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Simultaneous determination of a novel antifibrotic agent and three metabolites in human urine by LC–MS/MS

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ABSTRACT

A robust and validated high performance liquid chromatography tandem mass spectrometry (LC–MS/MS) method has been developed for simultaneous determination of F351 (5-methyl-1-(4-hydroxylphenyl)-2-(1H)-pyridone) and three major metabolites in human urine sample. This assay method has also been validated in terms of selectivity, linearity, lower limit of quantification (LLOQ), accuracy, precision, stability, matrix effect and recovery. Chromatography was carried out on an XTerra RP 18 column and mass spectrometric analysis was performed using an API 4000 mass spectrometer coupled with electrospray ionization (ESI) source in the positive ion mode. The MRM transitions of m/z 202 → 109, 232 → 93, 282 → 202 and 378 → 202 were used to quantify F351 and three metabolites, respectively. Retention times for F351 and three metabolites were 2.54, 1.38, 1.53 and 1.34 min, respectively. The assay was validated from 20 to 4000 ng/mL for F351 and M1, from 80 to16,000 ng/mL for M2 and M3. Intra- and inter-day precision for all analytes was <6.3%, method accuracy was between −11.2 and 0.3%. This assay was used to support a clinical study where multiple oral doses were administered to healthy subjects to investigate the pharmacokinetics, safety, and tolerability of F351.

1. Introduction

Hepatic fibrosis is a scarring response that occurs in almost all patients with liver injury, including hepatitis B or C viral infection, excessive alcohol ingestion, non-alcoholic steatohepatitis (NASH), and iron overload [1]. The progression of liver fibrosis frequently leads to end-stage liver cirrhosis associated with nodule formation and organ contraction [2,3]. Liver fibrosis is observed microscopically as excessive deposition and abnormal distribution of extracellular matrix (ECM) components [4], which is primarily associated with the activation of hepatic stellate cells [2]. With the great progress made over the past 20 years in understanding hepatic fibrosis, antifibrotic therapies is likely to emerge as an important option in patients with fibrotic liver disease [5–7].

F351 (5-methyl-1-(4-hydroxylphenyl)-2-(1H)-pyridone), a new derivative of pyridine, show obvious antifibrotic effect and reduced the hepatocyte necrosis in rat liver fibrosis models established by induction with CCl4 and DMN (dimethyl nitrosamine) respectively, it also significantly improved the liver function in those liver fibrosis models (unpublished results). In 2007, F351 was approved by China State Food and Drug Administration as an investigational new drug for treatment of liver fibrosis. The chemical structures of F351 and its three major metabolites, M1 (phase I metabolite), M2 (sulfonate metabolite), and M3 (glucuronide metabolite) are shown in Fig. 1.

Selective and sensitive analytical method for the quantitative evaluation of drug and their metabolites are critical for the successful conduct of clinical study. In this paper, we have developed an LC–MS/MS analytical method and validated in terms of selectivity, linearity, lower limit of quantification (LLOQ), accuracy, precision, stability, matrix effect and recovery. To our knowledge, this is the first study for the simultaneous determination of F351 and three metabolites using an LC–MS/MS method in human urine. All the validation terms were assessed according to FDA guideline [8].

2. Experimental

2.1. Chemicals

F351 (5-methyl-1-(4-hydroxylphenyl)-2-(1H)-pyridone), M1, M2 and M3 were obtained from Shanghai Genomics Inc. (Shanghai, China). Internal standard, N-(P-hydroxyphenyl) propionamide (Fig. 1) was kindly donated by SmithKline Beecham (London, UK). Ammonium acetate was purchased from Sigma–Aldrich chemicals (St. Louis, MO, USA). Acetonitrile and formic acid (FA) were of HPLC grade and were purchased from Fisher Scientific (NJ, USA). HPLC grade water was obtained using a Milli Q system.

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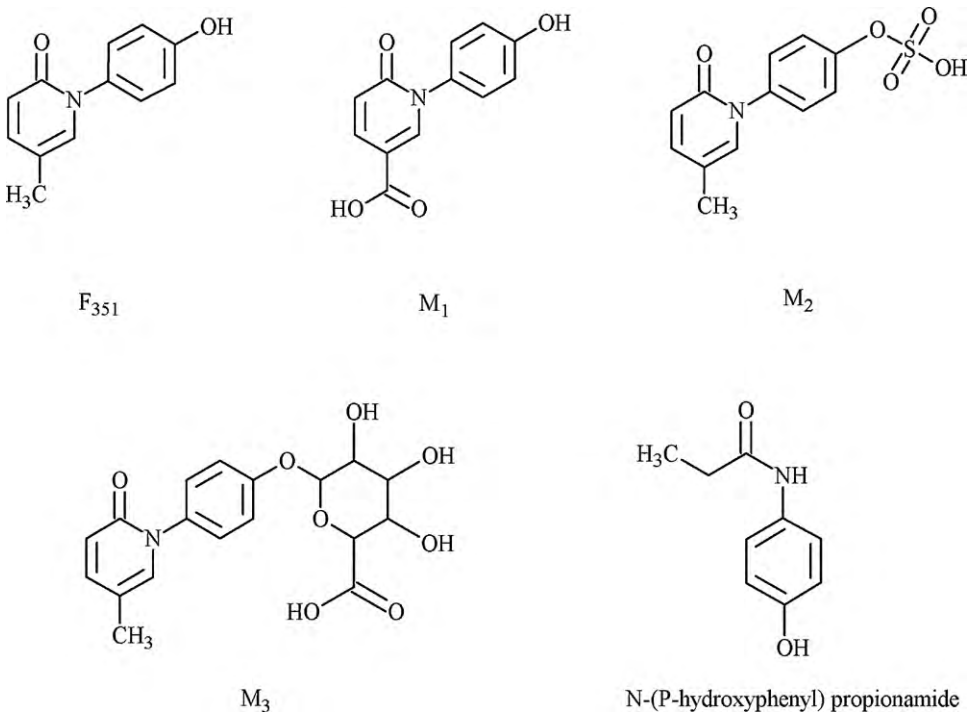

Fig. 1. Chemical structures of M1 (phase I metabolite), M2 (sulfonate metabolite), and M3 (glucuronide metabolite) and N-(P-hydroxyphenyl) propionamide (IS).

2.2. Instrumentation

The Shimadzu HPLC system equipped with two LC-20AD pumps, a SCL-10A controller, a DGU-20 solvent degasser, a CTO-20A column oven and a SIL-20AC autosampler were used. Mass spectrometric analysis was performed using API 4000 triple quadrapole mass spectrometer from Applied Biosystems MDS Sciex (Toronto, Canada) equipped with Turbo V source. Analyst (version 1.4.1) software from Applied Biosystems MDS Sciex was used for data acquisition and handling.

2.3. LC conditions

Separation was achieved on an XTerra RP18 analytical column (2.1 mm × 50 mm, 3.5 μm) from Water corporation (Milford, MA, USA). Chromatography was carried out via an isocratic system with 3.0 min sample run time after an injection volume of 5 μL. The mobile phase consisted of acetonitrile with 0.1% formic acid and ammonium formate with 0.1% formic acid (20:80, v/v) flowing at 0.2 mL/min. A 0.1% formic acid in acetonitrile/water mixture (50:50, v/v) was used as the needle wash solvent. The column and the samples were kept at 35 °C and 15 °C, respectively.

2.4. Mass spectrometer conditions

An API 4000 triple quadrapole instrument was used for the mass spectrometric detection using an electro-spray ionization (ESI) source in the positive mode. The detection was operated in the multiple reaction monitoring (MRM) mode under unit mass resolution (0.7 amu @ FWHH) in both the Q1 and Q3 mass analyzers, and the dwell time set to 150 ms for each MRM transition. After optimization, the source parameters were set as follows: curtain gas, 15 psi; nebulizer gas, 60 psi; turbo gas, 70 psi; ion spray voltage, 5 kV; and temperature, 300 °C. The MRM transition and compound specific parameters for all analytes and internal standard can be found in Table 1. Data acquiring and processing were performed using analyst version 1.4.1.

2.5. Standard solutions and sample preparation

Two separate (#1 and #2) combined stock solutions were prepared at concentrations of 0.5 mg/mL for F351 and M1, 2 mg/mL for M2 and M3 in methanol/water (50:50, v/v). Combined stock solutions #1 and #2 were used for the preparation of calibration standards and quality controls, respectively. Eight working standard solutions in 50:50 methanol/water (v/v) were prepared from the stock solution #1 and three working QC solutions from stock solution #2. Calibration standards and quality control samples were prepared by spiking blank (drug free) human urine. Concentrations in calibration standards were 20–4000 ng/mL for F351 and M1, 80–16,000 for M2 and M3; and concentrations in quality controls were 60, 600, 3000 ng/mL for F351 and M1, 240, 2400 and 12,000 ng/mL for M2 and M3. The stock internal standard (IS) solution was prepared by dissolving10 mg of N-(P-hydroxyphenyl) propionamide in 10 mL of 50:50 methanol/water (v/v). The working IS solution (5 μg/mL) was prepared by the appropriate dilution of the stock IS solution in water. All stock solutions, working solutions, calibration standards and quality controls were immediately stored at −30 °C.

2.6. Sample preparation

After thawing, a 20 μL internal standard working solution was added to 100 μL of standard and QC urine samples. Then, 300 μL

Table 1
MRM transitions and compound specific parameters for: F351, three metabolites and internal standard.

Compound	Parent ions (*m*/*z*)	Product ions (*m*/*z*)	Declustering potential (V)	Collision energy (V)
F351	202	109	86	33
M1	232	93	81	35
M2	282	202	66	40
M3	378	202	66	58
Internal standard	166	110	36	25

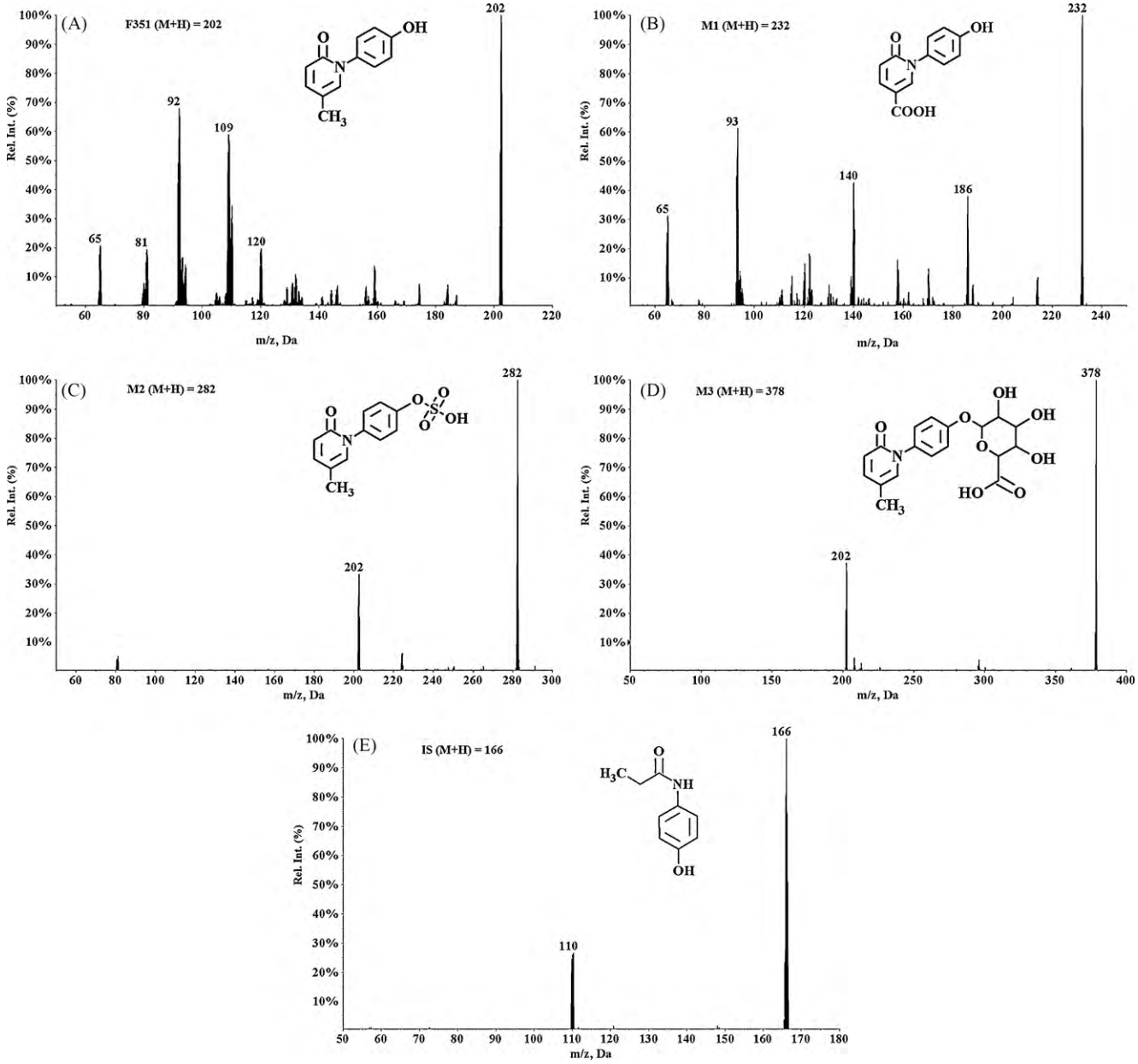

Fig. 2. The product ion mass spectra of (A) F351, (B) M1, (C) M2, (D) M3 and (E) IS.

of water was added into the polypropylene centrifuge tube and was shaken for10 s on a shaker. Subsequently, the mixture was centrifuged at 13,000 rpm for 5 min. The clear supernatants were transferred to vials and loading onto the autosampler tray maintained at 15 °C.

2.7. Method validation

Validation of the method included the assessment of selectivity, calibration curve performance, accuracy and precision, LLOQ, stability of the analytes at various test conditions, matrix effect and recovery. All terms of assay validation were undertaken according to FDA guidelines [8], and follow the recommendations provided by Viswanathan et al. [9].

The assay selectivity was assessed by analyzing six lots of analyte-free human urine from different sources. Endogenous interference at any of the retention time of the analytes was observed in any of the urine lots evaluated. In the mean while, "cross-talk" between MRM transitions was evaluated by injecting separately each analyte at the highest concentration on calibration standard and monitoring the response in all other MRM transition at LLOQ of respective analyte. The "cross-talk" effect may originate from the (i) slow removal of ions from the collision cell, that was quite common in the early designed collision cells [10]; or (ii) may occur when an analog, used as an IS and/or metabolite(s) that are not chromatographically separated from the analyte of interest, are converted in the interface of the mass spectrometer to the analyte of interest or are contaminated chemically with each other.

The intra-day precision and accuracy for the method was determined by analyzing five sets of quality control samples at three different levels on the same day. For inter-day precision and accuracy, five sets of quality control samples at three different levels were analyzed on four separate days. The precision for quality controls must be within 15%, and accuracy between −15 and 15%. LLOQ can be determined by several approaches (i) visual eval-

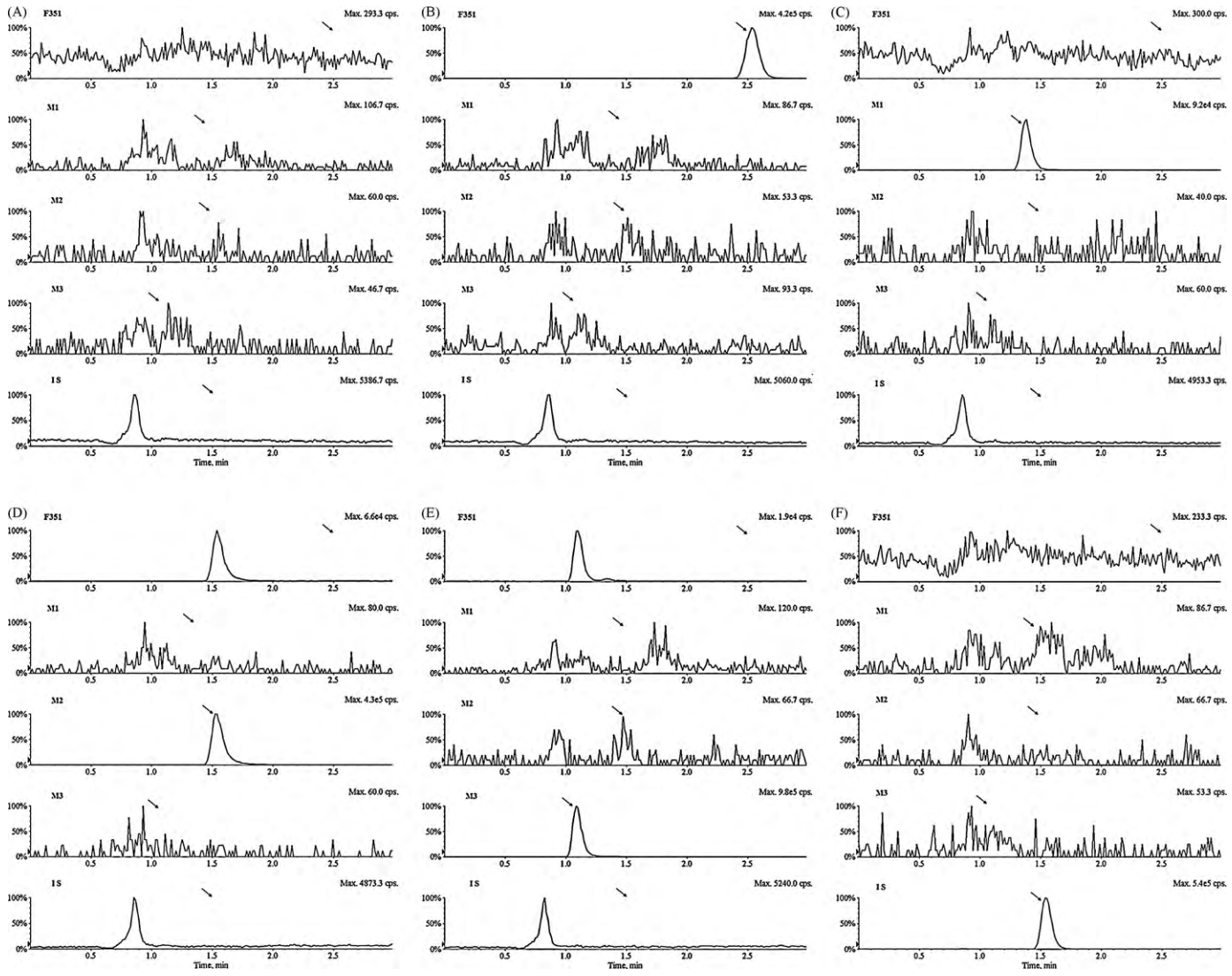

Fig. 3. Representative chromatograms (A) representative chromatograms of blank urine sample and monitored at all MRM transition; (B) representative chromatograms of urine sample spiking with F351 and monitored at all MRM transition; (C) representative chromatograms of urine sample spiking with M1 and monitored at all MRM transition; (D) representative chromatograms of urine sample spiking with M2 and monitored at all MRM transition; (E) representative chromatograms of urine sample spiking with M3 and monitored at all MRM transition; (F) representative chromatograms of urine sample spiking with IS and monitored at all MRM transition.

uations; (ii) signal-to-noise ratio of 10:1; and (iii) based on the precision and accuracy, wherein, the acceptable limit is 20%. In this assay, LLOQ are calculated by the approach of precision and accuracy.

Stability was tested by comparing the freshly prepared QC at the same level to the QC which had been maintained in different test condition and analyzed in the same analytical run. Conditions used in stability assessment included after long-term and short-term storage, after going through freeze and thaw cycles and in the autosampler.

The assessment of matrix effect is critical when analogs rather than stable isotope-labeled analytes, are used as internal standards [11–17]. The undetected co-eluting endogenous impurities may affect the ionization efficiency of the analytes. The approach we used to evaluate matrix effects was following a suggestion of Matuszewski et al. [12]. Matrix effect was assessed by comparing the peak areas of analytes in different lots of urine, and by comparing peak area ratios of analytes to IS spiked into urine after extraction to similar ratios of neat sample. The recovery was determined by comparing the mean peak areas of urine samples spiked before and after extraction.

3. Results and discussion

3.1. LC–MS/MS method development

During the development of any LC–MS/MS method, it is critical to confirm assay selectivity in the presence of metabolites. If metabolites are not chromatographically separated from the parent drug, they can fragment in the interface of the mass spectrometer to give the same protonated molecular ion as the one originating from the drug, these ions having the same m/z values could produce common product ions detected by the MS/MS system [17]. The product ion mass spectra of M2 and M3 (Fig. 2) indicated they both generated the product ion that had same m/z values with F351. Thus, in an effort to ensure assay selectivity, chromatographic conditions were developed to separate F351 from M2 and M3. The metabolic pathway and Log D calculation indicated those three metabolites are more polar than F351. The LC optimization was carried out using columns with different stationary phases, including C18, RP18 and HILIC. From C18 to HILIC, the retention of polar compound is increasing. The optimization results showed that except the C18 column, the RP18 and HILIC columns

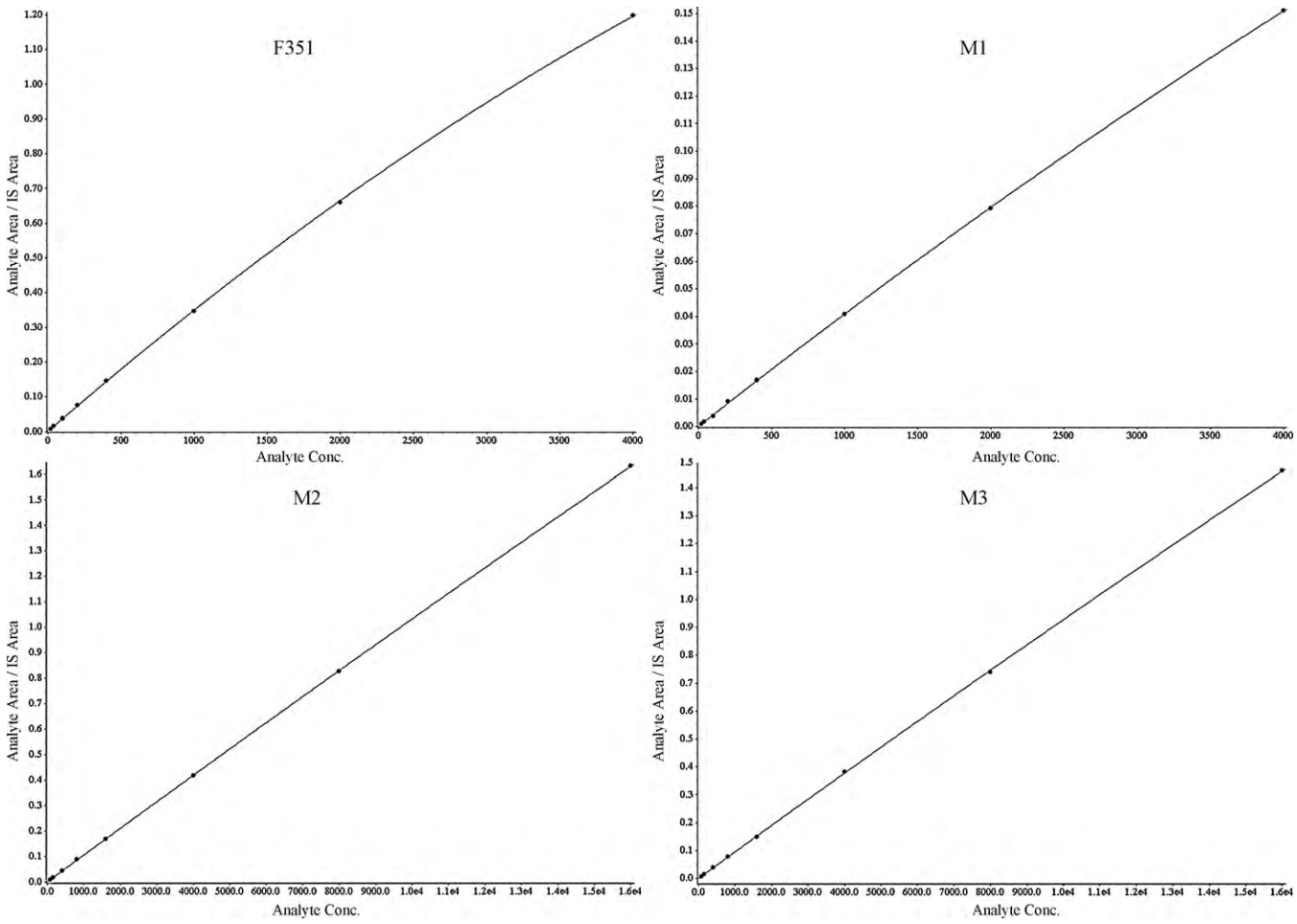

Fig. 4. Typical calibration curve plots (peak area ratios versus nominal concentration) (A) F351, (B) M1, (C) M2 and (D) M3.

can retain both F351 and three metabolites, also provided excellent peak shape and good chromatographic separation. Because the injection solvent for HILIC should be 100% organic solvent, there must be a dry-out procedure or using SPE technique in sample pretreatment. So, besides chromatographic separation, the RP column system has the advantage of simple and low-cost sample pretreatment. The final chromatographic system was carried out with XTerra RP18 analytical column (2.1 mm × 50 mm, 3.5 μm) with acetonitrile with 0.1% formic acid and ammonium formate with 0.1% formic acid (20:80, v/v). We also tested several compounds as internal standards. Among those tested, N-(P-hydroxyphenyl) propionamide was found to be a suitable internal standard for all analytes, eluting at 1.59 min without interference in any other MRM transitions.

Table 2
Back-calculated calibration standards of F351 and three metabolites.

		Calibration standards (ng/mL)[a]								*R*-squared
		1	2	3	4	5	6	7	8	
F351	Mean (n = 5)	18.4	39.1	104.2	214.2	404.1	990.2	1971.7	4020.1	0.9993
	SD	1.0	3.1	5.6	8.2	18.5	16.2	43.6	20.6	
	%CV	5.4	7.9	5.4	3.8	4.6	1.6	2.2	0.5	
	%Deviation	−8.0	−2.3	4.2	7.1	1.0	−1.0	−1.4	0.5	
M1	Mean (n = 5)	20.3	38.3	100.9	206.5	399.0	983.4	2016.2	3995.3	0.9996
	SD	1.5	0.5	6.5	4.7	14.4	15.6	40.5	18.2	
	%CV	7.4	1.3	6.4	2.3	3.6	1.6	2.0	0.5	
	%Deviation	1.5	−4.3	0.9	3.3	−0.3	−1.7	0.8	−0.1	
M2	Mean (n = 5)	78.4	154.9	411.7	835.0	1576.3	3959.6	8026.9	15,997.3	0.9996
	SD	4.0	10.8	19.2	29.8	80.8	30.8	115.3	48.1	
	%CV	5.1	7.0	4.7	3.6	5.1	0.8	1.4	0.3	
	%Deviation	−2.0	−3.2	2.9	4.4	−1.5	−1.0	0.3	0.0	
M3	Mean (n = 5)	79.9	151.9	407.2	836.1	1589.9	3985.2	7971.6	16,018.5	0.9995
	SD	2.5	9.4	20.7	30.1	59.5	93.7	197.8	76.3	
	%CV	3.1	6.2	5.1	3.6	3.7	2.4	2.5	0.5	
	%Deviation	−0.1	−5.1	1.8	4.5	−0.6	−0.4	−0.4	0.1	

[a] Concentrations in calibration standards were 20, 40, 100, 200, 400, 1000, 2000, 4000 ng/mL for F351 and M1, 80, 160, 400, 800, 1600, 4000, 8000, 16,000 ng/mL for M2 and M3.

Table 3
LLOQ, intra- and inter-day precision and accuracy for F351 and three metabolites.

		LLOQ (ng/mL)[a] $n=20$	Intra-day (ng/mL)[b] $n=5$			Inter-day (ng/mL)[b] $n=20$		
			Low	Mid	High	Low	Mid	High
F351	Mean	18.9	53.3	534.1	2894.5	55.3	550.0	2734.0
	SD	0.7	1.7	26.5	68.1	2.1	16.7	56.4
	Precision	3.7	3.2	5.0	2.4	3.8	3.0	2.1
	Accuracy	−5.6	−11.2	−11.0	−3.5	−7.8	−8.3	−8.9
M1	Mean	20.6	53.6	553.6	2802.2	54.1	555.0	2850.9
	SD	2.4	3.4	8.4	81.5	3.2	18.0	136.3
	Precision	12.2	6.3	1.5	2.9	5.9	3.2	4.8
	Accuracy	2.9	−10.7	−7.7	−6.6	−9.8	−7.5	−5.0
M2	Mean	80.6	227.3	2254.2	11,926.9	227.9	2312.0	12,031.8
	SD	3.9	5.9	43.4	219.2	9.6	112.3	451.0
	Precision	4.9	2.6	1.9	1.8	4.2	4.9	3.7
	Accuracy	0.7	−5.3	−6.1	−0.6	−5.0	−3.7	0.3
M3	Mean	82.7	234.9	2319.9	11,614.0	226.8	2311.3	11,918.5
	SD	4.3	6.1	39.0	260.8	8.0	90.1	526.2
	Precision	5.4	2.6	1.7	2.2	3.5	3.9	4.4
	Accuracy	3.3	−2.1	−3.3	−3.2	−5.5	−3.7	−0.7

[a] Concentrations in LLOQ was 20 ng/mL for F351 and M1, 80 ng/mL for M2 and M3.
[b] Concentrations in QC were 60, 600, 3000 ng/mL for F351 and M1, 240, 2400, 12,000 ng/mL for M2 and M3.

In this study, electro-spray ionization was chosen as the ionization source. The highest signal intensity for all analytes was found when using the ESI source in a positive ionization mode. The maximum abundance of the parent and product ions for all analytes was obtained by optimizing the mass spectrometric parameters. Product ions scan spectra are shown in Fig. 2. The compound specific parameters are listed in Table 1. Under these LC–MS/MS condition, the retention times of F351, M1, M2 and M3 were 2.54, 1.38, 1.53, 1.34 and 1.54 min, respectively. Representative LC–MS/MS chromatograms are shown in Fig. 3.

3.2. Method validation

3.2.1. Selectivity

Endogenous peak at the retention time of analytes of interest were not observed in all urine samples evaluated. In addition, the "cross-talk" between MRM transitions used for monitoring F351 and metabolites was evaluated. Although, M2 and M3 had responses in the MRM transition of F351, they did not have any interference to F351 via chromatographic separation. Representative chromatograms in Fig. 3 clearly show the absence of any interference at the retention time for F351, three metabolites and IS.

3.2.2. Calibration curve

The calibration curves were created by plotting the peak area ratios of the various analytes to internal standard versus nominal concentration of the analyte standards. A non-linear relationship for response with concentration is observed more often when ESI-MS detection is used for concentration series extending over wide dynamic rang. This is a direct consequence of saturation of ESI response at concentrations above ±10 μM (19.9, 17.3, 56.9, 42.4 μM for F351, M1, M2 and M3, respectively), a quadratic regression can be used in this situation [18,19]. In this work, a $1/x$ weighted quadratic regression of the type $Y = aX^2 + bX + c$ was used. Calibration analyses were performed on different days and the back-calculated values for each level are shown in Table 2. The firm relationship between peak area ratios and concentrations were demonstrated (Fig. 4), the %CV at each level varied from 0.3 to 7.9 and the %deviation from the theoretical value varied from −8.0 to 7.1 for all analytes. The R-squared for all analytes were greater than 0.9985 for daily runs.

3.2.3. Precision, accuracy and LLOQ

The intra- and inter-day precision and accuracy of the method were determined from the analysis of quality control samples at three different concentrations with 5 replicates and the results are summarized in Table 3. All values of accuracy and precision were found within recommended limits. Intra- and inter-day precisions were less than 5.0, 6.3, 4.9 and 4.4% for F351, M1, M2 and M3, respectively. Intra- and inter-day accuracies measured were between −11.2 and −3.5%, −10.7 and −5.0%, −6.1 and 0.3% and −5.5 and −0.7% for F351, M1, M2 and M3, respectively. The LLOQ

Table 4
Stability results of F351 and three metabolites in different test conditions.

	Nominal conc.[a] (ng/mL)	Stability QCs in mean conc. (%deviation)[b]			
		F351 (ng/mL)	M1 (ng/mL)	M2 (ng/mL)	M3 (ng/mL)
Auto sampler (15 °C, 24 h)	60/240	56.1 (−4.8)	53.1 (−1.7)	237.5 (0.1)	224.7 (−2.7)
	600/2400	542.3 (−6.2)	526.1 (−3.0)	2316.9 (−3.0)	2280.2 (−2.8)
	3000/12,000	2760.6 (−3.4)	2742.0 (−2.8)	11,955.9 (−1.6)	11,849.1 (−1.2)
Freeze–thaw (3 cycles)	60/240	55.3 (−1.9)	53.4 (−2.8)	216.8 (−5.7)	217.3 (−2.9)
	600/2400	562.7 (−3.8)	535.3 (−5.9)	2363.1 (−1.9)	2278.9 (−3.0)
	3000/12,000	2857.1 (−3.5)	2818.5 (−3.1)	12,165.4 (−1.7)	11,858.8 (−1.0)
Short-term (room temperature, 24 h)	60/240	56.4 (0.0)	53.0 (−3.8)	238.2 (3.7)	220.7 (−1.4)
	600/2400	559.7 (−4.4)	539.1 (−5.2)	2354.3 (−2.3)	2274.2 (−3.3)
	3000/12,000	2878.4 (−2.7)	2815.2 (−3.2)	12,176.9 (−1.5)	11,711.8 (−2.3)
Long-term (−30 °C, 18 weeks)	60/240	51.1 (−7.6)	52.9 (−1.1)	209.1 (−3.5)	211.5 (−2.7)
	600/2400	528.1 (−3.9)	542.2 (−2.1)	2156.5 (−1.5)	2207.8 (−0.6)
	3000/12,000	2658.1 (−2.7)	2852.5 (0.0)	11,688.0 (0.3)	12,040.7 (0.2)

[a] Nominal concentrations were 60, 600, 3000 ng/mL for F351 and M1, 240, 2400, 12,000 ng/mL for M2 and M3.
[b] $n=5$; %deviation = (stability QC − freshly prepared QC)/freshly prepared QC × 100.

Table 5
Peak areas of analytes spiked into six lots of different human urine before extraction.

	Nominal conc. (ng/mL)	Peak area mean (n = 6)	SD	%CV
F351	60	45,350.0	1972.6	4.3
	600	429,333.3	5278.9	1.2
	3000	1,870,000.0	20,000.0	1.1
M1	60	3828.3	378.4	9.9
	600	39,666.7	977.1	2.5
	3000	196,500.0	2073.6	1.1
M2	240	37,916.7	868.1	2.3
	2400	389,333.3	5278.9	1.4
	12,000	1,971,666.7	25,625.5	1.3
M3	240	34,650.0	1493.7	4.3
	2400	368,666.7	10,500.8	2.8
	12,000	1,881,666.7	22,286.0	1.2

for all analytes can be reliably quantified with both precision <5.3% and accuracy between −4.5 and 13.0% for 20 replicates (Table 3).

3.2.4. Stability

Conditions in stability assessment included after long-term (−30 °C for 18 weeks) and short-term (room temperature for 24 h) storage, after going through three freeze and thaw cycles and in the autosampler (15 °C for 24 h). By comparing the initial mean values at three different concentrations of QC in different test condition to the similar mean values of freshly prepared QC, the different stability of all analytes in urine was determined. The stability results are summarized in Table 4. There were no significant differences (<10%) in the mean values and indicating analytes stability.

3.2.5. Matrix effect and recovery

The approach used to assess matrix effect among different lots of urine was a modification of that described by Matuszewski et al. [12]. The "relative" matrix effect, possibility of matrix differences between the various lots, was assessed by comparing the analyte peak area corresponding to the different lots at each concentration level. As shown in Table 5, the %CV of the mean peak areas of all analytes at any given concentration in six different urine lots were <10%, indicating little or no difference in ionization efficiency of analytes from different urine lots. Furthermore, the "absolute" matrix effect was estimated by comparing mean peak area ratios of all analytes to IS for samples spiked after extraction from urine with the similar peak area ratios obtained by injecting neat samples at same concentration directly. As shown in Table 6, the matrix effects of F351, M1, M2 and M3 were 105.6, 91.9, 101.4 and 96.0%, respectively.

Table 6
Matrix effect results of F351 and three metabolites.

	Nominal conc. (ng/mL)	Matrix effect[a] (n = 5)	Mean (n = 15)	SD (n = 15)	%CV (n = 15)
F351	60	100.8	105.6	4.5	
	600	107.3			4.3
	3000	108.6			
M1	60	83.5	91.9	8.6	
	600	93.8			9.3
	3000	98.3			
M2	240	98.5	101.4	3.2	
	2400	101.0			3.2
	12,000	104.8			
M3	240	89.2	96.0	5.8	
	2400	96.4			6.1
	12,000	102.3			

[a] Matrix effect (%) expressed as the ratio of the mean peak area ratio of analyte to IS spiked into urine samples after extraction to the mean peak area ratio of the analyte to IS in neat samples and multiplied by100.

Table 7
Recovery results of F351 and three metabolites.

	Nominal conc. (ng/mL)	Recovery[a] (n = 5)	Mean (n = 15)	SD(n = 15)
F351	60	102.7	100.8	
	600	100.8		5.1
	3000	98.9		
M1	60	78.7	85.8	
	600	88.5		6.2
	3000	90.1		
M2	240	93.8	93.3	
	2400	94.0		2.8
	12,000	92.0		
M3	240	108.9	103.5	
	2400	103.4		5.2
	12,000	98.1		

[a] Recovery (%) expressed as the ratio of the mean peak area of analyte spiked into urine before extraction to the mean peak areas of the same analyte spiked into urine after extraction and multiplied by 100.

The recovery was calculated by comparing the mean peak areas of analytes spiked before extraction divided by the areas of analytes of samples spiked after extraction and multiplied by100. Results summarized in Table 7, mean recovery were 100.8, 85.8, 93.3 and 103.5% for F351, M1, M2 and M3, respectively.

4. Conclusion

A LC–MS/MS method for the simultaneous determination of F351 and three metabolites in human urine was developed and validated. The limit of reliable quantification of F351 and M1 was 20 ng/mL, and 80 ng/mL for M2 and M3. The assay affords the sensitivity, accuracy and precision needed for quantitative measurements of F351 and three metabolites; it also demonstrates the necessity for the careful evaluation of the assay selectivity when multiple analytes are quantified.

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