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AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF ANTIPYRINE AND ITS METABOLITES IN URINE

SOME PRELIMINARY RESULTS OBTAINED FROM SMOKERS AND NON-SMOKERS

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SUMMARY

A simple, accurate and fully automated high-performance liquid-chromatographic method was developed for the simultaneous determination of antipyrine (AP), 3-hydroxymethylantipyrine (3HMA), 4-hydroxyantipyrine (4OHA) and norantipyrine (NORA) in urine. This method requires no extraction step and only one chromatographic run with the use of a reversed-phase system. The coefficient of variation (%) (n=8 each) was: 4.14 for AP, 2.31 for 3HMA, 3.48 for 4OHA, and 2.71 for NORA. The method was applied to studies on AP metabolism in three smokers and three non-smokers who received an oral 10 mg/kg dose of AP. These preliminary results suggest that smokers appear to excrete more 4OHA and NORA in the urine than non-smokers.

INTRODUCTION

Antipyrine (AP) plasma or saliva half-life ($t_{1/2}$), or the derived metabolic clearance rates, are widely used to assess activities of the hepatic monooxygenase system in man [1,2]. The elimination rate or clearance of AP alone, however, does not seem to be an absolute indicator of an individual's capacity

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to metabolize drugs. For example, the overall metabolic clearance rate of AP and the clearance of many other drugs that are known to be mainly oxidized are poorly correlated [3]. Several investigators have demonstrated the absence of correlation between the microsomal cytochrome *P*-450 content and the metabolic clearance rate of AP in animals [4,5] and in humans [6—8].

A possible explanation for the observed lack of correlation between the rates of drug metabolism in vivo may be related to the heterogeneity of multiple forms of cytochrome *P*-450 [9], where each metabolic pathway of AP may be involved differently, since the AP metabolism is rather complicated. The primary metabolites of AP are 4-hydroxyantipyrine (4OHA), 3-hydroxymethylantipyrine (3HMA), and norantipyrine (NORA or N-desmethylantipyrine) [10—18]. Previous investigators have documented that a deficiency or absence of one biotransformation in a multimetabolized drug may be overlooked if one measures only the disappearance of the parent drug but not the appearance of its metabolites [19,20]. Hence, to assess AP as a hepatic probe for measuring the oxidative process of drug metabolism in man, investigation of the urinary excretion of the primary AP metabolites should be very important.

Until recently, studies on AP metabolism have been hampered by the lack of convenient methods for determining the different metabolites. The published gas—liquid chromatographic (GLC) methods have precluded the simultaneous determination of AP, 4OHA, 3HMA and NORA [11,17]. The use of high-performance liquid chromatography (HPLC), however, offers a useful tool to overcome difficulties involved in the GLC methods and avoids derivatization of the metabolites [14,15,18]. Among the HPLC methods recently reported, the method of Danhof and Breimer [16] requires two extraction steps and two separate chromatographic runs for the determination of AP, 4OHA, 3HMA and NORA, while that of Eichelbaum et al. [18] involves only one extraction step and one chromatographic run.

We report here on a fully automated HPLC system for the determination of AP and its three major metabolites in human urine which involves no extraction step and only one chromatographic run. This system permits the simple, rapid, sensitive, and quantitative determination of AP, 4OHA, 3HMA, and NORA in the urine. This method was also applied to test the urinary excretion of these metabolites in each of three healthy smoking and non-smoking subjects. Our method can be used as a clinical routine to examine hepatic drug metabolism in man.

EXPERIMENTAL

Chemicals

AP and NORA were obtained from Aldrich (Milwaukee, WI, U.S.A.). 3HMA and 4OHA were synthesized according to the methods described by Yoshimura et al. [21], and Knorr and Pshorr [22], respectively. The structure of the compounds synthesized was confirmed by mass spectrometry (JMS D-300, JEOL, Tokyo, Japan). Glusulase was obtained from Endo Labs. (Garden City, NY, U.S.A.). β-Glucuronidase from bovine liver type B-1, sulfatase from limpet type V, and saccharo-1,4-lactone were purchased from Sigma (St. Louis, MO, U.S.A.). All solvents and chemicals used were of analytical grade and were purchased from Wako (Osaka, Japan).

Apparatus

The automated high-performance liquid chromatograph was constructed with commercially available and laboratory-made components. A solvent-delivery system (M-45 pump) and an automatic sampler (WISP-710B) from Waters Assoc. (Milford, MA, U.S.A.) were used. A variable-wavelength UV detector, UVIDEC-100-III from JASCO (Tokyo, Japan), was operated at 252 nm. The chromatographic data were processed by an HP-3388 reporting integrator from Hewlett-Packard (Avondale, PA, U.S.A.). The interface between the WISP-710B sampler and HP-3388 integrator was newly contrived. To maintain the sample temperature at 37°C during enzymatic hydrolysis or at 5°C for the actual run, a thermostatic bath unit was built in the WISP-710B sampler. The available temperature ranged from −5°C to +50°C within ±1°C. The flow diagram of the total system developed is shown in Fig. 1.

Chromatographic conditions

For rapid and simple HPLC determination of a drug in biological specimens without the tedious clean-up procedure, it is considered advantageous to use a reversed-phase system. We intended to separate AP, 4OHA, 3HMA, and NORA in a single reversed-phase HPLC run. Although we tested and compared several reversed-phase columns (μBondapak C_{18}, Waters; Hibar RP-2 and RP-8, Merck, Darmstadt, G.F.R.; and Radial-Pak C_8 and C_{18}, Waters), only Radial-Pak C_8 (10 μm, 5 mm I.D.) was found to achieve this goal. The mobile phase was methanol—150 m*M* ammonium acetate solution (33.5:66.5) and the flow-rate was set at 1.5 ml/min (ca. 70 kg/cm^2). The concentration of the acetate buffer of the eluent was found to be critical for the separation of AP and 4OHA. The quantitation of each compound was based on the external area method using the HP-3388 integrator (Fig. 1).

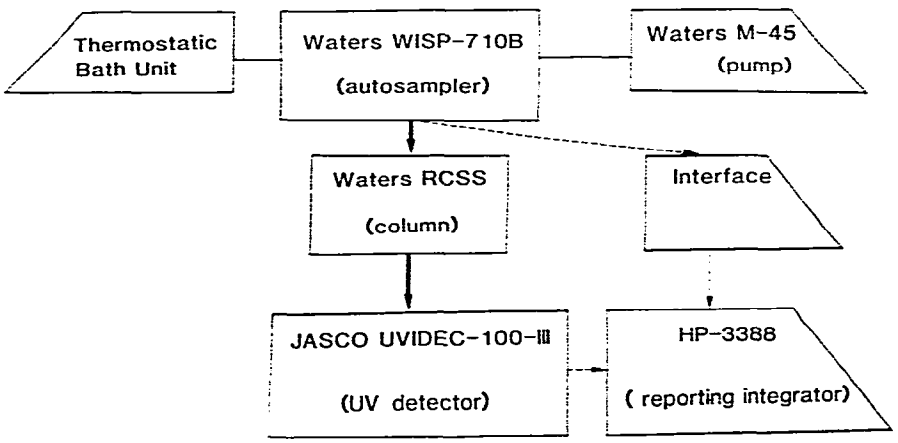

Fig. 1. Flow diagram of automated HPLC system. Thick arrows indicate the sample flow system; broken arrows indicate the operational sequence undergoing the signal.

Sample preparation and calibration curves

To 1.0 ml of urine sample, 1.0 ml of 1.0 *M* acetate buffer (pH 5.2) and 2.0 ml of *n*-hexane were added successively. The solution was mixed for 30 sec with a vortex-type mixer. After centrifuging at 1400 *g* for 5 min, the organic phase was discarded. A few hundred (200—400) microlitres of the aqueous phase were transferred to a micro-sample tube for the analysis of AP and 3 HMA excreted in urine as the free form. To 1.0 ml of the aqueous phase in a sample bottle, 0.5 ml of 10% sodium metabisulfite ($Na_2S_2O_5$) and 1.0 ml of Glusulase solution containing 10,000 Fishman Units (FU) per millilitre of β-glucuronidase were added. The solution was mixed for a few seconds and incubated at 37°C for 2 h in the sampler. Then the temperature of the bath was cooled down to 5°C for the analysis of total 3HMA, and 4OHA- and NORA-glucuronide. The injection volume of each sample was set at 10 μl.

To determine the amount of AP and its three major metabolites excreted in the urine, absolute calibration curves were prepared as follows. The urine was adjusted to pH 5.2 by the same volume of 1.0 *M* acetate buffer and was washed with *n*-hexane. Known amounts of AP and 3HMA, 4OHA and NORA were added and these standard urine solutions were then analyzed according to the procedures described above. The linear calibration curves were obtained by the least-squares method and tested for coefficient correlations.

RESULTS AND DISCUSSION

Sample clean-up procedure

No interfering peaks were eluted under the chromatographic conditions after a 10-μl injection of urine without *n*-hexane treatment. The enzymatic hydrolysis did not affect the separation. However, the life span of the column was found to be prolonged and the analysis time was shortened to 12 min by the pre-treatment. The non-polar endogenous substances were found to be removed effectively by the *n*-hexane washing. The loss by employing this step was negligibly small for AP. The recovery was 100.7 ± 1.2% (14.1 μg/ml of urine, $n=5$). Even for 3HMA excreted as the free form, the recovery was 96.8 ± 0.3% (18.5 μg/ml of urine, $n=5$).

Fig. 2 shows typical chromatograms of urine treated with and without enzymatic hydrolysis by Glusulase after an oral administration of 10 mg/kg AP to a volunteer. The Radial-Pak C_8 column with a radial compression separation system (RCSS) seemed to be suitable for human experiments; the theoretical plate was rather high (Fig. 2), the life span was about 500 runs of hexane-treated samples, and the operating pressure was constantly low.

Enzymatic hydrolysis

The time courses of the enzymatic hydrolysis are shown in Fig. 3A, B and C. The stability of AP metabolites under the enzymatic hydrolysis used was examined. First, in comparison of the two sources of β-glucuronidase, Glusulase and type B-1 β-glucuronidase, the former (Fig. 3B) showed a higher yield and faster equilibrium than the latter (Fig. 3A). Second, in the case of 3HMA and 4OHA conjugates, 1 m*M* saccharo-1,4-lactone perfectly inhibited the enzyme, so that these two metabolites were identified as the corresponding

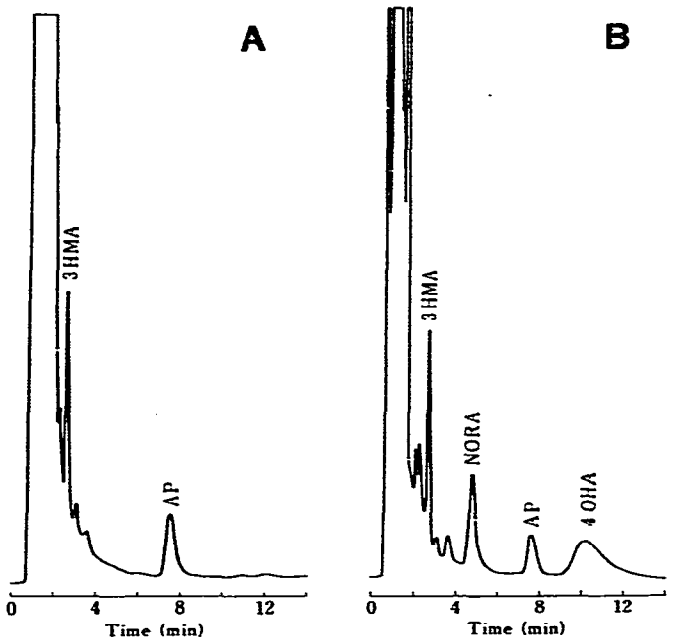

Fig. 2. Chromatograms of urine sample treated (A) without and (B) with enzymatic hydrolysis by Glusulase. The chromatograms indicate peaks corresponding to antipyrine and its metabolites. The drug concentrations are (μg/ml of urine): AP, 15.84; 3HMA free, 20.54; 3HMA total, 40.18; 4OHA, 94.4; NORA, 53.13.

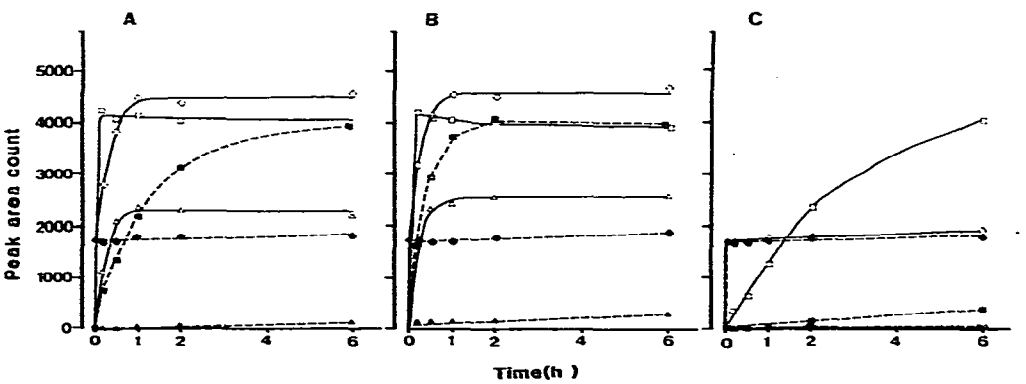

Fig. 3. Time courses of enzymatic hydrolysis under treatment conditions of (A) β-glucuronidase (20,000 FU/ml of urine), (B) Glusulase (20,000 FU β-glucuronidase and 2500 U sulfatase per ml of urine), and (C) sulfatase (100 FU β-glucuronidase and 1000 U sulfatase per ml of urine). ◇, 3HMA, ◆, 3HMA + saccharo-1,4-lactone 1 m*M*, △, 4OHA; ▲, 4OHA + saccharo-1,4-lactone 1 m*M*; □, NORA; ■, NORA + saccharo-1,4-lactone 1 m*M*. $Na_2S_2O_5$, 100 mg/ml of urine, was added to each treatment as an antioxidant.

β-glucuronide form. The behaviour of the NORA conjugate against saccharo-1,4-lactone inhibition was quite different from that of 3HMA- and 4OHA-glucuronide; the rate of hydrolysis was very fast, and this reaction was not inhibited by the generally used amount of saccharo-1,4-lactone (1 m*M*). However, the hydrolysis by sulfatase which contained 100 FU of

β-glucuronidase produced only a trace amount of free NORA, if any, as shown in Fig. 3C. These results indicate that the compounds to be quantitated in human urine include AP, 3HMA-free, 3HMA-glucuronide, 4OHA-glucuronide, and NORA-glucuronide. These findings are in good agreement with those of Inaba and Fischer [17], and Eichelbaum et al. [18], even though the former workers did not measure 3HMA.

We took into account other parameters to be optimized in the quantitative enzymatic hydrolysis, i.e. pH, incubation time, and amount of antioxidant as follows. (1) The favorable pH for hydrolysis with Glusulase ranged from 4.0 to 5.2 for a 3-h incubation with antioxidant ($Na_2S_2O_5$), and under this condition the yield of 4OHA, 3HMA, and NORA was found to be constant. However, since white precipitates were produced below pH 4.6, pH 5.2 was considered optimum. (2) The amounts of Glusulase and antioxidant, and the incubation time correlated to each other during the hydrolysis, so that each variable had to be approximately adjusted. To optimize these variables, the amounts of Glusulase were changed from 2500 to 20,000 FU of β-glucuronidase per ml of urine, and those of $Na_2S_2O_5$ from 0 to 100 mg/ml of urine for 0- to 4-h incubation. Fig. 4A shows the effect of the amount of antioxidant on the stability of NORA, the most unstable metabolite of AP [18], with 20,000 FU of β-glucuronidase per ml of urine. The optimum amount of $Na_2S_2O_5$ was found to be 100 mg/ml of urine, whereas 1 mg $Na_2S_2O_5$ was sufficient to prevent decomposition for 4OHA. (3) The effect of Glusulase potency on the hydrolysis of 3HMA- and 4OHA-glucuronide at 100 mg of $Na_2S_2O_5$ per ml of urine is illustrated in Fig. 4B. The hydrolysis of NORA-glucuronide did not depend upon the potency of Glusulase, and it reached a maximum immediately

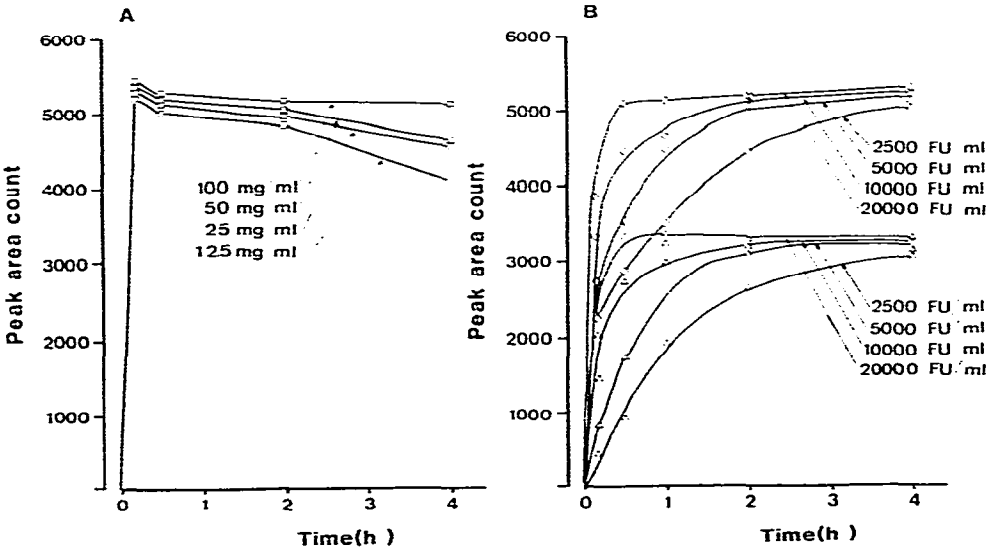

Fig. 4. Effect of (A) $Na_2S_2O_5$ amount on the stability of NORA during enzymatic hydrolysis with Glusulase, 20,000 FU/ml of urine, and (B) Glusulase potency on the hydrolysis of 3HMA-glucuronide (◇) and 4OHA-glucuronide (△) with 100 mg/ml $Na_2S_2O_5$. The amounts of $Na_2S_2O_5$ (A) and Glusulase (B) per millilitre of urine are indicated in the figure.

after the incubation, which is consistent with the finding of Inaba and Fischer [17]. The hydrolysis rates of 3HMA- and 4OHA-glucuronide were time-dependent at each amount of enzyme. At 20,000 FU/ml of urine, these two conjugates gave maximum yields after a 2-h incubation. Thus, the optimum conditions required for the stability of NORA were: 20,000 FU β-glucuronidase plus 100 mg of $Na_2S_2O_5$ per ml of urine plus 2-h incubation at 37°C.

Reproducibility, linearity, and efficiency

This assay differed from other studies, the use of an internal standard was unnecessary since there was no complicated solvent extraction, nor were there evaporation and reconstitution processes. We applied an external standard method by means of a simple clean-up procedure with high recovery and an automatic sampler with good precision and reproducibility on the injection procedures.

The data for reproducibility and linearity obtained from this method are set out in Table I. The results for AP and its three metabolites all gave satisfactory values for coefficients of variation and correlation coefficients. Only 1.0 ml of urine from human subjects, who were administered orally 10 mg/kg AP, was required for the determination of all the compounds, including free and conjugated forms.

TABLE I

REPRODUCIBILITY AND LINEARITY OF THE ANALYSIS OF ANTIPYRINE AND METABOLITES IN URINE

Substance*	Concentration (μg/ml)	Coefficient of variation** (%)	Concentration ranges examined (μg/ml)	Correlation coefficient (r)
AP	10.19	3.95	2.55—40.76	0.9987
	20.38	4.14		
3HMA	25.84	2.02	6.46—103.36	0.9994
	51.68	2.31		
4OHA	50.43	4.33	12.61—201.72	0.9972
	100.86	3.48		
NORA	50.55	3.06	12.64—202.20	0.9993
	101.10	2.71		

*AP = Antipyrine; 3HMA = 3-hydroxymethylantipyrine; 4OHA = 4-hydroxyantipyrine; NORA = norantipyrine.

**Calculated from eight replicates for each substance.

By using our HPLC method, the analysis time required for AP and its metabolites was 12 min, which is fairly compatible with other HPLC methods reported [14,15,18]. Furthermore, in order to evaluate the efficiency of our automated HPLC method, the number of samples that could be analyzed per day was also tested. It was found that a total of 43 measurements for urine specimens (≈ maximum efficiency), which included the parent drug and its

TABLE II

SUBJECT CHARACTERISTICS AND PHARMACOKINETIC PARAMETERS OF ANTIPYRINE IN PLASMA

Subjects	Age (years)	Sex	Body weight (kg)	Average cigarette consumption (No. per day)	Kinetic parameters of antipyrine*		
					$t_{1/2}$ (h)	V_d (l/kg)	MCR (ml per h per kg)
1. T.I.	39	M	65	30—40	11.5	0.622	37.4
2. T.S.	27	M	62	20--30	12.0	0.632	36.6
3. K.S.	23	M	60	20—30	11.7	0.681	40.4
Mean ± S.E.M.	29.7 ± 4.8		62.3 ± 1.5	—	11.7 ± 0.2	0.645 ± 0.022	38.1 ± 1.4
4. T.F.	26	F	54	0	14.7	0.608	28.7
5. T.E.	25	M	56	0	15.2	0.602	27.5
6. S.K.	34	M	78	0	14.9	0.539	25.2
Mean ± S.E.M.	28.3 ± 2.8		62.7 ± 7.7	—	14.9 ± 0.2	0.583 ± 0.027	27.1 ± 1.1

*Antipyrine half-life ($t_{1/2}$) was determined from the linear portion of the plot of plasma concentration versus time on semilog paper. The apparent volume of distribution (V_d) was calculated from the dose of the drug administered divided by the extrapolated y-intercept for drug concentration in plasma at time zero. The metabolic clearance rate (MCR) was calculated as $MCR = 0.693 \cdot V_d / t_{1/2}$.

metabolites in free form and as conjugates, were successfully accomplished with the present method.

Human study

The characteristics of six subjects (three smokers and three non-smokers), who received an oral dose of 10 mg/kg AP with 200 ml of orange juice after an overnight fast, and AP disposition data calculated from a one-compartment model analysis are summarized in Table II. A smoker was defined as a person taking more than 20 cigarettes per day for a year or more. Blood samples (3—5 ml) were collected at time 0, 2, 4, 6, 8, 12 and 24 h. AP in plasma had been measured with the GLC method previously reported [23]. A shorter $t_{1/2}$ or higher metabolic clearance rate in smokers than in non-smokers is compatible with the findings observed previously [24,25].

The automated HPLC method was applied to obtain a preliminary result for ascertaining if our method could be used for human study, and if the enhanced metabolic rate of AP in plasma in relation to the smoking habit may be reflected in the primary metabolites excreted in their urine. Urine samples were collected at the following time intervals: 0—4, 4—8, 8—12, 12—24 and 24—48 h. Urine was frozen at −20°C until analysis.

Shown in Fig. 5 are the mean data of the plasma concentration—time curves of AP and of the urinary excretion rates of AP and metabolites obtained from the three smokers and three non-smokers. Individual data of the amounts (percentage dose of AP) of AP, 3HMA-free, 3HMA-glucuronide, 3HMA-total, 4OHA-glucuronide, and NORA-glucuronide excreted in 0—48 h urine are given in Table II. Smokers tended to excrete higher amounts of 4OHA- and NORA-

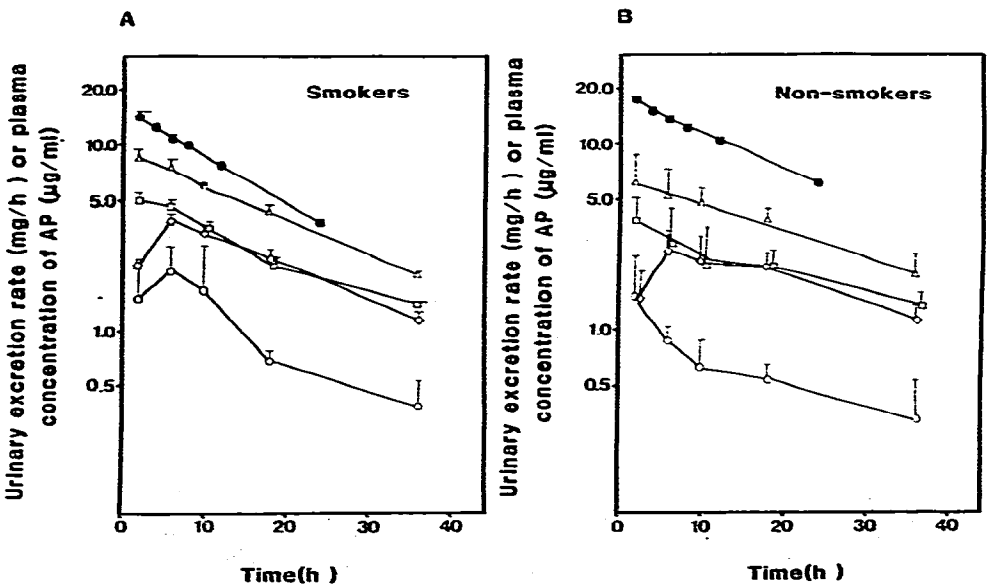

Fig. 5. Plasma concentration—time curves of antipyrine (●) and urinary excretion rates of antipyrine (○), 3HMA (◇), 4OHA (△) and NORA (□) in (A) smokers and (B) non-smokers. The mean ± S.E.M. values obtained from three smokers and three non-smokers are plotted.

TABLE III

CUMULATIVE URINARY EXCRETION OF ANTIPYRINE AND METABOLITES IN 48 h AS PERCENTAGE OF THE DOSE

Subjects*	AP, Free	3HMA			4OHA, Glucuronide	NORA, Glucuronide	Total
		Free	Glucuronide	Total			
Smokers							
1. T.I.	8.55	7.26	7.41	14.67	30.51	17.78	71.51
2. T.S.	6.16	5.90	8.94	14.84	31.04	19.20	71.24
3. K.S.	3.34	6.07	9.25	15.32	28.53	17.20	64.39
Mean ±	6.02	6.41	8.53	14.94	30.03	18.06	69.05
± S.E.M.	±1.84	±0.52	±0.70	±0.24	±0.94	±0.73	±2.85
Non-smokers							
4. T.F.	5.00	5.06	5.62	10.68	18.73	15.97	50.38
5. T.E.	2.75	4.60	8.57	13.17	26.61	11.21	53.74
6. S.K.	4.38	4.19	8.75	12.94	27.63	17.37	62.32
Mean	4.04	4.62	7.65	12.26	24.32	14.85	55.48
± S.E.M.	±0.82	±0.31	±1.24	±0.97	±3.44	±2.28	±4.35

*Subject number is the same as in Table II.

glucuronide. For AP and 3HMA, the differences between smokers and non-smokers appeared to be small. The total amount excreted in urine as a percentage of the AP dose administered tended to be greater in smokers (mean ca. 69%) than in non-smokers (ca. 55%), and the remaining amount (ca. 31—45%) of the administered dose was not detected with the assay method we employed (Table III). Although no exact explanation be offered for these phenomena, we are tempted to assume that AP may likely be metabolized via other pathway(s), as yet uncharacterized or unidentified. Eichelbaum et al. [18] found that on average 67—73% of the dose can be accounted for as AP and its three metabolites plus another minor metabolite, 3-carboxyantipyrine (4—5%). Excluding the latter, our figure of percentage dose seems to be in reasonable agreement with the finding of Eichelbaum et al. [18]. However, the trend towards the observation that AP also tends to be less in non-smokers than in smokers (Table III) remains totally unexplainable with the limited data of the present study.

The slopes of the excretion rates of 4OHA and NORA were fairly coincident with plasma AP disappearance in both smokers and non-smokers (Fig. 5). These preliminary results from human study suggest that the fundamental differences in the metabolic disposition of a drug such as AP, which is known to be oxidized by the hepatic microsomal system [3—6,8,9], exist in relation to the smoking habit, as has been indicated for other oxidized drugs [26—29]. Undoubtedly, whether our automated HPLC method will have a wide applicability to human study awaits further investigation using a large number of subjects with diverse clinical conditions where AP is used as a model marker to test the oxidative process of drug metabolism.

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