Simplified Determination of Antipyrine Clearance by Liquid Chromatography of a Microsample of Saliva or Plasma

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We describe a simplified and rapid liquid chromatographic determination of antipyrine clearance (CL_{AP}) calculated from peak height ratios of drug/internal standard. Saliva or plasma was collected 24 hr after the oral administration of 1 g of antipyrine to the subject. A 25-μl aliquot of the sample is deproteinized with acetonitrile containing 3-nitrophenol (internal standard) and injected into a radial compression module equipped with a 10-μm, 8 mm × 10-cm C_{18} cartridge, using a 0.025 *M* aqueous solution of sodium acetate and acetonitrile (88.5:11.5). The minimum measurable concentration was 0.2 μg/ml. The obtained CL_{AP} values in five healthy subjects and five patients with chronic liver disease coincided well ($r > 0.9994$) with those generated by the use of an established method. The antipyrine clearance in the healthy subjects ranged from 2.203 to 5.721 liters/hr, while in patients with chronic liver disease it was significantly ($P < 0.0027$) less (range, 0.544 to 1.103 liter/hr). We also determined antipyrine clearance in two of these subjects given lower doses of this drug and found that the dose has no significant impact on this parameter.

KEY WORDS: antipyrine; drug metabolism; metabolic liver function; hepatitis; P-450 activities; liquid chromatography.

INTRODUCTION

The microsomal enzyme activity of the liver is normally examined by measuring the rate of biotransformation of a model drug that is exclusively metabolized by the hepatic P-450 isoenzymes. This activity is genetically determined and is modified by environmental factors including diet (e.g., charcoal-grilled meat), smoking (due to polycyclic hydrocarbons), drugs (barbiturates, phenytoin, glucocortoids, cimetidine, disulfarm, etc.), alcohol consumption, and occupational exposure to certain chemicals such as aromatic hydrocarbons (1). Within a certain population, a polymorphic distribution of the metabolic, oxidative activities can be found. Ideally, the model compound used to probe for these activities should be administered orally and should be rapidly and completely absorbed. Further, it should have no side effects at the dose used and should not induce or inhibit its own metabolism.

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In addition to meeting all but the last criterion indicated above, antipyrine is secreted in saliva with a plasma/saliva concentration ratio close to unity (1). It is negligibly bound to plasma protein, and its volume of distribution is equal to that of the total-body water (1), so that antipyrine clearance can be accurately estimated with a single saliva sample in humans (2,3) and in rats (4). This method is commonly used in lieu of the traditional approach for measurement of antipyrine clearance which involves the collection of multiple plasma or saliva samples within 12–24 hr after the administration of the dose.

Antipyrine has been analyzed in biological fluids by spectrophotometry (5), radioimmunoassay (6), gas–liquid chromatography (7–10), and high-performance liquid chromatography (11–18). While the nonchromatographic assays suffer from lack of specificity or sensitivity, most of the chromatographic methods involve multiple or single extraction or derivatization steps and require plasma aliquots which can be obtained only by venipuncture (0.1–1 ml). Also, the retention capacity with the previously reported HPLC methods for antipyrine is relatively small ($k' = 0.4$ to 1.6), which renders them prone to interference from other drugs.

In this report, we describe a simplified and rapid liquid chromatographic method for the determination of antipyrine clearance by the use of a plasma or saliva microsample collected 24 hr after dosing.

MATERIALS AND METHODS

Materials. Pharmaceutical-grade antipyrine was recrystallized twice from benzene before use. An analytical sample of 3-nitrophenol from Fisher Scientic Co., Pittsburph, PA (internal standard) was used as received. Acetonitrile and sodium acetate (Fisher Scientific Co., Pittsburgh, Pennsylvania) were either HPLC or analytical reagent grade. The water employed was generated by passing "reverse-osmosis" water through a trace-organic removal cartridge and 0.45-μm membrane filter (Millipore Co., Milford, MA).

Instrument. We used a liquid chromatograph (Waters Associates, Milford, MA) consisting of a solvent delivery pump (Model 501), a manual Rheodyne injector, a Guard-PAK precolumn module with a C_{18} insert, a radial compression module (Model Z) equipped with a 10-μm 8 mm × 10-cm C_{18} cartridge, a variable-wavelength UV/visible detector (Model 481), and a data module (Maxima 820) with a printer. We detected the compounds in the effluent spectrophotometrically at 270 nm.

Mobile Phase. The mobile phase consisted of a mixture of a 0.025 *M* aqueous solution of sodium acetate and acetonitrile (88.5:11.5), filtered and degassed before use. The flow rate was 6 ml/min at a pressure ≤8.26 MPa (1200 psi).

Deproteinization of Plasma or Saliva Sample. The saliva or plasma sample (25 to 100 μl) was deproteinized by adding an equal volume of acetonitrile containing 10 μg/ml of internal standard and vortex-mixing for 15 sec. After centrifugation for 10 min at 2400 rpm, 50 μl of the supernatant is injected.

Linearity. To examine the linearity of the described analytical method, we constructed calibration curves by sup-

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plementing blank saliva or plasma with different amounts of antipyrine to yield the following concentrations: 0.2, 0.4, 1, 2, 4, 7, 10, and 20 μg/ml. These samples were deproteinized prior to injection by adding equal volumes of acetonitrile containing 10 μg/ml of internal standard as described above.

Also, we investigated the reliability of using a single standard sample to determine the antipyrine clearance by comparing the concentrations of antipyrine in the "unknown" samples obtained with this approach to those generated by the use of a calibration curve as described above.

Determination of Antipyrine Clearance

Administration of Antipyrine and Specimen Collection. To each of five healthy subjects and five patients with chronic liver disease, an oral dose of 1 g of antipyrine diluted in 50–100 ml of orange juice was given at least 3 hr after ingestation of the last meal. A saliva sample (0.05 to 0.5 ml) was collected 24 hr after dosing from each subject while chewing parafilm to stimulate saliva secretion, and stored frozen at −80°C until analysis. To examine the reproducibility of this method, one healthy subject and one patient were given, after a week washout interval, an additional dose of antipyrine under the same conditions described above.

Estimation of Antipyrine Clearance. We calculated antipyrine clearance (CL_{AP}) according to the following equation:

$$CL_{AP} = \frac{\ln(D/V_d) - \ln(PHR_t/PHR_s) - 2}{t} \times V_d \quad (1)$$

where D is the dose, V_d is the volume of distribution as estimated from Eq. (2) or (3), PHR_t is the peak height ratio (drug/internal standard) obtained for the saliva sample collected at time = t, and PHR_s is the peak height ratio obtained for a standard sample containing 7.389 μg/ml of antipyrine and the same amount of internal standard as in the unknown sample. This equation can be derived by substituting for the concentration of antipyrine in saliva at time = t in the equation presented by Dossing *et al.* (2).

$$V_d = 0.3625 \times BW + 0.2239 \times BH - 0.1387 \times Age - 14.17 \quad \text{(for men)} \quad (2)$$

and

$$V_d = 0.2363 \times BW + 0.1962 \times BH - 0.0272 \times Age - 10.26 \quad \text{(for women)} \quad (3)$$

where BW is body weight (kg) and BH is body height (cm) and age is in years.

Antipyrine Clearance by the Use of a Smaller Dose. To examine the feasibility of using a smaller dose to estimate antipyrine clearance, two healthy subjects received, at a weekly interval, two or three additional doses equivalent to 0.5, 0.25, and 0.125 g of antipyrine, and a saliva sample was collected from the subject following each dose and processed as described above.

RESULTS AND DISCUSSION

Analytical Procedure

Representative chromatograms for blank saliva, saliva supplemented with antipyrine and 3-nitrophenol (internal standard), and saliva sample collected from a healthy subject 24 hr after receiving an oral dose of 1 g of this drug are depicted in Fig. 1. Similarly, Fig. 2 shows chromatograms of blank plasma and plasma to which 0.2 μg/ml antipyrine (the minimum concentration measured) and internal standard were added. Both compounds exhibited, under the chromatographic conditions used, sharp peaks with mean retention times of 6.14 min for antipyrine and 10.96 min for the internal standard. While a rapid elution for antipyrine was maintained, the capacity factor (k') exceeded 8.6, which is 5- to 21-fold greater than any of the previously reported values for this agent. Undoubtedly, this makes the assay less susceptible to interferences from other drugs which may concomitantly be taken by the patient. Indeed, none of such drugs listed in Table I interfered. Additionally, despite the lack of extraction procedure from the assay, we observed no interference from any endogenous substance, and the recovery of antipyrine from plasma or saliva after deproteinization with acetonitrile was complete.

The standard curves of the peak height ratio (drug/internal standard) versus concentration of antipyrine in both plasma and saliva were highly linear, with the correlation coefficient (r) ranging from 0.9962 to 0.9998 (mean = 0.999; n = 10) for saliva and from 0.995 to 0.9998 (mean = 0.9985; n = 5) for plasma.

We determined the analytical recovery of antipyrine by analyzing 10 samples of saliva at each of the following concentrations: 0.25, 2, 10, and 15 μg/ml. The analytical recovery estimated as 100 × amount found/amount added at these low, medium, above-normal, and high concentrations was 111, 99.4, 100.5, and 96.0%, respectively, and the coefficient of variation (CV) did not exceed 4.73% (Table II). The between-day precision for antipyrine concentrations (Table III)

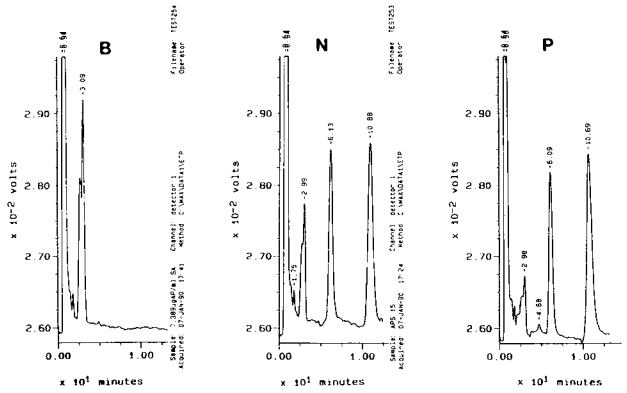

Fig. 1. Representative chromatograms of (B) a blank saliva, (N) a blank saliva to which 7.389 μg/ml of antipyrine and 10 μg/ml of 3-nitrophenol (internal standard) were added, and (P) a healthy subject's saliva sample collected 24 hr after oral administration of 1 g of antipyrine and to which 10 μg/ml of internal standard was added. A 50-μl diluted (1:1) sample was injected. Retention times: 6.09–6.13 min (antipyrine) and 10.69–10.88 min (internal standard). 1.0 V = 1.000 AU.

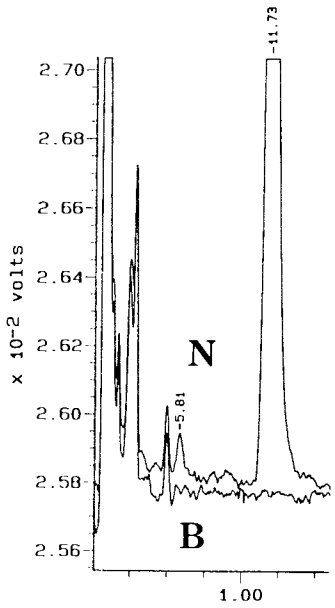

Fig. 2. Representative chromatograms of (B) a blank plasma and (N) a blank plasma to which 0.2 μg/ml of antipyrine and 10 μg/ml of internal standard were added. A 50-μl diluted (1:1) sample was injected. Retention times: 5.81 min (antipyrine) and 11.73 min (internal standard). 1.0 V = 1.000 AU.

was also good; the CV values ranged from 2.43 to 8.82% in saliva and from 2.27 to 9.51% in plasma.

Despite the small sample size (25 μl) used the method is sensitive, with a minimum measurable concentration of 0.2 μg/ml. If a signal-to-noise ratio of 3 is to be used as a criterion for sensivitity, smaller concentrations can be detected. While a few previously reported methods offer such sensitivity, none matches this small sample size limit.

Table I. Retention Times of Drugs Which May Be Concomitantly Administered with Antipyrine

Drug	Retention time (min)
Antipyrine	6.14
3-Nitrophenol	10.96
Bleomycin sulfate	ND[a]
Carboplatin	ND
Fluorouracil	ND
Carmustine	18.8
Cytidine	ND
Methotrexate	ND
Lomustine	ND
Cyclophosphamide	ND
Thiotepa	ND
Acetaminophen	1.5
Aspirin	ND
Cisplatin	ND
Adriamycin	ND
Vincristine sulfate	ND
Etoposide	ND
Prednisone	ND
Ranitidine	13.09
Cimetidine	ND

[a] Nondetectable during 20-min run.

Table II. Analytical Recovery and Within-Run Precision of the Described Assay for Antipyrine in Saliva

Amount added (μg)	Amount found (μg)	Coefficient of variation (%)	Analytical recovery (%)
0.25	0.277	4.73	111.0
2.0	2.11	1.99	99.4
10.0	10.05	3.32	100.5
15.0	14.4	3.33	96.0

Antipyrine Clearance

To validate the use of Eq. (1) to estimate antipyrine clearance, we compared the concentrations obtained with the use of a single standard sample to those obtained with a calibration curve. The values for each subject generated by both of these approaches were similar (Table IV) (no significant difference by a paired *t*-test statistical analysis). This result supports the use of Eq. (1) to estimate antipyrine clearance. Further, there was a strong correlation (r = 0.9994 and slope = 1.046) between the estimates generated according to Eq. (1) and those obtained by the established method of Dossing *et al.* (2), from which our simplified approach for estimation of antipyrine clearance was derived (Fig. 3). However, with the use of one standard sample, a minor bias (i.e., <5%) may occur, but its significance is offset by the expediency of our method. Further, the variation in estimates of antipyrine clearance obtained for the two subjects in whom the determination of antipyrine clearance was repeated the following week was relatively small [CV = 16.5% (healthy subject) and CV = 5.8% (patient with chronic liver disease)], indicating a good reproducibility.

The dose of antipyrine did not significantly alter the estimate of its clearance in the two healthy subjects. In fact, in the subject who received 1, 0.5, 0.25, and 0.125 g at weekly intervals, the values were 3.96, 3.89, 3.75, and 3.89 liters/hr, respectively, and in the subject who received the first three of the above doses; they were 2.36, 2.5, and 3.13 liters/hr. This result confirms the linearity of antipyrine pharmacokinetics and suggests that a smaller dose can be employed to determine the clearance of this agent using the method presented in this report, thereby avoiding any self-inducing metabolic effect of antipyrine observed at large doses when given at intervals shorter than one week.

Table III. Between-Day Precision of the Described Assay of Antipyrine in Saliva or Plasma

	Concentration (μg/ml)		
	0.4	2.0	7.0
Saliva			
Mean	0.415	1.95	7.0
SD	0.0366	0.11	0.17
CV (%)	8.82	5.63	2.43
Plasma			
Mean	0.406	1.92	7.1
SD	0.0386	0.057	0.161
CV (%)	9.51	2.97	2.27

Table IV. Effect of Quantitation Approach on the 24-hr Salivary Concentration of Antipyrine in Subjects who Received 1 g of this Agent

Subject No.	Diagnosis	Concentration (μg/ml)	
		Calibration curve	Single standard sample
1	H[a]	5.34	5.53
2	H	6.60	6.88
3	H	2.49	2.48
4	H	0.86	0.73
5	H	2.55	2.54
6	P[b]	15.05	15.6
7	P	13.05	13.76
8	P	12.04	12.48
9	P	14.86	15.46
10	P	15.98	16.64

[a] Healthy.
[b] Patient with chronic liver disease.

As demonstrated in Fig. 3, the antipyrine clearance in the healthy subjects ranged from 2.203 to 5.721 liters/hr (mean = 3.728; SD = 1.471), while in patients with chronic liver disease it was significantly ($P < 0.0027$) less (range = 0.544 to 1.103 liters/hr; mean = 0.87; SD = 0.242), presumably as a result of the hepatic deficit associated with this disease.

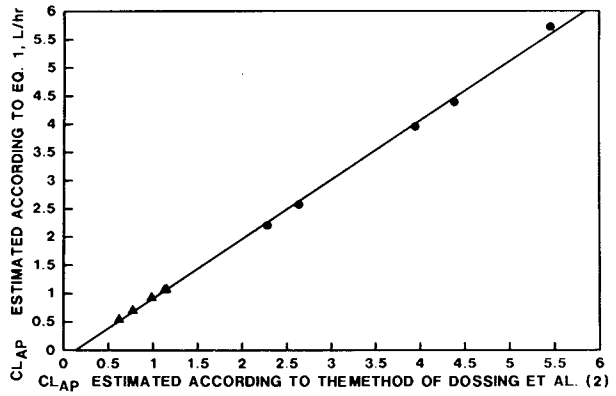

Fig. 3. Comparison between the antipyrine clearance values obtained for the subjects included in this study according to Eq. (1) and those generated according to the method previously described by Dossing *et al.* (2). Healthy (●); patient (▲).

In conclusion, the method presented here is a simplified procedure for the determination of antipyrine clearance from a single saliva or plasma sample. The excellent linearity of the assay has made it possible to use the peak height ratio in lieu of the concentration, which eliminates the need of a calibration curve. This coupled with the rapidity, sensitivity, and small sample size used in the liquid chromatographic analysis of antipyrine makes this method highly advantageous for testing the drug metabolizing capacity of the liver.

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