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Note

Reversed-phase high-performance liquid chromatographic assay for the determination of the in vitro acetylation of *p*-aminobenzoic acid by human whole blood

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N-Acetylation of arylamines is catalysed by the group of enzymes known as N-acetyltransferases (EC 2.3.1.5) and requires acetyl coenzyme A (coA) as the acetyl group donor. In man, the extent of acetylation of certain drugs such as isoniazid, sulphamethazine and hydralazine is genetically determined and individuals may therefore be classified as either rapid or slow acetylators of these compounds. Polymorphic acetylation is often clinically important with respect to both drug therapy and as a predisposing host factor to a variety of clinical conditions [1]. Several investigators have unsuccessfully attempted to develop in vitro methods of acetylator phenotyping using human whole blood as the source of N-acetyltransferase [2–4]. However, preliminary studies with certain human red blood cell fractions indicate that the in vitro acetylation of *p*-aminobenzoic acid (PABA), a compound which is monomorphically acetylated in vivo, is reciprocally related to acetylator phenotype [5].

The analytical method used in previous studies [6–8] for determination of the

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percentage of PABA acetylated was the Bratton-Marshall colorimetric assay. This method is non-specific for N-acetyl-*p*-aminobenzoic acid (NAPABA) which is determined as unconjugated PABA after acidic hydrolyis of the amide group at 100°C [9]. Additional problems associated with this method are incomplete breakdown of NAPABA [10] and degradation of the parent compound which may result in apparently negative values of percentage acetylation [11, 12].

We are currently interested in comparing the in vitro acetylation capacities of human whole blood and washed red cells and considered that the development of a liquid chromatographic method would enable more sensitive and specific analysis of this reaction than the colorimetric assay.

Several high-performance liquid chromatographic (HPLC) methods for the determination of PABA in serum and urine samples from human subjects administered the synthetic peptide N-benzyl-L-tyrosyl-*p*-aminobenzoic acid (NBT-PABA) in vivo have been reported [10,13,14]. However, all of these methods measure NAPABA non-specifically after either acidic or basic hydrolysis. More recently, an ion-pair HPLC method for measurement of PABA and its metabolites, including NAPABA, in urine and plasma samples from healthy individuals administered NBT-PABA has been reported [15]. However, the pretreatment of plasma samples necessary to achieve the required sensitivity is complex and the resolution between the extracted compounds is incomplete.

The first HPLC assay for determination of PABA and its conjugated metabolites in urine samples of subjects administered PABA itself was reported in 1974 [16]. This method employed ion-exchange chromatography but exhibited poor resolution between the parent compound and its metabolites. A more recent method used reversed-phase HPLC and enabled specific analysis of NAPABA in urine samples [17]. However, interference prevented direct measurement of PABA by this method. The total amount of PABA was therefore determined after acidic hydrolysis of the sample and the amount of PABA in the unhydrolysed sample calculated indirectly by subtracting the amount of NAPABA present from this value.

This paper describes an HPLC method for the analysis of PABA and NAPABA which has greater resolution and specificity and easier sample preparation than any of the above methods. The application of this method to the measurement of the in vitro N-acetyltransferase activities of whole blood and washed red blood cells from human subjects is also reported.

EXPERIMENTAL

Materials

PABA and NAPABA were supplied by Aldrich (Gillingham, U.K.). *p*-Hydroxybenzoic acid (PHBA) was obtained from Sigma (Poole, U.K.). Sodium citrate and perchloric acid were purchased from BDH (Poole, U.K.). HPLC-grade acetonitrile was obtained from Rathburn (Walkerburn, U.K.).

HPLC analysis of PABA and NAPABA

A Gilson 302 reciprocating pump (Gilson Medical Electronics, Villiers-le-Bel, France) was used to deliver a mobile phase of acetonitrile (12%, v/v) in 0.1 mol/

l citrate buffer (pH 3.5) at 1.5 ml/min. Samples were introduced through a Rheodyne injection valve (20 μl, Rheodyne, Cotati, CA, U.S.A.). A column (250 mm×4.6 mm I.D.) packed with 5-μm Spherisorb ODS2 (Phase Separations, Queensferry, U.K.) was used. Ultraviolet detection was carried out with a Pye Unicam LC3 UV detector (Pye Unicam, Cambridge, U.K.) having a flow cell volume of 12 μl and at a wavelength of 266 nm. The detector signal was monitored by an LDC Milton Roy CI-10 integrator (Laboratory Data Control, Stone, U.K.).

In vitro blood N-acetyltransferase activity

Blood samples were obtained from healthy male and female volunteers with a mean age of 24 years (range 23–25 years). The subjects fasted overnight and did not consume any beverages containing caffeine prior to donating blood. Blood was taken by venepuncture at 9.00 a.m. into lithium heparin tubes before its immediate use. The incubation protocol was as follows: 0.2 ml of heparinised whole blood and 0.2 ml of PABA (50 nmol) in 66.6 mmol/l phosphate buffer (pH 7.4) were mixed in 1.5-ml plastic Eppendorf tubes. The samples were incubated for periods of 1 and 24 h at 37°C in a Gallenkamp Model 1 incubator. After incubation, 0.1 ml of 2 mol/l perchloric acid and 0.1 ml of 66.6 mmol/l phosphate buffer (pH 7.4) were added. For analysis using PHBA as internal standard, 0.1 ml of this compound (50 nmol) in 66.6 mmol/l phosphate buffer (pH 7.4) was used in place of phosphate buffer in the protein precipitation procedure. After vortex-mixing, the extracts were centrifuged at 6000 *g* for 5 min at 4°C (Eppendorf 5412 centrifuge) and the resulting supernatant was analysed by HPLC.

To calibrate the assay, mixtures of PABA and NAPABA were prepared such that the total amount of PABA and NAPABA in these mixtures was maintained constant (50 nmol) although the amounts of each were varied to correspond to values of PABA acetylated of between 0 and 7.5%. These standards were then added to human whole blood and treated with perchloric acid as described above. Calibration lines were obtained by plotting the amount of NAPABA present against the peak-height ratio of NAPABA to PABA expressed as a percentage. For standards prepared using PHBA as internal standard, the amount of NAPABA present was plotted against the NAPABA/PHBA peak-height ratio.

Preparation of washed red blood cells

Whole blood was separated into plasma and packed cells by centrifugation at 2000 *g* for 10 min at 4°C. After removal of the plasma, an equivalent volume of isotonic (0.9 g/l) saline solution was added and the red blood cells were resuspended by gentle inversion. The red blood cell suspension was then washed three times with isotonic saline solution. The washed red blood cells were finally resuspended in isotonic saline solution and the N-acetyltransferase activity was determined as described above.

RESULTS

The calibration lines of PABA and NAPABA were linear ($y=3.04x-0.47$; $r>0.99$) over the ranges studied. There was good agreement between the amount

of NAPABA measured by analysis with or without internal standard. The correlation between the two methods was determined on 40 samples ($y=1.01x-0.23$) and gave a correlation coefficient $r>0.99$. The intra-assay precision of the method, expressed as the coefficient of variation ($n=6$), at the lowest (0.25 nmol) and highest (10 nmol) amounts of NAPABA studied were 5 and 2%, respectively. The values for the inter-assay precision ($n=5$) at these levels were 7 and 4%, respectively.

Fig. 1 illustrates a chromatogram from an extract of a human blood sample incubated with PABA (50 nmol) for 24 h. There were no interfering peaks in the blank samples, provided the subjects abstained from any caffeine-containing beverages for several hours prior to donating blood. This precaution was only important if PHBA was used as an internal standard since caffeine interfered with the analysis of this compound. The limit of detection of this method, i.e. the concentration of NAPABA resulting in a signal three times the noise level, is 0.28 μmol/l. This represents injection of approximately 1 ng on-column and corresponds to approximately 0.01% acetylation of the parent compound at the substrate level studied. NAPABA was not degraded by extraction with perchloric acid, an analytical problem which has been reported previously for other compounds [18].

Table I shows the mean in vitro blood N-acetyltransferase activities of whole

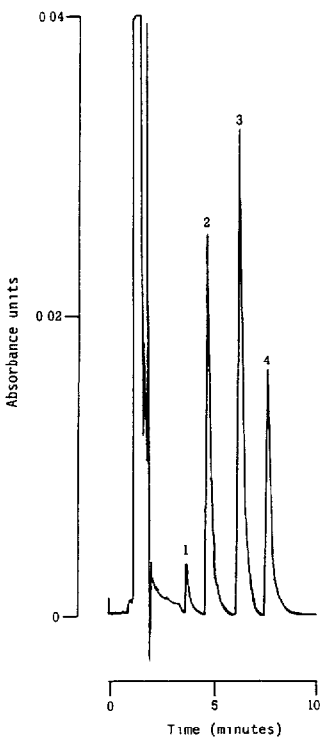

Fig. 1. Reversed-phase HPLC profile of an extract of human blood incubated with PABA (50 nmol). For HPLC conditions see text The identity of peak 1 was unknown but was also present in blank blood extracts. Other peaks: 2 = PABA; 3 = PHBA (internal standard); 4 = NAPABA.

TABLE I

IN VITRO ACETYLATION OF *p*-AMINOBENZOIC ACID BY HUMAN WHOLE BLOOD AND WASHED RED BLOOD CELLS

Incubation time (h)	N-Acetyl-*p*-aminobenzoic acid produced (nmol)			
	Whole blood		Washed red cells	
	Mean	Range	Mean	Range
1	0.78	0.56–1.10	0.55	0.44–0.74
24	6.81	4.53–8.88	2.89	1.56–4.08

blood and washed red cells from six subjects. The acetylation capacity of whole blood is significantly ($p < 0.05$, two-tailed Student's t-test) higher than that of washed red blood cells at each of the incubation times studied.

DISCUSSION

In contrast to the colorimetric method for determination of the amount of total aromatic amines and their acetylated metabolites present, the HPLC method described in this paper is selective for PABA and NAPABA. The selective detection and quantitation of the acetylated derivative as well as the parent compound is an important development compared to many of the previously published HPLC assays. The complete chromatographic resolution of PABA and NAPABA, the use of PHBA as an internal standard and the simple sample preparation procedure reported here are also considerable improvements on the existing HPLC methods reported for analysis of these compounds [15–17].

Another advantage of the specificity of the current HPLC method is that the in vitro blood N-acetyltransferase activity of subjects undergoing drug therapy for particular clinical conditions can be determined without necessitating discontinuation of these drugs provided these compounds do not interfere with the analysis of PABA and NAPABA. We have previously used an HPLC method to study the in vitro acetylation of the antibacterial drug sulphamethazine by blood samples from diabetic patients, some of which were being treated with insulin or various hypoglycaemic agents and others which were also concurrently receiving other drugs for other clinical conditions [19].

In addition to the greater selectivity of the HPLC method, the detection limit for NAPABA of this assay is approximately 18 000 times lower than that reported for the Bratton-Marshall assay [7]. This increased sensitivity should enable study of the in vitro N-acetyltransferase activity at lower initial substrate concentrations or using separated blood fractions containing lower activities of this enzyme. In this study, the acetylation of PABA by both human whole blood and washed red cell preparations in the absence of added acetyl-CoA was demonstrated. This result contrasts those reported by several previous groups [8,20] and is probably due to the increased sensitivity and selectivity of the HPLC method

used to detect NAPABA compared to the colorimetric assay used in previous studies. The results also demonstrate a significant difference between the in vitro N-acetyltransferase activities of whole blood and washed red cels. The reasons for this difference are currently being investigated.

In addition to its application to studying the in vitro acetylation of PABA by human blood preparations, the resolution and selective detection of the developed HPLC method also permits investigation of the in vitro deacetylation capacity of these samples. Preliminary studies using blood samples from three human subjects have demonstrated in vitro deacetylation (approximately 3%) of NAPABA by plasma, whole blood and washed red blood cells incubated with this compound. A previous study did not observe deacetylation of NAPABA by human whole blood [6] although the small amount of PABA produced may have been below the detection limit of the analytical method used.

Although the current report is restricted to an in vitro application, it may be possible to use the HPLC method described to detect and quantitate the other metabolites of PABA (for example, *p*-aminohippuric acid and N-acetyl-*p*-aminohippuric acid) produced in vivo [21].

REFERENCES

1 W.W. Weber and D W Hein, Pharmacol. Rev., 37 (1985) 25
2 D.J. Hearse, R. Szabadi and W.W. Weber, Pharmacologist, 12 (1970) 274.
3 D.E. Drayer, J.M. Strong, B. Jones, A. Sandler and M. Reidenberg, Drug Metab. Dispos., 2 (1974) 65.
4 R.M. Lindsay and J.D Baty, Biochem. Pharmacol., in press.
5 W W. Weber, H.E. Radtke and R.H. Tannen, in T.E Gram (Editor), Extrahepatic Metabolism of Drugs and Other Foreign Compounds, MTP Press, Lancaster, 1980, p. 493.
6 S.H. Blondheim, Arch. Biochem. Biophys, 55 (1955) 365.
7 D.J Hearse and W.W. Weber, Biochem. J., 132 (1973) 519.
8 F. Mandelbaum-Shavit and S.H. Blondheim, Biochem. Pharmacol., 30 (1981) 65.
9 A C. Bratton and E.K. Marshall, Jr., J. Biol. Chem., 128 (1939) 537.
10 S. Ito, K. Maruta, Y. Imai, T. Kato, M Ito, S. Nakajima, K. Keisuke and T. Kurahashi, Clin Chem., 28 (1982) 323.
11 R. Whelpton, G. Watkins and S.H. Curry, Clin. Chem., 27 (1981) 1911.
12 H.H. Andres, A.J. Klem, S.M. Szabo and W.W. Weber, Anal. Biochem., 145 (1985) 367.
13 J.D. Berg, I. Chesner and N. Lawson, Ann. Clin. Biochem, 22 (1985) 586.
14 N Lawson, J.D. Berg and I. Chesner, Clin. Chem., 31 (1985) 1073.
15 C.T. Hung, D.G. Perrier, A.M. Schicker and A.R. Zoest, Biomed. Chromatogr., 2 (1987) 13.
16 N.D. Brown, R.T. Lofberg and T.P. Gibson, J. Chromatogr., 99 (1974) 635.
17 K. Chan, Eur. J. Drug Metab. Pharmacokin., 11 (1986) 129.
18 H.J.E.M. Reeuwijk and U.R. Tjaden, J. Chromatogr., 353 (1986) 339.
19 R.M. Lindsay, J.D. Baty and N.R. Waugh, in G. Piemonte, F. Tagliaro, M. Marijo and A. Frigerio (Editors), Developments in Analytical Methods in Pharmaceutical, Biomedical and Forensic Sciences, Plenum, New York, 1987, p 303.
20 G S. Drummond, H.C. Kelker and W.W. Weber, Biochem. J., 187 (1980) 157.
21 H.G. Mandel, C.-Y. Yen and P.K. Smith, Fed. Proc., Fed. Am. Soc. Exp. Biol., 11 (1952) 372.