Journal of Chromatography, 338 (1985) 377—388
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2441

ANALYSIS OF N-BENZOYL-L-TYROSYL-*p*-AMINOBENZOIC ACID (BENTIROMIDE) METABOLITES IN URINE BY ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

H. THOMAS KARNES*

Department of Pharmacy and Pharmaceutics, Medical College of Virginia, Box 581 MCV Station, Richmond, VA 23298 (U.S.A.)

and

C.M. RILEY, STEPHEN H. CURRY and S.G. SCHULMAN

College of Pharmacy, University of Florida, Box J-4, J. Hillis Miller Health Center, Gainesville, FL 32610 (U.S.A.)

(First received July 24th, 1984; revised manuscript received November 5th, 1984)

SUMMARY

A high-performance liquid chromatographic method is described for the analysis of bentiromide metabolites in urine. The procedure involves no more than direct injection of the diluted urine sample, obviating the need for an extraction step or an internal standard. A μBondapak C_{18} column is used with a mobile phase of 0.01 *M* tetrabutylammonium chloride (pH 7.4)—methanol (9:1). A flow-rate of 1.4 ml/min, detection at 254 nm and column temperature of 40°C are employed. These conditions were achieved by investigating the effects of mobile phase pH, and concentrations and types of organic modifiers, buffers and ion-pairing agents on the resolution of the metabolites. The analysis time is 18 min per sample and the coefficient of variation on replicate assays is less than 10% for most concentrations studied. Analytical recoveries were between 95 and 100% throughout the appropriate concentration ranges and no interferences were obtained with the exception of *p*-acetamidobenzoyl glucuronide which could be eliminated by treatment of the samples with β-glucuronidase. Concentration profiles of the metabolites were studied in normal subjects, and the method was found to be potentially useful for clinical situations in which the existing bentiromide test leads to ambiguous results because of small bowel and hepatic dysfunctions.

INTRODUCTION

Urine analyses of *p*-aminobenzoic acid (PABA) derived from N-benzoyl-L-

0378-4347/85/$03.30

tyrosyl-*p*-aminobenzoic acid (bentiromide, Fig. 1) have been proposed as a screening test for human pancreatic function [1—3]. The cleavage of PABA from bentiromide takes place through the action of the pancreatic endopeptidase (E.C. 3.4.21.1, chymotrypsin) in the small bowel following an oral dose of the drug. The PABA marker is then absorbed from the small bowel, metabolized by the liver and excreted in the urine. The urinary concentrations of PABA and its metabolites are therefore reflective of chymotrypsin activity. Exocrine pancreatic insufficiency is indicated by low recoveries of these substances.

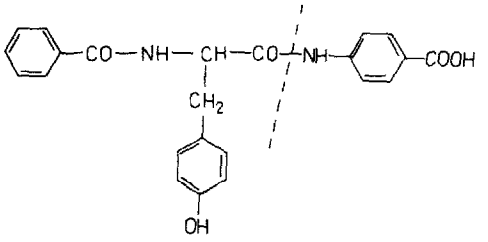

Fig. 1. Structure of bentiromide showing the point of cleavage by chymotrypsin (- - - - - -).

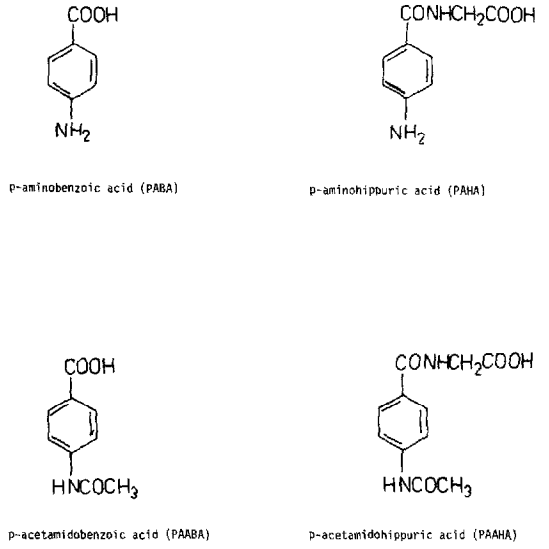

Fig. 2. Structures of *p*-aminobenzoic acid and metabolites.

Quantification of total urinary PABA may be accomplished by hydrolysis of PABA metabolites to the parent compound followed by treatment with chromogenic reagents and spectrophotometric measurement [4, 5]. More recently a liquid chromatography method has been described for the determination of PABA using electrochemical detection [6]. Abnormalities in small bowel and hepatic function however, may give rise to ambiguous results in vivo [7], since current methods detect only total PABA and it is not possible to differentiate low recovery caused by pancreatic insufficiency from that caused by small bowel or liver diseases. If individual PABA metabolites are quantified, concentration patterns or fingerprints may be established that

would indicate the source of low PABA recovery and thus differentiate false positives from true pancreatic dysfunction.

p-Acetamidobenzoic acid (PAABA), *p*-aminohippuric acid (PAHA) and *p*-acetamidohippuric acid (PAAHA) (Fig. 2) have been identified as PABA metabolites in animals [8, 9] and are likely candidates as human metabolites. We have developed an ion-pair high-performance liquid chromatographic (HPLC) method for urinary analysis of these metabolites as well as the parent compound and applied it to a study of metabolite concentration profiles in normal subjects.

EXPERIMENTAL

Chemicals and reagents

PABA, PAABA and PAHA were obtained from Sigma, PAAHA was synthesized by the reduction of acetic anhydride by PAHA as described by Vogel [10]. The tetrabutylammonium (TBA) chloride was 95% pure from Fisher Scientific as were the tetramethylammonium chloride, tetraethylammonium chloride and hexadecyltrimethylammonium bromide. HPLC-grade organic modifiers (methanol and acetonitrile) were also supplied by Fisher. All other chemicals (KH_2PO_4, phosphoric acid and sodium hydroxide) were analytical grade from various sources. The water was distilled and deionized by a Watts Model M (Lawrence, MA, U.S.A.) water purifier.

Human studies

Bentiromide (500 mg) (Chymex, Adria Labs. Columbus OH, U.S.A.) was administered in 250 ml of water to five healthy adult volunteers (four males, one female, ages 21—31 years) who were fasted overnight. Prior to dosing, the subjects were instructed to empty their bladders and consume 500 ml of water. An additional 250-ml volume of water was given at 2 h and 4 h after administration of the drug and the subjects fasted throughout the test. Urine samples were collected at 30 min and 1, 2, 3, 4, 5 and 6 h after receiving the dose. Each sample was mixed by inversion, the volume was measured, and aliquots stored at 4°C for no more than three days prior to analysis.

Sample preparation

Individual urine samples were centrifuged and then diluted with deionized water so that the metabolite concentrations were within the range of the standard curve. The extent of dilution was based on the results of preliminary experiments. Samples smaller than 500 ml were diluted 100-fold whereas volumes voided which were between 50 and 100 ml, 100 and 150 ml and above 150 ml were diluted 50-, 20- and 10-fold respectively. The diluted samples were injected without further preparation.

Chromatography

A Waters Model M-6000A liquid chromatograph (Milford, MA, U.S.A.) equipped with a Rheodyne 7105 loop injector and fixed wavelength detector (254 nm) was used for the analyses. Chromatography was performed on a Waters μBondapak (30 × 0.4 cm, 10 μm) column. An Omniscribe Model 3532

strip chart recorder (Houston Instruments, Austin, TX, U.S.A.) was used. The column temperature (40°C) was controlled with an Alltech water jacket (Deerfield, IL, U.S.A.) connected to a Temptrol 153 (Precision Scientific, Chicago, IL, U.S.A.) circulating-water bath. The ultraviolet spectra of PABA and its metabolites were obtained using a Beckman Model 35 spectrophotometer (Beckman Instruments, Irvine, CA, U.S.A.). The chromatographic conditions used for analysis of bentiromide metabolites in urine are summarized in Table I.

TABLE I

CHROMATOGRAPHIC CONDITIONS

Parameters	Conditions
Column	μBondapak C_{18}
Mobile phase	Methanol—0.01 *M* TBA (10:90)
Wavelength	254 nm
Flow-rate	1.4 ml/min
Temperature	40°C
Chromatography time	18 min
a.u.f.s.	0.01
Sample volume	20 μl

RESULTS AND DISCUSSION

Separation

Organic modifier. The influence of methanol and acetonitrile concentration on the retention of the compounds of interest by the column was studied. The aqueous component of the mobile phase was buffered at pH 4 with a 0.01 *M* phosphate buffer and no pairing ions were added at this stage. In all cases, decreasing the concentration of organic modifiers increased the retention of the solutes by the column (Figs. 3 and 4). However, acetonitrile had a more selective effect on retention than had methanol. For example, lowering the acetonitrile concentration from 15 to 5% increased the capacity ratios (k') of PAABA and PAAHA from 1.0—2.4 and 0.4—1.3, respectively. The corresponding increase in the k' values of PABA and PAHA were only 1.0—1.3 and 0.4—0.6 respectively. Consequently, whereas 5% acetonitrile provided satisfactory overall retention characteristics, the resolution of PABA and PAAHA was poor (Fig. 3). In comparison, methanol demonstrated a less selective effect on the k' values of PAABA and PAAHA which increased from 2.6—4.1 and 0.9—2.3, respectively, when the methanol concentration was decreased from 20 to 5% (Fig. 4). The corresponding increases in the k' values of PABA and PAHA were 1.0—1.4 for PABA and 0.4—0.5 for PAHA. With concentrations of either organic modifier below 5%, the solutes eluted too slowly from the column to be analytically useful.

The results indicate that methanol is a stronger solvent, having less effect on analyte retention, than acetonitrile for PAABA and PAAHA but not for their non-acetylated counterparts. A possible explanation for this is that the amides (PAABA and PAAHA) are better proton acceptors than the amines

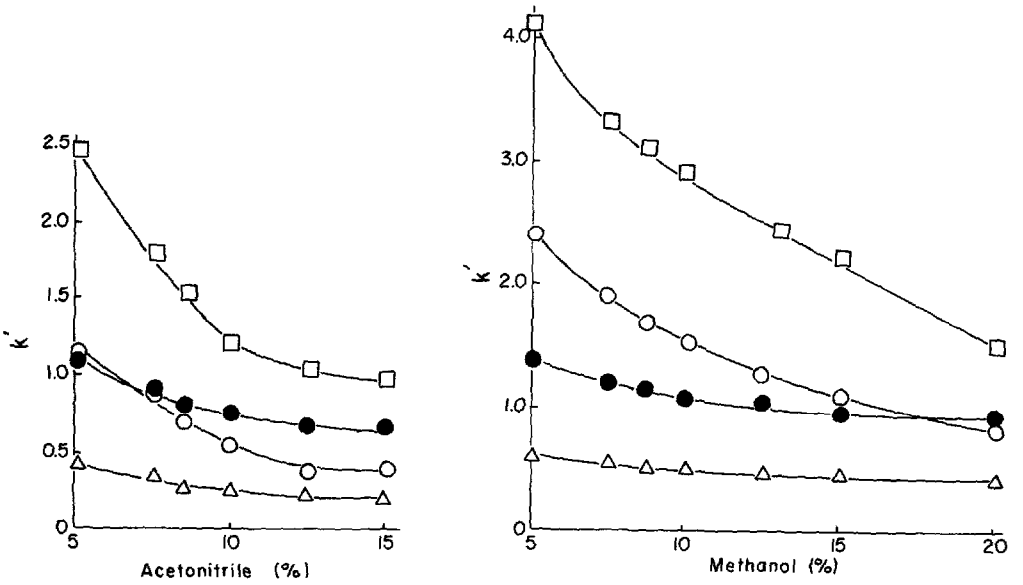

Fig. 3. The effect of mobile phase acetonitrile concentration on capacity factors for *p*-aminobenzoic acid and metabolites. The chromatographic conditions were the same as those listed in Table I except the flow-rate was 1.2 ml/min and the column temperature was 25°C. • = PABA; △ = PAHA; ○ = PAAHA; □ = PAABA.

Fig. 4. The effect of mobile phase methanol concentration on capacity factors for *p*-aminobenzoic acid and metabolites. The symbols and conditions are the same as for Fig. 3.

(PABA and PAHA) [11] and could be expected to interact more strongly with a proton donating solvent. Methanol can serve as both a proton donor and acceptor [12] making it a stronger solvent than acetonitrile for hydrogen-bonding interactions [13].

With a mobile phase containing 10% methanol the resolution [14] of all of the peaks of interest was greater than 1.00. Although a mobile phase containing 10% methanol in 0.01 *M* phosphate buffer (pH 4.0) adequately resolved the peaks of interest, interference from endogenous components of urine was observed. Resolution was optimized by investigating the effect of pH and the addition of cationic ion-pairing agents to the mobile phase.

pH Optimization. The dependence of retention volume on pH was studied with a mobile phase containing 10% methanol. The pH of the aqueous component of the mobile phase (0.01 *M* KH_2PO_4) was adjusted by addition of phosphoric acid or sodium hydroxide. Fig. 5 shows that the retention of all the compounds decreased with increasing pH above 3.5 because of deprotonation of the carboxylic groups of the solutes [15]. Changes in elution order with changing pH may be attributed to differences in the pK_a values of the carboxyl groups. For example, the acidic pK_a values for PABA and PAHA are 4.9 and 3.6, respectively [16]. No literature values for the pK_a values of PAABA and PAAHA are available.

Good separation of the metabolites was achieved at pH 4.0. However, under these conditions components of human urine persisted, indicating that an alternative strategy was necessary, to selectively enhance the retention of the

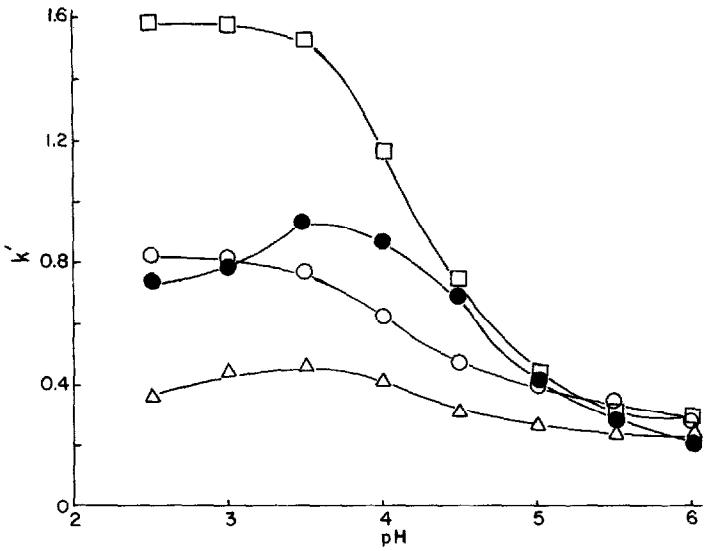

Fig. 5. The effect of pH on capacity factors for *p*-aminobenzoic acid and metabolites. Conditions and symbols as in Fig. 3 except the mobile phase contained 10% methanol and 0.01 *M* KH_2PO_4.

compounds of interest without affecting the endogenous components of urine.

Ion-pair chromatography. The four solutes of interest possess ionizable carboxylate groups suggesting that their retention may be selectively enhanced by the addition of an oppositely charged, cationic, pairing-ion to the mobile phase [17]. The retention of all the solutes was found to increase in a sigmoidal manner [17] with increasing concentration of TBA chloride (0.001—0.01 *M*) in a mobile phase consisting of methanol—0.01 *M* phosphate buffer

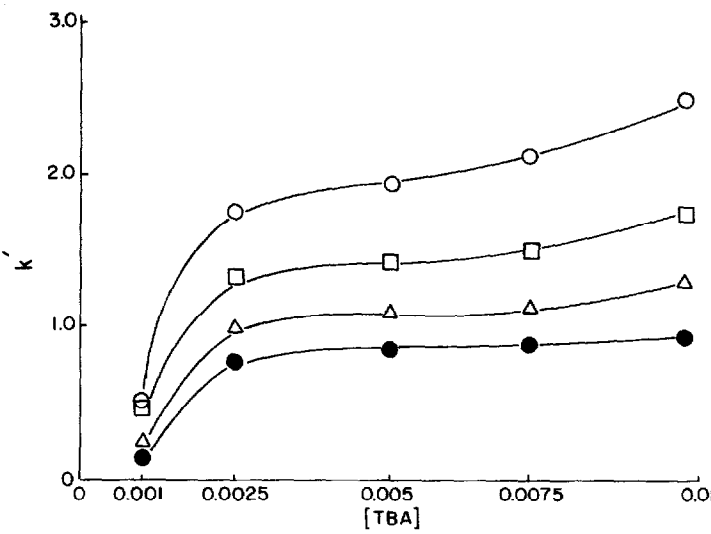

Fig. 6. The effect of tetrabutylammonium chloride concentration on the capacity factors of *p*-aminobenzoic acid and metabolites. The symbols and conditions are the same as those for Fig. 5 and the pH of the mobile phase was adjusted to 6.0.

(pH 6.0) (10:90) (Fig. 6). No changes in elution order were observed with changes in the TBA concentrations. This is consistent with previous observations [17] that selectivity is independent of pairing-ion concentration, provided that the solutes are fully ionized. The influence of ion-pairing on the retention of the solutes was investigated further by studying the effect of pairing-ion hydrophobicity (carbon number) at a constant concentration (0.01 *M*) in a mobile phase consisting of methanol—0.01 *M* phosphate buffer (pH 6.0) (10:90) (Fig. 7). Three symmetrical cationic pairing ions (TMA, TEA, TBA) of general formula $(C_nH_{2n+1})_4N^+$ ranging in total carbon number from 4 to 16 (n = 1—4) were studied and compared with a long chained cationic surfactant, hexadecyltrimethylammonium bromide (HTAB). In the case of the symmetrical quaternary ammonium ions there was a linear relationship between the logarithm of the capacity ratio of the solutes and the total number of carbon atoms in the pairing ions. In contrast with the effect of changing pairing-ion concentration (Fig. 6), which produced no changes in selectivity, the selectivity of the system and hence, the elution order of the solutes was markedly influenced by the hydrophobicity of the pairing ion. The two hippuric acid analogues (PAHA, PAAHA) exhibited a stronger dependence on pairing-ion size than their benzoic acid counterparts (Fig. 7). This effect may be attributed to the greater distances between the ionized carboxylate groups and the phenyl ring in PAHA and PAAHA than in PABA and PAABA. The influence of steric effects on the interaction between the solutes and the pairing ions is further demonstrated by the effect of HTAB on the retention of the solutes which is greater than would have been predicted by the relationships between log k' the number of carbons in the symmetrical pairing ions (TMA, TEA and TBA).

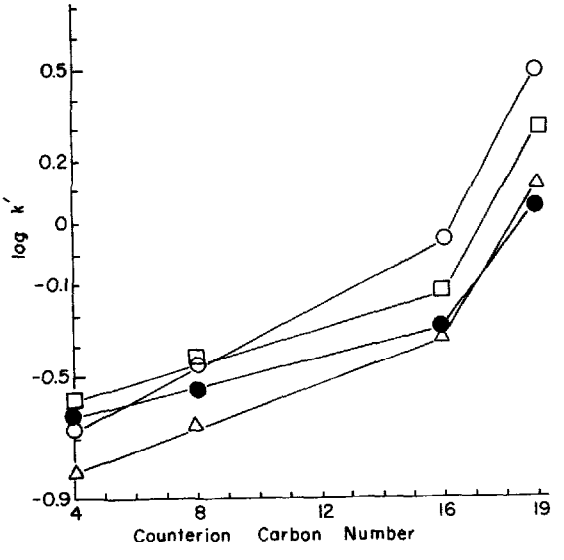

Fig. 7. Counter-ion size effect on capacity factors for *p*-aminobenzoic acid and metabolites. Tetramethylammonium (TMA) chloride = 4 carbons; tetraethylammonium (TEA) chloride = 8 carbons; tetrabutylammonium chloride = 16 carbons and hexadecyltrimethylammonium bromide = 19 carbons. The symbols and conditions are the same as for Fig. 6.

In the presence of TBA, retention of the solutes was much less influenced by changing the pH of the mobile phase (pH 4—5.8) (Fig. 8). Over the pH range studied, the degrees of ionization of the solutes could be expected to change with decreasing pH of the mobile phase and a change in retention mechanism would take place. At higher pH values the solutes would be retained as a result of interaction with the ion-pairing agent added to the mobile phase (0.01 *M* TBA), and at lower pH values the solutes would be retained by the column as neutral or positively charged species. Fig. 8 indicates that for three of the solutes (PABA, PAHA, PAAHA) the two contributions to retention appear to be about equal. In contrast the neutral form of PAABA is retained to a greater extent than its corresponding ion-pair with TBA.

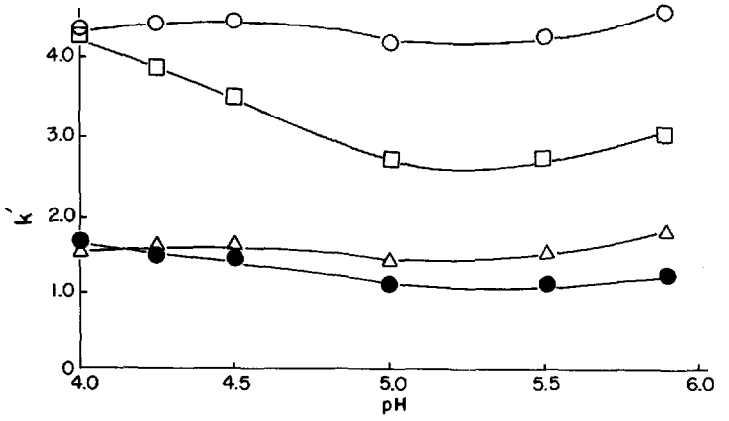

Fig. 8. The effect of pH on capacity factors for *p*-aminobenzoic acid and metabolites with 0.01 *M* tetrabutyl ammonium chloride added to the mobile phase. Other conditions as in Fig. 6; symbols as in Fig. 3.

The addition of 0.01 *M* TBA to the mobile phase at pH values above 5.8 had little if any effect on the elution of the components of urine which previously interfered with the compounds of interest. The optimum conditions for the separation and determination of bentiromide metabolites in urine are summarized in Table I. The optimization of the chromatographic conditions was performed with a phosphate buffer in the mobile phase. It was found that this component could be eliminated from the mobile phase without any detrimental effect on the separation and peak shapes (Fig. 9). Under these conditions the pH of the aqueous component of the mobile phase (0.01 *M* TBA) was found to be 7.4.

The studies described above were performed at ambient temperature (25°C) with a flow-rate of 1.2 ml/min. A reduction in the overall analysis time and a slight improvement in peak shape was obtained by operating at a flow-rate of 1.4 ml/min and 40°C. Further increases in flow-rate (up to 2 ml/min) and temperature (up to 70°C) resulted in incomplete resolution of the compounds of interest.

Linearity. There was a linear relationship between peak height and concentration of the solutes in aqueous solutions over the following ranges: PABA (0.13—1.0 mg/l), PAHA (0.18—1.42 mg/l), PAABA (1.63—13.04 mg/l) and PAAHA (3.23—25.86 mg/l). The mean slopes of the graphs and corresponding

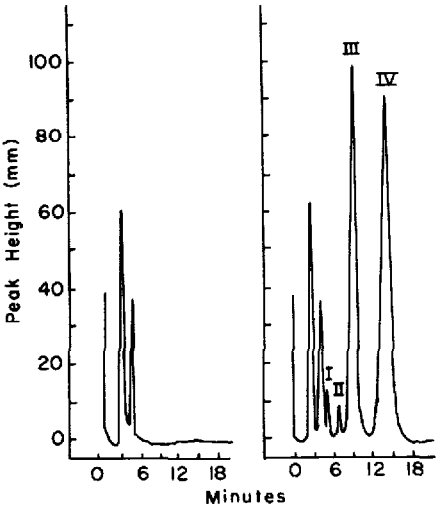

Fig. 9. Typical chromatograms under the conditions in Table I, of a pooled blank urine (left) and a spiked urine containing 0.5 mg/l PABA (I), 0.71 mg/l PAHA (II), 6.52 mg/l PAABA (III), and 12.93 mg/l PAAHA (IV).

standard deviations of the mean of six measurements were 31.7 ± 0.44 for PABA; 17.1 ± 0.20 for PAHA; 17.7 ± 0.19 for PAABA and 8.5 ± 0.06 for PAAHA. The limits of detection, taken as the peak height corresponding to twice the baseline noise, were found to be 0.06 mg/l for PABA, 0.12 mg/l for PAHA, 0.11 mg/l for PAABA and 0.24 mg/l for PAAHA.

Recovery. Various amounts of each compound were added to pooled blank urine collected from fasting subjects under test conditions. The recoveries were determined by replicate analyses ($n = 6$) at each concentration. The mean slopes of the peak height versus concentration curve for spiked urine were compared with those curves obtained by direct injection of identical aqueous standards. The results are summarized in Table II.

TABLE II

RECOVERY STUDIES ($n = 6$)

Compound	Concentration range (mg/l)	Mean slope	Standard error	Recovery (%)
PABA	0.13— 1.0	30.0	0.23	95
PAHA	0.18— 1.42	16.7	0.38	98
PAABA	1.63—13.04	17.7	0.13	100
PAAHA	3.23—25.86	8.4	0.03	100

Precision. The between-day precision was assessed by analysing samples containing four different concentrations of each solute on five consecutive days. The coefficient of variation (C.V.) for each compound was less than 10% for all urine concentrations tested (Table III) except for very low concentrations of PABA and PAHA. The within-day variation in peak height was less than 5% (C.V.) at the lowest and highest standard concentrations for all compounds tested ($n = 5$).

TABLE III

BETWEEN-DAY PRECISION (n = 6)

Compound	Mean concentration (mg/l)	C.V. (%)
PABA	0.98	3.0
	0.48	9.2
	0.25	9.2
	0.13	16.7
PAHA	1.40	3.6
	0.67	2.3
	0.32	5.4
	0.18	14.0
PAABA	12.4	6.5
	6.5	3.8
	3.3	3.2
	1.6	8.3
PAAHA	26.0	2.9
	12.8	1.4
	6.5	1.4
	3.3	5.3

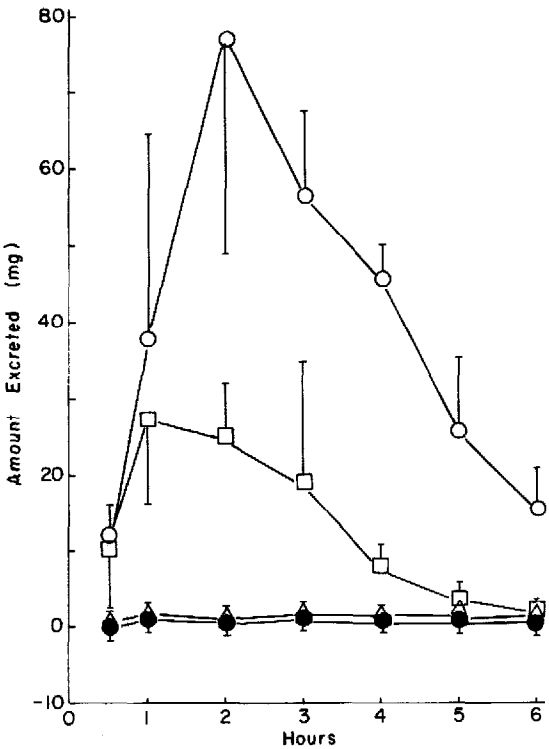

Fig. 10. Urinary excretion versus time curves for p-aminobenzoic acid and metabolites (n = 5). See Fig. 3 for symbols and Table I for conditions.

Selectivity. Good selectivity of the method under test conditions was indicated by the fact that no interfering peaks were observed in pooled blank urine (Fig. 9). The y intercept of curves constructed from spiked urine was 0.039 ± 0.13 for PABA, −0.66 ± 0.32 for PAHA, 1.78 ± 0.98 for PAAHA and −0.059 ± 0.50 for PAAHA (n = 6 in each case) also indicating no significant interferences. Blank urine from non-fasting subjects who were also receiving medication, however, did demonstrate several interfering peaks which suggests that test conditions must be rigorously controlled for good selectivity.

Patient samples

Bentiromide (500 mg) was administered to five normal volunteers and the amount of each metabolite excreted in urine versus time is presented in Fig. 10. While the concentration profiles of PAABA and PAHA are too low to be useful in the normal subjects tested, it is possible that a study of hepatic or small bowel diseased patients may produce higher levels. The profiles of PAAHA and PAABA demonstrate concentration maxima at 3 and 2 h, respectively and potential differentiation of liver and small bowel versus pancreatic disease in a single sample would probably be best achieved over a collection interval of 0–3 h, since any metabolic lag time could best be detected prior to the concentration maximum, and levels would be high enough for easy quantitation. Also, a peak which interfered with PAABA was observed in samples collected after 4 h. This was confirmed as PAABA glucuronide by treatment of the sample with β-glucuronidase which eliminated the interfering peak and caused an increase in PAABA peak height.

CONCLUSIONS

An accurate and reproducible method is described for the measurement of PABA and its metabolites in human urine from patients undergoing the bentiromide test for pancreatic function. This method was applied to a study in normal subjects to determine the appropriate sampling interval for metabolite differentiation. This work will permit clinical studies involving small bowel, hepatic and pancreas diseased patients to determine whether differential diagnosis can be made on the basis of metabolite concentration patterns. The large standard error found in normal subjects reflects individual variation. The methodology described may also be applicable to pharmacokinetic studies designed to reveal a better understanding of the mechanism of PABA metabolism.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from Adria Labs., Columbus, OH, U.S.A. We would also like to thank Dr. Phillip P. Toskes and Dr. Don Campbell for helpful discussions.

REFERENCES

1 C. Arvanitakis and N. Greenberger, Lancet, ii (1976) 663.

2 C. Lang, K. Gyr, G.A. Stalder and D. Gillessen, Brit. J. Surg., 68 (1981) 771.
3 P.P. Toskes, Gastroenterology, 85 (1983) 565.
4 H.W. Smith, N. Finkelstein, L. Aliminosa, B. Crawford and M. Graber, J. Clin. Invest., 24 (1945) 388.
5 C. Yamato and K. Kinoshita, Anal. Biochem., 98 (1979) 13.
6 S. Ito, K. Maruta, Y. Imai, T. Kato, M. Ito, S. Nakajima, K. Fujita and T. Kurahashi, Clin. Chem., 28 (1982) 323.
7 P.P. Toskes, Pharmacother., 4 (1984) 74.
8 M. Kitamura, M. Nakao and L. Yangisawa, J. Biochem., 47 (1960) 60.
9 C. Yamato and K. Kinoshita, J. Pharmacol. Exp. Ther., 206 (1978) 468.
10 A.I. Vogel, Practical Organic Chemistry, Wiley and Sons, New York, 1956.
11 R.W. Taft, D. Gurka, L. Joris, P. von Schleyer and J.W. Rakshys, J. Amer. Chem. Soc., 9 (1969) 4801.
12 C.R. Snyder, J. Chromatogr., 92 (1974) 223.
13 L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley-Interscience, New York, 2nd ed., 1979.
14 L.R. Snyder, J. Chromatogr. Sci., 10 (1972) 200.
15 P.J. Twitchett and A.C. Moffat, J. Chromatogr., 111 (1975) 149.
16 D.W. Newton and R.B. Kluza, Drug. Intel. Clin. Pharm., 12 (1978) 546.
17 C.M. Riley, E. Tomlinson and T.M. Jefferies, J. Chromatogr., 185 (1979) 197.