Research article

Biomedical Chromatography

Received: 29 August 2015, Revised: 16 November 2015, Accepted: 2 December 2015 Published online in Wiley Online Library: 19 January 2016

(wileyonlinelibrary.com) DOI 10.1002/bmc.3666

A quantitative determination of fluorochloridone in rat plasma by UPLC-MS/MS method: application to a pharmacokinetic study

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ABSTRACT: A precise, high-throughput and sensitive ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method has been developed for the determination of fluorochloridone (FLC) in rat plasma. The extraction of analytes from plasma samples was carried out by protein precipitation procedure using acetonitrile prior to UPLC-MS/MS analysis. Verapamil was proved as a proper internal standard (IS) among many candidates. The chromatographic separation based on UPLC was well optimized. Multiple reaction monitoring in positive electrospray ionization was used with the optimized MS transitions at: *m/z* 312.0 → 292.0 for FLC and *m/z* 456.4 → 165.2 for IS. This method was well validated with good linear response ($r^2 > 0.998$) observed over the investigated range of 3–3000 ng/mL and with satisfactory stability. This method was also characterized with adequate intra- and inter-day precision and accuracy (within 12%) in the quality control samples, and with high selectivity and less matrix effect observed. Total running time was only 1.5 min. This method has been successfully applied to a pilot FLC pharmacokinetic study after oral administration. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: UPLC-MS/MS; fluorochloridone; rat plasma; pharmacokinetics

Introduction

With the growing use of herbicides, human health is faced with chronic harmful effects of herbicide residues in vegetables and other foods, such as hormone imbalance (Rieke *et al.*, 2014), nervous system damage (Wu *et al.*, 2012) and fertility disorder (Lotter, 2015). Fluorochloridone (FLC) is a herbicide widely used for pre-emergence control of broad-leaved weeds. It is a mixture of *cis*- and *trans*- diastereoisomer pairs. The European Food Safety Authority has reported that FLC is considered a potential endocrine disruptor and the no observed adverse effect level is 20 mg/kg b.w./day, with the lowest observed adverse effect level 25 mg/k.g. bw/day (European Food Safety Authority, 2010). An assay of 90 days toxicity of FLC by repeated oral administration in rats showed that liver and hematopoietic systems were the main target organs except for lesions of testis and epididymis depending on the dose of FLC (Zhang *et al.*, 2015).

As regards the analytical methods for determination of FLC, several studies have been carried out to identify the concentration of FLC residues in soil (Marković *et al.*, 2010), plants (Chen *et al.*, 2011), and water (Leandro *et al.*, 2006), mainly using solid-phase extraction or solid-phase micro-extraction sample pretreatment and subsequent detection with gas chromatography–mass spectrometry. To our knowledge, there has been no report involving FLC analysis in biological sample. This study was initiated by the need to determine the concentration of FLC in biosamples. Therefore, the aim of this work was to establish and validate a reliable, rapid and sensitive ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method for the quantitation of FLC in the rat plasma.

Experimental

Chemical and reagents

Fluorochloridone (99.0% purity) was purchased from Sigma, USA. Verapamil (internal standard, IS) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China). Acetonitrile, methanol and formic acid of HPLC grade were purchased from Merck (German) and high-purity nitrogen (Shanghai Success Gas Corporation) was used. Ultrapure water (>18 MΩ) was prepared by Millipore A10 system. All the other reagents and chemicals were of analytical grade.

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†† Correction added on 1 March 2016, after first online publication: the author correspondence details have been corrected

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Abbrevitions used: ESI, electrospray ionization; FLC, fluorochloridone

UPLC-MS/MS conditions

The samples were analyzed using a tandem quadrupole mass spectrometer (Sciex, USA) coupled with an Acquity UPLC system (Waters Corp., Milford, MA, USA). An Acquity UPLC™ C_{18} column (BEH 2.1 × 100 mm, 1.7 μm; Waters Corp., Milford, MA, USA) was employed for separation with the temperature maintained at 40°C. An elution was used to achieve chromatographic separation with a mobile phase composed of 50% mobile phase A (acetonitrile) and 50% mobile phase B (water, containing 0.1% formic acid). Throughout the UPLC process, the flow rate was set at 0.6 mL/min and the autosampler temperature was set at 4°C. The injection volume was 1 μL and the running time for each sample was 1.5 min.

Mass spectrometry was operated in electrospray ionization positive (ESI^+) mode and mass spectral data was acquired with multiple reaction monitoring. The optimized MS transitions were *m/z* 312.0 → 292.0 and *m/z* 456.4 → 165.2 for FLC and IS, respectively. The ionization source conditions were set as follows: source temperature, 450°C; capillary voltage, 5 kV; cone voltage, 35 V. The cone and desolvation gas flow rates were both 45 L/h. The optimal collision energy was 31 and 41 eV for FLC and IS, respectively. Nitrogen was used as collision gas at a pressure of 4 psi. Analyst software version 1.5.1 supplied by Sciex was used for instrument control and data acquisition and analysis.

Preparation of standards and quality control samples

Stock standard solutions of FLC and IS were prepared in methanol at a concentration of 0.3 and 0.05 mg/mL, respectively. The FLC working solutions (0.03–30 μg/mL) and IS working solution (0.05 μg/mL) were separately prepared by serially diluting their stock solutions with the appropriate amount of methanol. Matrix-matched calibration standard solutions (3, 15, 30, 150, 300, 1500 and 3000 ng/mL) and quality control samples (5, 150 and 2400 ng/mL) were prepared by redissolving 10 μL dried FLC solutions of different concentrations with 100 μL drug-free rat plasma. All standard solutions were stored at 4°C until use.

Sample preparation

The sample pretreatment was performed based on the previous studies with a little change (Li *et al.*, 2012; Fillion *et al.*, 2000). The rat plasma samples (100 μL, including calibration samples, QC samples and real samples) were added 10 μL of IS solution (verapamil, 0.05 μg/mL in methanol) and mixed for 30 s, and then 300 μL acetonitrile added for protein precipitation. The extraction was performed by vortexing for 3 min and centrifugation at 20,000 rpm for 10 min at 4°C (Sartorius SIGMA 3K30 Centrifuge, Göttingen, Germany). An aliquot of the supernatant (200 μL) was subsequently transferred into a glass auto-sampler vial with plastic inner tube and 1 μL was injected for UPLC-MS/MS analysis.

Method validation

The analytical method for quantification of FLC in rat plasma sample was validated for selectivity, linearity, LLOQ (lower limit of quantitation), precision, matrix effect, extraction recovery and stability in accordance with the guidelines of the US Food and Drug Administration (2013 and the *Pharmacopoeia of the People's Republic of China* (China Pharmacopoeia Administration, 2010) for bioanalytical methods validation. The selectivity of the method was tested by analyzing six batches of blank plasma samples obtained from six rats. The selectivity was evaluated by comparing chromatograms of blank plasma with those of standard blood samples spiked with FLC and IS and those of plasma samples obtained after oral administration.

Dynamic linearity range, expressed as regression coefficient (r^2), and LLOQ, expressed by the lowest FLC concentration with <20% relative standard deviation (RSD), were investigated using matrix matched calibration standards (3–3000 ng/mL). The calibration curves in the form of $y = a + bx$ were generated by plotting the peak area ratio (y) of FLC to IS against paclitaxel concentration (x) with weighted ($1/x^2$) least squares linear regression.

Extraction efficiency of FLC was calculated as the ratio between the peak areas of the extracted plasma ($n = 3$) at QC concentrations and those of blank plasma samples ($n = 3$) spiked with corresponding FLC after protein precipitation. The extraction efficiency of IS was calculated in the same way.

The intra-day precision and accuracy were investigated by analyzing replicates ($n = 6$) of blank plasma spiked at LLOQ concentration (3 ng/mL) and three QC concentrations (5, 150 and 2400 ng/mL) on the same day. The inter-day precision and accuracy were tested by analyzing replicates ($n = 6$) of QC samples over three consecutive days.

Based on the previous study (Matuszewski *et al.*, 2003), matrix effect was determined at QC levels by comparing the peak areas from six parallel post-spiked plasma samples with those of pure standard solution. The matrix effect of IS was measured in the same manner.

Different conditions were investigated for the study of FLC stability in plasma by analyzing replicates ($n = 3$) at two QC concentration levels, including 24°C for 6 h, 4°C for 24 h, −80°C for 90 days and three cycles of freeze–thaw to stimulate the worst operation conditions. The area responses were compared with those of freshly prepared QC samples and the ratio of concentration deviation was calculated.

Pharmacokinetic study

Five male Wistar rats (180–220 g for each), purchased from Shanghai Slack Laboratory Animal Company, were acclimatized in a room (temperature 21–23°C, humidity 40–60%) with a 12 light/dark cycle for 5 days before the experiment at Shanghai Institute for Food and Drug Control, with free access to standard laboratory food and water. The rats were fasted for 12 h before the experiment. The animal studies were conducted according to the ethical principles for animal use and care.

In the pharmacokinetics study, the rats were administered intragastrically 10 mg/kg of FLC suspended in 0.5% (w/v) sodium carboxymethyl cellulose (CMC-Na). To measure FLC concentrations, blood samples of 500 μL were collected from the retinal venous plexus before administration, and 0.5, 1, 3, 5, 8,10 and 24 h after oral administration. Plasma samples were prepared by centrifuging the blood samples at 4500 rpm for 10 min at 4°C and collecting the supernatant. During analysis phase, each analytical run included double sets of calibration standard curves at the start and the end, a set of QC samples in duplicate at intervals per batch and the prepared blood samples.

Pharmacokinetic parameters were calculated by a noncompartmental method describing the FLC concentrations in plasma over time by DAS 2.1.1 software (Mathematical Pharmacology Professional Committee of China).

Results and discussions

Method development

Initially three possible internal standards were investigated for accurate quantitative determination of FLC, including propranolol, metformin and verapamil. The former two were observed with adverse effect on the MS signal suppression of FLC, but such effect was not observed for verapamil. Additionally, verapamil exhibited similar chromatographic behavior to FLC, resulting in the nearly same extraction efficiency. Thus, verapamil was selected as the internal standard for FLC in this study.

Both FLC and IS presented a higher sensitivity in ESI source compared with atmospheric-pressure chemical ionization and produced strong signals in the positive-ion mode rather than the negative-ion mode. Therefore, ESI^+ mode was appropriate for the detection of FLC and IS. Through full-scan product ion spectra, the transitions *m/z* 312.0 → 292.0 and *m/z* 456.4 → 165.2 were chosen for the quantitation of FLC and IS, respectively. Parameters such as ESI source temperature, capillary and cone voltage were

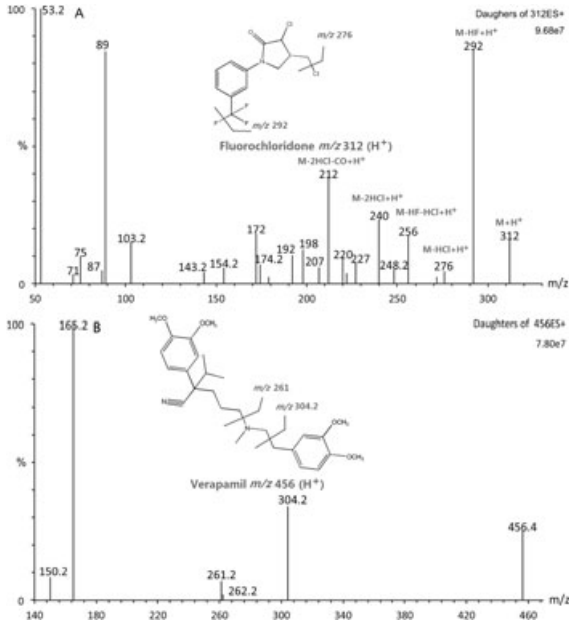

Figure 1. Full-scan product ion spectra of $[M + H]^+$ for (A) fluorochloridone (FLC) and (B) verapamil.

optimized to capture the strongest ion intensity. The product ion spectra are shown in Fig. 1.

In the chromatographic optimizing process, acetonitrile–water (50: 50, v/v) solution was used in the mobile phase instead of methanol–water solution (50: 50, v/v) because of its lower background noise. Adding formic acid could obviously increase detection sensitivity by enhancing ionization of the analyte. Therefore, water (0.1% formic acid) and acetonitrile (50:50, v/v) were selected as the mobile phases in this study. The total running time was 1.5 min. During the sample preparation, acetonitrile was selected as the precipitation solvent for higher extraction and lower background noise compared with methanol.

Method validation

Selectivity. It is clear from Fig. 2 that FLC and IS were highly selectively detected with no significant interference observed from endogenous substances in plasma at the retention time of the two compounds.

Linearity and LLOQ. Dynamic linearity was well established in an investigated rang of 3–3000 ng/mL. The mean calibration curve regression equation was: $y = 0.0062x + 0.000641$ ($r^2 = 0.9989$), where y represents the peak area ratio of FLC to IS and X is the drug concentration. This concentration range showed an excellent linear relationship ($r^2 > 0.99$) in all analytical runs. The accuracy and precision values of LLOQ are summarized in Table 1, indicating that the LLOQ of this method is suitable for the quantitative determination of FLC plasma concentration.

Precision and accuracy. Table 1 summarized the accuracy and precision for FLC of QC samples, showing the satisfactory results with relative standard deviation (RSD) <12.54% and recoveries ranging from 92.67 to 112.00%.

Matrix effect. The results of matrix effect, expressed as the ratios of peak area between the spiked plasma and the blank plasma, are shown in Table 2. The average matrix effect at QC concentration levels ranged from 95.24 to 100.34%. The matrix effect for the IS was 91.25%. These values indicated that no substance significantly influenced the ionization of FLC and IS.

Extraction recovery and stability. The extraction recoveries were determined in triplicate. All of the results are summarized in Table 2. Mean extraction recoveries of FLC at 5, 150 and 2400 ng/mL were 90.78, 93.33 and 89.35%, respectively. The extraction recovery of IS was 93.79%. The results of the stability study are shown in Table 3, indicating that FLC in rat plasma is stable under different conditions [24°C for 6 h, −4°C for 24 h, −80°C for 90 days and three freeze (−80°C) and thaw (24°C) cycles].

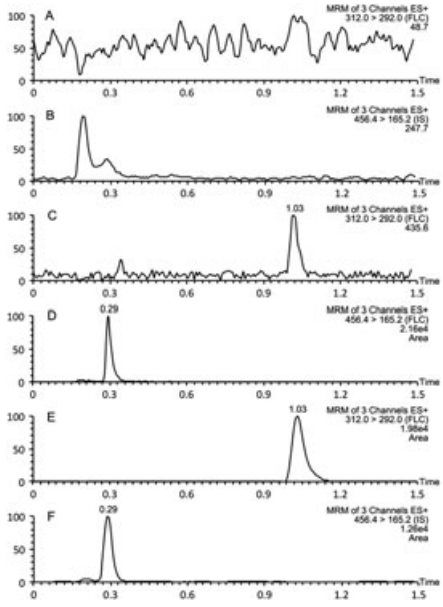

Figure 2. Chromatograms of blank plasma of rat (A and B), blank plasma spiked with FLC at LLOQ (C) and IS (D), and plasma obtained 5 h after oral administration of FLC (E and F).

Table 1. Intra- and inter-day precision and accuracy of fluorochloridone (FLC) in rat plasma

Nominal concentration (ng/mL)	Intra-day precision			Inter-day precision		
	Mean ± SD	Precision (%)	Accuracy (%)	Mean ± SD	Precision RSD (%)	Accuracy Recovery (%)
3	2.78 ± 0.32	11.54	92.7	—	—	—
5	4.97 ± 0.48	9.71	99.3	5.04 ± 0.23	10.05	100.7
150	168.0 ± 3.74	3.74	112.0	157.5 ± 9.66	10.05	101.7
2400	2447 ± 70.9	2.90	101.9	2351 ± 107.3	6.08	97.96

Table 2. Extraction recovery and matrix effect of FLC in rat plasma ($n = 3$)

Nominal concentration (ng/mL)	Extraction recovery		Matrix effect	
	Mean ± SD(%)	RSD (%)	Mean ± SD(%)	RSD (%)
5	90.78 ± 6.33	6.97	96.93 ± 3.79	3.91
150	93.33 ± 8.17	8.75	100.34 ± 3.84	3.83
2400	89.35 ± 0.74	0.83	95.24 ± 4.18	4.39
IS	93.79 ± 2.23	2.38	91.25 ± 0.72	3.91

Table 3. Stability of FLC in rat plasma under different conditions ($n = 3$)

Concentration (ng/mL)	Measured concentration (Mean ± SD)	Accuracy (%)	Precision (%)
24°C for 6 h			
5	5.35 ± 0.23	100.07	4.38
2400	2277 ± 57.74	94.86	2.54
4°C for 24 h			
5	4.58 ± 0.45	99.92	9.78
2400	2087 ± 55.08	86.94	2.64
Third freeze–thaw			
5	5.07 ± 0.43	100.01	8.46
2400	2350 ± 154.0	97.92	6.55
−80°C for 90 days			
5	4.73 ± 0.16	99.95	3.36
2400	2520 ± 60.83	105.0	2.41

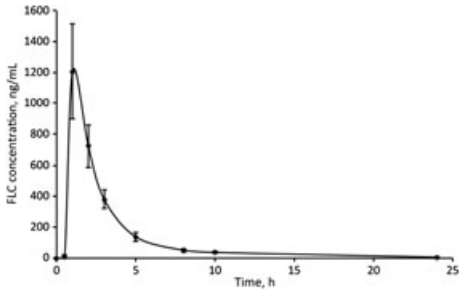

Figure 3. Mean plasma concentration–time profiles for FLC in rats after oral administration at a dose of 10 mg/kg FLC.

Table 4. Pharmacokinetic parameters after oral administration of FLC (10 mg/kg) to rat ($n = 5$)

Parameters	Unit	Mean ± SD
C_{max}	μg/L	1206 ± 307.8
T_{max}	h	1.00 ± 0
$t_{1/2z}$	h	5.47 ± 3.19
Vz/F	L/kg	23.87 ± 11.40
CLz/F	L/h/kg	3.17 ± 0.57
$MRT_{(0-t)}$	h	3.33 ± 0.16
$MRT_{(0-\infty)}$	h	4.07 ± 1.09
$AUC_{(0-t)}$	μg/L h	3136 ± 546.0
$AUC_{(0-\infty)}$	μg/L h	3227 ± 543.8

[a]C_{max}, Peak (maximum) plasma concentration; T_{max}, time to reach C_{max}; $t_{1/2z}$, half-life; *Vz/F*, apparent volume of distribution; *CLz/F*, total body clearance; MRT, mean residence time; AUC, area under the concentration–time curve.

Application

This developed method was successfully applied in the determination of FLC in rat plasma collected after oral administration (10 mg/kg). The mean curve of FLC plasma concentration vs time is presented in Fig. 3. The major pharmacokinetic parameters of FLC are shown in Table 4, showing that FLC reached a peak concentration in the plasma of rats 1 h after oral administration. FLC eliminated relatively quickly and widely in rats with a half-life ($t_{1/2z}$) of 5.47 ± 3.19 h and the apparent volume of distribution (*Vz/F*) is 23.87 ± 11.4 L/kg. The total body clearance (*CLz/F*) was 3.17 ± 0.57 L/h/kg and FLC could still be detected in oral plasma with a quantitation close to LLOQ 24 h after oral administration. These results indicate that the pharmacokinetics of FLC in rats is characterized rapidly for absorption and elimination and broadly for distribution.

Conclusion

A rapid and sensitive UPLC-MS/MS method has been developed and validated for the determination of FLC in rat plasma. Based on a simple protein-precipitation extraction process, this method has been proved applicabile for the pharmacokinetic study of FLC in rats after oral administration. Additionally, this method can be employed in monitoring programs of populations intentionally or accidentally exposed to FLC and in investigating potential pharmacokinetics in humans or animals. The method also exhibited its feasibility for expanded application in toxicokinetics and in studying FLC distribution in different tissues.

Acknowledgments

This study was financially supported by the National Natural Science Foundation of China (grant no. 81373040).

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