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High-sensitivity liquid chromatography–tandem mass spectrometry method for the simultaneous determination of sodium picosulfate and its three major metabolites in human plasma

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ABSTRACT

Sodium picosulfate (PICO-Na) is a member of the polyphenolic group of stimulant laxatives. Its major metabolites in humans are its active aglycone BHPM (bis-(p-hydroxyphenyl)-pyridyl-2-methane), the monoglucuronide (M1) and the monosulfate (M2) of BHPM. A sensitive, specific and rapid liquid chromatography–tandem mass spectrometry method was established and validated for the simultaneous determination of picosulfate (PICO) and its three major metabolites in human plasma to investigate the pharmacokinetics of PICO and its major metabolites. Following protein precipitation with acetonitrile, chromatographic separation was achieved on a Luna 5u $C_{18}(2)$ column using gradient elution starting with 10% of 10 mM ammonium acetate followed by increasing percentages of acetonitrile to eliminate interferences due to in-source conversion of the conjugated metabolites. Detection was performed on a tandem mass spectrometer equipped with an electrospray ionization source operated in the positive mode, using the transitions of m/z 438.1 → m/z 278.1 for PICO, m/z 278.1 → m/z 184.2 for BHPM, m/z 454.1 → m/z 184.2 for M1, and m/z 358.1 → m/z 184.2 summed with m/z 358.1 → m/z 278.1 for M2. Deuterium labeled compounds of the analytes were used as the internal standard, two of which, M1-d_{12} and M2-d_{12}, were synthesized in-house. The method was validated in concentration ranges of 0.150–40.0 ng/mL for PICO and M2, 0.600–160 ng/mL for BHPM, and 0.045–12.0 ng/mL for M1 with acceptable accuracy and precision. The method was successfully applied to characterize the pharmacokinetic profiles of PICO and its metabolites in healthy volunteers after a single oral administration of 5 mg PICO-Na.

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1. Introduction

Sodium picosulfate (PICO-Na, disodium (pyridin-2-ylmethylene)di-4,1-phenylene disulfate), is a member of the polyphenolic group of stimulant laxatives (Fig. 1A). PICO-Na is a prodrug and has no intrinsic laxative activity itself. After the oral administration of PICO-Na (5–10 mg once daily), it is hydrolyzed into its aglycone bis-(p-hydroxyphenyl)-pyridyl-2-methane (BHPM) by bacteria naturally present in the large intestine [1–3]. Thereafter, BHPM inhibits the absorption of water and electrolytes and increases their secretion into the intestinal lumen, which then causes laxative effects [4]. After an extensive absorption in the large intestine, BHPM is further metabolized to BHPM monoglucuronide (M1), and then excreted in bile and urine [3,5,6].

To the best of our knowledge, although PICO-Na has been widely used in clinic for over 30 years, and information on the clinical pharmacokinetic properties of picosulfate (PICO) and its metabolites remain lacking, and the absorption levels and circulating exposure of PICO and BHPM remain incompletely understood. No study concerning the individual or simultaneous determination of PICO and its metabolites in a biological matrix has yet been reported. Possibly constrained by assay sensitivity, the published procedures for the toxicological screening of PICO only determine the total concentration of BHPM in a biological matrix after enzymatic cleavage of its conjugates [7,8].

A human metabolism study carried out in our laboratory revealed that the major metabolite of PICO in human plasma is M1. Besides this finding, the monosulfate of BHPM (M2) could also be detected. M1 has been reported to be produced by the glucuronidation of BHPM [5]. The formation mechanism of M2 is unknown. Further research should be undertaken to determine whether the exposure of M2 originates from the hydrolysis of PICO or the further sulfation of BHPM.

In this research, a sensitive, rapid, and simple liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed for the simultaneous quantification of PICO, BHPM, M1, and M2 in human plasma. The method was validated according to the US Food and Drug Administration (FDA) guidelines and

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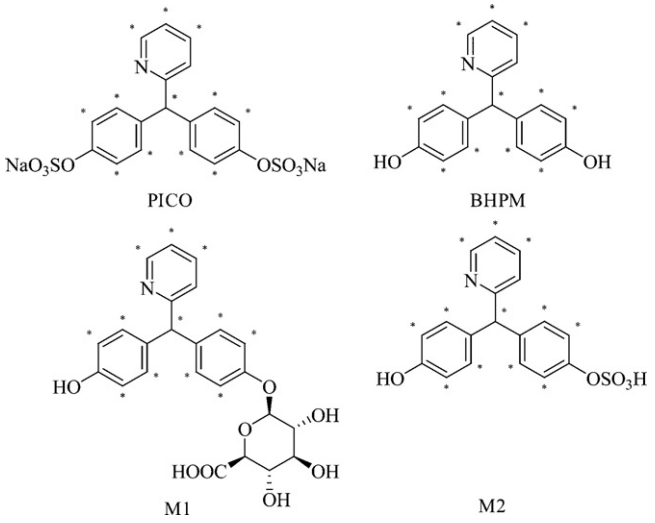

Fig. 1. Structures of PICO, BHPM, M1, M2 and their stable isotopically labeled ISs. The "*" was position of deuterium labeled.

successfully applied to a pharmacokinetic study following a single oral administration of 5 mg of PICO-Na. The pharmacokinetic profiles of PICO and its metabolites in humans were reported for the first time.

2. Experimental

2.1. Materials

PICO-Na (purity > 99%) and BHPM (purity 100%) were kindly provided by Chongqing LUMMY Pharmaceutical Co., Ltd. (Chongqing, China). The two deuterium labeled internal standards, PICO-d_{12} and BHPM-d_{12}, were purchased from Toronto Research Chemicals Inc. (Ontario, Canada). Acetonitrile and methanol (HPLC grade) were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Ammonium acetate (HPLC grade) was purchased from Tedia (Fairfield, OH, USA). Deionized water (18.2 mΩ and TOC ≤ 50 ppb) was prepared by a Millipore Milli-Q Gradient Water Purification System (Molsheim, France).

2.2. Reference compound syntheses

M1 and M1-d_{12} was biosynthesized by the incubation of BHPM (1 mM) or BHPM-d_{12} (1 mM) with uridine 5′-diphospho glucuronic acid (4 mM) and $MgCl_2$ (5 mM) in rat liver homogenates at 37 °C for 1 h. M2 and M2-d_{12} were produced by the incubation of PICO (6.2 mg/mL) or PICO-d_{12} (1.0 mg/mL) in aqueous hydrochloric acid solutions (1.9 M) at 40 °C for 2 h. All of the syntheses were accomplished in our laboratory. Further isolation and purification procedures were performed by WuXi AppTec Co., Ltd. (Shanghai, China).

2.3. Instruments

The liquid-chromatographic system consisted of a LC-30AD binary pump, a DGU-20A_5 vacuum degasser, a SIL-30AC autosampler, and a CTO-30A column oven (Shimadzu Corporation, Kyoto, Japan). An AB Sciex (Foster City, CA, USA) 5500 Qtrap triple-quadrupole mass spectrometer equipped with a Turbolon Spray (ESI) source was used for mass analysis and detection. Data acquisition and processing were performed using Analyst version 1.5.1 software (AB Sciex).

Table 1
The MS parameters for each transition.

Analyte	Q1 (*m/z*)	Q3 (*m/z*)	Dwell time (ms)	CE (eV)
PICO	438.1	278.1	80	36
BHPM	278.1	184.2	80	64
M1	454.1	184.2	80	47
M2	358.1	278.1/184.2	80	20/37
PICO-d_{12}	450.1	290.1	80	36
BHPM-d_{12}	290.1	192.2	80	64
M1-d_{12}	466.1	192.2	80	47
M2-d_{12}	370.1	290.1/192.2	80	20/37

2.4. LC–MS/MS conditions

Chromatographic separation was carried out on a Luna 5u C_{18}(2) column (50 mm × 2.0 mm i.d., 5 μm; Phenomenex, Torrance, CA, USA) equipped with a Security-Guard C_{18} column (4.0 mm × 3.0 mm i.d.; Phenomenex, Torrance, CA, USA). The column temperature was maintained at 40 °C. The mobile phase used for gradient elution consisted of (A) 10 mM ammonium acetate and (B) acetonitrile. The initial mobile phase was composed of 10% B. During the first 2.5 min of elution, the composition of B was increased to 25%. Then, it was stepwise increased to 70% in 1.5 min and to 100% in 0.1 min. These conditions were maintained for 0.4 min, after which the column was equilibrated with the initial mobile phase. The initial flow rate was 0.6 mL/min, which was maintained for the first 4.4 min. Afterwards, the flow rate was increased to 1.0 mL/min in 0.1 min, which was maintained from 4.5 min to 6.0 min. The eluents were diverted to waste for the first 1.0 min and after 4.5 min to avoid excessive contamination of the ionization source. The injection volume was 20 μL.

The mass spectrometer was operated in the positive ion mode. The ion spray voltage and the source temperature were set to 3000 V and 600 °C, respectively. The nebulizer gas (Gas 1), heater gas (Gas 2), and curtain gas were set to 70, 70, and 30 psi, respectively. The collision activated dissociation gas level was set to medium. MRM transitions were recorded and used for quantification, and their relative MS parameters are listed in Table 1.

2.5. Preparation of calibration standard and quality control (QC) samples

Stock solutions for all analytes were prepared in duplicate; one set was used for the preparation of calibration standards and the other for QC samples. Stock solutions of PICO, BHPM, M1, and M2 were prepared by weighting appropriate amounts of the analytes and dissolving them in water or methanol to obtain concentrations of 962 (calculated as free base), 1000, 87.0, and 500 μg/mL, respectively. Standard combined dilutions were prepared in methanol–water (50:50, v/v) at 800/3200/800/240, 160/640/160/48.0, 60/240/60/18.0, and 6.00/24.0/6.00/1.80 ng/mL for PICO, BHPM, M1, and M2. Calibration standards were prepared at 0.150/0.600/0.150/0.045, 0.300/1.20/0.300/0.090, 1.00/4.00/1.00/0.300, 3.00/12.0/3.00/0.900, 8.00/32.0/8.00/2.40, 20.0/80.0/20.0/6.00, and 40.0/160/40.0/12.0 ng/mL by adding small volumes (a maximum of 5% of the total volume) of dilutions to analyte-free plasma. QC samples were similarly prepared at concentrations of 0.150/0.600/0.150/0.045, 0.300/1.20/0.300/0.090, 3.00/12.0/3.00/0.900, and 32.0/128/32.0/9.60 ng/mL for the lower limit of quantification (LLOQ), low QC (LQC), medium QC (MQC), and high QC (HQC), respectively. A combined internal standard (IS) working solution (PICO-d_{12}/BHPM-d_{12}/M1-d_{12}/M2-d_{12}, 325/80.0/250/150 ng/mL) was prepared by diluting the IS stock solutions (PICO-d_{12}, 1.00 mg/mL; M1-d_{12}, 17.0 μg/mL; M2-d_{12},

77.3 μg/mL; BHPM-d_{12}, 368 μg/mL) with methanol–water (50:50, v/v). All solutions were kept refrigerated at 4 °C and brought to room temperature before analysis. Calibration standards and QC samples were stored at −20 °C.

2.6. Sample preparation

To a 100 μL aliquot of the plasma samples, 20 μL of the IS solution and 400 μL of acetonitrile were added. The mixture was vortex-mixed for 1 min and centrifugated for 5 min at 11,000 × *g*. The supernatant was transferred to another tube and evaporated to dryness under a stream of nitrogen at 40 °C. The residue was reconstituted in 100 μL of acetonitrile–water (10:90, v/v), and a 20 μL aliquot of the resulting solution was injected onto the LC–MS/MS system for analysis.

2.7. Method validation

The method was validated for selectivity, linearity, precision and accuracy, stability, recovery, and matrix effect according to US FDA guidelines [9].

The selectivity of the method was evaluated by analyzing blank plasma samples and spiked plasma samples at LLOQ level from six different sources. The peak areas of coeluting interferences should be <20% of the peak areas of the LLOQ standards.

The calibration standards were prepared and assayed in duplicate on three consecutive days. The peak area ratios (each analyte to the IS) were plotted against the nominal analyte concentrations to construct the calibration curves. The linear least-squares regression model was used for curve fitting, with $1/x^2$ as the weighting factor, where *x* stands for the nominal concentration.

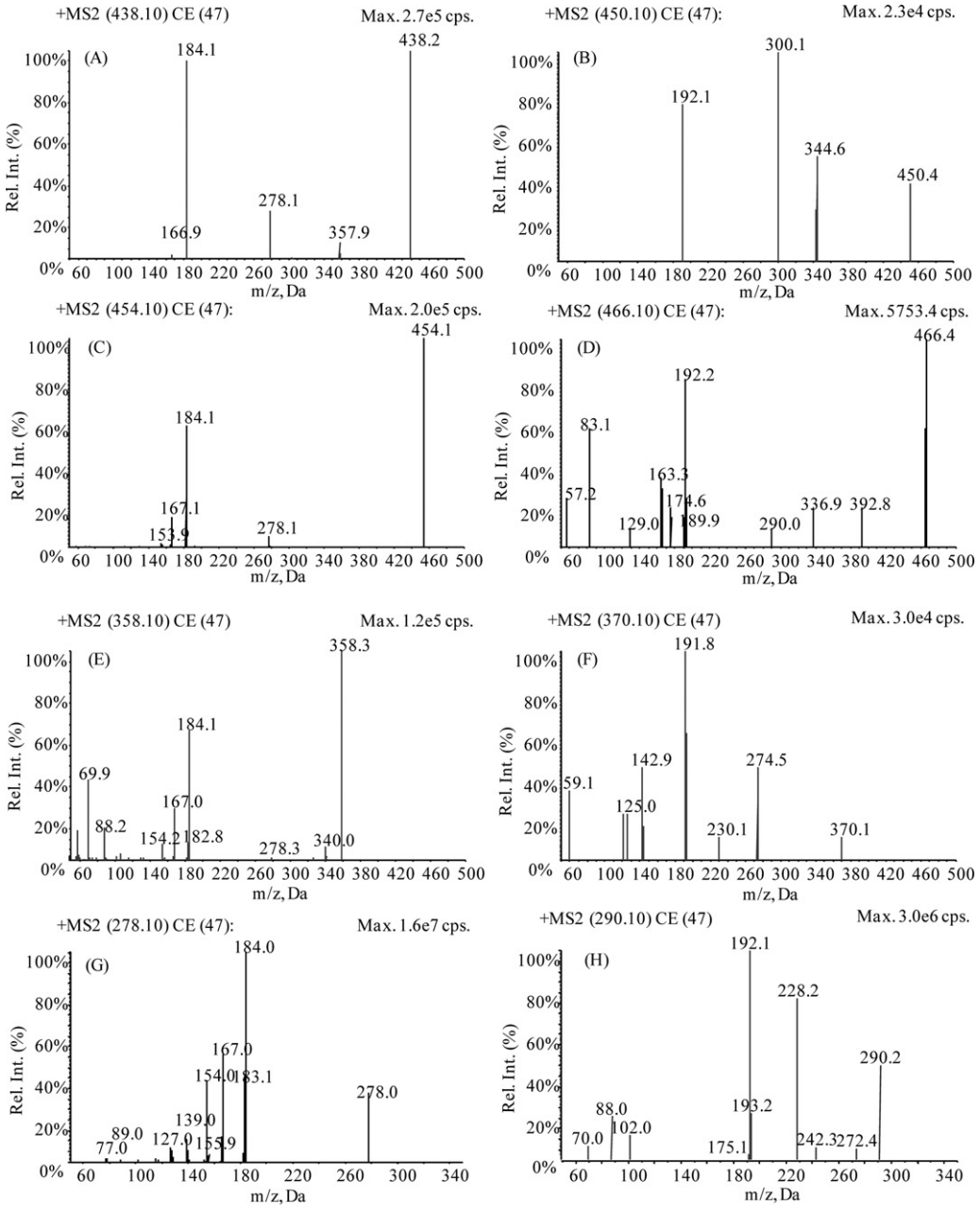

Fig. 2. Product ion spectra of $[M+H]^+$ ions: (A) PICO; (B) PICO-d_{12}; (C) M1; (D) M1-d_{12}; (E) M2; (F) M2-d_{12}; (G) BHPM; (H) BHPM-d_{12}.

The accuracy and precision were evaluated by the determination of QC samples at four concentrations in six replicates over three consecutive days. The intra- and inter-day precision expressed by the relative standard deviation (RSD), should not exceed 20% for the LLOQ samples and 15% for the other QC samples. The accuracy expressed by relative error (RE) should be within ±20% for LLOQ samples and ±15% for the other QC samples.

The stability of each analyte was investigated by analyzing replicates ($n = 3$) of plasma samples at low QC and high QC, which were exposed to different conditions (time and temperature). The following stability conditions were investigated: room temperature stability (2 h at 25 °C), long-term storage stability (30 days at −20 °C), and three freeze/thaw cycles stability (−20 °C). All analytes were considered stable in plasma when 85–115% of the nominal concentrations were found.

The recoveries of each analyte were determined at three QC levels by comparing the mean peak areas of the regularly pretreated QC samples ($n = 6$) with those of the blank plasma samples ($n = 3$) spiked with working solutions after extraction. The recoveries of the ISs were determined in a similar method, using the QC samples at the medium concentrations as the references.

Two sets of samples at LQC and HQC levels were prepared to evaluate matrix effects (suppression or enhancement of ionization) on the analytes and ISs: (A) blank plasma samples extracted from six different donors and spiked with standards and (B) standards in the mobile phase at the same concentration levels. The mean peak area ratios (analyte to the IS) were compared [10]. The ratio (A/B × 100%) is defined as the IS-normalized matrix effect. Inter-subject variability in the matrix effect should be <15% [11].

2.8. Pharmacokinetic study

The validated LC–MS/MS method was applied to investigate the circulating profiles of PICO and its three major metabolites (BHPM, M1, and M2) after a single oral administration of 5 mg of PICO-Na to 12 healthy volunteers. The clinical study was approved by the Ethics Committee of the Hospital. Venous blood (2–3 mL) samples were collected in heparinized tubes 5 min before dosing and 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 16, 24, and 36 h thereafter. Following centrifugation (2000 × *g* for 10 min) the plasma was separated and stored at −20 °C until analysis.

3. Results and discussion

3.1. Reference compound syntheses

Reference standards of M1 and M2 were not commercially available. Therefore, the present study utilized an *in vitro* metabolic system for the biosynthesis of M1 followed by its isolation and purification. M2 was synthesized by the acidic hydrolysis of PICO with 1.9 M HCl. The purity of the synthesized reference standards was >99% for both M1 and M2 based on HPLC-UV data. M1-d_{12} and M2-d_{12} were obtained by the same methods described above using BHPM-d_{12} and PICO-d_{12} as raw materials, respectively. Since the reference standards of M1-d_{12} and M2-d_{12} were not obtained in sufficient quantities, their fractions from a semi-preparative HPLC system were collected and further concentrated. The concentrations of the M1-d_{12} and M2-d_{12} stock solutions were 17.0 and 77.3 μg/mL, respectively, as determined by HPLC-UV using M1 and M2 as references. No apparent non-deuterated impurities were observed.

3.2. Mass spectrometry

In the Q1 full scan experiment, the ionization polarity was optimized by comparing the analyte responses in positive and negative ESI. A strong and stable MS signal for each analyte was observed in positive ionization mode. The protonated molecules of PICO, BHPM, M1, and M2 were observed at *m/z* 438.1, 278.1, 454.1, and 358.1, respectively. In the product ion spectrum of PICO, two major fragment ions at *m/z* 358.1 and 278.1 were formed by serial neutral losses of the SO_3 (−80 Da) moiety from the $[M+H]^+$ ion. The further loss of the hydroxyphenol moiety from *m/z* 278.1 produced a fragment ion at *m/z* 184.2 (Fig. 2). For BHPM, the major fragment ion was observed at *m/z* 184.2. For M2 and M1, the major product ions were observed at *m/z* 278.1 and 184.2. The proposed mass spectral fragmentation patterns of all four analytes are shown in Fig. 3. The ion transition of *m/z* 438.1 → *m/z* 278.1 provided a better signal-to-noise ratio compared with the ion transition of *m/z* 438.1 → *m/z* 184.2 and was thus selected for the quantitation of PICO. Two ion transitions (*m/z* 358.1 → *m/z* 278.1 and *m/z* 358.1 → *m/z* 184.2) were selected and summed for the quantitation of M2 to enhance sensitivity. For both M1 and BHPM, the predominant fragment ion at *m/z* 184.2 was used in the MRM transitions. The method for selecting MRM transitions of isotopic ISs was in accordance with that for their corresponding analytes. A short scan-cycle was required to obtain an adequate number of data points. The dwell time for monitoring each transition was set to 80 ms. The instrument parameters, including the collision energy (CE), declustering potential (DP), and source temperature, were carefully optimized to maximize the MS responses.

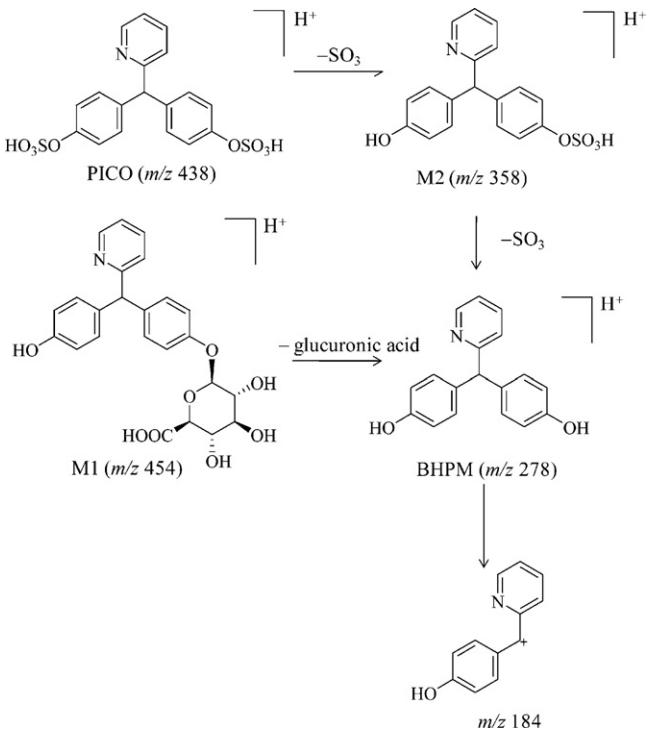

Fig. 3. The proposed mass fragmentation pathways for the analytes.

3.3. Chromatography

In recent years, there has been an increasing awareness of the risks associated with unstable metabolites that contribute to the parent drug concentration during the analysis of biological samples [12–15]. These unstable drug metabolites, such as glucuronides and sulfates, could undergo in-source conversion to generate an ion that is isobaric with the aglycone and thus interfere with the quantitation of the drug. A good strategy to avoid this type of interference is chromatographic separation. In the present study, we also encountered interferences due to in-source conversion, which

Table 2
Putative interference substances of analytes of interest and their respective in-source conversion reactions.

Interference substance	Protonated ion	Analyte of interest	In-source conversion reaction
PICO	*m/z* 358.1	M2	*m/z* 438.1 → *m/z* 358.1
PICO	*m/z* 278.1	BHPM	*m/z* 438.1 → *m/z* 278.1
M1	*m/z* 278.1	BHPM	*m/z* 454.1 → *m/z* 278.1
M2	*m/z* 278.1	BHPM	*m/z* 358.1 → *m/z* 278.1

are summarized in Table 2. Ensuring sufficient chromatographic retention and baseline separation of the analytes is thus essential for the analysis.

Several normal and reversed-phase chromatographic columns, such as Zorbax XB-phenyl, Luna HILIC, Ultimate XB-CN, Luna NH_2, Luna C_{18}, and Zorbax SB-C_{18} columns, were tested. Only the C_{18} columns could provide acceptable retention of the analytes. The mobile phase systems were then optimized. A decrease in the percentage of the organic phase resulted in the retention enhancement of all analytes; the retention time of BHPM was much longer than those of other analytes. For example, when the separation was performed on a Luna 5u $C_{18}(2)$ column (50 mm × 2.0 mm, i.d., 5 μm; Phenomenex) at a flow rate of 0.6 mL/min using acetonitrile–water (10:90, v/v) as the mobile phase, the retention times of PICO, M1, and M2 ranged from 2 min to 5 min and that of BHPM was more than 10 min. A gradient elution program was then employed to shorten the chromatographic run time, and after the analytes were eluted, a column wash program using 100% acetonitrile at a flow rate of 1 mL/min was applied to rapidly remove the endogenous substances from the column. The column was then brought to equilibrium with the initial mobile phase. Different aqueous phases containing acidic or basic modifiers were tested to improve the MS responses of the analytes. The retention times of all analytes ranged from 1.6 min to 1.9 min when 5 mM ammonium acetate–formic acid (100:0.1, v/v) was used as the aqueous phase. The retention time of PICO was 1.6 min, with a broad peak that was not separated from M2. The aqueous phase was then changed to water:1% ammonia solution (100:0.1, v/v), which provided a higher MS response but showed poorer retention. Afterwards, 10 mM ammonium acetate (pH 6.88) was tested, and the mobile phase provided an MS response similar to the basic aqueous phase without sacrificing the chromatographic retention of the analytes. Baseline separation of all analytes was achieved under the chromatographic conditions employed. A coeluting endogenous interference of PICO

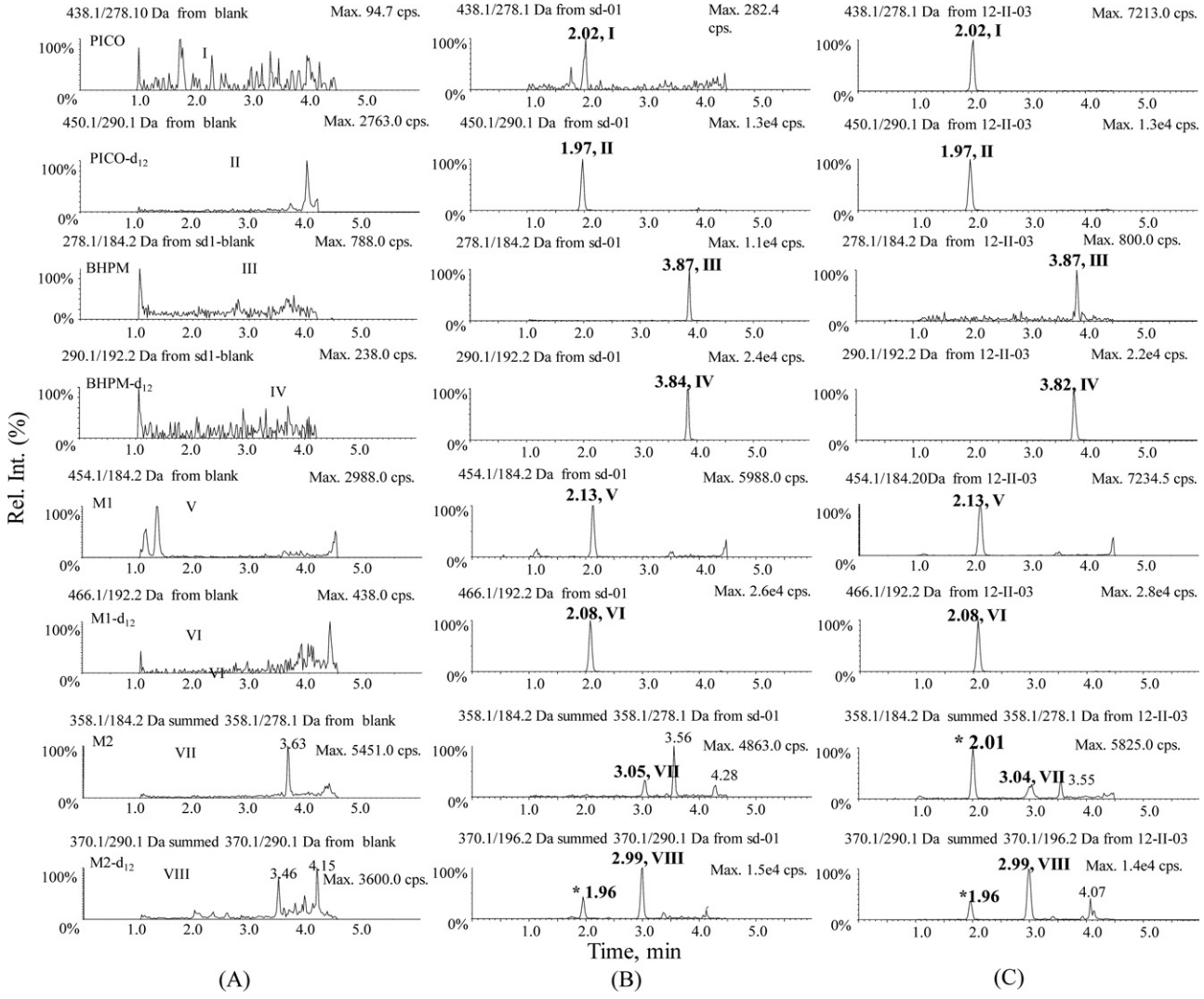

Fig. 4. Typical MRM chromatograms of PICO, M1, M2 and ISs in the plasma of CML patients. (A) Blank plasma sample; (B) blank plasma spiked with PICO (0.150 ng/mL), M1 (0.150 ng/mL), M2 (0.045 ng/mL), BHPM (0.600 ng/mL) and ISs (PICO-d_{12}/M1-d_{12}/M2-d_{12}/BHPM-d_{12}, 325/80.0/250/150 ng/mL); (C) plasma sample 1 h after an oral administration of 5 mg of PICO-Na to a volunteer. Peaks I, II, III, IV, V, VI, VII and VIII correspond to PICO, PICO-d_{12}, M1, M1-d_{12}, M2, M2-d_{12}, BHPM and BHPM-d_{12}, respectively. The peaks labeled with "*" arise due to in-source conversion.

was observed in the MRM transition of m/z 438.1 → m/z 278.1. Thus, the gradient elution program was slightly adjusted, and separation of PICO from the interference was achieved.

3.4. Sample preparation

Considering the high polarity of PICO, M1, and M2, protein precipitation (PPT) was chosen as the sample preparation procedure. Further concentration was performed by employing an evaporation/reconstitution step. The reconstitution solution was optimized by varying the proportion of water. When the proportion of water increased, the peak shape of PICO was distinctly sharpened and the peak height of PICO was raised. Therefore, acetonitrile–water (10:90, v/v) was employed as the reconstitution solution.

3.5. Validation

3.5.1. Selectivity

Fig. 4 depicts the typical chromatograms of PICO, M1, M2, BHPM, and the ISs resulting from the analysis of extracts of 100 μL of plasma from a blank plasma sample (Fig. 4A), a blank plasma sample spiked with PICO, M1, M2, and BHPM at the LLOQ and ISs (Fig. 4B), and a plasma sample obtained 1 h after a single oral administration of 5 mg of PICO to a healthy volunteer (Fig. 4C). The interferences caused by in-source conversion and other endogenous substances (t_R = 1.8 min) were avoided by baseline chromatographic separation. The retention times for PICO, PICO-d_{12}, M1, M1-d_{12} M2, M2-d_{12}, BHPM, and BHPM-d_{12} were 2.0, 2.0, 2.1, 2.1, 3.0, 3.0, 3.9, and 3.8 min, respectively.

3.5.2. Linearity of calibration curve and lower limit of quantification

The linear regression curves were fitted over the concentration ranges of 0.150–40.0 ng/mL for PICO and M1, 0.0450–12.0 ng/mL for M2, and 0.600–160 ng/mL for BHPM in human plasma. The mean equations of the calibration curves for each analyte generated during the validation were as follows:

$$\text{PICO}: \quad y = (0.0781 \pm 0.0106)x + (0.00275 \pm 0.00305) \quad (r = 0.9982 \pm 0.0008);$$
$$\text{M1}: \quad y = (1.29 \pm 0.19)x + (0.0113 \pm 0.0168) \quad (r = 0.9982 \pm 0.0010);$$
$$\text{M2}: \quad y = (0.517 \pm 0.080)x + (0.00351 \pm 0.00406) \quad (r = 0.9979 \pm 0.0017);$$
$$\text{BHPM}: \quad y = (0.165 \pm 0.003)x + (0.00198 \pm 0.00297) \quad (r = 0.9973 \pm 0.0015);$$

where y represents the peak area ratios of the analytes to their corresponding IS, and x represents the nominal concentrations of the analytes. The LLOQs of PICO, M1, M2, and BHPM were 0.150, 0.150, 0.0450, and 1.20 ng/mL, respectively, with acceptable accuracy and precision (Table 3). These results indicate that the proposed method was sensitive enough to determine the plasma concentration of PICO and its metabolites.

3.5.3. Precision and accuracy

The intra- and inter-day precision and accuracy values for the QC samples are summarized in the Table 3. The intra- and inter-day precisions for PICO were less than 10.3%, while accuracy was within ±3.5%. The inter- and intra-day precisions for M1 were less than 10.6%, while accuracy was within ±3.7%. The inter- and intra-day precisions for M2 were less than 13.0%, while accuracy was within ±2.7%. For BHPM, the inter- and intra-day precisions were less than 11.9%, while accuracy was within ±6.7%. These results indicate that the proposed method is accurate and precise.

3.5.4. Recovery and matrix effect

The mean extraction recoveries of PICO were 89.1%, 93.9%, and 84.5% at concentrations of 0.300, 3.00, and 32.0 ng/mL, respectively.

Table 3
Accuracy and precision for the analysis of PICO, M1, M2, and BHPM in human plasma (n = 3 days, six replicates per day).

Analyte	Concentration (ng/mL)		RSD (%)		RE (%)
	Added	Found	Intra-day	Inter-day	
PICO	0.150	0.155	8.7	7.6	3.5
	0.300	0.294	7.9	5.8	−2.1
	3.00	2.97	5.3	2.7	−1.2
	32.0	31.5	2.5	10.3	−1.5
M1	0.150	0.156	5.3	7.6	3.7
	0.300	0.298	4.0	3.4	−0.8
	3.00	3.02	3.5	3. 7	0.6
	32.0	32.4	1.5	10.6	1.1
M2	0.045	0.046	4.9	9.3	2.6
	0.090	0.092	7.7	8.3	2.7
	0.900	0.893	3.5	5.8	−0.8
	9.60	9.48	4.8	13.0	−1.2
BHPM	0.600	0.586	9.3	8.5	−2.3
	1.20	1.12	6.6	8.8	−6.7
	12.0	11.6	4.5	11.9	−3.2
	128	126	3.2	10.0	−1.5

The recovery levels of M1 were 99.2%, 96.4%, and 90.3% at concentrations of 0.300, 3.00, and 32.0 ng/mL, respectively. The recovery levels of M2 were 110%, 106%, and 104% at concentrations of 0.0900, 0.900, and 9.60 ng/mL, respectively. For BHPM, the mean extraction recoveries were 71.2%, 70.1%, and 83.1% at concentrations of 1.20, 12.0, and 128 ng/mL, respectively. The mean extraction recoveries of PICO-d_{12}, M1-d_{12}, M2-d_{12}, and BHPM-d_{12} were 88.7%, 98.7%, 103%, and 75.4%, respectively.

The IS-normalized matrix effects for PICO determined at 0.300 and 32.0 ng/mL were 106% and 98.1%, respectively. The IS-normalized matrix effects for M1 determined at 0.300 and 32.0 ng/mL were 102% and 95.7%, respectively. The IS-normalized matrix effects for M2 determined at 0.0900 and 9.60 ng/mL were 105% and 101%, respectively. The IS-normalized matrix effects for BHPM determined at 1.20 and 128 ng/mL were 111% and 105%, respectively. The IS-normalized matrix effects for PICO-d_{12}, M1-d_{12}, M2-d_{12}, and BHPM-d_{12} were 98.5%, 104%, 99.4%, and 89.9%, respectively. The inter-subject variability of the matrix effects for each analyte was below 13.1%. As a result, the matrix effect could be ignored in the present experimental conditions.

3.5.5. Stability

The analytes were stable in plasma stored at room temperature for 2 h (RE in the range of −1.8% to 11.7% for each analyte), in plasma after three freeze–thaw cycles at −20 °C (RE in the range of −5.7% to 4.4% for each analyte), and in plasma stored at −20 °C for 30 days (RE in the range of −11.4% to 6.0% for each analyte).

3.5.6. Dilution test

For some plasma samples collected near T_{max}, the M1 concentration extended beyond the upper limit of quantification (ULOQ). The attempt to expand the ULOQ failed because the MS response of M1 tended to become saturated when spiked samples at high concentrations were analyzed. Therefore, a 10-fold dilution was performed to analyze these plasma samples.

Blank plasma samples were spiked with M1 at 320 ng/mL and further diluted with blank plasma (20 μL in 200 μL) prior to

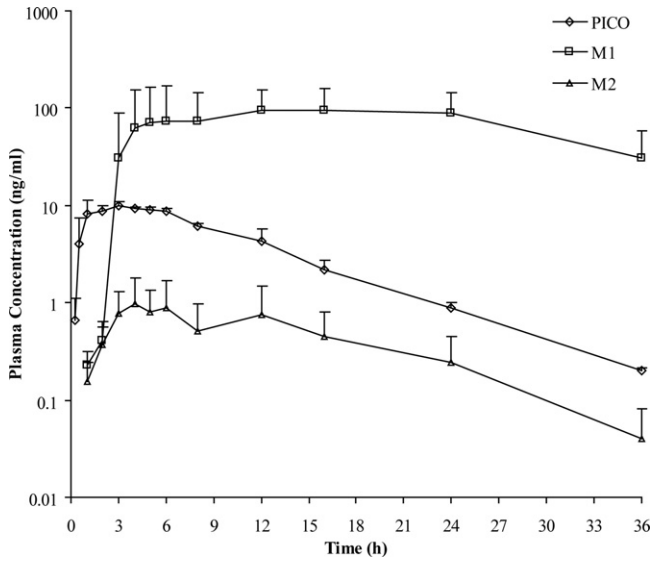

Fig. 5. Mean plasma concentration–time curves of PICO, M1 and M2 in healthy subjects after an oral administration of 5 mg PICO-Na. All data indicate mean ± SD.

analysis ($n = 6$). The precision was less than 7.5%, and accuracy ranged from −6.4% to 10.7%. The results indicate that 10-fold dilution with blank human plasma does not yield significant deviations in the quantification results of M1.

3.5.7. *Method application*

The validated method was successfully applied to simultaneously determine the plasma concentrations of PICO, M1, M2, and BHPM in 12 healthy human volunteers after a single oral administration of 5 mg of PICO-Na. The mean plasma concentration *versus* time curves of PICO, M1, and M2 are presented in Fig. 5. In human plasma, the parent drug PICO and its metabolites M1 and M2 were detected for at least 36 h after the oral dose. The plasma concentration of BHPM was lower than the LLOQ (0.600 ng/mL). The quantification of BHPM is helpful in two applications. After incubation (37 °C, 12 h) of PICO clinical plasma samples with a cocktail of glucuronidase and sulfatase, most of the conjugates were converted into BHPM. The concentration of BHPM could then be determined by analyzing the extracted mixture using the validated LC–MS/MS method. For bioanalysts and laboratories constrained by a lack of reference standards for M1 and M2, the method is very helpful in the toxicological screening of PICO. Moreover, the method was further developed and validated for the determination of BHPM in human urine after enzymatic cleavage and successfully applied to an excretion study of PICO.

4. Conclusion

The validated LC–MS/MS method for the simultaneous quantification of PICO and its three major metabolites in human plasma was sensitive, selective, simple, and rapid. The LLOQs for PICO, M1, M2, and BHPM were 0.150, 0.150, 0.0450, and 0.600 ng/mL, respectively. These results ensure the quantification of PICO and its metabolites after an oral administration of 5 mg PICO-Na. A time-saving protein precipitation procedure made the method readily applicable in a clinical study. The application of stable isotopically labeled ISs ensured the accurate quantification of PICO and its major metabolites in human plasma. The pharmacokinetic profiles of PICO, M1, and M2 in humans were characterized for the first time.

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