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Note

Simultaneous determination of dipyrone metabolites in plasma by high-performance liquid chromatography

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Dipyrone {sodium [N-(1,5-dimethyl-3-oxo-2-phenylpyrazolin-4-yl)-N-methylamino]methanesulphonate monohydrate} is an effective analgesic, antipyretic and anti-inflammatory drug widely used for nearly 60 years. During the last decade several attempts were made to study dipyrone metabolism; however, many aspects of the drug pharmacokinetics have not been fully elucidated. Initial studies revealed that after oral administration of [^{14}C]dipyrone, absorption was rapid and distribution uniform. The half-life of elimination of the radioactivity from the plasma was 7 h and 90% of the dose was recovered in the urine [1]. Further studies, using thin-layer chromatography, have shown that following oral and intramuscular administration in man, dipyrone as such is undetectable in the plasma since absorption is preceded by hydrolysis to 4-methylaminoantipyrine (MAA), which is further metabolized to 4-aminoantipyrine (AA) [2]. Another MAA metabolite, 4-formylamino-

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antipyrine (FAA), was identified by Volz and Kellner [3]. It is formed by the oxidation of the N-methyl side-chain of MAA [4]. AA is further metabolized to 4-acetylaminoantipyrine (AAA), which is the major metabolite appearing in the urine [3] (Fig. 1). All these metabolites (MAA, AA, AAA and FAA) are also metabolites of aminopyrine [4].

Additional methods to study pyrazolone derivatives have employed spectrophotometric [5], gas chromatographic [6] and mass spectrometric [7] determinations.

High-performance liquid chromatography (HPLC) was first applied for the determination of antipyrine in biological fluids [8] and more recently for the quantitation of aminopyrine and MAA, AA and AAA after it had been added to urine [9].

We hereby report an HPLC method for the simultaneous determination of four dipyrone metabolites (MAA, AA, AAA, FAA) in human plasma, without using derivatization steps. Employing this method, a preliminary study on single-dose kinetics of dipyrone metabolites in a healthy volunteer is presented.

MATERIALS AND METHODS

Chemicals

All of the chemicals used were of analytical grade. The solvents were LiChrosolv grade from Merck (Darmstadt, F.R.G.). The pure metabolites MAA, AA, FAA, AAA and the internal standard (4-propylaminoantipyrine, PAA) were provided by Hoechst (Frankfurt/M., F.R.G.).

Apparatus

The HPLC system consisted of a Spectra Physics Model 3500 liquid chromatograph with an M6K injector. A JASCO Model UVIDEC-100-III spectrophotometric detector (cell volume, 5 μl; path length, 10 mm) was operated at 257 nm. A stainless-steel column (300 mm × 3.9 mm I.D.) packed with 10-μm μBondapak C_{18} (part No. 27324, Waters Assoc., Milford, MA, U.S.A.) was used. The mobile phase was 8% methanol in 0.01 *M* sodium acetate, adjusted to pH 3.0 with concentrated hydrochloric acid. The column was maintained at room temperature and the flow-rate was 1.6 ml/min. The recorder was operated at 10 cm/h.

Standard solutions

Stock solutions of 500 μg/ml MAA, AA, FAA and AAA were prepared in methanol. A 50 μg/ml PAA internal standard solution was also prepared in methanol. Normal plasma was spiked with known amounts of the metabolites over the range 0.625—5.0 μg/ml and with 2.5 μg of PAA. For the range of 5—20 μg/ml, 10 μg of PAA were added as internal standard.

Extraction procedure

To 1.0 ml of plasma sample or standard in a 15-ml glass conical tube, 50 or 200 μl of internal standard solution (PAA, 50 μg/ml), 100 μl of 1 *M* sodium hydroxide and 5 ml of chloroform were added. The contents of the tubes were mixed for 1 min on a Vortex-type mixer and then centrifuged for 10 min at

850 *g*. The organic phase was transferred to a tapered centrifuge tube and evaporated to dryness in a 40°C water bath under a stream of air. The aqueous phase was re-extracted with another 5 ml of chloroform following the same procedure. To concentrate the residues at the bottom of the conical tubes, 0.5 ml of chloroform was added, vortexed and again evaporated. The residue was redissolved in 50 μl of methanol and 5–10 μl aliquots were injected into the HPLC system.

RESULTS AND DISCUSSION

The structure of dipyrone and the four major metabolites which can be identified in plasma are shown in Fig. 1. A representative chromatogram obtained when a plasma sample containing 10 μg/ml MAA, AA, AAA, FAA and 10 μg PAA as internal standard was extracted by the method described, is shown in Fig. 2. Complete separation of the four metabolites was obtained, with the following retention times: MAA 14, AA 17, FAA 21, AAA 25, and PAA 41 min. When drug-free plasma was extracted in the same manner, no

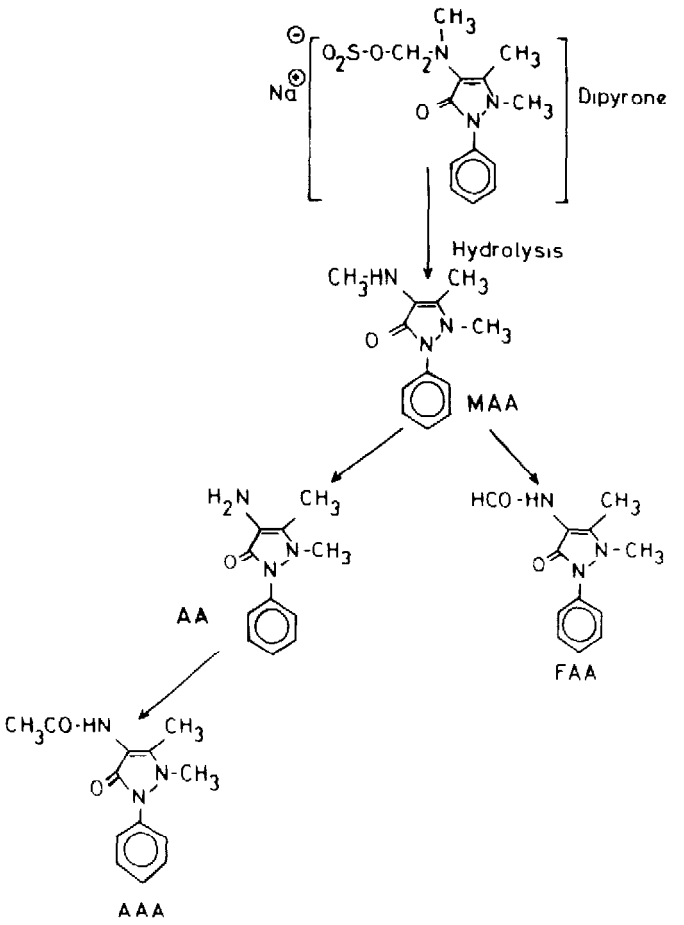

Fig. 1. Structure of dipyrone and its metabolites in man. MAA = 4-methylaminoantipyrine; AA = 4-aminoantipyrine; AAA = 4-acetylaminoantipyrine; FAA = 4-formylaminoantipyrine.

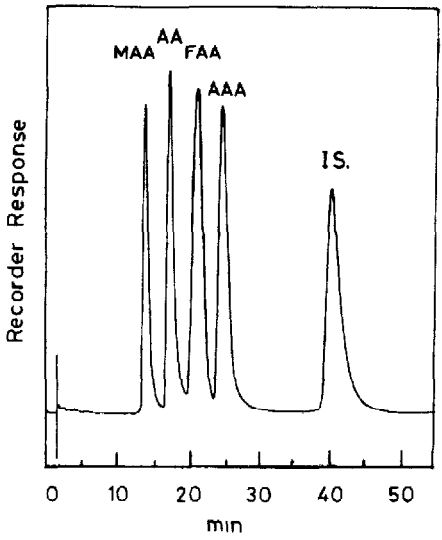

Fig. 2. A representative HPLC chromatogram of human plasma containing 10 μg/ml MAA, AA, FAA, AAA and 10 μg PAA as internal standard (I.S.).

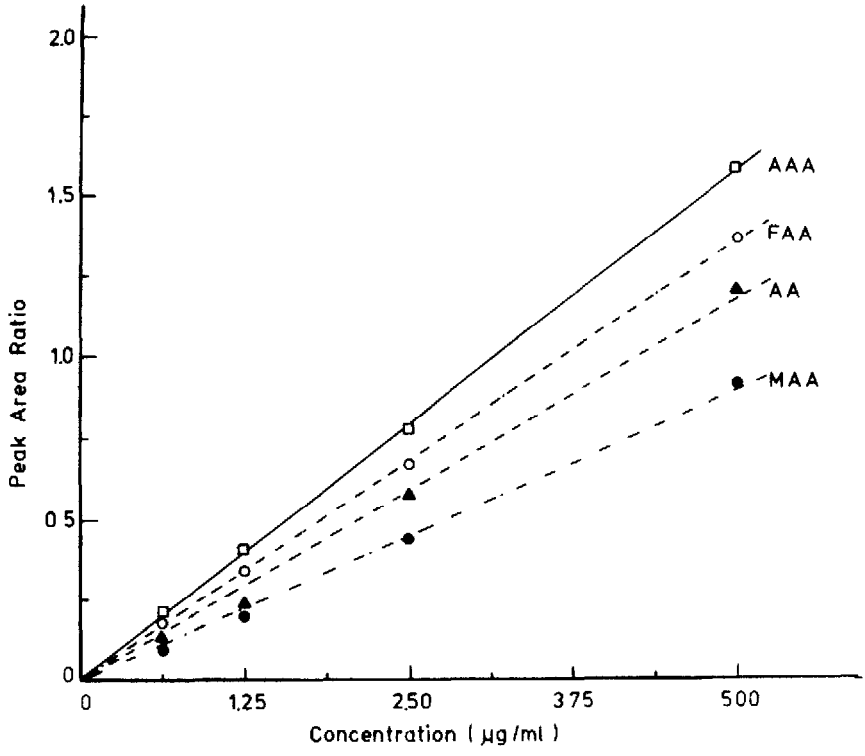

Fig. 3. Calibration curves for dipyrone metabolites with 2.5 μg PAA as internal standard.

interfering peaks from endogenous plasma constituents were observed. Plasma samples obtained after drinking coffee showed a peak with a retention time of 36 min which was identified as caffeine; this did not interfere with the assay.

The complete separation of the four metabolites was possible only when an appropriate mobile phase was found; 8% methanol in 0.01 *M* sodium acetate, adjusted to pH 3.0, resulted in the optimum resolution. From our previous experience, when the pH is raised to 5.0, FAA and AAA appeared as one peak eluting first from the column, followed by PAA, AA and MAA. The conditions employed in previously reported methods [8, 9] did not allow the simultaneous separation of the four metabolites.

Results of the calibration curves for each metabolite are given in Figs. 3 and 4. We observed that the linearity is better over the range 0.625—5.0 μg/ml if 2.5 μg of internal standard (PAA) were used. Correlation coefficients were 0.996, 0.998, 0.999 and 0.999 for MAA, AA, FAA and AAA, respectively. For higher concentrations of the metabolites (5.0—20.0 μg/ml), 10 μg of PAA were used and the correlation coefficients obtained were 0.999, 0.998, 0.999 and 0.999, respectively.

The precision of the method was determined by repeated analyses of plasma specimens containing known concentrations of the metabolite. As shown in Table I, within-day precision varied between 1.5% and 3.6% and day-to-day precision between 2.4% and 6.7%.

We measured the analytical recovery from plasma of the four metabolites. To obtain a good extraction reproducibility the double-extraction method was selected mainly to improve the recovery of MAA and AA. The recovery was calculated from a plot of the absolute method against the extractive method,

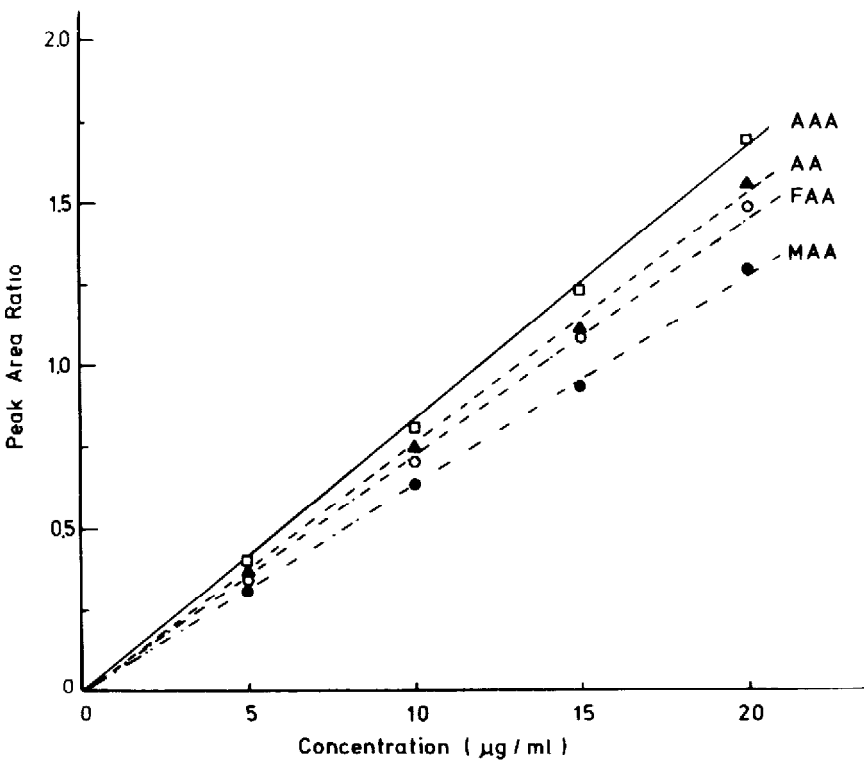

Fig. 4. Calibration curves for dipyrone metabolites, with 10 μg PAA as internal standard.

TABLE I

PRECISION OF THE DETERMINATION OF DIPYRONE METABOLITES APPLIED TO SPIKED HUMAN PLASMA SAMPLES

$n = 6$.

Metabolite	Within-day precision range		Day-to-day precision range	
	μg/ml (± S.D.)	C.V. (%)	μg/ml (± S.D.)	C.V. (%)
MAA	9.99 ± 0.33	3.3	9.99 ± 0.66	6.7
	5.00 ± 0.18	3.6	4.99 ± 0.31	6.2
AA	9.99 ± 0.15	1.5	10.00 ± 0.43	4.3
	5.00 ± 0.10	2.0	5.00 ± 0.12	2.4
FAA	9.99 ± 0 16	1.6	10.00 ± 0.51	5.1
	5.00 ± 0.16	3.1	4.99 ± 0.14	2.8
AAA	10.0 ± 0.25	2.5	10.00 ± 0.50	5.0
	4.99 ± 0.13	2.5	5.01 ± 0.15	3.2

exactly the same amount of internal standard being added after the extraction. The recovery was essentially complete for the FAA and AAA metabolites (96.0—100%) and ranged from 70.6% to 87.8% for MAA and AA. With a plasma sample volume of 1.0 ml, concentrations as low as 0.1 μg/ml for the four metabolites can be detected.

HUMAN EXPERIMENT

A healthy male volunteer (age 29 years, weight 100 kg) gave written consent to participate in the study. He was given 1.0 g of dipyrone [two Novalgin (Hoechst) tablets] orally with 200 ml of water following an overnight fast. Venous blood samples (10 ml) were drawn at 0, 1, 2, 4, 6, 8, 10, 14, 24, 36, and 48 h after ingestion; the plasma was separated and analysed as described. Chromatograms of the samples drawn at 4, 10, and 24 h are shown in Fig. 5. Results of the analysis were plotted semilogarithmically as concentration—time curves (Fig. 6). The major metabolite detected in the first 6 h following administration was MAA. Peak values were measured at 2 h. Corresponding with the decrease in MAA levels, AA and FAA concentration increased between 4 and 6 h after dipyrone administration. AAA appeared later than the other metabolites. These observations served as preliminary data for the planning of further pharmacokinetic studies.

A quantitative determination of dipyrone metabolites in plasma is needed for studies on the pharmacokinetics of the drug. The HPLC method described is easy to perform giving a good separation between all metabolites. This could be achieved without any derivatization steps. The separation of FAA and AAA depends, according to our previous experience, on the characteristics of the mobile phase.

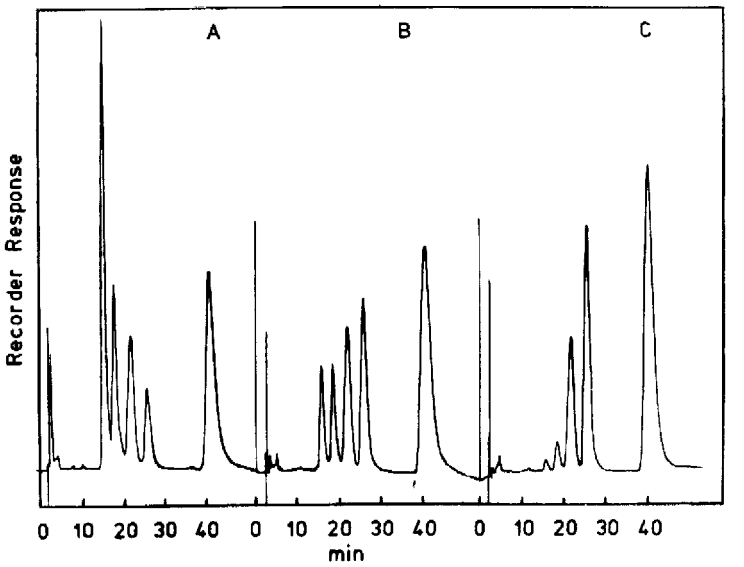

Fig. 5. Chromatograms of plasma samples drawn at 4 (A), 10 (B) and 24 (C) h following the oral administration of 1 g of dipyrone to a healthy volunteer. The metabolite concentrations measured were: (A) MAA 6.0, AA 2.0, FAA 1.3 and AAA 0.4 μg/ml; (B) MAA 1.3, AA 1.0, FAA 1.2 and AAA 1.6 μg/ml; (C) MAA 0.12, AA 0.24, FAA 0.9 and AAA 1.8 μg/ml. A constant 2.5 μg internal standard (PAA) was included in each sample. For retention times see text.

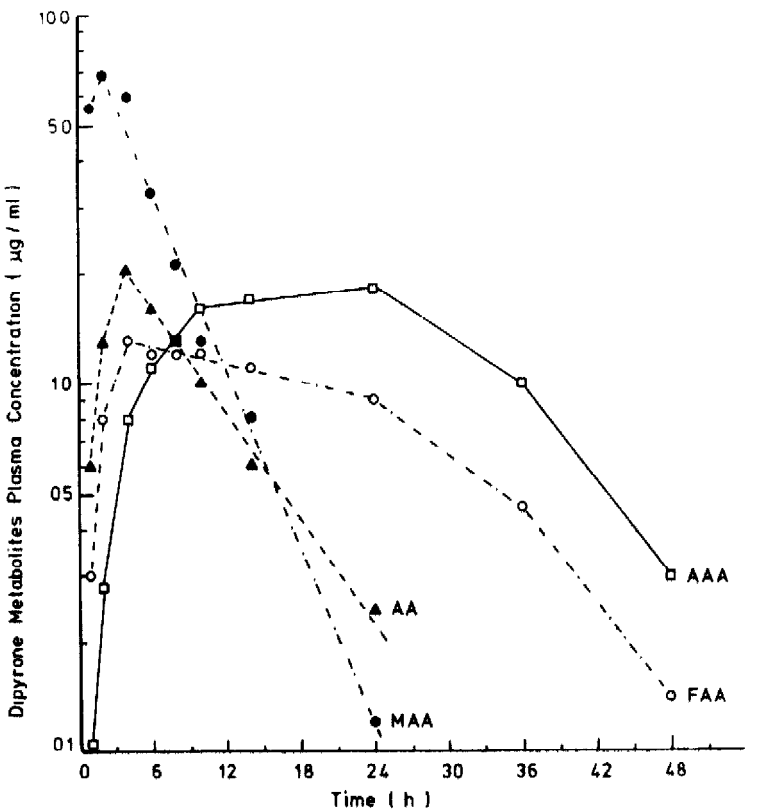

Fig. 6. Plasma concentration—time curves of dipyrone metabolites following the oral administration of 1.0 g of dipyrone to a healthy volunteer.

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