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Determination of *p*-aminobenzoic acid and its metabolites in rabbit plasma by high-performance liquid chromatography with fluorescence detection

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Abstract

A simple, accurate and sensitive high-performance liquid chromatographic method with fluorescence detection was used for measuring plasma concentrations of *p*-aminobenzoic acid (PABA) and its three metabolites: *p*-acetaminobenzoic acid (PAABA), *p*-aminohippuric acid (PAHA) and *p*-acetaminohippuric acid (PAAHA). A Cosmosil MS-C_{18} column (250×4.6 mm, 5μm) was used under temperature control at 40°C. The mobile phase was H_2O–CH_3CN–CH_3COOH (100:3:1, pH 4.0) with a flow-rate of 1.5 ml/min. The excitation and emission wavelengths for fluorescence detection were set at 270 and 350 nm, respectively. Plasma samples (200 μl) were acidified by the addition of 150 μl of 1 *M* $HClO_4$ solution containing salicylic acid (SA) as the internal standard. After centrifugation, 30 μl of the supernatant were injected onto the column. Using this method, PABA and its three metabolites could be determined within 25 min. Within the investigated concentration ranges of PABA (0.1–50 μg/ml), PAABA (0.2–50 μg/ml), PAHA (0.1–50 μg/ml) and PAAHA (0.5–50 μg/ml), good linearity (r>0.99) for the standard curves was obtained. The validation of this method showed coefficient of variance (C.V.) that was well below 15% for all compounds. After intravenous (i.v.) administration of PABA (20 mg/kg) to rabbits (n=7), PABA followed a one-compartment open model elimination with a half-life of 10.90±1.03 min. The mean half-lives for PAABA, PAHA and PAAHA were 24.61±6.42, 12.81±6.04 and 11.27±2.77 min, respectively.

Keywords: *p*-Aminobenzoic acid; *p*-Aminohippuric acid; *p*-Acetaminobenzoic acid; *p*-Acetaminohippuric acid

1. Introduction

p-Aminobenzoic acid (PABA) is widely included as a member of the vitamin B complex in nutritional supplements. It is also an essential metabolite for certain microorganisms in the synthesis of folic acid. In a laboratory environment, it is often used as a reference drug in acetylation studies [1]. In clinical use, PABA is used for determining pancreatic function after oral administration of N-benzoyl-L-tyrosyl-*p*-aminobenzoic acid [2]. At present, it is also popularly used as a sun-screen agent in cosmetics [3].

The metabolism of PABA is mainly metabolized by acetylation and glycine conjugation in the liver to form *p*-acetaminobenzoic acid (PAABA), *p*-aminohippuric acid (PAHA) and *p*-acetaminohippuric acid (PAAHA), as shown in Fig. 1 [4]. Like other aromatic amine and hydrazide drugs, PABA undergoes acetylation in vivo, but shows a different

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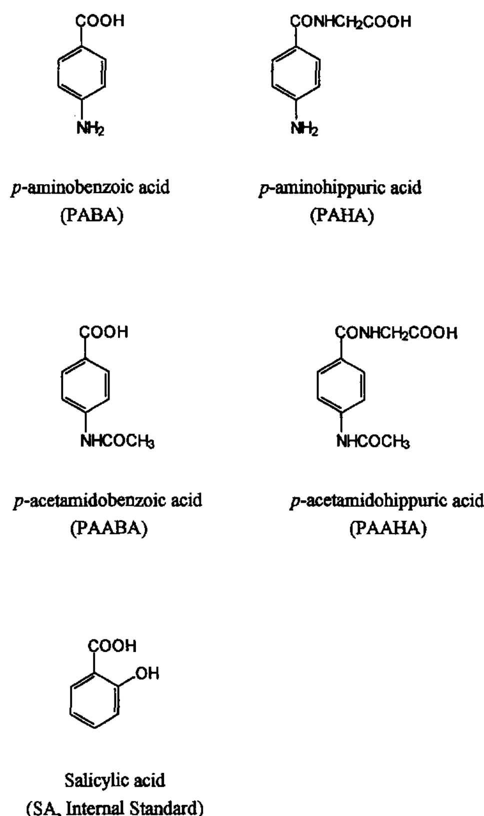

Fig. 1. Structures of PABA, PAABA, PAHA, PAAHA and SA.

acetylation phenomenon [5]. Acetylation of sulfonamide and isoniazid can be classified as rapid and slow acetylation phenotypes in humans and rabbits. This bimodal distribution of acetylation is genetically controlled. In the acetylation of PABA, only the monomorphic phenotype can be found in humans and rabbits. On the contrary, in hamsters, acetylation of PABA shows genetic polymorphism, but only monomorphism can be found in sulfonamide and isoniazid acetylation [6].

In past years, several methods had been developed for quantitation of the concentrations of PABA and its metabolites in plasma [7–10]. These include a colorimetric method and HPLC with UV detection. The colorimetric method based on the Bratton-Marshall procedure is not a specific method [11]. Although HPLC–UV methodology has been developed by many researchers, the low recovery of analytes in sample preparation procedures, the relatively long chromatographic retention times of analytes in the chromatogram and the low sensitivity for quantitation in plasma are weak points in detailed PABA metabolic pharmacokinetic studies [9–11]. In this study, a more simple, specific, accurate and precise method was developed for determining the concentration of PABA and its metabolites in plasma with HPLC–fluorescence detection. By using the present analytical method, PABA and its three metabolites can be determined within 25 min and the limit of quantitation in plasma is lower than that described in any previously published results.

2. Experiments

2.1. Chemicals and reagents

All chemicals were reagent grade and all solvents were HPLC grade. PABA, PAABA, PAHA, SA and heparin were obtained from Sigma (St. Louis, MO, USA). PAAHA was prepared according to the method of Yung-Jato et al. [11].

2.2. Instruments

Chromatographic analysis was performed by using an LC-10AD high-pressure pump, an RF-551 fluorescence detector, a CR-7A integrator and an SIL-9A auto-injector (all from Shimadzu, Kyoto, Japan).

2.3. Analytical conditions

A Cosmosil MS-C_{18} column (250×4.5 mm, 5 μm) was used for analysis and the column temperature was set at 40°C. The mobile phase was H_2O–CH_3CN–CH_3COOH (100:3:1) adjusted to pH 4.0 with 10 *M* NaOH solution. PABA and its metabolites were separated under a flow-rate of 1.5 ml/min. The excitation and emission wavelengths for fluorescence detection were set at 270 and 350 nm, respectively.

2.4. Preparation of the calibration curves and quality control (QC) samples

2.4.1. Preparation of the calibration curves for PABA, PAABA, PAHA and PAAHA

200-μl Aliquots of blank rabbit plasma samples were spiked with PABA concentrations of 0.1 to 50

μg/ml (nine points were used in this curve), PAABA concentrations of 0.2 to 50 μg/ml (eight points used), PAHA concentrations of 0.1 to 50 μg/ml (nine points used) and PAAHA concentrations of 0.5 to 50 μg/ml (seven points used). The spiked plasma samples were treated according to the described sample preparation procedure. All of the calibration samples were prepared freshly before daily analysis.

2.4.2. Preparation of QC samples

200-μl Aliquots of blank rabbit plasma samples were spiked with PABA concentrations of 0.15, 0.30, 1.50, 3.00, 15.00 and 30.00 μg/ml, PAABA concentrations of 0.30, 1.50, 3.00, 15.00 and 30.00 μg/ml, PAHA concentrations of 0.150, 0.300, 1.50, 15.0 and 30.0 μg/ml and PAAHA concentrations of 1.50, 3.00, 15.0 and 30.0 μg/ml. The QC samples were also treated according to the described sample preparation procedure and all of the QC samples were prepared freshly before daily analysis.

2.5. Sample preparation

An aliquot containing 200 μl of rabbit plasma was acidified with 150 μl of 1 *M* $HClO_4$ solution containing SA as the internal standard. After centrifugation at 10 000 *g* for 5 min, 30 μl of the supernatant were injected onto the HPLC.

2.6. Accuracy, precision and recovery

The accuracy and precision of the method were assessed by within- and between-run validation. During each run, the QC samples were positioned between the unknown plasma samples and calibration samples. By substituting the peak-height ratio into the calibration curve obtained from the same run, the concentration of PABA and its three metabolites could be measured. By comparing calculated and theoretical concentrations, the relative errors and C.V. could be obtained.

Recovery (extraction efficiency) of PABA and its three metabolites was calculated by comparing the peak-height ratios obtained from plasma samples to those obtained from analysis of equivalent amounts of aqueous solution.

2.7. Animal experiments

Male New Zealand White rabbits, weighing 1.6–2.5 kg, were used in these experiments. There was a wash-out period of at least 7 days and an overnight fast between each experiment. Before each experiment, a blank blood sample was drawn for calibration curve usage. All syringes and microtubes were rinsed with heparin (500 units/ml) before use to prevent clotting. A 20 mg/kg dose of PABA was dissolved in normal saline and injected into the marginal vein of the rabbit's ear. Blood samples (1 ml) were drawn from the ear vein on the other ear at 1, 3, 5, 7, 9, 12, 15, 20, 30, 45, 60, 80, 100, 120, 140, 160 and 180 min after injection. After centrifugation, 200 μl of plasma was used for analysis.

2.8. Data analysis

The calibration curve was calculated using the least-square linear regression with a weighting factor of $1/C$. The concentrations of the spiked QC samples and rabbit samples were calculated from this linear equation.

The plasma concentrations of PABA and its three metabolites obtained from the experiments were fitted in a one-compartment open model by the PCNONLIN computer program [13]. Pharmacokinetic parameters of area under the plasma concentration time curve (AUC), elimination half-life, volume of distribution (V_d) and systemic clearance (*Cl*) were obtained. All data were expressed as mean ± S.D.

3. Results and discussion

Several methods have been developed for the quantitation of PABA and its metabolites in biological fluids. A colorimetric method based on the Bratton-Marshall procedure was developed first [7]. However, this method does not differentiate between PABA and its metabolites, and the result obtained is a combination of the concentrations of PABA and its metabolites [11]. The HPLC–UV method was developed in 1980 and has a greater specificity than the colorimetric method. The large polarity differences between PABA, PAABA, PAHA and PAAHA, the

long retention time of analytes in the chromatogram, the low recovery of analytes and the low sensitivity of the quantitation of analytes in plasma are common weak points in every report [9–11]. Analysis of PABA derived from N-benzoyl-L-tyrosyl-p-amino-benzoic acid has been proposed as a screening test for human pancreatic function [2]. Insufficient quantitation of PABA and its metabolites may lead to misjudgement about pancreatic function [12]. Therefore, a rapid, accurate and sensitive method for PABA, PAABA, PAHA and PAAHA analysis is needed for clinical use.

Fluorescence is a more specific and sensitive detection method than UV, and the aromaticity of PABA, PAABA, PAHA and PAAHA lends itself to detection by fluorescence. Following the sample preparation procedures and under HPLC–fluorescence detection, the chromatograms for PABA, PAABA, PAHA and PAAHA are shown in Fig. 2. PABA and its three metabolites were resolved well in 25 min with retention times for PAHA, PABA, PAAHA and PAABA of 4.01, 8.32, 12.63 and 21.28 min, respectively. There were no endogenous substances interfering with the analysis of PABA and its three metabolites, which indicated that the chromatographic method used here has good specificity. Justification of internal standard should be noticed that the similarity of fluorescent property and the separation of parent and endogenous compounds. Here, it was found that SA was well-separated from endogenous and parent compounds and it also had a excellent fluorescent property under these chromatographic conditions.

The quantitation ranges for these compounds were PABA (0.1–50 μg/ml), PAABA (0.2–50 μg/ml), PAHA (0.1–50 μg/ml), and PAAHA (0.5–50 μg/ml). In order to obtain a better precision and accuracy in the lower concentration ranges, the calibration curves were performed under the least square linear regression with a weighting factor of $1/C$. These calibration curves all showed excellent linearity, precision and accuracy, as shown in Table 1. The detection limits for PABA, PAABA, PAHA and PAAHA were 0.05, 0.10, 0.05 and 0.20 μg/ml,

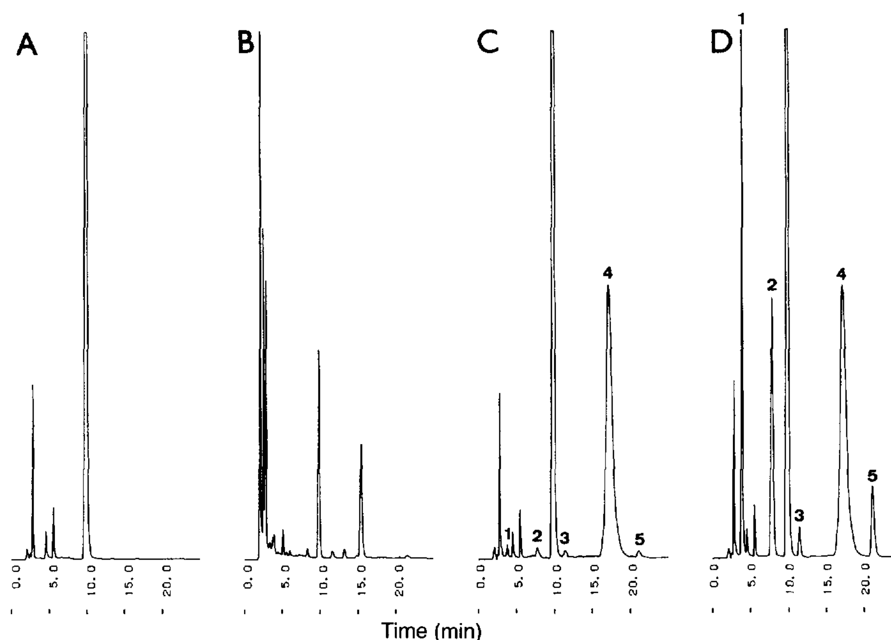

Fig. 2. Chromatograms of PABA and its metabolites. (A) Blank plasma sample, the excitation and emission wavelengths for fluorescence detection were set at 270 and 350 nm, respectively. (B) Blank plasma sample, the wavelength for UV detection was set at 270 nm. (C) Spiked plasma sample with PAHA (0.10 μg/ml), PABA (0.10 μg/ml), PAAHA (0.50 μg/ml) and PAABA (0.20 μg/ml). (D) Plasma sample after 60 min of i.v. administration of PABA (20 mg/kg) using fluorescence detection. Peaks: 1=PAHA, 2=PABA, 3=PAAHA, 4=SA and 5=PAABA.

Table 1
Precision and accuracy of the calibration curves for PABA and its metabolites (n=6)

Compound	Concentration range (μg/ml)	Linear regression equation[a]	r	S.D. of slope	C.V. of slope (%)
PABA	0.1–50	$y = -3.772 \cdot 10^{-2} + 6.351 \cdot 10^{-1}x$	0.9975	$4.817 \cdot 10^{-2}$	7.58
PAABA	0.2–50	$y = -5.229 \cdot 10^{-3} + 7.901 \cdot 10^{-2}x$	0.9983	$4.878 \cdot 10^{-3}$	6.17
PAHA	0.1–50	$y = -8.940 \cdot 10^{-3} + 8.481 \cdot 10^{-1}x$	0.9994	$7.482 \cdot 10^{-2}$	8.82
PAAHA	0.5–50	$y = -5.551 \cdot 10^{-3} + 3.550 \cdot 10^{-2}x$	0.9937	$4.189 \cdot 10^{-3}$	11.80

[a] y=peak height ratio; x=spiked concentration (μg/ml).

respectively. The validation of this method was performed using different concentrations of the four compounds in rabbit plasma by within- and between-run assays. As shown in Table 2, the C.V.s were found to be between 0.67% to 11.33% and 1.33% to 10.67%, the relative errors ranged from −7.33% to 7.00% and −2.00% to 6.00% in within- and between-run assays. The average recoveries of PABA and its 3 metabolites were: PABA 88.4%, PAABA 89.4%, PAHA 90.2% and PAAHA 91.3%. These results are higher than in other papers published previously.

The method was applied in a pharmacokinetic study by i.v. administration of PABA (20 mg/kg) to rabbits. Typical plasma concentration-time profiles are shown in Fig. 3. The plasma concentrations of PABA and its three metabolites were all within the range of the standard curves. After PCNONLIN program [13] treatment, PABA followed a one compartment open model elimination. Its three metabolites, PAABA, PAHA and PAAHA, also fitted within first order elimination. The pharmacokinetic parameters of PABA, PAABA, PAHA and PAAHA are shown in Table 3. The concentration of PABA,

Table 2
Validation of PABA and its metabolites in rabbit plasma (n=6)

Compound	Within-run				Between-run			
	Spiked concentration (μg/ml)	Measured concentration[a] (μg/ml)	Error (%)	C.V. (%)	Spiked concentration (μg/ml)	Measured concentration[a] (μg/ml)	Error (%)	C.V. (%)
PABA	0.150	0.139±0.017	−7.33	11.33	0.150	0.149±0.016	−0.67	10.67
	0.300	0.299±0.017	−0.33	5.67	0.300	0.294±0.021	−2.00	7.00
	1.50	1.50±0.06	0.00	4.00	1.50	1.48±0.07	−1.33	4.67
	3.00	3.10±0.10	3.33	3.33	3.00	3.01±0.10	0.33	3.33
	15.0	14.8±0.8	−1.33	5.33	15.0	14.9±0.4	−0.67	2.67
	30.0	29.7±1.5	−1.00	5.00	30.0	29.9±1.0	−0.33	3.33
PAABA	0.300	0.305±0.012	1.67	4.00	0.300	0.301±0.014	0.33	4.67
	1.50	1.53±0.03	2.00	2.00	1.50	1.56±0.11	4.00	7.33
	3.00	3.16±0.05	5.33	1.67	3.00	3.07±0.14	2.33	4.67
	15.0	15.9±0.1	6.00	0.67	15.0	15.3±0.9	2.00	6.00
	30.0	32.1±0.4	7.00	1.33	30.0	30.7±1.7	2.33	5.67
PAHA	0.150	0.149±0.004	−0.67	2.67	0.150	0.159±0.007	6.00	4.67
	0.300	0.298±0.013	−0.67	4.33	0.300	0.296±0.015	−1.33	5.00
	1.50	1.51±0.04	0.67	2.67	1.50	1.54±0.06	2.67	4.00
	3.00	2.89±0.11	−3.67	3.67	3.00	3.04±0.05	1.33	1.67
	15.0	15.1±0.7	0.67	4.67	15.0	15.4±0.7	2.67	4.67
	30.0	30.6±1.3	2.00	4.33	30.0	30.3±0.4	1.00	1.33
PAAHA	1.50	1.53±0.03	2.00	2.00	1.50	1.54±0.04	2.67	2.67
	3.00	3.16±0.05	5.33	1.67	3.00	3.04±0.05	1.33	1.67
	15.0	15.8±0.1	5.33	0.67	15.0	15.7±0.7	4.67	4.67
	30.0	31.8±0.6	6.00	2.00	30.0	31.0±0.6	3.33	2.00

[a] Data are shown as mean ± S.D.

Table 3
Pharmacokinetic parameters of PABA after i.v. administration of PABA (20mg/kg) to rabbits (n=7)

Pharmacokinetic parameter	Rabbit No.							Mean±S.D.
	R01	R03	R04	R05	R06	R07	R08	
PABA								
AUC (μg·min/ml)	604.56	608.53	503.82	670.03	936.50	755.09	835.83	702.05±149.58
Half-life (min)	12.22	11.39	9.92	9.23	11.57	11.33	10.67	10.90±1.03
Cl (ml/min·kg)	33.08	32.87	39.70	29.85	21.36	26.49	23.93	29.61±6.25
Vd (ml/kg)	583.25	540.25	568.50	397.44	356.50	432.99	368.43	463.91±97.50
PAABA								
AUC (μg·min/ml)	1126.37	887.46	1451.17	510.56	554.29	1085.29	1125.80	962.99±337.62
Half-life (min)	30.10	29.77	31.61	19.45	22.15	14.19	25.02	24.61±6.42
PAHA								
AUC (μg·min/ml)	51.28	90.18	42.91	571.13	688.64	206.81	243.46	270.63±258.97
Half-life (min)	21.23	14.66	1.32	13.88	15.61	11.51	11.43	12.81±6.04
PAAHA								
AUC (μg·min/ml)	174.14	137.92	184.98	[a]	[a]	311.02	407.18	243.05±112.62
Half-life (min)	8.42	11.83	8.52	[a]	[a]	14.78	12.82	11.27±2.77

[a] PAAHA are not found in these rabbits.

PAABA and PAHA could be detected beyond three elimination half-lives of each compound in plasma, although the concentration of PAAHA could not be detected at this level. PAAHA, formed from glycine conjugation of PAABA or acetylation of PAHA, was difficult to detect in plasma after 45 min of dosing with PABA. There were also two rabbits in this study that had no detectable levels of PAAHA in their plasma.

These results demonstrate that this analytical method is rapid, sensitive and accurate. From the data obtained after PABA administration to rabbits, it is concluded that this method can be used in pharmacokinetic studies or in pancreatic functional studies.

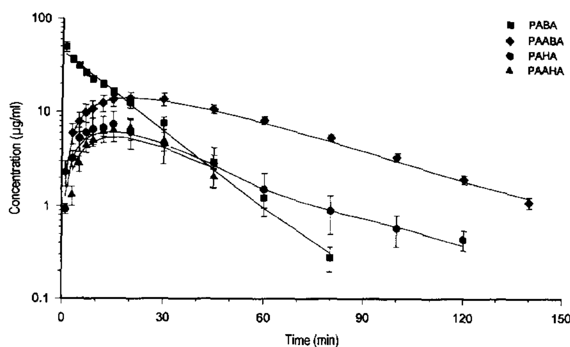

Fig. 3. Plasma concentration-time profile after i.v. administration of PABA (20 mg/kg) to rabbits (n=7).

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