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Method development and validation of the simultaneous determination of a novel topoisomerase 1 inhibitor, the prodrug, and the active metabolite in human plasma using column-switching LC–MS/MS, and its application in a clinical trial

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ABSTRACT

A robust and sensitive method using liquid chromatography–tandem mass spectrometry was developed and validated for the simultaneous determination of a novel topoisomerase 1 inhibitor CH0793076 (3076), the prodrug CH4556300 (TP300), and the active metabolite CH0793011 (3011) in human plasma. All plasma analyzed with this method was acidified with 1 M HCl and 46% citric acid solution in a ratio of 100:10:1 (v:v:v) to avoid the pH-based degradation of TP300 and to shift the equilibria of 3076 and 3011 between the lactone and carboxylate forms towards the lactone forms. After the plasma proteins were precipitated with methanol:acetonitrile:HCl 1 M (50:50:1, v:v:v) containing stable isotopic internal standards, the analytes were trapped on an Xterra MS C18 column (10 × 2.1 mm i.d., 5 μm) and separated on a Gemini C18 column (50 × 2.0 mm i.d., 5 μm) using column-switching liquid chromatography. Electrospray ionization in the positive-ion mode and multiple reaction monitoring were used to quantify the analytes with transitions *m/z* 587.2 > 441.2 for TP300, 459.1 > 415.2 for 3076, and 475.1 > 361.1 for 3011. The inter- and intra-day precisions were below 12%, and the accuracy was between −16% and 16% at the lower limit of quantitation (LLOQ) and between −11% and 14% at the other quality controls. The LLOQs of TP300, 3076, and 3011 were 0.8, 0.04, and 0.04 ng/mL, respectively. The validated method was successfully applied to clinical sample analysis and incurred sample reanalysis was also conducted.

1. Introduction

CH0793076 (3076) is a new hexacyclic camptothecin analogue, identified as a novel topoisomerase 1 inhibitor. It exhibited in vivo antitumor activities superior to CPT-11 in human cancer xenograft models in mice [1]. CPT-11 is the prodrug of the active form SN-38, which is converted by carboxylesterase [2]. It has been reported that the interindividual variability in its pharmacokinetic properties and toxicity is related in part to multiple polymorphic pathways, including the biotransformation of CPT-11, notably a cytochrome P450 3A4-mediated route for the parent drug, and inactivation of SN-38 by UGT1A1, leading to the formation of SN-38 glucuronide [2]. Because 3076 is poorly soluble in water, CH4556300 (TP300), a water-soluble prodrug that can be activated by a nonenzymatic process, was designed to minimize the variability observed in CPT-11 treatments. TP300 is stable at acidic pH, but is rapidly converted chemically to 3076 at physiological pH [3]. This conversion is not an enzymatic reaction. As a relationship between carboxyesterase levels and the cytotoxicity of CPT-11 in human lung cancer cell lines are suggested [4], the profile of TP300 is expected to reduce the interindividual variability in the treatment response.

The severe diarrhea caused by SN-38 and its involvement as a substrate of breast cancer resistance protein (BCRP) are also factors that limit its therapeutic use. BCRP is a half ATP-binding cassette (ABC) efflux transporter that is associated with resistance to camptothecin agents [5,6]. The inhibitor 3076 is not a substrate of BCRP and the antitumor activity of 3076 is higher than that of SN-38 [1,7], regardless of the level of BCRP expression. No acute diarrhea, which results from acetylcholinesterase inhibition, has been observed during treatment with TP300 [7]. Based on this favorable profile, the intravenous administration of TP300 has been under clinical investigation for the treatment of advanced solid tumors, and is expected to have greater utility in cancer therapy than similar classes of compounds.

Our in vitro metabolism study in a human hepatic subcellular fraction suggested that 3076 is metabolized to CH0793011 (3011), the active metabolite, by aldehyde oxidase. Although 3011 is not as potent as 3076, it also has antiproliferative activity [1]. It has been reported that predicting the intrinsic clearance of drugs metabolized by aldehyde oxidase from in vitro–in vivo correlations is

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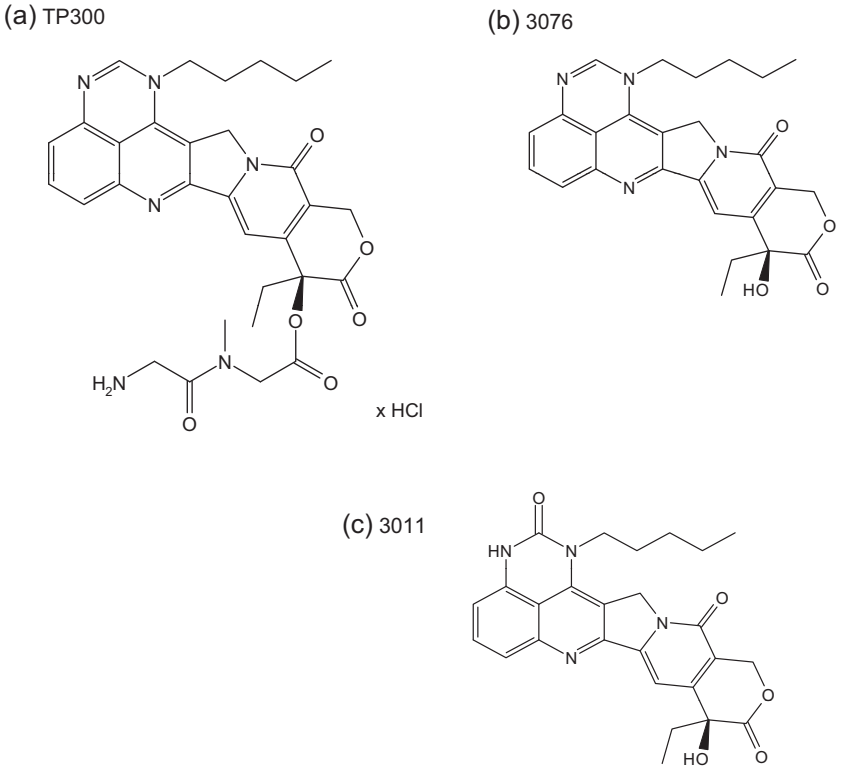

Fig. 1. Structures of (a) TP300, (b) 3076, and (c) 3011.

challenging [8]. To determine the pharmacokinetic profile of TP300 appropriately, not only TP300 and 3076 require quantitation, but also 3011.

CPT-11 and SN-38 are sensitive to a pH-dependent reversible conversion between a pharmacologically active lactone form and its inactive carboxylate form. Similarly, the lactone forms of 3076 and 3011 can be hydrolyzed to the carboxylate forms, depending on the ambient pH [9]. At acidic pH, the interconversion is shifted to the lactone form. Regarding CPT-11 and SN-38, most of the published bioanalytical methods require complicate sample preparation steps include solid phase extraction or liquid–liquid extraction [10]. In addition, some bioanalytical methods applied to the camptothecin analogues have been used to quantify the total concentrations of the two forms of the drugs under acidification or alkalization [11–17]. Other methods have been used to analyze the lactone and carboxylate forms separately in plasma [18–21]. However, careful sample collection, such as with dry ice/methanol, is required to minimize the interconversion between the two forms of the drug.

High sensitivity, high throughput, robustness, and easy handling are generally required for the bioanalysis of clinical samples. High sensitivity is crucial, especially in evaluating the pharmacokinetic profiles of a drug in a first-in-humans study compared with a toxicokinetic study, and high throughput is also required to allow timely decisions to be made regarding dose escalation. Robustness and easy handling are required to ensure system reliability, because it sometimes takes a long time to conduct a clinical trial.

To satisfy these requirements, we developed and validated a quantitation method for the simultaneous determination of TP300, 3076, and 3011 in human plasma using column-switching liquid chromatography–tandem mass spectrometry (LC–MS/MS). On-line sample extraction with a column-switching system has contributed to improvements in the selectivity, sensitivity, robustness and throughput, and a reduction in the cost of bioanalysis [22–24].

2. Materials and methods

2.1. Chemicals and reagents

TP300, 3076, 3011, and their stable isotopic internal standards, d_3-TP300, d_9-3076, and d_3-3011, respectively, were synthesized by Chugai Pharmaceutical Co., Ltd (Tokyo, Japan) (Fig. 1). High-performance liquid chromatography (HPLC)-grade methanol, HPLC-grade acetonitrile, and all the other reagents used were of the highest quality and purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

2.2. Biological samples

Human blood was collected from six healthy individuals in accordance with the Chugai Ethical Guidelines, using heparin sodium as the anticoagulant. The blood from each volunteer was centrifuged at 1500 × *g* for 10 min at 4 °C and used as the blank individual plasma samples. Human blank pooled plasma was purchased from KAC Co., Ltd (Kyoto, Japan). All plasma was mixed with 1 M HCl and 46% citric acid solution in a ratio of 100:10:1 (v:v:v) and stored in a freezer at −20 °C until analysis.

Samples for the clinical study were acidified at the bedside. Immediately following the collection of the blood, it was transferred into a tube containing heparin and 46% citric acid solution. The tube was stored in a refrigerator or on ice until centrifugation. The volumes of blood and 46% citric acid solution were in the ratio of 100:1 (v:v). The tube contents were mixed, and the sample immediately centrifuged at 1500 × *g* for 10 min at 4 °C. The plasma supernatant was then mixed with 1 M HCl in a ratio of 10:1 (v:v). The samples were stored at −70 °C until analysis.

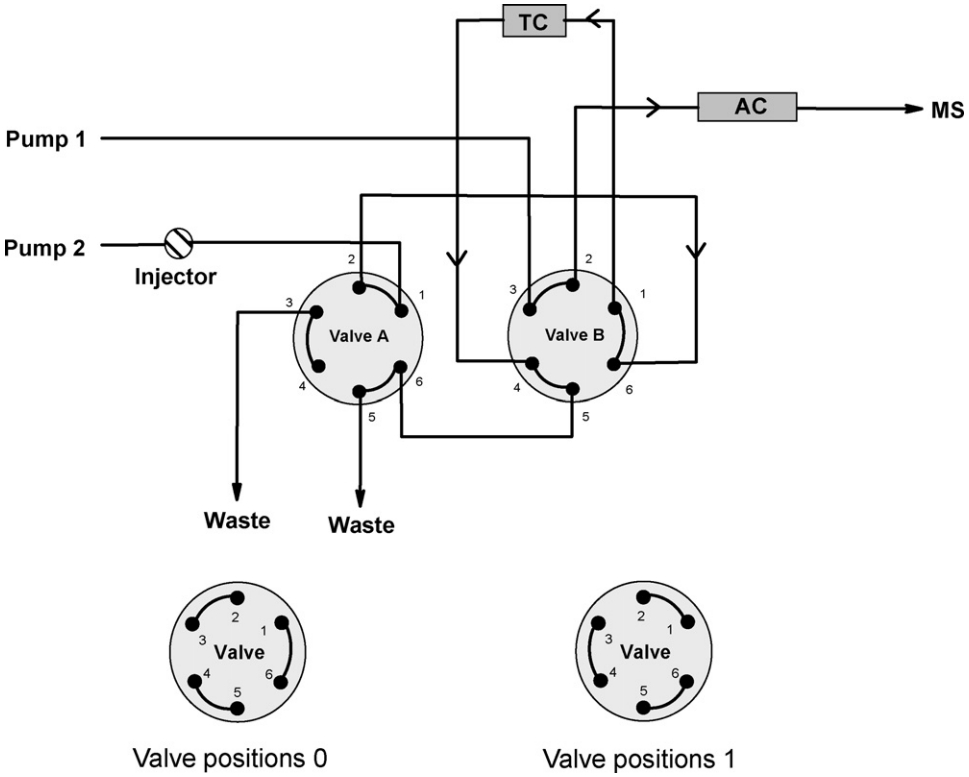

Fig. 2. Scheme of the column-switching LC–MS/MS system. AC, analytical column; TC, trapping column.

2.3. Chromatography and mass spectrometry conditions

The column-switching system is shown in Fig. 2. LC–MS/MS analysis was conducted on an AB SCIEX 4000 Qtrap triple quadrupole mass spectrometer (AB SCIEX, Forster City, CA), equipped with a TurboIonSpray® ionization source and two Shimadzu LC-20 chromatographs (Kyoto, Japan). Two columns were used in the system: a Gemini C18 column (50 × 2.0 mm i.d., 5 μm particle size; Phenomenex Inc., Torrance, CA) as the analytical column and an Xterra MS C18 column (10 × 2.1 mm i.d., 5 μm particle size; Waters, Milford, MA) as the trapping column. The mobile phase consisted of (A1) 0.1% formic acid in water, (B1) 0.1% formic acid in methanol, (A2) 0.1% formic acid in water:methanol (8:2, v:v), and (B2) methanol:acetonitrile (1:1, v:v). The linear gradient conditions and switching valve positions are shown in Table 1. The autosampler was maintained at 5 °C. The injection volume was 200 μL. The injected sample was trapped on the Xterra MS C18 column conditioned with a mobile phase of 0.1% formic acid in water:methanol (8:2, v:v) delivered by pump 2. At 1.5 min, the position of valve A was switched from 1 to 0 for back flash, to remove any unwanted material on the column frit. At 1.7 min, the positions of valves A and B were switched from 0 to 1 for sample elution and separation. At 3.01 min, the position of valve B was switched from 1 to 0 for washing of the trapping column with 100% organic solvent. At 4.01 min, the gradient condition of the mobile phase and the valve were switched to the initial values for the next sample injection. The TurboIonSpray® ionization source was operated in positive mode at a spray voltage of 5500 V and a temperature of 600 °C. The compounds were determined in multiple-reaction-monitoring (MRM) mode. The transitions for MRM were: TP300: 587.2 > 441.2; d_3-TP300: 590.2 > 444.2; 3076: 459.1 > 415.2; d_9-3076: 468.2 > 424.3; 3011: 475.1 > 361.1; and d_3-3011: 478.1 > 361.1.

Table 1
LC time program and corresponding valve positions.

Time (min)	Pump 1		Pump 2		Valve position	
	Flow rate (mL/min)	B1%	Flow rate (mL/min)	B2%	A	B
0	0.2	80	0.05	0	1	0
0.1	0.2	80	2	0	1	0
1.5	0.2	80	2	0	0	0
1.7	0.2	80	2	0	1	1
1.8	0.2	80	2	100	1	1
2.5	0.2	95	2	100	1	1
3.01	0.2	95	2	100	1	0
4.0	0.2	95	2	100	1	0
4.01	0.2	80	2	0	1	0
5.0	0.2	80	2	0	1	0

2.4. Data evaluation

All data were acquired and analyzed using the Analyst® 1.4 software (AB SCIEX). The calibration standards were analyzed and the calibration curves were calculated with the least squares method (weighting of $1/x^2$) using the relationship between the ratio of the analyte peak area to the internal standard (IS) peak area (peak area ratio) and the nominal analyte concentration.

2.5. Preparation of stock solutions and working solutions

Stock solutions of TP300, 3076, d_3-TP300, and d_9-3076 were prepared separately by dissolving the compounds in standard solution solvent A, consisting of acetonitrile, water, and 1 M HCl mixed in a ratio of 50:50:1 (v:v:v), to produce 1 mg/mL stock solutions. The stock solutions of 3011 and d_3-3011 were prepared by dissolving the compounds in standard solution solvent B, consisting of acetonitrile, DMSO, and 1 M HCl mixed in a ratio of 50:50:1 (v:v:v), to produce 1 mg/mL stock solutions. Working stock solutions of 50 μg/mL TP300 and 50 μg/mL 3076 were prepared by

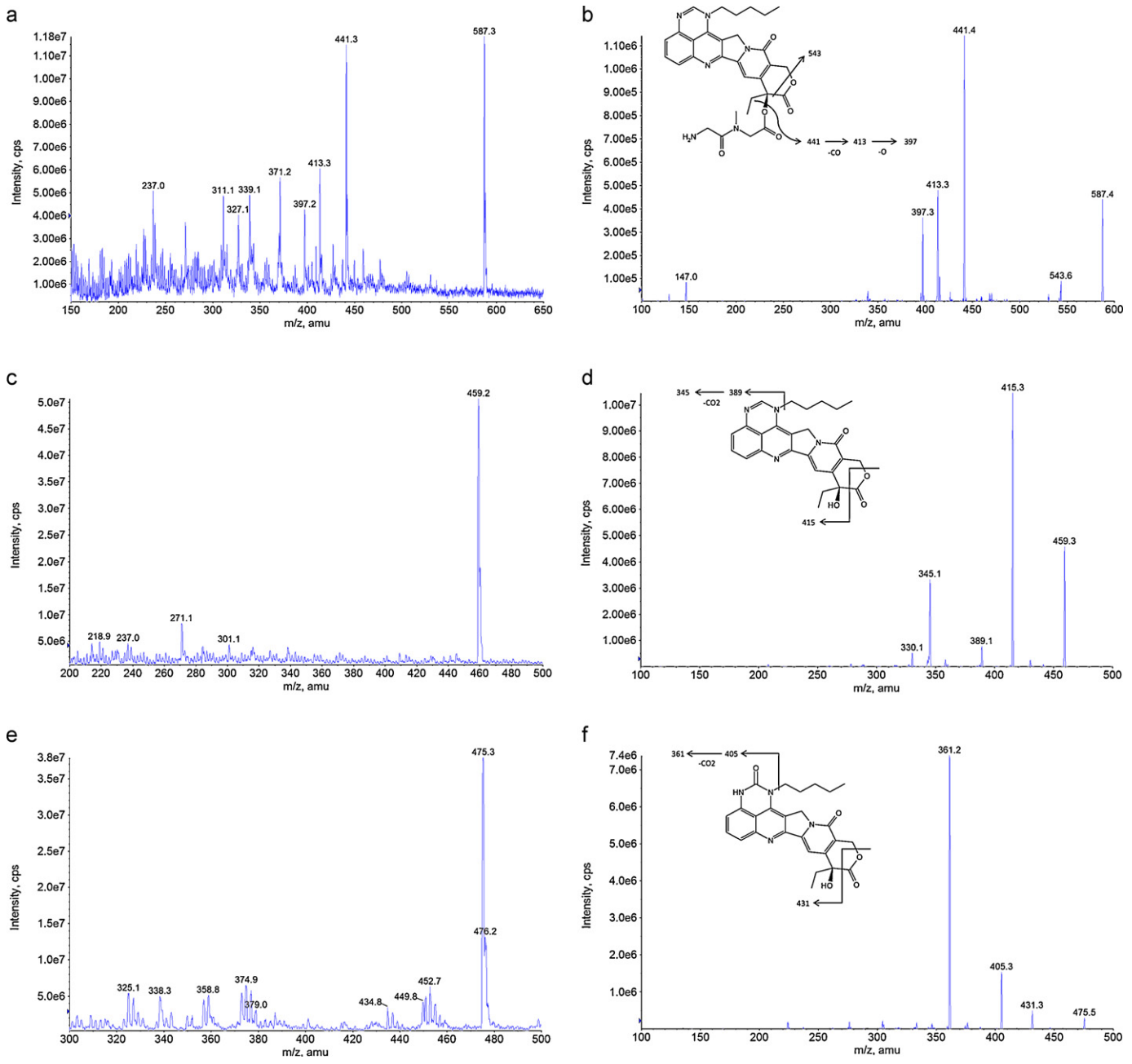

Fig. 3. Q1 full-scan spectra of TP300 (a), 3076 (c), and 3011 (e) and the product-ion spectra of the precursor at *m/z* 587 (b), 459 (d), and 475 (f).

diluting the stock solution with standard solution solvent A. A working stock solution of 50 μg/mL 3011 was prepared by diluting the stock solution with standard solution solvent B. IS solutions of d_3-TP300, d_9-3076, and d_3-3011 were prepared by dissolving the stock solutions in methanol:acetonitrile:HCl 1 M (50:50:1, v:v:v). All the stock solutions were stored at −20 °C and used within 60 days of preparation. Stock solution stability under these conditions was confirmed with our in-house data.

2.6. Calibration standard and quality control (QC) preparation

Working stock solutions of the calibration standards and QCs were diluted with standard solution solvent A to prepare the standard solutions. The calibration standards and QCs were prepared by spiking acidified human blank pooled plasma with them. The calibration standards for TP300 were prepared at concentrations of 0.8, 2, 5, 10, 25, 62.5, 125, and 313 ng/mL and the QC samples were prepared at concentrations of 0.8, 2, 20, and 250 ng/mL. The calibration standards for 3076 and 3011 were prepared at concentrations of 0.04, 0.1, 0.25, 0.5, 1.25, 3.13, 6.25, and 15.6 ng/mL and the QC samples were prepared at concentrations of 0.04, 0.1, 1, and 12.5 ng/mL. The calibration standards were freshly prepared during the validation study.

2.7. Sample preparation

A 200 μL aliquot of the IS solution was added to 100 μL of human plasma and vortexed. For preparing blank sample, the same volume of methanol:acetonitrile:HCl 1 M (50:50:1, v:v:v) was used instead of the IS solution. After centrifugation at 20,400 × *g* for 15 min at 4 °C, the supernatant was transferred to a new 2 mL 96-well plate and mixed with 300 μL of 1 M HCl:water (1:1000, v:v).

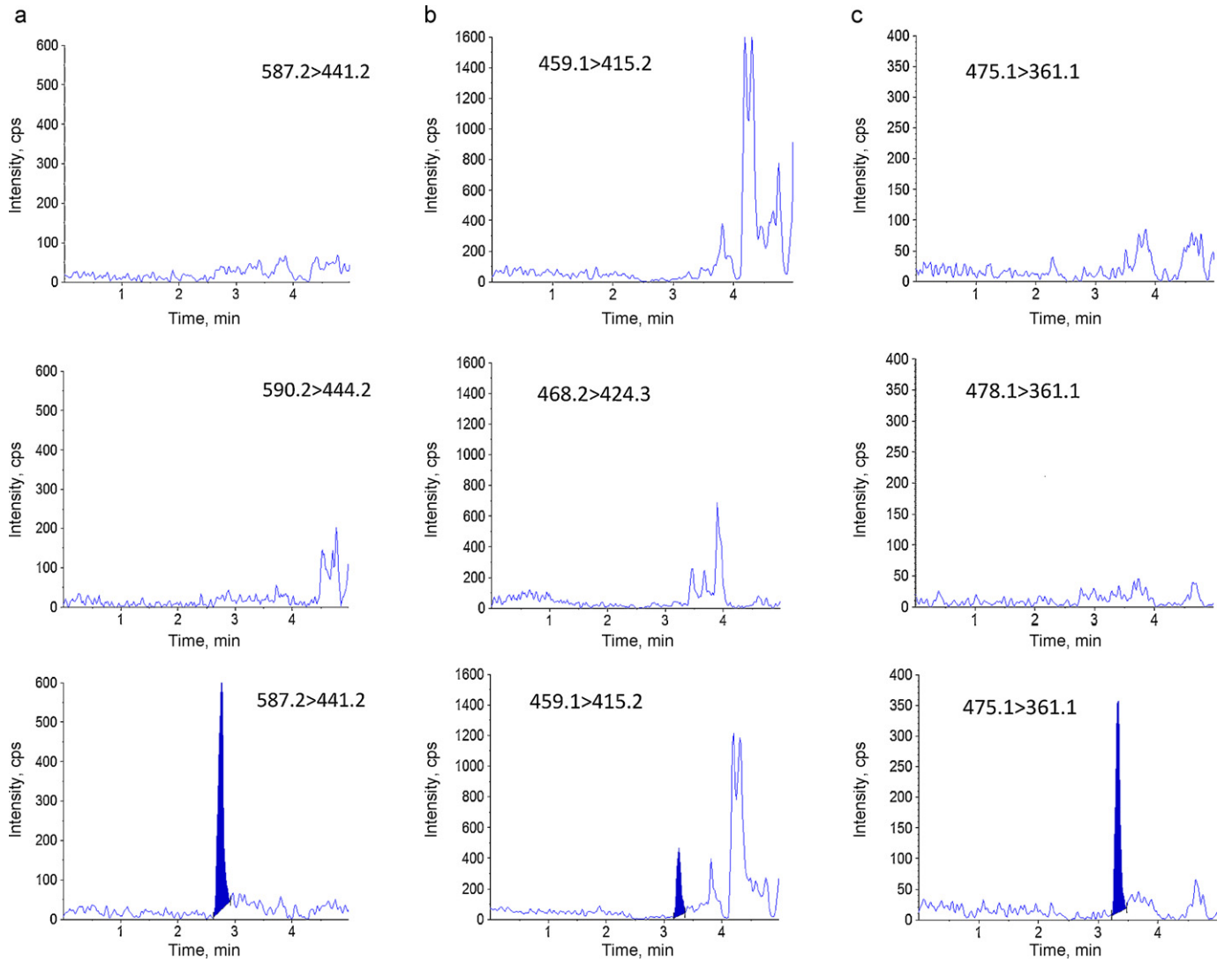

Fig. 4. Representative MRM chromatograms: (a) TP300 and d_3-TP300 in blank plasma (upper and middle) and TP300 at LLOQ (lower); (b) 3076 and d_9-3076 in blank plasma (upper and middle) and 3076 at LLOQ (lower); and (c) 3011 and d_3-3011 in blank plasma (upper and middle) and 3011 at LLOQ (lower).

2.8. Validation procedure

Validation was conducted according to the FDA guidance for bioanalytical method validation [25]. Selectivity was evaluated by analyzing six independent individual human plasmas. Eight points were analyzed for the calibration standards, excluding the blank and zero, and the calibration curves were calculated with the least squares method (weighting of $1/x^2$) using the relationship between the ratio of the analyte peak area to the IS peak area (peak area ratio) and the analyte concentration. The linearity of the calibration curves was evaluated by analyzing the calibration standards on three different days and calculating the accuracy of the back-calculated concentrations. Interday and intraday reproducibility were evaluated by analyzing four levels of QC samples as five replicates on three different days. Intraday reproducibility was determined each day.

LLOQ was defined as the lowest concentration on the calibration curve that met the criteria for selectivity, linearity of the calibration curve, and intraday and interday reproducibility. Dilution integrity was evaluated by analyzing the 10 μg/mL TP300 sample and 1 μg/mL 3076 and 3011 sample. The samples were diluted 100- and 1000-fold, respectively, with blank human plasma. The diluted samples were processed and analyzed as five replicates. The concentrations were calculated by multiplying the assay values by the dilution ratio.

The recovery and matrix effects were evaluated by analyzing the samples prepared in the three sets: (1) prespiking sample: the analyte was spiked into blank plasma and the sample was extracted with IS solution; (2) postspiking sample: the analyte and the IS were spiked into blank plasma extracts with methanol:acetonitrile:HCl 1 M (50:50:1, v:v:v); and (3) solution sample: the analyte and the IS were spiked into the matrix-free solution. The concentrations used for these evaluations were the same as those designated low QC (2 ng/mL of TP300 and 0.1 ng/mL of 3076 and 3011), middle QC (20 ng/mL of TP300 and 1 ng/mL of 3076 and 3011), and high QC (250 ng/mL of TP300 and 12.5 ng/mL of 3076 and 3011). The recovery of the analyte was calculated by dividing the peak area of (1) by that of (2). The absolute matrix effects for the analyte and IS were calculated by dividing the peak area of (2) by that of (3). The IS-normalized matrix effect for the analyte was calculated by dividing the peak area ratio (analyte/IS) of (2) by that of (3). Five replicates of three concentrations per sample were analyzed and the averages of the responses were used for the calculation.

Bench-top stability, freeze/thaw stability, long-term freezer storage stability, and extracted sample stability were evaluated by comparing the peak area ratio of the freshly prepared low-QC and

high-QC samples with that of the samples stored under the relevant storage conditions in triplicate. Freshly prepared duplicate low-QC, middle-QC, and high-QC samples were analyzed to ensure control of the assay for the stability evaluation.

3. Results and discussion

3.1. Method development

To optimize the MS conditions for TP300, 3076, 3011, and the corresponding ISs, each infusion solution was prepared in 0.1% formic acid:MeOH (1:1, v:v) at a concentration of 100 ng/mL from each working stock solution, and injected into the mass spectrometer. Positive-ion mode was used for all compounds. The Q1 full-scan spectra and product-ion spectra for TP300, 3076, and 3011 are presented in Fig. 3. The most intense ions on the Q1 full scan were observed at m/z 587, 459, and 475 for TP300, 3076, and 3011, respectively, and those ions were identified as $[M+H]^+$ ions. The product-ion spectra of these precursor ions were obtained at collision energies (CEs) of 37 eV, 41 eV, and 47 eV for TP300, 3076, and 3011, respectively, as shown in Fig. 3. Major fragments were observed at m/z 441, 415, and 361 for TP300, 3076, and 3011, respectively. The CEs and collision cell exit potentials were adjusted to obtain the best ion intensity on MRM. The MRM transitions of TP300, 3076, and 3011 were selected at 587.2 > 441.2, 459.1 > 415.2, and 475.1 > 361.1, respectively.

The column-switching LC conditions were developed by evaluating the intensity and selectivity of the analytes. To prevent the pH-dependent degradation of TP300 and interconversion of 3076 and 3011, acidic conditions were selected for the mobile phase. The ratio of aqueous solution in mobile phase A2 to show maximum adsorption of the analytes on a trapping column was determined to be 80%, at which the peak areas for TP300, 3076, and 3011 were most intense when passed through an Xterra MS C18 trapping column compared with other C18 columns. An injected sample was transferred to the trapping column at the flow rate of 2 mL/min for 1.5 min to retain the analyte on the column. After selecting the mobile phase and gradient conditions for the trapping column, the analytical column was selected. A Gemini C18 column was used as the analytical column because it gave the best peak shape and selectivity compared with other C18 columns.

Human plasma was acidified with 46% citric acid solution and 1 M HCl to avoid the degradation of TP300 to 3076 during sample handling and to shift the equilibrium between the lactone and carboxylate forms of 3076 and 3011 towards the lactone forms.

3.2. Selectivity

Aliquots of blank plasma from six different individuals were tested for endogenous interference. Representative chromatograms for blank plasma and LLOQ are shown in Fig. 4. No significant interference peak was observed in any individual plasma at the retention times and MRM channels for TP300, 3076, 3011, or their ISs.

3.3. Calibration curve linearity

The back-calculated concentrations and accuracies are presented in Table 2. The calibration curves were linear and valid for human plasma in the range from 0.8 ng/mL to 313 ng/mL for TP300 and from 0.04 ng/mL to 15.6 ng/mL for 3076 and 3011. The accuracies for TP300, 3076, and 3011 ranged from −3.9% to 7.2%, from −6.4% to 12.8%, and from −6.0% to 13.0%, respectively.

Table 2
Linearity of the calibration curves for TP300, 3076, and 3011 in human plasma.

TP300 nominal conc.	Accuracy (%)		
(ng/mL)	Day 1	Day 2	Day 3
0.8	−3.1	−0.9	2.0
2	6.0	2.5	−3.0
5	3.6	1.0	−3.2
10	2.0	−3.9	−3.6
25	−2.0	0.4	−3.6
62.5	−2.7	0.3	1.4
125	−0.8	0.8	7.2
313	−3.5	−0.3	3.2
3076 Nominal conc.	**Accuracy (%)**		
(ng/mL)	Day 1	Day 2	Day 3
0.04	−2.3	−4.8	−0.3
0.1	3.0	8.0	1.0
0.25	6.0	12.8	0.0
0.5	0.0	−1.4	0.0
1.25	2.4	−6.4	−2.4
3.13	−1.9	−0.3	1.3
6.25	−3.8	−6.1	0.0
15.6	−3.2	−1.9	0.0
3011 Nominal conc.	**Accuracy (%)**		
(ng/mL)	Day 1	Day 2	Day 3
0.04	−5.3	1.5	−0.5
0.1	13.0	−6.0	2.0
0.25	0.0	7.2	−2.4
0.5	0.4	−0.6	1.0
1.25	1.6	−4.0	−1.6
3.13	−2.2	2.2	0.6
6.25	−4.8	−1.6	0.0
15.6	−2.6	1.3	1.3

3.4. Reproducibility and dilution integrity

The intraday and interday reproducibility values are presented in Table 3. Accuracy and precision were evaluated to establish the method reproducibility. All the accuracy values for TP300, 3076, and 3011 were within ±15% (±20% at LLOQ), and all the precision values for TP300, 3076, and 3011 were less than 15%.

The LLOQs for TP300, 3076, and 3011 were 0.8 ng/mL, 0.04 ng/mL, and 0.04 ng/mL, respectively, determined from the results for selectivity, calibration curve linearity, and reproducibility.

The dilution integrity data are presented in Table 4. The samples used to evaluate dilution integrity, at concentrations of 10 μg/mL TP300 and 1 μg/mL 3076 and 3011, were diluted 100- and 1000-fold, respectively, with blank plasma. All the accuracy values for TP300, 3076, and 3011 were within ±15% and all the precision values for TP300, 3076, and 3011 were less than 15%. This confirms that samples at concentrations above the calibration range can be diluted 100-fold for TP300 and 1000-fold for 3076 and 3011 with acidified blank human plasma.

3.5. Recovery and matrix effect

The recovery and matrix effect data are presented in Table 5. The investigation was conducted at three concentrations. The recovery data were uniform for all concentrations but were not very high. No modification of the method was made to improve the recovery because we judged from previous results that the intensity was sufficient for the quantitation and the assay was performed with stable isotopic ISs. The absolute matrix effect values for TP300 and the IS were between 0.6 and 0.7, which means ion suppression was observed. The absolute matrix effect values for 3076, 3011, and

Table 3
Intraday and interday accuracy and precision of QC samples for TP300, 3076, and 3011 in human plasma.

	Nominal concentration (ng/mL)	Intraday accuracy (%)	Interday accuracy (%)	Intraday precision (%)	Interday precision (%)
TP300	0.8	−2.0 to 16.3	9.5	2.8 to 8.9	9.5
	2	−1.5 to 13.5	8.0	1.3 to 9.4	8.0
	20	−5.0 to 10.0	5.0	1.0 to 1.9	6.9
	250	−4.4 to 12.8	4.8	2.8 to 3.9	7.7
3076	0.04	−15.5 to 7.8	−0.3	3.1 to 7.5	12.1
	0.1	−10.2 to 10.0	3.0	4.1 to 7.2	10.5
	1	−3.1 to 12.0	7.0	1.4 to 2.0	7.1
	12.5	−5.6 to 10.4	4.8	0.4 to 1.4	7.1
3011	0.04	−15.3 to 6.5	−1.3	2.9 to 6.2	11.4
	0.1	−10.5 to 10.0	2.0	0.8 to 6.4	9.9
	1	−4.0 to 14.0	7.0	1.0 to 3.3	8.0
	12.5	−7.2 to 12.8	4.0	1.1 to 1.4	8.4

Table 4
Dilution integrity of TP300, 3076, and 3011 in human plasma.

	Dilution factor	Accuracy (%)	Precision (%)
TP300	100	−5.1	1.9
3076	1000	7.0	2.2
3011	1000	7.0	1.4

the ISs were between 1.0 and 1.3, which means ion enhancement was observed. However, the stable isotopic ISs compensated these effects. The IS-normalized matrix effect values were between 0.9 and 1.2.

3.6. Stability

The stability results are presented in Table 6. The study was conducted using low- and high-QC samples to evaluate the stability of the extracted samples in the autosampler tray at 5 °C for 24 h, their bench-top stability at room temperature for 24 h, their freeze/thaw stability after four cycles, and their long-term freezer storage stability at −20 °C and −70 °C for 252 days. All results were within 15% of the initial values, showing sufficient stability for sample analysis and storage.

3.7. Application to clinical sample analysis

The validated method was successfully applied to the sample analysis in a clinical trial. All extractions and analyses were conducted under secure and stable conditions. Representative data for the first-in-humans study are presented in Fig. 5, which shows the time–plasma concentration profiles for 3076 and 3011 after the intravenous administration of TP300 to a subject at a dose of 1 mg/m^2. In all analyses, TP300 was below the limit of quantitation, which suggests that TP300 was converted to 3076 at physiological pH, as previously observed [3]. The sensitivity of the quantitation of 3076 and 3011 was sufficient to evaluate the time–plasma concentration profiles of the compounds in the first-in-humans study.

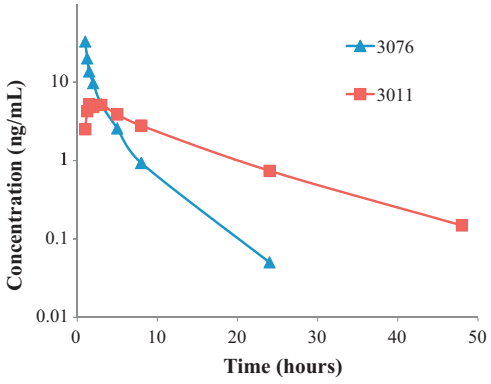

Fig. 5. Representative time–plasma concentration profiles of 3076 and 3011.

Incurred sample reanalysis (ISR) of 3076 and 3011 was conducted to confirm the robustness and reproducibility of the

Table 5
Recovery and matrix effect.

Analyte	TP300			3076			3011		
Nominal conc. (ng/mL)	2	20	250	0.1	1	12.5	0.1	1	12.5
Recovery (%)	72.8	65.7	62.1	72.3	67.2	72.5	70.8	65.0	68.2
Absolute matrix effect (analyte)	0.7	0.6	0.7	1.2	1.3	1.3	1.0	1.3	1.3
Absolute matrix effect (IS)	0.7	0.6	0.6	1.3	1.2	1.2	1.1	1.1	1.0
IS-normalized matrix effect	1.1	1.1	1.1	0.9	1.1	1.1	1.0	1.2	1.2

Table 6
Stability of TP300, 3076, and 3011 in extracted sample and human plasma (shown as % difference from the initial sample).

		Extracted sample/autosampler tray (5 °C, 24 h)	Bench top (room temperature, 24 h)	Freeze/thaw (−20 °C, 4 times)	Freeze/thaw (−70 °C, 4 times)	Freezer storage (−20 °C, 252 days)	Freezer storage (−70 °C, 252 days)
TP300	Low QC	−2.3	−8.6	−11.8	−4.5	−9.5	−7.7
	High QC	11.4	−10.6	−2.7	−1.5	−0.4	2.3
3076	Low QC	−3.4	−0.9	−5.1	−12.8	−12.8	−14.5
	High QC	6.6	−1.5	−2.2	−1.5	−10.2	−6.6
3011	Low QC	−0.9	4.6	4.6	−0.9	−13.0	−11.9
	High QC	4.5	−2.3	1.5	3.0	−8.3	−8.3

method. The ISR criterion was that two-thirds of the repeated samples should vary less than 20%. The variability was calculated with the following formula: [(repeated value−original value)/average of the two] × 100 [26]. In both the 3076 and 3011 analyses, 77.5% of the repeated samples met the criterion for ISR.

4. Conclusions

We have developed and validated a method for the simultaneous determination of TP300, 3076, and 3011 in human plasma using column-switching LC–MS/MS, and applied it to the analysis of clinical samples. Because of the high sensitivity and robustness of this method, the time–concentration profiles for 3076 and 3011 were successfully observed in a first-in-humans study and there was no need to modify the method throughout the analysis of the whole study sample. Bioanalysis requires laborious work, especially in sample preparation, and a column-switching system can be a useful tool not only to improve the capacity for sample analysis, including the sensitivity and reproducibility of the method, but to increase the throughput of the whole process of sample preparation.

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