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## Note

# Antipyrine determination in human plasma by gas—liquid chromatography using nitrogen—phosphorus detection

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Antipyrine, used as a marker of human hepatic oxidative drug metabolism, has been measured spectrophotometrically since 1949 [1]. The need for a more specific method, particularly in subjects with renal disease [2], has led to development of techniques using gas—liquid chromatography (GLC) with flame ionization detection [2—4], high-performance liquid chromatography [5], and radioimmunoassay [6]. Heterocyclic nitrogens in the antipyrine molecule suggest that GLC coupled to a nitrogen—phosphorus detector (NPD) may offer a specific, sensitive method of antipyrine quantitation in plasma [7].

Here we describe such a method which, when coupled with an automated injection system, permits analysis of up to 100 samples per 24 h. Extraction is simple and requires minimum technical time (1—1.5 h) for complete sample preparation. Comparison of this method to the spectrophotometric method is made and application to a single-dose pharmacokinetic study is demonstrated.

## EXPERIMENTAL

### *Apparatus and chromatographic conditions*

The analytic instrument is a Hewlett-Packard Model 5840A gas chromatograph equipped with a nitrogen—phosphorus detector and electronic integrator, coupled to a Hewlett-Packard Model 7672A automatic injection system. The column is coiled glass, 1.83 m × 2 mm I.D. packed with 3% SP-2250 on 80—100 mesh Supelcoport (Packing 1-1767, Supelco, Bellefonte, PA, U.S.A.). The carrier gas is ultra high pure helium (Matheson Gas Products, Gloucester, MA, U.S.A.) at a flow-rate of 30 ml/min. The detector purge is ultra high pure hydrogen (Matheson) at a flow-rate of 3 ml/min mixed with dry air (Matheson) at 50 ml/min. Operating temperatures are: injection port, 310°C; column,

230°C; detector, 275°C. Before being connected to the detector, a new column is conditioned at 285°C for 48 h with a carrier flow-rate of 30 ml/min.

At the beginning of each working day, the column is primed by injection of 5 μg asolectin in benzene. Use of this phospholipid as a priming agent gives greater peak resolution, presumably by coating surface-active sites on the column.

*Reagents*

The following reagents are used: pesticide grade ethyl acetate (Fisher Scientific, Fair Lawn, NJ, U.S.A.), analytical reagent grade toluene (Mallinkrodt, St. Louis, MO, U.S.A.), certified isoamyl alcohol (Fisher), analytical reagent grade methanol (Fisher), analytical reagent grade benzene (Mallinkrodt), and analytical reagent grade sodium hydroxide (Mallinkrodt). Isoamyl alcohol is glass distilled prior to use; other solvents are used without further distillation.

*Reference standards*

Pure antipyrine is obtained from Aldrich (Milwaukee, WI, U.S.A.). Pure diazepam was kindly supplied by Hoffmann-LaRoche (Nutley, NJ, U.S.A.). The antipyrine standard is prepared by dissolving 100 mg in 100 ml methanol. Sequential dilutions to 100 μg/ml are made. Diazepam is dissolved in 100 ml benzene, with sequential dilutions in benzene to 100 μg/ml. These solutions are stored in amber bottles at 4°C and are stable for at least six months.

*Preparation of samples*

Diazepam is used as the internal standard. A 100-μl volume of stock solution (100 μg/ml), containing 10 μg diazepam, is added to a series of 15-ml glass culture tubes with PTFE-lined screw top caps. A 1-ml sample of unknown plasma is added to each tube. Calibration standards are prepared by adding 1, 2.5, 5.0, 10, 25, and 50 μg antipyrine to consecutive tubes. Drug-free control plasma is added to each of the calibration tubes. One blank sample, taken from the subject prior to drug administration, is analyzed with calibration standards and each set of unknown samples.

*Extraction procedure*

One ml 1 *M* sodium hydroxide is added to each tube. To this 6 ml ethyl acetate are added and the tubes are agitated gently in the upright position on a vortex mixer for 10 min. The samples are centrifuged at room temperature for 10 min at 400 *g* (Portable Refrigerated Centrifuge Model PR-2; head No. 269, International Equipment, Boston, MA, U.S.A.). The organic layer is transferred to a conical 13-ml centrifuge tube. The organic extracts are evaporated to dryness at 40°C under conditions of mild vacuum. The final dry residue is redissolved in 1 ml toluene—isoamyl alcohol (85:15), of which 6 μl is injected into the chromatograph.

*Clinical pharmacokinetic study*

A healthy 24 year old female volunteer participated after giving written informed consent. A single 1.2-g dose of antipyrine was administered by constant intravenous infusion over 5 min. Mutiple venous blood samples were drawn

over the following 24 h. Concentrations of antipyrine in plasma were determined spectrophotometrically [1,8], and by the method described above.

Plasma antipyrine concentrations determined by each method were analyzed by iterative weighted non-linear least-squares regression analysis as described in detail elsewhere [9]. After correction for the time of infusion [10], the following pharmacokinetic variables were determined: distribution half-life, elimination half-life, total volume of distribution, and total clearance.

## RESULTS

### *Evaluation of method*

Under the described conditions the retention times of antipyrine and diazepam are 2.35 and 12.35 min, respectively (Fig. 1). The relation between antipyrine plasma concentration and antipyrine-to-diazepam peak areas is linear to at least 50 μg/ml. Analysis of 26 standard curves constructed on different days over a 3-month period indicated that the correlation is always 0.99 or greater. Day-to-day coefficient of variation in the slopes of the calibration curves was 6.0%.

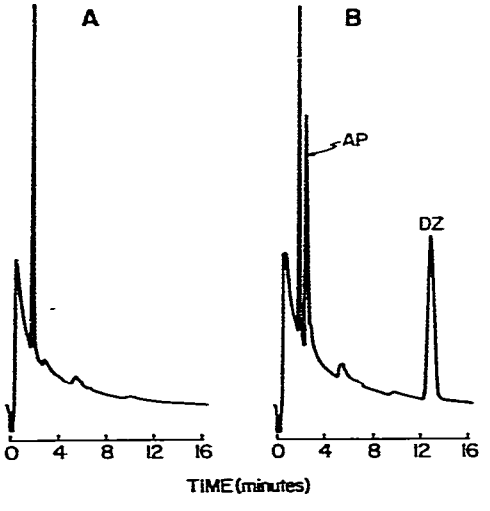


Fig. 1. Gas—liquid chromatogram of (A) extract of 1 ml of drug-free blank plasma and (B) the same plasma after addition of 5 μg/ml antipyrine (AP) and 10 μg/ml diazepam (DZ), the internal standard.

The sensitivity limit of the method is 1 μg per ml of the original sample. Coefficients of variation for identical samples were: at 50 μg/ml, 3.7% ($n$=6); at 25 μg/ml, 2.7% ($n$=6); at 10 μg/ml, 3.2% ($n$=6); at 5 μg/ml, 2.6% ($n$=5); at 2.5 μg/ml, 5.7% ($n$=6); and at 1 μg/ml, 6.6% ($n$=6). Residue analysis indicated that extraction of antipyrine is greater than 95% complete.

A series of 72 plasma samples from pharmacokinetic studies was analyzed by both GLC—NPD and spectrophotometric methods to establish comparability of methods (Fig. 2). The correlation coefficient between methods was 0.98, and the slope of the regression was 1.07.

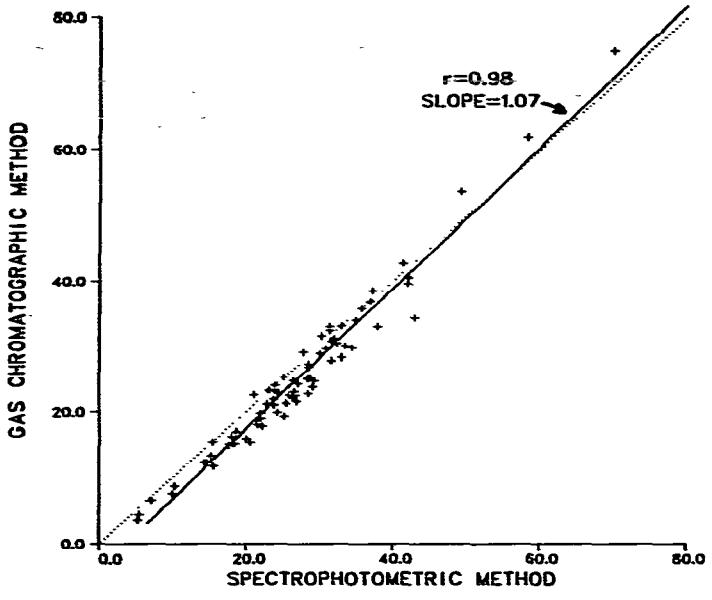


Fig. 2. Comparison of antipyrine determination using spectrophotometric and GLC—NPD methods on 72 plasma samples obtained from pharmacokinetic studies. The solid line was determined by linear regression analysis; the dotted line is the line of identity ($y=x$).

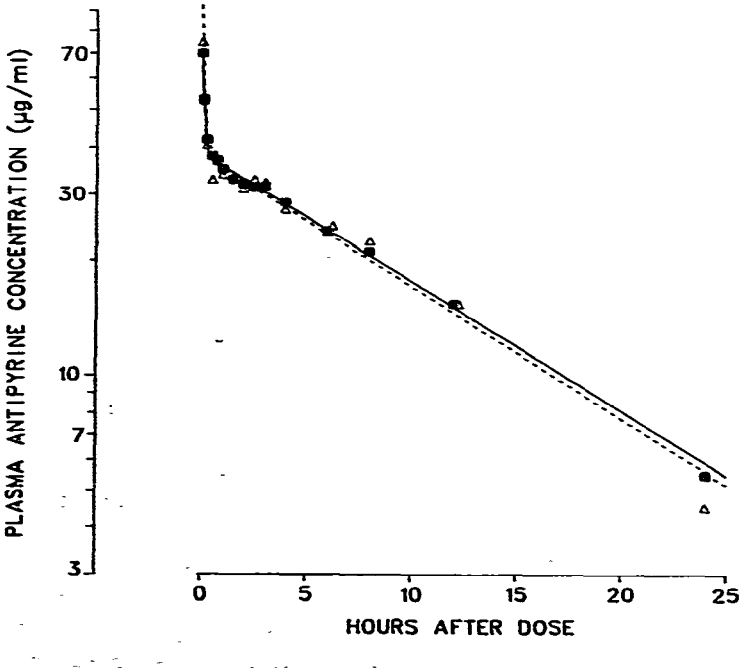


Fig. 3. Plasma antipyrine concentrations and pharmacokinetic functions from single-dose study of a 24 year old female with analysis of each sample using both spectrophotometric (■——■) and GLC—NPD (△ — — — △) methods. See Table I for kinetic parameters.

*Pharmacokinetic study*

Fig. 3 shows plasma antipyrine concentrations and pharmacokinetic functions for the subject as determined by each method. Derived pharmacokinetic parameters obtained from each method of analysis were nearly identical (Table I).

TABLE I

COMPARISON OF PHARMACOKINETIC PARAMETERS DERIVED FROM ONE STUDY* WITH PLASMA ANTIPYRINE DETERMINED BY SPECTROPHOTOMETRIC AND GLC—NPD METHODS

| Parameter | Methods | |
|---|---|---|
| | GLC-NPD | Spectrophotometric |
| Distribution half-life (min) | 4.0 | 4.9 |
| Elimination half-life (h) | 8.7 | 8.9 |
| Volume of central compartment (l/kg) | 0.22 | 0.29 |
| Total volume of distribution (l/kg) | 0.53 | 0.52 |
| Total metabolic clearance (ml/min/kg) | 0.70 | 0.68 |

*See Fig. 3.

## DISCUSSION

This report describes a rapid, specific, and sensitive method for quantitation of antipyrine in plasma using GLC—NPD. A single basic extraction from plasma, evaporation of the organic phase, and injection of the redissolved residue directly into the chromatograph is the method utilized. Blank plasma samples are consistently free of contaminants in the areas corresponding to retention times of antipyrine and diazepam. Structural analogues of antipyrine were evaluated as potential internal standards. However, their retention times were similar to contaminant peaks attributable to endogenous plasma constituents or to blood collection artifacts appearing with the NPD system. Although the retention time of diazepam is long, it is well separated from contaminants and has excellent chromatographic properties.

Injection of the known major metabolites of antipyrine (4-hydroxy, 3-hydroxymethyl, and N-desmethyl derivatives) yielded no chromatographic peaks under the described conditions. Thus the method is highly specific for intact antipyrine. Furthermore, the high degree of comparability between GLC and spectrophotometric methods suggests that the latter technique, widely used for pharmacokinetic studies of antipyrine, also is essentially specific for the intact drug in the healthy persons with normal renal function that we have

studied to date. The value of the GLC method is in the short sample preparation time, its sensitivity and reproducibility, and the ease of adapting it to automated techniques for analysis of more than 100 samples per 24 h.

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