Biochemical Pharmacology, Vol. 23, pp. 197–201. Pergamon Press, 1974. Printed in Great Britain.

CORRELATION OF THE PLASMA ELIMINATION OF ANTIPYRINE AND THE APPEARANCE OF 4-HYDROXY ANTIPYRINE IN THE URINE OF MAN*

DAVID H. HUFFMAN,† DON W. SHOEMAN and DANIEL L. AZARNOFF

Clinical Pharmacology-Toxicology Center, Departments of Medicine and Pharmacology, University of Kansas Medical Center, Kansas City, Kans., U.S.A.

(*Received* 12 *March* 1973; *accepted* 15 *June* 1973)

Abstract—A new gas chromatographic method for the determination of antipyrine and 4-hydroxy antipyrine in biological fluids is described. Utilizing these methods, we find a significant correlation ($r = 0{\cdot}89$, $P < 0{\cdot}001$) between the plasma elimination half-life of antipyrine and the rate of appearance of 4-hydroxy antipyrine conjugate in the urine. This observation validates previous studies utilizing the rate of plasma decay of antipyrine as a measure of drug metabolism in man.

ANTIPYRINE has several properties which make it suitable for the evaluation of drug metabolism in man. It is bound less than 10 per cent to plasma proteins at pharmacological plasma levels, distributed in total body water, and metabolized by the hepatic microsomal drug-oxidizing system. Many of the reported studies which evaluate drug metabolism in man are predicated on the plasma disappearance of antipyrine.[1–3] In these studies, it has been assumed that the plasma disappearance of antipyrine is a direct function of its rate of metabolism. Since the plasma elimination of a drug may reflect processes other than metabolism, we determined whether or not the rate of elimination of antipyrine from plasma correlated with the rate of appearance of 4-hydroxy antipyrine, a major metabolite, in the urine.

In our hands, the spectrophotometric method for antipyrine[4] has a high blank and poor reproducibility. These factors tend to limit the reliability of pharmacokinetic studies. Therefore, we have developed a gas chromatographic method for determining antipyrine and 4-hydroxy antipyrine in biological samples. This report will describe these methods as well as the pharmacokinetics of antipyrine in man utilizing them.

MATERIALS AND METHODS

Patient selection. Nine patients with a variety of illnesses as well as eight healthy volunteers participated in this study. Informed consent was obtained and the volunteers were given antipyrine, 10 mg/kg orally with 200 ml water, following an overnight fast. The healthy volunteers had taken no other drugs including alcohol for at least 48 hr prior to the study. Blood was collected at 0, 4, 6, 8, 10 and 25 hr in 10-ml tubes containing 143 units of Na heparin. The blood was centrifuged, the plasma separated and stored frozen (−18°) until assayed. After obtaining a baseline sample, urine was collected from 0–2, 2–4, 4–6, 6–8, 8–12, 12–24, 24–36 and 36–48 hr following administration of antipyrine.

* Supported by Grant GM 15956 from the United States Public Health Service.

† United States Public Health Service postdoctoral trainee in clinical pharmacology (GM 01342).

Chemicals. All chemicals and solvents were reagent grade with the exception of chloroform, which was nanograde quality. The antipyrine was dispensed in soft, gelatin capsules without added ingredients. 4-Hydroxy antipyrine was purchased from the Aldrich Chemical Co., Madison, Wis.; Regisil [bis-(trimethylsilyl) trifluoroacetamide plus 1% trimethylchlorosilane] from the Regis Chemical Co., Chicago, Ill.; and beta-glucuronidase (limpets, soluble type L-1) from the Sigma Chemical Co., St. Louis, Mo.

Gas chromatography. Antipyrine was assayed on a 6-foot, 3% OV-17 on 80/100 mesh Gas Chrom Q column in a Hewlett Packard 5750 research chromatograph. Helium was the carrier gas and the column temperature was 190°. The 4-hydroxy antipyrine was assayed on a 5-ft, 2·5% SE 30 column on the same support in a Varian Aerograph series 1400 gas liquid chromatograph. Nitrogen was used as the carrier and the column temperature was 240°.

Assay procedures. Plasma antipyrine was assayed as follows: duplicate 1-ml samples of plasma made basic with 0·5 ml of 1 N NaOH were extracted with 8 ml chloroform. After slow shaking for 10 min, the extracts were centrifuged at 3000 rev/min for 10 min. Erratic results were obtained if emulsions were formed. The aqueous phase was aspirated and a 6-ml aliquot of the chloroform extract evaporated to dryness under a stream of air. The sides of the tube were washed down with 1 ml acetone and the solvent was again evaporated. The tube was placed in ice water, the residue reconstituted in 25 μl ice-cold acetone, and a 5-μl aliquot injected into the OV-17 column. For each analysis, a standard curve was prepared with concentrations of 4, 5, 10 and 25 μg/ml. These standards were prepared in blank plasma and were carried through the entire procedure along with the unknown samples. Standards were injected both at the beginning and at the end of each run.

Appropriately diluted aliquots of urine were assayed for 4-hydroxy antipyrine in duplicate. To 1 ml urine, 0·2 ml of 12 N HCl was added and the sample heated in the water bath at 100° for 1 hr. The tubes were chilled in ice water, the hydrolyzed urine was made basic with 2 g K_2HPO_4–$3H_2O$ and extracted with 4 ml ethylene dichloride by shaking for 15 min. After centrifuging, a 1-ml aliquot of the organic phase was evaporated at 55° to dryness under a stream of air. When cooled to room temperature, 0·1 ml Regisil was added to each tube and allowed to react for 1 hr. A 1-μl aliquot was injected onto the SE-30 column. For each analysis, a standard curve was prepared by adding a methanolic solution of 4-hydroxy antipyrine (1 mg/ml) to blank urine to achieve concentrations of 25–200 μg/ml. The standards were carried through the procedure along with the unknown samples.

RESULTS

Typical standard curves for antipyrine and 4-hydroxy antipyrine are shown in Fig. 1. The relative standard errors of the slope estimates, based on a least-squares regression analysis, were 2·6 and 4·4 per cent for antipyrine and 4-hydroxy antipyrine respectively.

Recovery of the 4-hydroxy antipyrine from urine was 94 per cent, whereas the recovery of antipyrine from plasma was 62 per cent. However, the recovery of antipyrine from different plasma samples was reproducible. After enzymatic hydrolysis of urine (0·5 ml urine + 0·5 ml of 0·1 M acetate buffer, pH 3·8, +20,000 units β-glucuronidase incubated for 20 hr at 37°), 4-hydroxy antipyrine levels were slightly lower

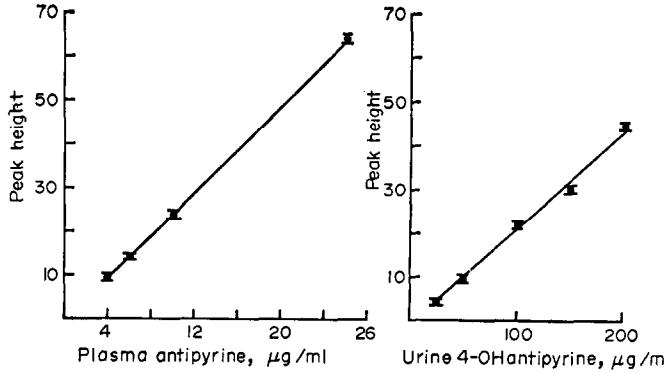

FIG. 1. Standard curves for plasma antipyrine and urinary 4-hydroxy antipyrine. Each point represents the mean ± S.E. of four determinations.

than those found following acid hydrolysis. This indicated that hydrolysis with acid was complete and that heating did not result in destruction of the 4-hydroxy antipyrine.

For most subjects, the plasma disappearance of antipyrine was in the log-linear phase by 4 hr, the time of the first sample following oral administration. In a few patients, however, the antipyrine concentration was highest at 6 hr. This was particularly true for patients with shorter plasma antipyrine half-lives. In each case, however, the antipyrine half-life was calculated from the log-linear portion of the curve both graphically and with the aid of a digital computer using a regression analysis of the log-drug concentration and the time of the sample. The results from the eight healthy subjects are given in Fig. 2. The mean plasma half-life was 11·4 ± 3 (S.D.) hr. In Fig. 3, the data for the excretion of 4-hydroxy antipyrine in urine are given. For the purposes of this study, the half-life of 4-hydroxy antipyrine in urine was

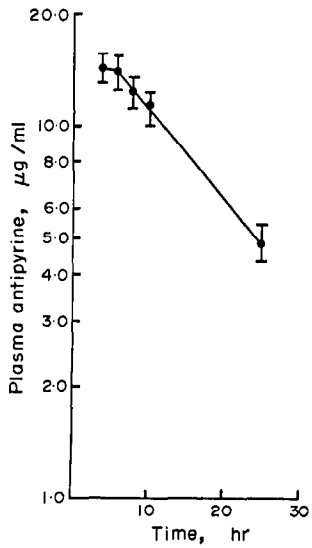

FIG. 2. Plasma disappearance of antipyrine. Each point represents the mean ± S.E. of the eight healthy volunteers.

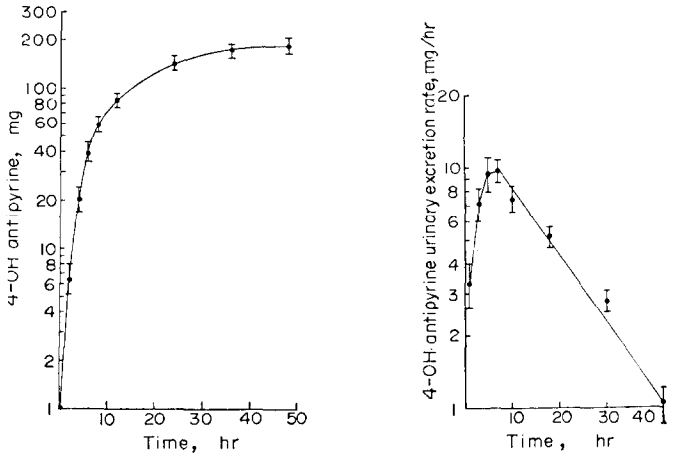

FIG. 3. Urinary excretion of 4-hydroxy antipyrine. The left panel is the cumulative excretion and the right panel is the excretion rate. Each point represents the mean ± S.E. of the eight healthy volunteers.

calculated utilizing the rate of excretion. The mean half-life of 4-hydroxy antipyrine was 10·7 ± 2·5 hr. There was a significant correlation ($r = 0{\cdot}89$, $P < 0{\cdot}001$) between the plasma half-life of antipyrine and the urinary excretion half-life of 4-hydroxy antipyrine (Fig. 4).

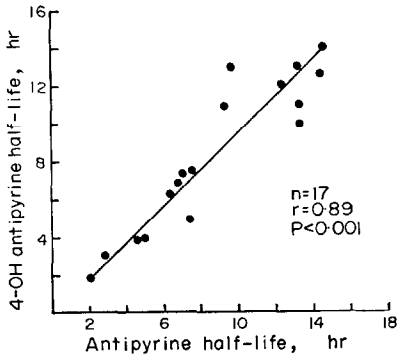

FIG. 4. Correlation between urinary 4-hydroxy antipyrine half-life and plasma antipyrine half-life.

DISCUSSION

We have described gas chromatographic methods for the determination of antipyrine and 4-hydroxy antipyrine in biological fluids which are suitable for the determination of the pharmacokinetics of antipyrine in man. In addition to being reproducible, sensitive and accurate, the methods lend themselves to semi-automation.

The plasma half-life and rate constant of drug disappearance have been interpreted as an index of drug metabolism. The utilization of substrate disappearance rather than product formation can lead to erroneous conclusions, since the plasma elimination may also reflect other processes such as changes in distribution, excretion, etc. We have demonstrated a good correlation between the plasma decay of antipyrine

and the appearance of a major metabolite, 4-hydroxy antipyrine conjugate, in urine. This observation validates previous studies utilizing the plasma elimination of antipyrine as a measure of drug metabolism in man. Even so, the determination of the rate of appearance of 4-hydroxy antipyrine in the urine would be the most valid method for evaluating antipyrine metabolism in man. We have determined that the excretion of antipyrine is less than 5 per cent and of hydroxy methyl antipyrine less than 10 per cent of the administered dose, and under normal conditions has little influence on the plasma elimination rate constant.

There are other advantages in utilizing the excretion of 4-hydroxy antipyrine in urine: (1) The efficiency of extraction is 94 per cent compared to 62 per cent for antipyrine in plasma, so that variability in recovery is reduced. (2) Complete urine collections are not necessary to make this method valid. It is possible to use rate of excretion in calculations to determine the half-life, making it possible to collect urine intermittently for abbreviated periods as long as the volunteer can empty his bladder completely each time, e.g. for 3 hr during each 6-hr period. (3) This greatly facilitates the measurement of antipyrine pharmacokinetics in outpatients who cannot be available for the entire 48 hr and, particularly, it avoids the requirement for unpleasant, repeated venipunctures and is particularly useful, therefore, in children.

A word of caution concerning the absorption of antipyrine is required. We find that the peak serum concentration may not occur in some people until 6 hr after oral administration. Therefore, data obtained at earlier periods (such as at 2 hr in the studies of O'Malley *et al.*[3]) should be disregarded, since they can lead to errors in the estimation of the plasma antipyrine half-life.

REFERENCES

1. G. Kolmodin, D. L. Azarnoff and F. Sjöqvist, *Clin. Pharmac. Ther.* **10,** 638 (1969).
2. E. S. Vesell, G. T. Passanti, F. E. Greene and J. G. Page, *Ann. N.Y. Acad. Sci.*, **179,** 752 (1971).
3. K. O'Malley, I. H. Stevenson and J. Crooks, *Clin. Pharmac. Ther.* **13,** 552 (1972).
4. B. B. Brodie, J. Axelrod, R. Soberman and B. B. Levy, *J. biol. Chem.* **179,** 25 (1949).