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Note

Rapid and sensitive method for the determination of antipyrine in biological fluids by high-pressure liquid chromatography

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Although no longer used as an antipyretic and analgesic drug, antipyrine has been widely employed recently as a drug for various pharmacokinetic studies. It has been used to study the influence of diseases[1–3], environmental factors[4], diet[5], drugs[6] and genetic factors[7,8] on drug metabolism in man. Antipyrine is well suited for this purpose as it is almost completely absorbed after oral administration, extensively metabolized, mainly by hydroxylation, and all of the metabolites formed are excreted in the urine[9,10]. Its rate of decline in plasma, which is taken as a measure of the drug-metabolizing capacity of the individual tested, correlates closely with the rate of formation of one of its main metabolites, 4-hydroxyantipyrine[11]. At doses between 1.8 and 20 mg per kg body weight its plasma half-life is not dose-dependent[12].

In most studies, the method of Brodie *et al.*[13] for the determination of antipyrine has been used. This method is rather time consuming, requires 2 ml of plasma and is of low sensitivity which means that, in spite of high doses of 20 mg/kg, levels can be measured for not longer than 18–24 h. Under certain conditions, for example uremia, some metabolites seem to interfere with the measurement of the parent compound, mimicking a prolongation of half-life where, in fact, when measured by a more specific gas–liquid chromatographic (GLC) assay, the half-life was appreciably decreased[3]. Several GLC assays have been described, requiring between 0.5 and 3 ml of plasma or saliva and an evaporation step, and the lower limit of detection is *ca.* 0.5–2.0 μg/ml (refs. 14–18).

Recently, a radioimmunoassay for the determination of antipyrine was reported which requires small plasma samples and is of high sensitivity[12]. Since the antibody is not commercially available, this method requires the preparation of the antibody which may cause some problems. Welch *et al.*[19] recently described a sensitive method using thin-layer chromatography and spectrodensitometry.

We report a method which we think is superior to the methods published. This method uses high-pressure liquid chromatography and is sensitive, specific, accurate and rapid. With this method, *ca.* 1500 samples have been analyzed to our satisfaction during the last 1.5 years.

MATERIALS AND METHODS

Chemicals

All of the chemicals and solvents used were of analytical grade and were purchased from Merck (Darmstadt, G.F.R.).

Apparatus

The liquid chromatographic system consisted of a Spectra Physics 3500 B liquid chromatograph. A Zeiss PM 2 LC spectraphotometer (cell volume, 10 μl; path length, 10 mm) was used as the UV detector and operated at 254 nm. A stainless-steel column (250 mm × 6 mm O.D. × 3 mm I.D.) packed with 5-μm spherical silica gel (Spherisorb 5 μ; Spectra Physics, Darmstadt, G.F.R.) was used. The mobile phase was dichloromethane–methanol–ammonia (25% in water) (98:1.8:0.2) with a flow-rate of 1.6 ml/min (pressure, 80 bar). The mobile phase was degassed by ultrasonification and the system was operated at room temperature.

Procedure

Plasma or saliva (0.5 ml) was pipetted into a tapered test-tube and 2.5 μg of the internal standard, 4-aminoantipyrine (dissolved in 20 μl of distilled water) and 0.1 ml of 0.1 *N* NaOH were added. The sample was mixed for a few seconds on a Vortex-type mixer and 1 ml of dichloromethane was added. The contents of the tube were again mixed for 2 min (*e.g.*, on a Heto Rotamix test-tube rotator). After centrifugation (1500 *g*), the aqueous phase was aspirated and an aliquot portion of the organic phase (*ca.* 100 μl) was injected on to the column.

RESULTS AND DISCUSSION

Under the chromatographic conditions described, the following chromatographic parameters were observed at a linear velocity of 0.40 cm/sec.

	antipyrine	*4-aminoantipyrine*
Retention time	4.6 min	6.4 min
Capacity factor, k'	3.5	5.0
Number of plates, N	2500	2400
HETP	0.1 mm	0.1 mm

The known metabolites of antipyrine, norantipyrine, 4-hydroxyantipyrine, 3-hydroxymethylantipyrine and 3-carboxyantipyrine, were all separated from antipyrine and 4-aminoantipyrine and did not interfere with the determination of antipyrine. Interfering peaks could not be detected in analyses of plasma samples of non-medicated members of the staff or of patients receiving a variety of drugs (phenytoin, primidone, phenobarbital, carbamazepine, phenacetin, aspirin, digoxin, vitamins or penicillin).

Quantitation was done by use of a Hewlett-Packard Model 3850 A integrator, from the peak-area ratios of antipyrine to the internal standard, 4-aminoantipyrine. A linear relation was obtained for the range tested (0.1–30 μg/ml). Almost the same accuracy was obtained by peak-height ratio calculation.

Fig. 1 shows the chromatograms obtained from plasma and saliva before and after the oral administration of an aqueous solution of 10 mg per kg antipyrine to a volunteer.

Precision

The precision of the method for the determination of antipyrine was obtained

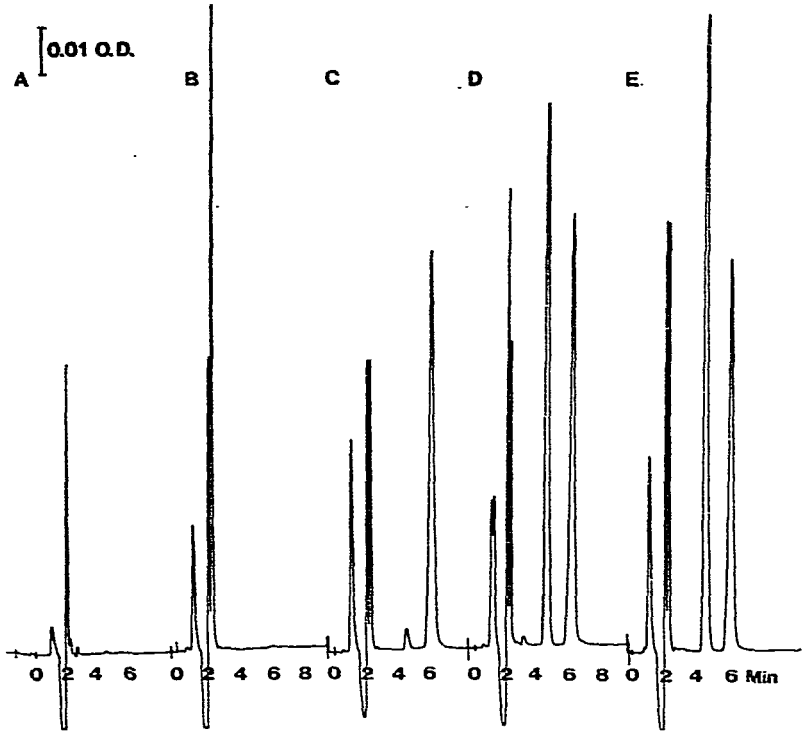

Fig. 1. Chromatograms of plasma and saliva extracts. Aliquots of 50 μl of plasma or saliva extract were injected. Serum (A) and saliva (B) blanks were recorded before the administration of antipyrine. The retention times were 4.6 min for antipyrine and 6.4 min for 4-aminoantipyrine. Extracts from plasma containing 0.12 μg/ml (C) and 2.5 μg/ml (D) of antipyrine, and from saliva containing 3.3 μg/ml of antipyrine (E) were then chromatographed.

by analyzing pooled plasma and saliva containing 5 and 0.5 μg/ml. The coefficient of variation (10 samples of each concentration were analyzed) was $\pm$ 3.5% for 5 μg/ml and $\pm$ 4.8% for 0.5 μg/ml antipyrine, respectively. Day-to-day precision obtained by analyzing pooled plasma and saliva containing 5 μg/ml antipyrine at different days over a 6-month period was $\pm$ 3.9% ($n = 30$).

Accuracy

The accuracy of the method was checked by adding known amounts of antipyrine to drug-free plasma and analyzing the samples as described. Table I shows the results of this experiment. The coefficient of correlation was 0.99, thus demonstrating the good accuracy of the method.

Sensitivity

With a sample volume of 0.5 ml of either plasma or saliva, antipyrine concentrations as low as 0.1 μg/ml can be detected.

TABLE I

ACCURACY OF THE DETERMINATION OF ANTIPYRINE

Sample	*Amount of antipyrine (μg/ml)*	
	added	*found*
A	0.30	0.28
B	5.00	4.90
C	2.50	2.35
D	0.60	0.57
E	10.00	9.90
F	0.50	0.53
G	15.00	14.90
H	0.40	0.40

Specificity

The specificity of our method was assessed by collecting the antipyrine peaks eluted from the column from different subjects and recording the mass spectra of these peaks (direct-inlet system). These mass spectra were identical with those obtained from reference compounds. Since only a single extraction step and no evaporation of organic solvent is required, in contrast to the published gas chromatographic (GC) methods, and retention times are short, 50–60 samples can be analyzed by one technician within a normal working day.

The results indicate that the method described is sensitive, specific, rapid, precise and accurate. The half-lives of antipyrine measured with this method are in close agreement with the published half-lives measured by different methods[10]. The high sensitivity of the method allows the exact measurement of antipyrine for several half-lives, or at lower doses of antipyrine than normally used. The main advantage of the method is its speed. It allows at least twice as many analyses to be carried out as compared with the method of Brodie *et al.* and the GC methods. The column has now been in use for more than 1.5 years. About 1500 samples have been analyzed without loss of column efficiency, which is also an advantage compared with GC methods.

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