Journal of Chromatography, 426 (1988) 103–109
Biomedical Applications
Elsevier Science Publishers B.V , Amsterdam — Printed in The Netherlands

CHROMBIO. 4073

DIRECT AND SIMULTANEOUS HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE DETERMINATION OF *p*-AMINOBENZOIC ACID AND ITS CONJUGATES IN HUMAN URINE

K. CHAN*

Department of Pharmacology, Faculty of Medicine, The Chinese University of Hong Kong , Shatin, N.T. (Hong Kong)

and

J.O. MINERS and D.J. BIRKETT

Department of Clinical Pharmacology, School of Medicine, Flinders University of South Australia, Bedford Park, Adelaide 5042, South Australia (Australia)

(Received October 20th, 1987)

SUMMARY

Procedures based on high-performance liquid chromatography (HPLC) were developed for identifying and measuring *p*-aminobenzoic acid (PABA) and its conjugate metabolites in human urine after oral doses of PABA. *p*-Aminohippuric acid (PAH), PABA, *p*-acetamidohippuric acid (PAHA) and *p*-acetamidobenzoic acid (PADB) in urine were resolved and determined by HPLC simultaneously and directly without extraction. A mobile phase consisting of 3% (v/v) acetonitrile in distilled water containing 0.005 *M* 1-heptanesulphonic acid in glacial acetic acid (PIC-B7) at pH 3.3 was eluted at 1 ml/min through a C_{18} Spherisorb column, followed by UV detection at 280 nm. After hydrolysis of urine samples at 37°C for 3 h with β-glucuronidase, the amounts of PABA-glucuronide and PADB-glucuronide were also determined. The retention times of PAH, a dominant peak which disappeared after hydrolysis, PABA, DABA (3,5-diaminobenzoic acid, as the internal standard), PAHA and PADB were 11.8, 14, 15, 18, 24 and 46 min, respectively. The 24-h urinary recoveries of PAH, PAHA, PADB, PADB-glucuronide, PABA and PABA-glucuronide after separate oral doses of 200 and 800 mg of PABA in one healthy subject were 43.4 and 48.1, 7 and 29 1, 11.2 and 11.8, 34.8 and 6.6, 0.2 and 0.3, and 1.0 and 2.4%, respectively. It is interesting that at high dose (800 mg) saturation of glucuronidation of PADB (N-acetylated PABA) appeared to occur, which resulted in an increase in the formation of PAHA, the glycine conjugate of PADB. Over 90% of the oral dose was accounted for by 8 h after administration.

INTRODUCTION

p-Aminobenzoic acid (PABA) is often included as a member of the vitamin B group in nutritional supplements available over-the-counter. It is an essential metabolite for certain microorganisms in the synthesis of folic acid; hence, sul-

0378-4347/88/$03.50

phonamides are effective chemotherapeutic agents. A deficiency of PABA in man and animals has not been demonstrated as they probably obtain their folic acid from dietary sources. PABA in high doses (up to 12 g daily in divided doses) has been used in the treatment of rickettsial infections. It has also been included in sunscreen agents for topical application [1].

The study of the routes of metabolism of PABA originated as a pharmacological practical exercise for medical students [2]. Using the Bratton and Marshall spectrophotometric method it was observed that in a group of 22 Chinese subjects the amount of acetylated PABA and its conjugate recovered in the urine [3] was greater than reported values [4]. A more specific high-performance liquid chromatographic (HPLC) method after organic solvent extraction under acidic conditions was used to measure PABA, *p*-aminohippuric acid (PAH) and *p*-acetamidobenzoic (PADB) in urine and it was reported that only 65% of the oral dose was recovered in 24 h [5]. It is conceivable that some of the more polar conjugates were not extracted. We now report a direct and simultaneous assay, without any organic solvent extraction, for measuring PAH, PABA, PAHA (*p*-acetamidohippuric acid) and PADB (*p*-acetamidobenzoic acid) using HPLC with an ion-pair agent.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a Waters 6000A pump, a U6K continuous-flow injector with a 25–2000 μl sample loop (Waters Assoc., Milford, MA, U.S.A.) and an M40 fixed-wavelength UV detector set at 280 nm and linked to a flat-bed chart recorder (SE 120/BBC Goerz Metrawatt). Analyses were performed on a reversed-phase C_{18} column (Spherisorb R, S5, ODS 2, 150 mm $\times$ 4.6 mm I.D., particle size 5μm; ICI Australia Operations, Dingley, Australia). Other apparatus used included a conical glass centrifuge tube (10 ml capacity), small glass test-tubes (5 ml capacity) and Hamilton microsyringes (100 μl capacity).

Materials and reagents

PAH, PABA, DABA (3,5-diaminobenzoic acid), PADB and β-glucuronidase (*Helix pomatia*) were purchased from Sigma (St. Louis, MO, U.S.A.). Glass-distilled water was used. The methanol and acetonitrile solvents were of HPLC grade from Ajax Chemicals (Auburn, Australia). Tetrabutylammonium solution (Baker, Phillipsburg, NJ, U.S.A.) and 1-heptanesulphonic acid solution (PIC-B7) (Waters Assoc.) were used as ion-pair agents in the mobile phase.

A small amount of PAHA was obtained from Hoffmann-La Roche (Basle, Switzerland). It was also prepared by dissolving PAH (200 mg) in glacial acetic acid (10 ml) followed by reaction with acetic anhydride (2 ml) in a tightly sealed centrifuge tube (15 ml) at 37°C for 3 h. The colourless solution turned yellow at the end of the reaction. The resulting mixture was evaporated to dryness under vacuum by the use of a Model RT-100 Speed Vac concentrator (Savant, Australia). The yellow solid was recrystallized from hot methanol–water. The off-

white PAHA was characterized by HPLC and had a retention time identical with that of the authentic compound supplied by Hoffmann-La Roche.

Standard solutions of PAH, PABA, DABA, PAHA and PADB (1 mg/ml) were prepared in methanol. Subsequent dilution was carried out with drug-free urine to cover the concentration range 2.5–30 μg/ml.

Choice of mobile phase. When a neat drug-free sample (20 μl) was injected into the HPLC system using the previously developed mobile phase [5] consisting of 40% methanol in phosphate buffer (10 m*M*) at pH 3.5, interfering peaks appeared that masked the analytical peaks of PAH and PABA. Ion-pair agents were included in the mobile phase with reduction of the percentage of organic solvent in order to separate interfering peaks from the analytical peaks. Tetrabutylammonium hydroxide (5 m*M*) in acetate buffer (10 m*M*) at pH 6.5 with 15% (v/v) methanol was not satisfactory. Subsequently, a mobile phase consisting of 3% (v/v) acetonitrile in distilled water containing 0.005 *M* 1-heptanesulphonic acid in glacial acetic acid (PIC-B7) at pH 3.3 was found to be satisfactory in withholding PAH long enough for its separation from interfering peaks near the solvent front. Therefore, this mobile phase was chosen with a flow-rate of 1 ml/min for the analysis of PABA and various metabolites.

Analytical procedures

To a 100-μl urine sample in a small glass test-tube was added sodium acetate buffer (0.1 m*M*, pH 5.0) (70 μl). To another 100 μl of the same urine sample were added sodium acetate buffer (50 μl) and β-glucuronidase (20 μl). These tubes were incubated at 37 °C for 3 h. At the end of the incubation period, glacial acetic acid (20 μl), a methanolic solution of DABA (50 μl, equivalent to 50 μg) and mobile phase (50 μl) were added. The mixture was vortexed for 30 s and centrifuged at 3000 rpm for 10 min. An aliquot of 20 μl was injected on to the chromatographic column for analysis with a mobile phase consisting of 3% acetonitrile containing 0.005 *M* 1-heptanesulphonic acid in water at pH 3.3 with a flow-rate of 1 ml/min. The eluates were detected at 280 nm.

Quantitation

Calibration graphs were constructed by introducing PAH, PABA, PAHA and PADB into drug-free urine. Dilutions of the stock standards were made to cover the concentration range 2.5–30 μg/ml for each of these compounds. The solutions were assayed as described under *Analytical procedures*. Between-day standards of concentration of 10 μg/ml were determined to obtain the between-batch variation of the assay. The calibration graph measurement was repeated six times during the course of study.

Stability on storage

Samples of test urine and drug-free urine spiked with drugs, even when stored at −20 °C for three months, did not show any appreciable loss of the drugs [5].

RESULTS AND DISCUSSION

Inclusion of ion pair in the mobile phase

The previously developed HPLC analysis, which could measure PAH, PABA and PADB simultaneously, did not require an ion pair [5]. However, in that assay, the preliminary organic solvent extraction of the compounds from acidified urine might not have extracted the more polar conjugates. Both PAH and PABA are zwitterions. Moreover, interference peaks were detected when drug-free urine samples were injected directly using the mobile phase without an ion-pair agent. During the development of the present assay, tetrabutylammonium chloride in 10 m*M* acetate buffer (pH 7.0) in 10% acetonitrile was used as the mobile phase. Interfering peaks from drug-free urine could not be separated from PAH. Further,

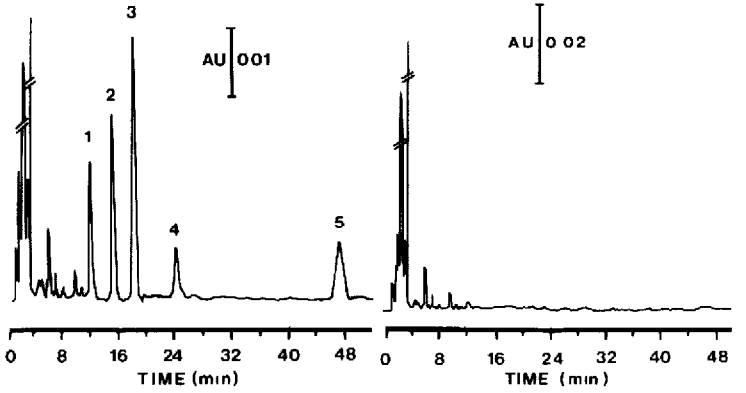

Fig. 1 Chromatograms showing (left) separation of (1) PAH, (2) PABA, (3) DABA (internal standard), (4) PAHA and (5) PADB and (right) blank urine.

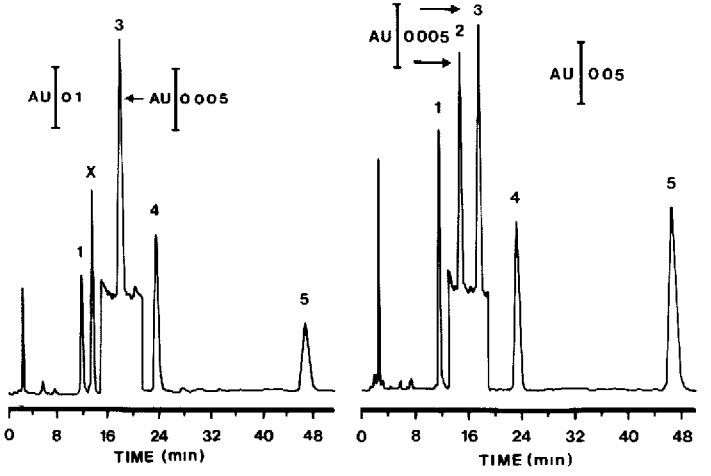

Fig. 2. Chromatograms of urine showing (left) 2-h sample after ingestion of 800 mg of PABA (X is an additional peak) and (right) same 2-h sample after β-glucuronidase hydrolysis (peak X disappeared). Note the appearance of peak 2 (PABA) and the increase in height of peak 5 (PADB).

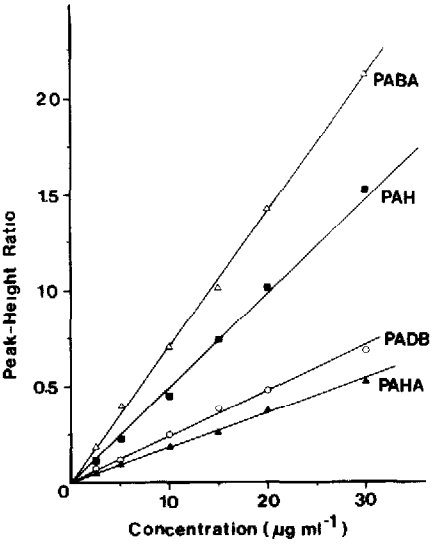

Fig. 3. Calibration graphs showing correlation of peak-height ratios with concentrations in urine of PABA, PAH, PADB and PAHA.

TABLE I

CALIBRATION AND PRECISION OF THE HPLC ASSAY ($n=6$)

Concentration (μg/ml)	PAH		PABA		PAHA		PADB	
	Peak-height ratio (mean ± S D)	C.V. (%)	Peak-height ratio (mean ± S.D.)	C.V. (%)	Peak-height ratio (mean ± S.D.)	C V. (%)	Peak-height ratio (mean ± S.D.)	C.V (%)
2.5	0.117 ± 0 003	2.57	0.181 ± 0.005	2 76	0 056 ± 0.003	5 36	0.077 ± 0.004	5.19
5.0	0.237 ± 0.011	4.64	0.396 ± 0.014	3.54	0.102 ± 0.008	7 84	0 112 ± 0.005	4 46
10.0	0.499 ± 0 018	3.61	0.701 ± 0 022	3.14	0.181 ± 0.012	6 63	0 249 ± 0.013	5.22
15.0	0.739 ± 0 026	3.52	1.059 ± 0 045	4.25	0.267 ± 0.010	3 75	0 384 ± 0 008	2.08
20.0	1.015 ± 0 038	3.74	1.417 ± 0 040	2.82	0.368 ± 0.020	5 43	0 475 ± 0.022	4.63
30.0	1.513 ± 0 042	2.78	2.125 ± 0 075	3.53	0.518 ± 0.031	5 98	0 674 ± 0 042	6.23
Batch standard* at 10 μg/ml ($n=8$)	0.512 ± 0.029	5.66	0.698 ± 0 031	4.44	0.200 ± 0.010	5 00	0 263 ± 0.018	6.84
Calibration graph:	$y=0.051x-0.009$ ($r=0$ 9999)		$y=0.07x-0.01$ ($r=0.9997$)		$y=0$ 017$x+0.01$ ($r=0.9990$)		$y=0.023x+0.015$ ($r=0.9973$)	

*An indication of between-day precision.

rapid increases in back-pressure was observed; at pH 7 in the presence of tetrabutylammonium ions the packing material deteriorated. This effect has been reported previously [6]. The present mobile phase system [3% (v/v) acetonitrile in distilled water containing 0.005 *M* 1-heptanesulphonate in glacial acetic acid (pH 3.3)] is less hostile to the column packing.

Performance of the HPLC procedure

The analytical peaks of PAH, PABA, DABA, PAHA, PADB and an unknown compound, which disappeared after β-glucuronidase hydrolysis, were well re-

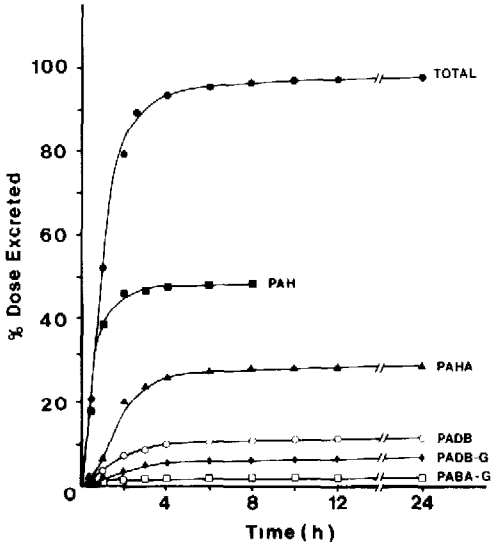

Fig. 4. Cumulative recovery of metabolites of PABA in urine over 24 h in one subject after oral ingestion of 800 mg of PABA.

TABLE II

RECOVERY OF PABA AND ITS CONJUGATES AFTER 200- AND 800-mg ORAL DOSES OF PABA OVER 24 h

Compound	Recovery (%)	
	After 200 mg	After 800 mg
PAH	43.4	48.1
PAHA	7.0	29.1
PADB	11.2	11.8
PADB-glu	34.8	6.6
PABA	0.2	0.3
PABA-glu	1.0	2.4
Total	97.6	98.3

solved with good symmetry (Figs. 1 and 2). The retention times were 11.8, 14, 15, 18, 24 and 46 min for PAH, the unknown compound that did not appear in a drug-free urine sample, PABA, DABA (the internal standard), PAHA and PADB, respectively.

Reproducibility and accuracy of the assay

Fig. 3 shows that the calibration graphs covering the concentration range 2.5–30 μg/ml were linear for all four compounds with acceptable coefficients of linear regression. The between-day coefficients of variation at 10 μg/ml were as given in Table I.

Application of the assay

A healthy non-smoker was given 200 and 800 mg PABA on two separate occasions seven days apart. On the first occasion, 0–8 and 8–24 h bulk urine collection was carried out. On the second occasion urine samples were obtained at various intervals until 48 h after dosing. The urine samples were assayed as described under *Analytical procedures*.

Fig. 4 shows the cumulative percentage of all compounds after an oral dose of 800 mg of PABA. Up to 8 h after administration more than 90% of the dose was recovered in the urine. Table II compares the percentage recovery of PABA and its conjugate over 24 h after separate doses of 200 and 800 mg PABA. It is interesting that with the higher dose of PABA the glucuronidation of the PADB metabolite was saturated and glycine conjugation was operating. Previously reported data on one subject, possibly a Caucasian [4], are not in agreement with the present findings. More work is required to substantiate our observations.

ACKNOWLEDGEMENT

One of us (K.C.) is grateful to the Flinders University of South Australia for allowing him to use the facilities at the University during his appointment as Honorary Senior Visiting Research Fellow in the Department of Clinical Pharmacology.

REFERENCES

1 A. Wade (Editor), Martindale, The Extra Pharmacopoeia, Pharmaceutical Press, London, 26th ed., 1973, p. 1940.
2 K. Chan, J.E. Gardiner, R.C.K. Pak and C.L. Wong, in Abstracts of the IUPHAR 9th International Congress of Pharmacology, London, July 29–Aug. 3, 1984, 706P.
3 K. Chan, in Abstracts of the 4th Southeast Asian and Western Pacific Regional Meeting of Pharmacologists, Penang, May 7–11, 1985, p 26.
4 C.W. Tabor, M.V. Freeman, J. Bailey and P.K. Smith, J Pharmacol. Ep. Ther., 102 (1951) 98.
5 K. Chan, Eur. J. Drug Metab. Pharmacokin., 11 (1986) 129.
6 A. Wehrli, J.C. Hildenbrand, H.P. Keller, R. Stampfli and R.W. Frei, J. Chromatogr., 149 (1978) 199.