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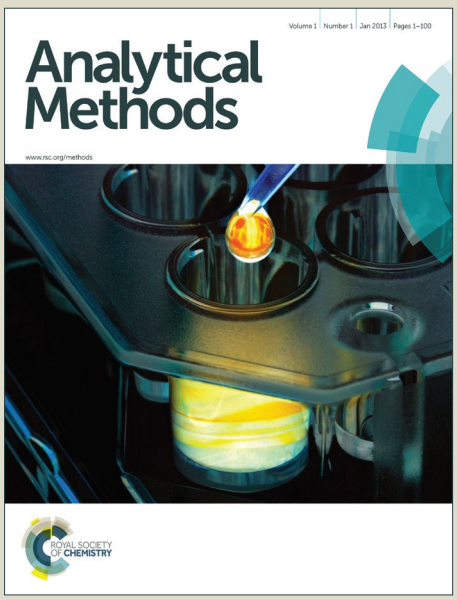

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Simultaneous determination of p-acetaminobenzoic acid and N,N-dimethylamino-2-propanol in human plasma by liquid chromatography–tandem mass spectrometry with positive/negative ion-switching electrospray ionization and its application to a pharmacokinetic study

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Abstract

A liquid chromatography-tandem mass spectrometric (LC-MS/MS) method using positive/negative electrospray ionization (ESI) switching for the simultaneous quantitation of p-acetaminobenzoic acid (PABA) and N,N-dimethylamino-2-propanol (DIP) in human plasma has been developed and validated. After simple protein precipitation using acetonitrile, the analytes and internal standard (IS) diazepam were separated on a CAPCELL PAK Phenyl column (150 × 2.0 mm, 5 μm) using formic acid:water (1:1000, v/v) as solvent A and acetonitrile as solvent B at a flow rate of 0.4 mL/min. N,N-dimethylamino-2-propanol and IS was detected by positive ion electrospray ionization followed by multiple reaction monitoring (MRM) of the transition at *m/z* 104.0→86.1 and 256.3→167.1, respectively. P-acetaminobenzoic acid was detected by negative ion electrospray ionization followed by MRM of the transitions at *m/z* 178.1→134.0. The method was linear for PABA and DIP in the concentration range 0.02-10 μg/mL with intra- and inter-day precisions (as relative standard deviation) of ≤ 5.81% and accuracy (as relative error) of ≤ 6.52% and limit of detection (LOD) values were 10 and 5 ng/mL, respectively. The method was successfully applied to a pharmacokinetic study of the analytes in human after oral administration of 1.0 g inosiplex

tablet.

Keywords:
LC-MS/MS; Inosine pranobex; p-acetaminobenzoicacid; N,N-dimethylamino-2-propanol; Pharmacokinetics; Human.

Introduction

Inosine pranobex (Isoprinosine or Methisoprinol) is a compound formulation composed of inosine, acetamidobenzoic acid (PABA), and N,N-dimethylamino-2-propanol (DIP) in molar ratio of 1:3:3(figure1).[1-4] As immunostimulant[5, 6] and analog of thymus hormones, Inosine pranobex could be used in the treatment of various viral infections. Many studies in vitro and in vivo have shown immunomodulating and antiviral activities of inosine pranobex.[6-13] It is most commonly used to treat the rare measles complication Subacute sclerosing panencephalitis,[13, 14] recalcitrant alopecia areata,[15] human papilloma virus (HPV) infection.[16] In order to better investigate the pharmacodynamic and pharmacokinetic behavior of Inosine pranobex, it is important to simultaneously analyse multiple active components in plasma.

Fig. 1

In order to meet the requirement for pharmacokinetic studies of Inosine pranobex, a selective, rapid, sensitive and robust analytical method is highly desirable for the active ingredients. Inosine, just as the endogenous substance, the concentration of it did not increase significantly with dose enhancement and was maintained in the range of 100–1000 ng/mL in healthy people.[17] Therefore, the determination of inosine was not emphasized in the pharmacokinetic study of Inosine pranobex. Several studies [17-19] have been reported concerned with the determination of PABA or DIP in biological matrix. However, due to the different polarities and solubilities of the analytes, no research was currently available concerned with the simultaneous determination of PABA and DIP in biological sample.

The present investigation aimed to develop an LC-MS/MS method to determine PABA and DIP in human plasma. This paper reports the first such assay and its application to a pharmacokinetic study of PABA and DIP after an oral administration dose of 1.0 g

inosiplex tablet.

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Experimental

Chemicals and reagents

PABA (purity > 99%), DIP (purity > 99%) provided by Sigma Co(Shanghai, China). Diazepam (purity>99 %) for use as internal standard (IS) was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The structures of the analytes and IS are shown in Fig. 1. Acetonitrile (HPLC-grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Distilled water, prepared from demineralized water, was used throughout the study. All other chemicals were of HPLC grade.

LC-MS conditions

The LC-MS system consisted of an Agilent 1100 Series HPLC (Agilent Technologies, Palo Alto, CA, USA) coupled to a Qtrap 5500 mass spectrometer (Sciex, Ontario, Canada) equipped with a TurboIonSpray source. Data acquisition and integration were controlled by Analyst Software1.5.2. (Sciex, Ontario, Canada)

Chromatography was performed on a Capcell Pak Phenyl column (150 × 2.0 mm, 5 μm) maintained at 40°C. Gradient elution utilized formic acid:water (1:1000, v/v) as solvent A and acetonitrile as solvent B delivered at a flow rate of 0.4 mL/min. The gradient elution program was as follows: 0-1 min 10% B; 1-2 min 10%→20% B; 2-2.1 min 20%→75% B; 2.1-4 min 75% B; 4-4.1 min 75%→10% B; 4.1-7 min 10% B. MS parameters optimized by infusing a standard solution of analytes and IS using a syringe pump and they were shown in table 1.

Table 1

Preparation of calibration standards and quality control (QC) samples

A mixed stock solution of PABA and DIP (both 1 mg/mL) was prepared in acetonitrile:water (50:50, v/v) and stored at 4°C when not in use. Standard solutions were prepared by diluting the stock solution with acetonitrile:water (50:50, v/v) to

concentrations of 1, 2.5, 5, 15, 50, 150 and 500 μg/mL. QC solutions at concentrations of 2.5, 15, and 150μg/mL were prepared independently in the same way. Calibration standards with concentrations of 0.02, 0.05, 0.1, 0.3, 1, 3 and 10 μg/mL were prepared by mixing 50 μL aliquots of standard solutions with 2450 μL blank plasma. Low, medium and high QC samples with concentrations of 0.05, 0.3, and 3 μg/mL were prepared from QC solutions in a similar manner. All plasma samples were stored at -80°C prior to use.

Sample preparation

Frozen human plasma samples were allowed to thaw in a water-bath at room temperature. To a 50 μL aliquot of plasma (or calibration standard or QC sample) was added 50 μL IS working solution and 200 μL acetonitrile to precipitate protein. The mixture was vortex-mixed for 1 min and centrifuged for 5 min at 15000 rpm.100 μL supernatant was collected and vortex-mixed with 200 μL 0.1% formic acid for 1 min, and 50 μL injected into the LC-MS system.

Method validation

The method was fully validated according to the Food and Drug Administration (FDA) guidance [20] for biological method validation.

Selectivity was proved using blank plasma samples from six healthy volunteers, which were individually analyzed and evaluated for interference. In addition, cross-talk and carryover phenomena among MS/MS channels were evaluated by injecting PABA, DIP and IS, separately, at the highest concentrations of the calibration curve and monitoring the responses in the other MS/MS channels.

Linearity was evaluated by linear least-squares regression with a weighting index of $1/x^2$ of calibration curves based on peak area ratios of analyte:IS prepared in duplicate on three separate days.

Accuracy (as relative error (R.E.)) and intra- and inter-day precision (as relative standard deviation (R.S.D.) were based on assay of six replicate QC samples on three different days.

The LOD and the LLOQ were calculated using the following formulas as reported in ICH guidelines[21]:

$LOD = 3.3 * \sigma / S$

$LLOQ = 10 * \sigma / S$

where σ = the standard deviation of the response.
S = the slope of the calibration curve.
The lower limit of quantitation (LLOQ) was defined as the lowest concentration that could be determined with accuracy±20% and precision < 15%.
Matrix effects were evaluated by comparing peak areas of analytes and IS in post-extraction spiked samples with those in standard solutions. Recovery was determined by comparing peak areas of QC samples with those of post-extraction blank plasma spiked at corresponding concentrations.
Stability of analytes in human plasma was evaluated in QC samples placed on storage for 1 month at -80°C, for 2 h at room temperature (25°C) and after three freeze/thaw cycles. Stability of analytes in processed samples on storage in autosampler vials at room temperature for 2 h was also evaluated.

Pharmacokinetic study

A group of healthy male volunteers (n=10, age 21.0 ± 0.8 years, body weight 62.7 ± 7.6 kg) were enrolled in the study. They were not allowed to consume alcohol or take any other medication during the study. The clinical protocol was approved by the Ethics Committee of the Second Clinical Hospital affiliated to Chinese Medical University, China. All healthy volunteers read the protocol and gave written informed consent before entering the study. Volunteers received a single oral dose of 1.0 g inosiplex tablet. Blood samples (4 mL) were collected into heparinized plastic tubes before administration and at 0.08, 0.16, 0.5, 0.75, 1.0,1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0 and 24.0 h post-dose. Plasma was separated immediately by centrifugation at 15000 rpm for 10 min and stored at –20°C prior to analysis. Pharmacokinetic parameters were calculated using the software DAS 3.0.

Results and discussion

Sample preparation

PABA and DIP gave robust MS response allowing the intended LLOQ to be attained with simple protein precipitation. Acetonitrile was selected as the protein precipitant since it gave superior recovery and reduced matrix effects compared to methanol or perchloric acid, the data were shown in Table2. It was found that adding 200 μL 0.1% formic acid to the

upper clear solution enhanced peak shape and avoided any solvent effect.

Table 2

LC-MS/MS conditions

PABA is hydrophobic compound containing a carboxylic acid group. In this study, PABA responded well to negative ionization giving deprotonated molecules $[M-H]^-$ as the major species. DIP is a alkaline compound which responded well in positive ionization mode. Full scan product ion spectra of DIP, PABA and diazepam (IS) are shown in Fig. 2. MRM acquisitions at unit resolution used the transitions at m/z 178.1→134.0 for quantitation for PABA. The transitions at m/z 104.0→86.1 for quantitation and the transition at m/z 104.0→71.0 as qualifier for DIP. The transition at m/z 285.2→193.1 was used for Diazepam.

Fig. 2

Chromatographic conditions

To determine the best chromatographic performance, numerous commercially available columns (Zorbax SB-C18, Zorbax SB-Aq, Zorbax extend-C18, Venusil MP-C18 and CAPCELL PAK Phenyl) and various mobile phase compositions were evaluated. The latter involved testing different ratios of organic solvent (methanol, acetonitrile) to water and inclusion of pH modifiers (ammonium acetate, ammonium formate, formic acid, ammonia) in the aqueous phase. The addition of formic acid decreased ionization efficiency and sensitivity of PABA and addition of ammonia decreased its retention. Finally, the CAPCELL PAK Phenyl column using gradient elution with acetonitrile-formic acid:water (1:1000, v/v) at 0.4 mL/min gave adequate retention, symmetrical peak shapes, excellent selectivity and satisfactory mass spectrometric responses for analytes and IS.

Selection of IS

Diazepam was adopted as IS due to the similarity of its extraction efficiency with that of analytes and its efficient ionization in the positive ionization mode.

Assay validation

Typical MRM chromatograms of blank plasma, a plasma sample spiked at the LLOQ and a plasma sample from a human 1h min after oral administration of inosiplex tablet are shown

in Fig. 3. The assay is free of interference from endogenous substances in plasma at the retention times of the analytes and IS, indicating that the method possesses good selectivity. No enhancement in the response for analytes and IS was observed in blank plasma samples indicating the negligible affection in carry-over evaluation. Meanwhile, cross-talk phenomena among MS/MS channels were not observed in quintuplicate assay samples. The assay was linear for PABA and DIP in the range 0.02-10 μg/mL with typical regression equations of y=0.0011x+0.0355 (r=0.9960) and y=0.0277x-0.0432 (r=0.9980), respectively. The LLOQ was 0.02 μg/mL for both analytes (0.1163 μmol/L for PABA, 0.1939 μmol/L for DIP). For PABA and DIP respectively, limits of detection (LOD) were 10ng/mL(0.0582 μmol/L) and 5 ng/mL(0.0485 μmol/L). Accuracy and precision for the analysis of PABA and DIP in human plasma are shown in Table 3, intra- and inter-day accuracies were <5.81 and 6.52% and precisions were < 15% at all concentrations.

Fig. 3

Table 3

The recoveries for each analyte are shown in Table 2. The results show that the recoveries were repeatable and consistent across the concentration range studied. The recovery for the IS was shown in Table 4.

Table 4

In terms of matrix effects, actual concentrations (mean±SD) as percentage of nominal concentrations for low, medium and high QC samples respectively were as follows: DIP, 87.9 ± 0.9%, 88.5 ± 0.8%, 89.1 ± 0.5%; PABA, 91.5 ± 0.4%, 90.3 ± 0.7%, 92.6 ± 1.1 %. In terms of stability, the data are shown in Table 5. Concentrations under the various test conditions were all within ± 11.2% of nominal concentrations indicating no significant degradation of the analytes occurred under any of the storage conditions tested.

Table 5

Pharmacokinetic study

Mean pharmacokinetic parameters for DIP and PABA in human plasma after single oral dose of 1.0 g inosiplex tablet (n = 10) are shown in Table 6. The mean plasma

concentration-time curves of DIP and PABA are shown in Fig.4. The maximum plasma concentration (Cmax) of DIP and PABA were 2081 ±694.1 ng·mL^{-1} and 7042±2610 ng·mL^{-1}, respectively. The area under the plasma concentration-time curve (AUC) of DIP and PABA were 8447 ±2241 ng·h·mL^{-1} and 7946 ±4474 ng·h·mL^{-1}. The plasma elimination half-life (t1/2) of DIP and PABA were 3.81 ± 2.35 h and 1.14 ± 0.41 h. In comparison,[22] an oral dose of 1 g inosiplex tablet to 10 healthy Chinese volunteers (5 males, 5 females) produced Cmax of DIP and PABA after the dose of 2240 ± 180 ng·mL^{-1} and 5180±1800 ng·mL^{-1}, AUC of DIP and PABA after the dose of 13280 ±2310 ng·h·mL^{-1} and 7830 ±1160 ng·h·mL^{-1}. The plasma elimination half-life (t1/2) of DIP and PABA of 4.417 ± 0.812 h and 0.952 ± 0.126 h. The assay was clearly sensitive enough for pharmacokinetic studies.

Table 6

Fig. 4

Conclusions

A simple, rapid and sensitive LC-MS/MS assay for the simultaneous determination of PABA and DIP in human plasma has been developed and validated. The method involves relatively simple sample preparation and gives high sensitivity using only 50 μL plasma. The analytes were separated on a CAPCELL PAK Phenyl column (150 × 2.0 mm, 5 μm) at a flow rate of 0.4 mL/min. The short running time of 7 minutes per sample for the simultaneous analysis of PABA and DIP meets the requirement of high throughput analysis. The method was linear for PABA and DIP in the concentration range 0.02-10 μg/mL with intra- and inter-day precisions (as relative standard deviation) of ≤ 5.81% and accuracy (as relative error) of ≤ 6.52% and limit of detection (LOD) values were 10 and 5 ng/mL, respectively. The method was successfully applied to a pharmacokinetic study in human after oral administration of 1.0g inosiplex tablet. The assay could also be used in therapeutic drug monitoring of PABA and DIP.

Acknowledgments

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Fig. 1. Chemical structures of A inosine pranobex, B diazepam, C inosine, D DIP, E PABA.

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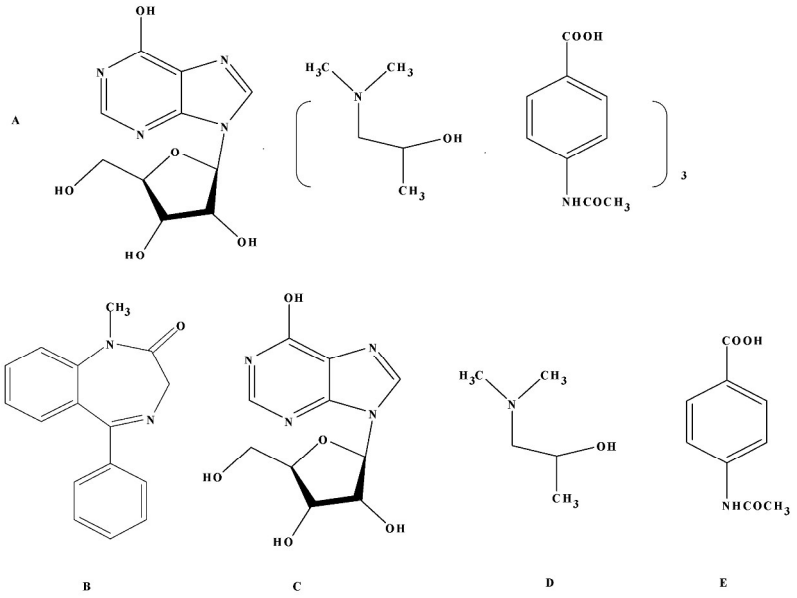
Fig. 2. Full scan product ion spectra of (A) DIP, (B) PABA and (C) diazepam (IS).

Fig. 3. Representative MRM chromatograms of I DIP, II PABA and III diazepam (IS) in left, blank plasma, middle, a plasma spiked at the LLOQs and right, a sample taken from a human 1h after oral administration of 1.0 g inosiplex tablet.

Fig. 4. Mean plasma concentration-time curve for DIP and PABA after oral administration of 1.0 g inosiplex tablet to humans (data are mean ± SD, n = 10).



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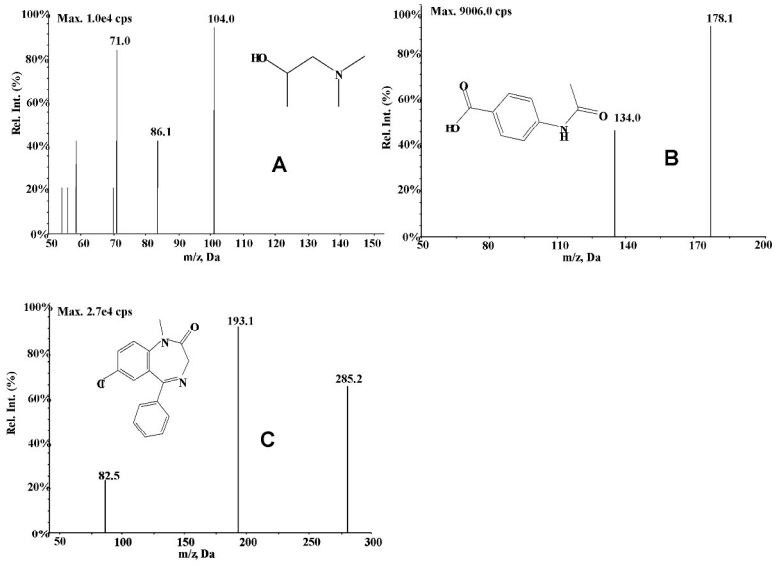

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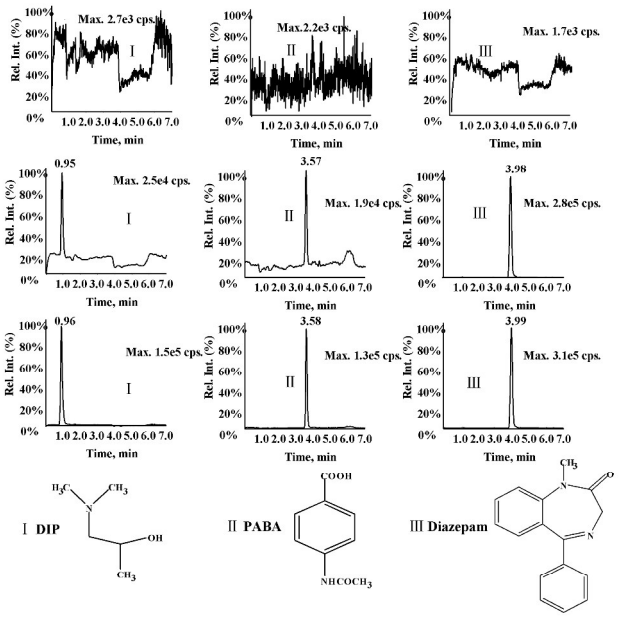

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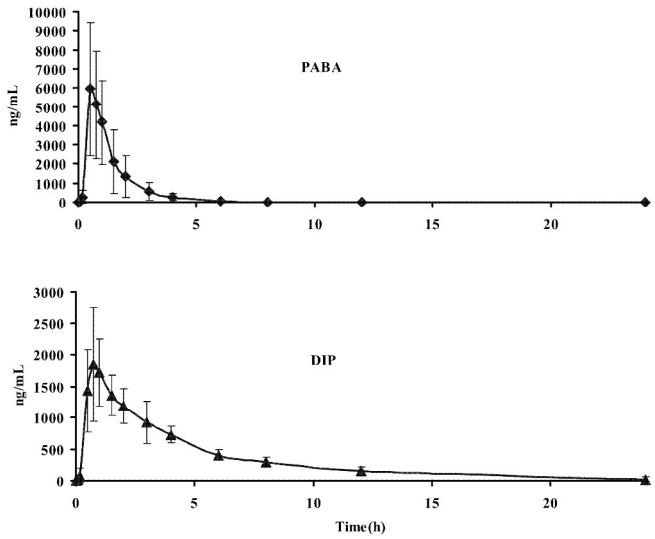

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Table 1. Multiple reaction monitoring (MRM) transitions and their mass spectrometry parameters.

	DIP		PABA	Diazepam
Ionization mode	Positive	Positive	Negative	Positive
MRM transition(precursor→product)	104.0→86.1	104.0→71.0	178.1→134.0.	285.2→193.1
Nebulizer gas (Unit)	40	40	40	40
Heater gas (Unit)	50	50	50	50
Ionspray needle voltage (V)	4500	4500	4500	4500
Heater gas temperature (°C)	500	500	500	500
Declustering potentials (V)	144	144	70	55
Collision energies (eV)	17	25	18	15

Table 2. Absolute recoveries (%) and Matrix Effect (%) for DIP and PABA in human plasma with different protein precipitant. (Data are mean ± S.D. for n=6)

		Recovery (%)			Matrix Effect (%)		
		low QC	medium QC	high QC	low QC	medium QC	high QC
Methanol	DIP	82.2±3.1	84.5±1.9	83.1±3.2	76.1±3.2	77.2±2.5	79.1±3.8
	PABA	83.1±4.2	80.8±2.4	81.7±2.1	72.1±2.5	74.8±3.1	78.7±2.7
Perchloric acid	DIP	87.1±4.4	90.1±2.6	88.1±1.9	77.9±3.8	83.3±2.9	84.1±5.1
	PABA	90.2±2.2	87.9±2.9	88.9±3.5	83.1±2.8	84.7±4.0	78.5±1.8
Acetonitrile	DIP	95.1±3.5	94.2±2.1	97.1±3.9	87.9 ± 0.9	88.5 ± 0.8	89.1 ± 0.5
	PABA	92.1±2.2	94.8±3.1	98.7±2.5	91.5 ± 0.4	90.3 ± 0.7	92.6 ± 1.1

Table 3. Accuracy and precision for the analysis of PABA and DIP in human plasma (Data are for analysis of 6 replicates on 3 different days)

Analyte	Concentration (μg/mL)		Accuracy (RE %)	Precision RSD (%)	
	Nominal conc.	Mean calculated	RE (%)	Intra-day	Inter-day
PABA	0.05	0.052	4.00	3.61	7.55
	0.3	0.307	2.33	4.07	8.63
	3	3.174	5.81	5.12	3.89
DIP	0.05	0.051	2.00	7.22	13.2
	0.3	0.313	4.33	9.31	10.4
	3	3.196	6.52	6.23	11.2

Table 4. Absolute recoveries (%) for diazepam in human plasma .

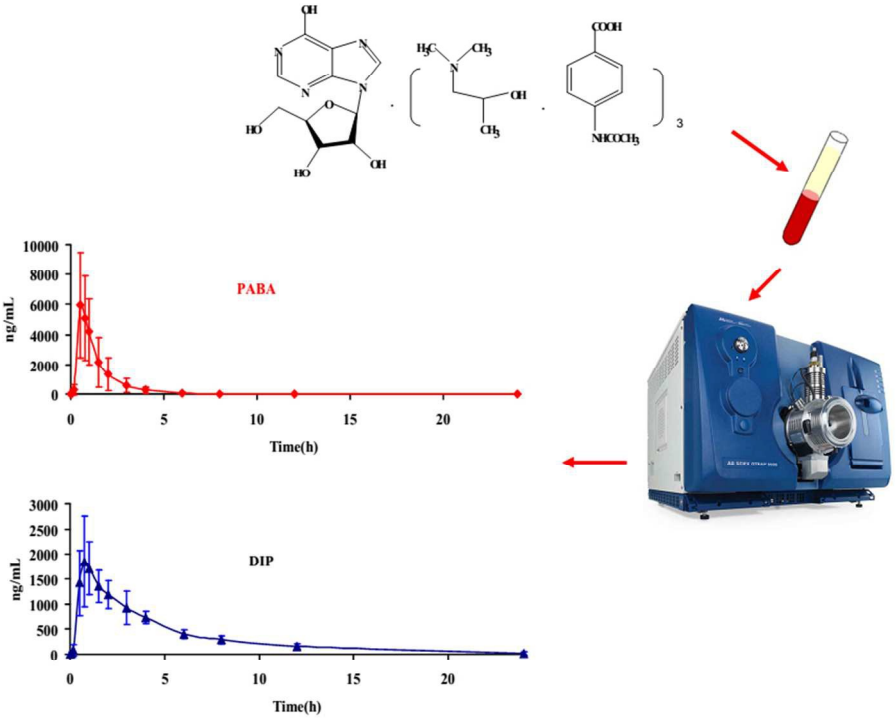
	Recovery (%)
Diazepam	96.3
	93.8
	97.1
	99.1
	95.5
	89.6
mean	95.2
SD	3.3

Table 5. Stability of DIP and PABA under various storage conditions (Data are mean ± SD, n = 3).

Compound	Nominal Conc. µg/mL)	Long term −80 °C	Short term	Freeze -thaw	Post-preparative
DIP	0.05	101.2 ± 2.1	101.6 ± 1.5	97.1± 2.2	100.2 ± 1.8
	0.3	100.1 ± 1.3	99.7 ± 1.2	96.2 ± 1.4	103.1 ± 1.7
	3	98.5 ± 2.2	97.9 ± 1.3	99.7 ± 3.0	105.5 ± 6.2
PABA	0.05	102.3 ± 5.5	102.0 ± 3.5	99.9 ± 2.3	102.3 ± 3.7
	0.3	99.1 ± 2.3	99.9 ± 2.1	100.1 ± 2.0	103.2 ±2.2
	3	97.4 ± 3.2	98.3 ±1.3	97.8 ± 1.8	101.4 ± 1.9

Table 6. Mean pharmacokinetic parameters for **DIP and PABA** in human plasma after single oral dose of 1.0 g inosiplex tablet **(n = 10)**.

	PABA		DIP	
time（h）	Mean	±SD	Mean	±SD
0	0	0	0	0
0.08	42.71	83.44	13.1	41.426
0.16	283.29	351.12	84.34	110.975
0.5	5946.8	3506.77	1424.6	657.032
0.75	5109	2807.35	1846.8	904.059
1	4186	2194.14	1717.9	534.336
1.5	2146.4	1657.82	1356.5	326.605
2	1349.8	1104.62	1183.5	279.842
3	566.4	470.79	924	336.304
4	282.8	199.05	730.4	127.513
6	61.3	54.71	401.8	82.974
8	23.73	25.31	290.3	74.318
12	3.63	7.78	161.02	55.263
24	0	0	20.39	33.47
AUC(0-t) (ug/L*h)	7912.17	4483.07	8215.4	1990.67
AUC(0-∞) (ug/L*h)	7946.12	4474.07	8447.85	2241.31
t1/2 (h)	1.15	0.42	3.816	2.354
Tmax (h)	0.63	0.18	0.725	0.184
Cmax (ug/L)	7042	2610.86	2081	694.141



253x197mm (96 x 96 DPI)