

Analytical Methods

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4 1 **Simultaneous determination of p-acetaminobenzoic acid and**
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6 2 **N,N-dimethylamino-2-propanol in human plasma by liquid**
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8 3 **chromatography–tandem mass spectrometry with positive/negative**
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10 4 **ion-switching electrospray ionization and its application to a**
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12 5 **pharmacokinetic study**

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29
30 **Abstract**

31 A liquid chromatography-tandem mass spectrometric (LC-MS/MS) method using
32 positive/negative electrospray ionization (ESI) switching for the simultaneous quantitation
33 of p-acetaminobenzoic acid (PABA) and N,N-dimethylamino-2-propanol (DIP) in human
34 plasma has been developed and validated. After simple protein precipitation using
35 acetonitrile, the analytes and internal standard (IS) diazepam were separated on a
36 CAPCELL PAK Phenyl column (150 × 2.0 mm, 5 μm) using formic acid:water (1:1000,
37 v/v) as solvent A and acetonitrile as solvent B at a flow rate of 0.4 mL/min.
38 N,N-dimethylamino-2-propanol and IS was detected by positive ion electrospray
39 ionization followed by multiple reaction monitoring (MRM) of the transition at *m/z*
40 104.0→86.1 and 256.3→167.1, respectively. P-acetaminobenzoic acid was detected by
41 negative ion electrospray ionization followed by MRM of the transitions at *m/z*
42 178.1→134.0. The method was linear for PABA and DIP in the concentration range
43 0.02-10 μg/mL with intra- and inter-day precisions (as relative standard deviation) of ≤
44 5.81% and accuracy (as relative error) of ≤ 6.52% and limit of detection (LOD) values
45 were 10 and 5 ng/mL, respectively. The method was successfully applied to a
46 pharmacokinetic study of the analytes in human after oral administration of 1.0 g inosiplex
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4
5 34 **Keywords:**

6
7 35 LC-MS/MS; Inosine pranobex; p-acetaminobenzoic acid; N,N-dimethylamino-2-propanol;
8
9 36 Pharmacokinetics; Human.

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11
12 38 **Introduction**

13
14 39 Inosine pranobex (Isoprinosine or Methisoprinol) is a compound formulation composed
15
16 40 of inosine, acetamidobenzoic acid (PABA), and N,N-dimethylamino-2-propanol (DIP) in
17
18 41 molar ratio of 1:3:3 (figure 1).¹⁻⁴ As immunostimulant^{5,6} and analog of thymus hormones,
19
20 42 Inosine pranobex could be used in the treatment of various viral infections. Many studies in
21
22 43 vitro and in vivo have shown immunomodulating and antiviral activities of inosine
23
24 44 pranobex.⁶⁻¹³ It is most commonly used to treat the rare measles complication Subacute
25
26 45 sclerosing panencephalitis,^{13,14} recalcitrant alopecia areata,¹⁵ human papilloma virus (HPV)
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28 46 infection.¹⁶ In order to better investigate the pharmacodynamic and pharmacokinetic
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30 47 behavior of Inosine pranobex, it is important to simultaneously analyse multiple active
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32 48 components in plasma.

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Fig. 1

51 In order to meet the requirement for pharmacokinetic studies of Inosine pranobex, a
52 selective, rapid, sensitive and robust analytical method is highly desirable for the active
53 ingredients. Inosine, just as the endogenous substance, the concentration of it did not
54 increase significantly with dose enhancement and was maintained in the range of
55 100–1000 ng/mL in healthy people.¹⁷ Therefore, the determination of inosine was not
56 emphasized in the pharmacokinetic study of Inosine pranobex. Several studies¹⁷⁻¹⁹ have
57 been reported concerned with the determination of PABA or DIP in biological matrix.
58 However, due to the different polarities and solubilities of the analytes, no research was
59 currently available concerned with the simultaneous determination of PABA and DIP in
60 biological sample.

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

64 inosiplex tablet.

65 .

66

67 **Experimental**

68 Chemicals and reagents

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

76

77 LC-MS conditions

78 The LC-MS system consisted of an Agilent 1100 Series HPLC (Agilent Technologies,
79 Palo Alto, CA, USA) coupled to a Qtrap 5500 mass spectrometer (Sciex, Ontario, Canada)
80 equipped with a TurboIonSpray source. Data acquisition and integration were controlled
81 by Analyst Software 1.5.2. (Sciex, Ontario, Canada)

82 Chromatography was performed on a Capcell Pak Phenyl column (150 × 2.0 mm, 5 μm)
83 maintained at 40°C. Gradient elution utilized formic acid:water (1:1000, v/v) as solvent A
84 and acetonitrile as solvent B delivered at a flow rate of 0.4 mL/min. The gradient elution
85 program was as follows: 0-1 min 10% B; 1-2 min 10%→20% B; 2-2.1 min 20%→75% B;
86 2.1-4 min 75% B; 4-4.1 min 75%→10% B; 4.1-7 min 10% B. MS parameters optimized by
87 infusing a standard solution of analytes and IS using a syringe pump and they were shown
88 in table 1.

89

Table 1

90

91 Preparation of calibration standards and quality control (QC) samples

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

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3 95 concentrations of 1, 2.5, 5, 15, 50, 150 and 500 µg/mL. QC solutions at concentrations of
4
5 96 2.5, 15, and 150µg/mL were prepared independently in the same way. Calibration
6
7 97 standards with concentrations of 0.02, 0.05, 0.1, 0.3, 1, 3 and 10 µg/mL were prepared by
8
9 98 mixing 50 µL aliquots of standard solutions with 2450 µL blank plasma. Low, medium and
10
11 99 high QC samples with concentrations of 0.05, 0.3, and 3 µg/mL were prepared from QC
12
13 100 solutions in a similar manner. All plasma samples were stored at -80°C prior to use.
14

101

102 Sample preparation

103 Frozen human plasma samples were allowed to thaw in a water-bath at room
104 temperature. To a 50 µL aliquot of plasma (or calibration standard or QC sample) was
105 added 50 µL IS working solution and 200 µL acetonitrile to precipitate protein. The
106 mixture was vortex-mixed for 1 min and centrifuged for 5 min at 15000 rpm. 100 µL
107 supernatant was collected and vortex-mixed with 200 µL 0.1% formic acid for 1 min, and
108 50 µL injected into the LC-MS system.
109

110

110 Method validation

111 The method was fully validated according to the Food and Drug Administration (FDA)
112 guidance²⁰ for biological method validation.

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

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

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

123 The LOD and the LLOQ were calculated using the following formulas as reported in ICH
124 guidelines²¹:

125 $LOD = 3.3 * \sigma/S$

126 $LLOQ = 10 * \sigma/S$

1
2
3 127 where σ = the standard deviation of the response.
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5 128 S = the slope of the calibration curve.
6
7 129 The lower limit of quantitation (LLOQ) was defined as the lowest concentration that could
8
9 130 be determined with accuracy $\pm 20\%$ and precision $< 15\%$.
10
11 131 Matrix effects were evaluated by comparing peak areas of analytes and IS in
12
13 132 post-extraction spiked samples with those in standard solutions. Recovery was determined
14
15 133 by comparing peak areas of QC samples with those of post-extraction blank plasma spiked
16
17 134 at corresponding concentrations.
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19 135 Stability of analytes in human plasma was evaluated in QC samples placed on storage for 1
20
21 136 month at -80°C , for 2 h at room temperature (25°C) and after three freeze/thaw cycles.
22
23 137 Stability of analytes in processed samples on storage in autosampler vials at room
24
25 138 temperature for 2 h was also evaluated.
26

27 139 28 140 Pharmacokinetic study

29 141 A group of healthy male volunteers ($n=10$, age 21.0 ± 0.8 years, body weight 62.7 ± 7.6 kg)
30
31 142 were enrolled in the study. They were not allowed to consume alcohol or take any other
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33 143 medication during the study. The clinical protocol was approved by the Ethics Committee
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35 144 of the Second Clinical Hospital affiliated to Chinese Medical University, China. All
36
37 145 healthy volunteers read the protocol and gave written informed consent before entering the
38
39 146 study. Volunteers received a single oral dose of 1.0 g inosiplex tablet. Blood samples (4
40
41 147 mL) were collected into heparinized plastic tubes before administration and at 0.08, 0.16,
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43 148 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
44
45 149 immediately by centrifugation at 15000 rpm for 10 min and stored at -20°C prior to
46
47 150 analysis. Pharmacokinetic parameters were calculated using the software DAS 3.0.
48

49 151 50 152 **Results and discussion**

51 153 Sample preparation

52 154 PABA and DIP gave robust MS response allowing the intended LLOQ to be attained with
53
54 155 simple protein precipitation. Acetonitrile was selected as the protein precipitant since it
55
56 156 gave superior recovery and reduced matrix effects compared to methanol or perchloric acid,
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58 157 the data were shown in Table2. It was found that adding 200 μL 0.1% formic acid to the
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4 158 upper clear solution enhanced peak shape and avoided any solvent effect.

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Table 2

160 LC-MS/MS conditions

161 PABA is hydrophobic compound containing a carboxylic acid group. In this study, PABA
162 responded well to negative ionization giving deprotonated molecules $[M-H]^-$ as the major
163 species. DIP is a alkaline compound which responded well in positive ionization mode.
164 Full scan product ion spectra of DIP, PABA and diazepam (IS) are shown in Fig. 2. MRM
165 acquisitions at unit resolution used the transitions at m/z 178.1 \rightarrow 134.0 for quantitation for
166 PABA. The transitions at m/z 104.0 \rightarrow 86.1 for quantitation and the transition at m/z
167 104.0 \rightarrow 71.0 as qualifier for DIP. The transition at m/z 285.2 \rightarrow 193.1 was used for
168 Diazepam.

169

Fig. 2

170
171 Chromatographic conditions

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

183 Selection of IS

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

186 Assay validation

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

189 in Fig. 3. The assay is free of interference from endogenous substances in plasma at the
190 retention times of the analytes and IS, indicating that the method possesses good selectivity.
191 No enhancement in the response for analytes and IS was observed in blank plasma samples
192 indicating the negligible affection in carry-over evaluation. Meanwhile, cross-talk
193 phenomena among MS/MS channels were not observed in quintuplicate assay samples.
194 The assay was linear for PABA and DIP in the range 0.02-10 µg/mL with typical regression
195 equations of $y=0.0011x+0.0355$ ($r=0.9960$) and $y=0.0277x-0.0432$ ($r=0.9980$),
196 respectively. The LLOQ was 0.02 µg/mL for both analytes (0.1163 µmol/L for PABA,
197 0.1939 µmol/L for DIP). For PABA and DIP respectively, limits of detection (LOD) were
198 10ng/mL(0.0582 µmol/L) and 5 ng/mL(0.0485 µmol/L). Accuracy and precision for the
199 analysis of PABA and DIP in human plasma are shown in Table 3, intra- and inter-day
200 accuracies were <5.81 and 6.52% and precisions were < 15% at all concentrations.

201

Fig. 3

202

Table 3

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

207

Table 4

208 In terms of matrix effects, actual concentrations (mean±SD) as percentage of nominal
209 concentrations for low, medium and high QC samples respectively were as follows: DIP,
210 $87.9 \pm 0.9\%$, $88.5 \pm 0.8\%$, $89.1 \pm 0.5\%$; PABA, $91.5 \pm 0.4\%$, $90.3 \pm 0.7\%$, $92.6 \pm 1.1 \%$.

211 In terms of stability, the data are shown in Table 5. Concentrations under the various test
212 conditions were all within $\pm 11.2\%$ of nominal concentrations indicating no significant
213 degradation of the analytes occurred under any of the storage conditions tested.

214

Table 5

216 Pharmacokinetic study

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

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4 219 concentration-time curves of DIP and PABA are shown in Fig.4. The maximum plasma
5
6 220 concentration (C_{max}) of DIP and PABA were 2081 ±694.1 ng·mL⁻¹ and 7042±2610
7
8 221 ng·mL⁻¹, respectively. The area under the plasma concentration-time curve (AUC) of DIP
9
10 222 and PABA were 8447 ±2241 ng·h·mL⁻¹ and 7946 ±4474 ng·h·mL⁻¹. The plasma
11
12 223 elimination half-life (t_{1/2}) of DIP and PABA were 3.81 ± 2.35 h and 1.14 ± 0.41 h. In
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14 224 comparison,²² an oral dose of 1 g inosiplex tablet to 10 healthy Chinese volunteers (5 males,
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16 225 5 females) produced C_{max} of DIP and PABA after the dose of 2240 ± 180 ng·mL⁻¹ and
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18 226 5180±1800 ng·mL⁻¹, AUC of DIP and PABA after the dose of 13280 ±2310 ng·h·mL⁻¹ and
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20 227 7830 ±1160 ng·h·mL⁻¹. The plasma elimination half-life (t_{1/2}) of DIP and PABA of 4.417
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22 228 ± 0.812 h and 0.952 ± 0.126 h. The assay was clearly sensitive enough for pharmacokinetic
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Table 6

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Fig. 4

32 232 33 233 **Conclusions**

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35 234 A simple, rapid and sensitive LC-MS/MS assay for the simultaneous determination of
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37 235 PABA and DIP in human plasma has been developed and validated. The method involves
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39 236 relatively simple sample preparation and gives high sensitivity using only 50 µL plasma.
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41 237 The analytes were separated on a CAPCELL PAK Phenyl column (150 × 2.0 mm, 5 µm) at
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43 238 a flow rate of 0.4 mL/min. The short running time of 7 minutes per sample for the
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45 239 simultaneous analysis of PABA and DIP meets the requirement of high throughput analysis.
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47 240 The method was linear for PABA and DIP in the concentration range 0.02-10 µg/mL with
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49 241 intra- and inter-day precisions (as relative standard deviation) of ≤ 5.81% and accuracy (as
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51 242 relative error) of ≤ 6.52% and limit of detection (LOD) values were 10 and 5 ng/mL,
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53 243 respectively. The method was successfully applied to a pharmacokinetic study in human
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55 244 after oral administration of 1.0g inosiplex tablet. The assay could also be used in
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57 245 therapeutic drug monitoring of PABA and DIP.

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3 246 **Acknowledgments**
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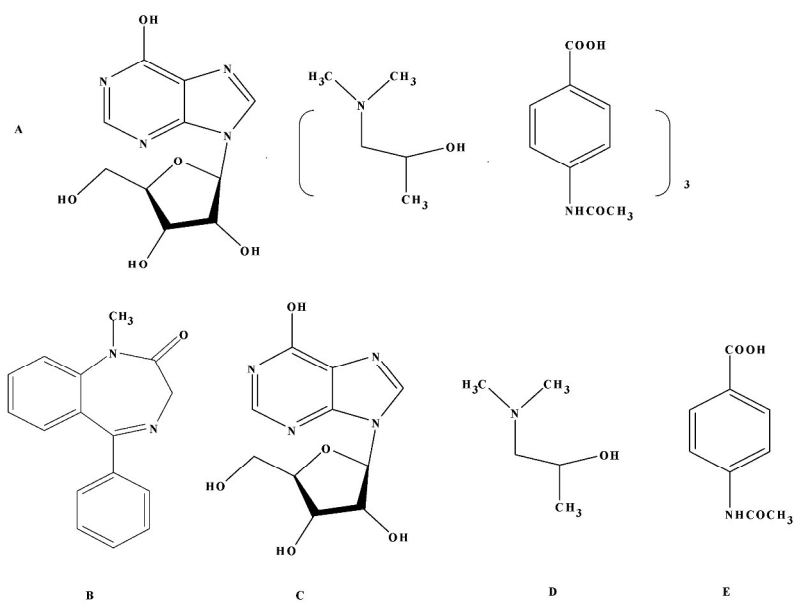
5 247 This research was supported by the National Natural Science Foundation of China (Grant
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7 248 No. 81430087, 81473142 and 81102383), the Science and Technology Major Specialized
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9 249 Projects for “significant new drugs creation” of the 12th five-year plan (2012ZX09303-015,
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11 250 2014ZX09303303) and the National Key Technology R&D Program of the Ministry of
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13 251 Science and Technology (2012BAI30B00), CERS-1-70 (CERS-China Equipment and
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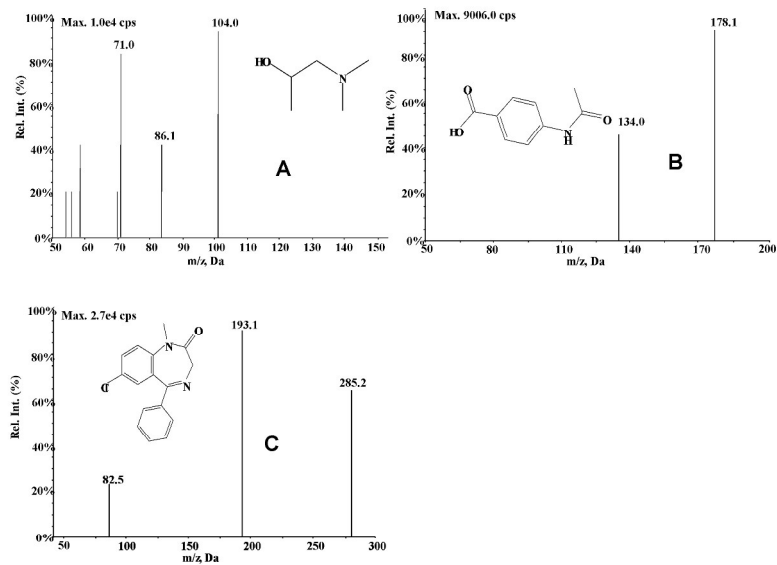
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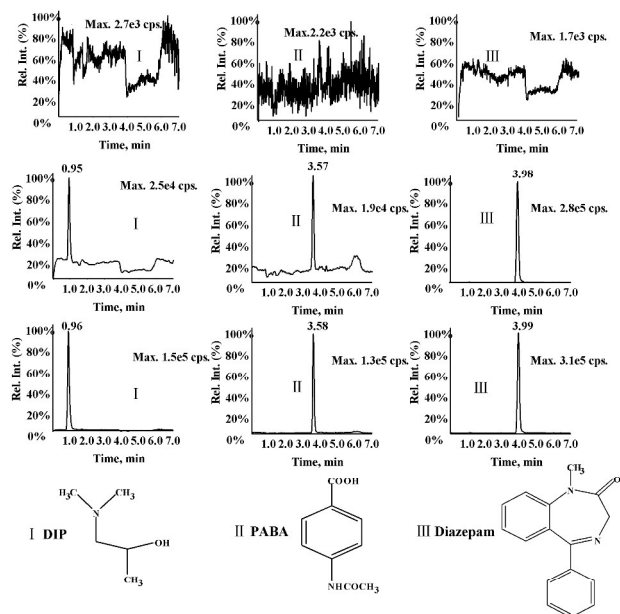
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3 291 Fig. 1. Chemical structures of A inosine pranobex, B diazepam, C inosine, D DIP, E PABA.
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6 293 Fig. 2. Full scan product ion spectra of (A) DIP, (B) PABA and (C) diazepam (IS).
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10 295 Fig. 3. Representative MRM chromatograms of I DIP, II PABA and III diazepam (IS) in left, blank
11 296 plasma, middle, a plasma spiked at the LLOQs and right, a sample taken from a human 1h after oral
12 297 administration of 1.0 g inosiplex tablet.
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14 298
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16 299 Fig. 4. Mean plasma concentration-time curve for DIP and PABA after oral administration of 1.0 g
17 300 inosiplex tablet to humans (data are mean \pm SD, n = 10).
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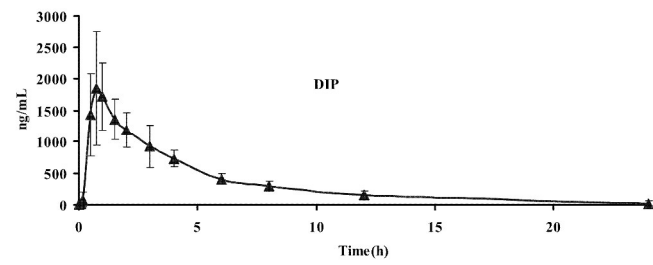
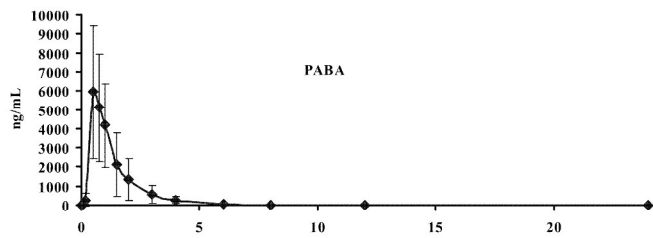


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Table 1. Multiple reaction monitoring (MRM) transitions and their mass spectrometry parameters.

	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

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

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

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

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

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

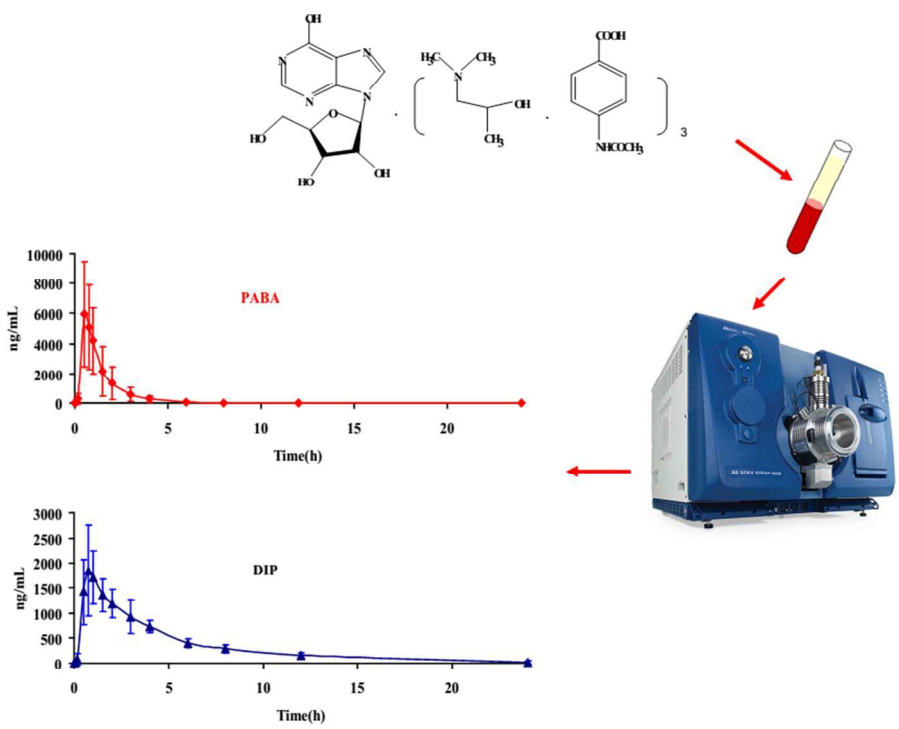
Compound	Nominal Conc. $\mu\text{g/mL}$	Long term $-80\text{ }^{\circ}\text{C}$	Short term	Freeze -thaw	Post- preparative
DIP	0.05	101.2 ± 2.1	101.6 ± 1.5	97.1 ± 2.2	100.2 ± 1.8
	0.3	100.1 ± 1.3	99.7 ± 1.2	96.2 ± 1.4	103.1 ± 1.7
	3	98.5 ± 2.2	97.9 ± 1.3	99.7 ± 3.0	105.5 ± 6.2
PABA	0.05	102.3 ± 5.5	102.0 ± 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

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

time (h)	PABA		DIP	
	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

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