720
Mean	30.9	124	719	736	2033	30.1	119	683
R.S.D. (%)	6.3	2.9	2.1	3.4	4.0	5.7	5.6	4.8
R.E. (%)	+2.8	+3.5	−0.2	+2.3	+1.7	+0.4	−1.0	−5.1
	DCL (pg/ml)							
	Intraday ($n = 6$)					Interday ($n = 18$)		
	75.0	300	1800	1800[a]	5000[a]	75.0	300	1800
Mean	84.1	318	1853	1878	5153	79.7	324	1872
R.S.D. (%)	9.5	4.3	4.3	6.4	5.0	9.4	5.9	3.5
R.E. (%)	+12.1	+6.1	+3.0	+4.4	+3.1	+6.2	+7.9	+4.0

[a] Samples were diluted 5-fold with blank plasma prior to analysis.

Table 3
Stability data (unit:% of theoretical value, mean of six determinations)

QC samples (pg/ml)	Three freeze–thaw cycle	Room temperature storage 24 h	−20 °C storage 3 months	Processed sample refrigerated 24 h
LOR				
30.0	87.8	104.6	89.7	96.8
120	97.5	101.8	ND	95.0
720	96.7	100.3	104.7	95.2
DCL				
75.0	86.4	104.9	90.6	108.2
300	107.9	101.9	ND	105.6
1800	97.6	103.3	99.4	102.1

ND, not determined.

eries for LOR-d_3 and DCL-d_3 were 57 and 52%, respectively. Recoveries obtained at three concentration levels for LOR/DCL were 41.4/44.8% at 30.0/75.0 pg/ml, 66.1/68.0% at 120/300 pg/ml, and 50.8/57.1% at 720/1800 pg/ml. The reproducibility of the recovery measurement was relatively high, as reflected by the R.S.D.% ranging from 15.0 to 25.0. The relatively low recoveries further emphasized the importance of using stable-labeled analytes I.S. so that any variability during the extraction can be compensated. Indeed, as demonstrated from the validation results, LOR-d_3 and DCL-d_3 tracked LOR and DCL very well and low R.S.D. values were obtained throughout the entire study. Extraction recovery could probably be improved by using more elaborated solid-phase extraction but since liquid/liquid extraction using hexane was very easy-to-perform and gave very clean extracts necessary for adequate sensitivity, further attempt on recovery improvement was not carried out.

3.2. Validation results

The accuracy and precision data were generated using four validation batches. Calibration batch parameters and data are listed in Table 1. The correlation coefficients of the four validation curves were all > 0.995 for LOR and > 0.999 for DCL. The standards show a linear range of 10–1000 and 25–2500 pg/ml for LOR and DCL, respectively, using weighted (1/concentration^2) least-square linear regression. Regression using non-weighted or weighted (1/concentration) gave less satisfactory results. The precision and accuracy data for QC samples are summarized in Table 2. The data show that this method is consistent and reliable with low R.S.D. and R.E. values. Method ruggedness was demonstrated by the validation work performed by two chemists on two LC/MS/MS systems using two analytical columns.

Shown in Table 3 are the stability results of sample storage, processing (freeze–thaw and bench–top), and chromatography (extracts). Three freeze/thaw cycles and ambient temperature storage of the QC samples for up to 24 h prior to analysis, appeared to have little effect on the quantitation. QC samples stored in a freezer at −20 °C remained stable for at least 3 months. Extracted calibration standards and QC samples were allowed to stand at 2–8 °C for 24 h prior to injection. No effect on quantitation of the calibration standards or QC samples was observed.

4. Conclusion

An easy, sensitive and reliable LC/MS/MS method for the measurement of LOR and its metabolite DCL in human plasma has been successfully developed and validated. Deuterated analytes were used as I.S. to improve the method ruggedness. A simple liquid/liquid extraction procedure was used to extract the analytes from plasma samples. A silica column and an

aqueous–organic mobile phase were used to improve the sensitivity. The LLOQ was 10 pg/ml for LOR and 25 pg/ml for DCL. The analytical run time was 3 min per sample.

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