High-Performance Liquid Chromatographic Analysis of Dipyrone Metabolites to Study Their Formation in Human Liver Microsomes

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INTRODUCTION

Dipyrone (Metamizol, sodium [N-(1,5-dimethyl-3-oxo-2-phenylpyrazolin-4-yl)-N-methylamino] methanesulphonate monohydrate) is a potent analgesic and antipyretic drug which has been used in many countries for more than 70 years. The pharmacokinetics of dipyrone are well established (1). It is non-enzymatically hydrolyzed in the gastric juice to 4-methyl-aminoantipyrine (MAA) which undergoes metabolism in the liver to 4-aminoantipyrine (AA) via demethylation and to 4-formylaminoantipyrine (FAA) by a not yet characterized oxidation of the N-methyl group (figure 1). The specific cytochrome P450 (CYP) enzymes involved in these reactions have not yet been identified. AA is further acetylated to acetylaminoantipyrine (AAA) by the polymorphic N-acetyltransferase (NAT2) system. *In vitro* studies on dipyrone metabolism in human liver microsomes are so far not available.

Several analytical procedures for the quantification of dipyrone metabolites from biological fluids have been described. These include thin-layer chromatographic (2), spectrophotometric (3), gas chromatographic (4) and high-performance liquid chromatographic methods (5–8). For the determination and quantification of dipyrone metabolites from *in vitro* studies with human liver microsomes none of these methods appears to be easily applicable. This is partly due to separation problems of rather high amounts of MAA (used as substrate) as compared to low concentrations of metabolites formed from MAA.

This paper describes a simple and rapid HPLC method addressed to the determination of dipyrone metabolites from *in vitro* microsomal studies.

MATERIALS AND METHODS

Chemicals

Chemically pure metabolites of dipyrone (MAA, AA, FAA, AAA) and the internal standard (antipyrine) were kindly supplied by Hoechst AG (Frankfurt/Main, Germany). Glucose-6-phosphate, NADP and glucose-6-phosphate dehydrogenase were purchased from Boehringer (Mannheim, Germany). Ketoconazole was obtained from Janssen Chemie. All other chemicals used were at least of analytical grade.

Chromatographic System

Due to the concentration differences of MAA relative to the formed metabolites isocratic elution was found to be unsatisfactory and a gradient system had to be employed. The HPLC system consisted of a Model PU 980 gradient pump (Jasko, Labor- und Datentechnik GmbH, Groß-Umstadt, Germany), a Model UV 975 UV monitor (Jasko) fitted with a Model 231 diluter-autosampler (Gilson/Abimed, Langenfeld, Germany) and the Borwin™ chromatography software (Labor- und Datentechnik GmbH, Groß-Umstadt, Germany). Separation was achieved with a prepacked Lichrospher 60 RP—select column (Ecochart, 125 mm × 3.0 mm I.D., 5 μm, Merck, Darmstadt, Germany). Owing to the temperature dependence of the separation, a column thermostat, set at 23 °C, was used (Labor- und Datentechnik GmbH, Groß-Umstadt, Germany). The following eluents were used: Eluent A: methanol, eluent B: sodium acetate buffer (0.05 M, pH 2.2). The delivery rate of the mobile phase was 0.9 ml min^{-1} and a typical run was performed as following. At time zero (sample injection) 1% eluent A and 99% eluent B were delivered. Over the next 6 min the percentage of eluent A was increased linearly to 4%. From 6 to 13 min, from 13 to 18 min, from 18 to 26 min and from 26 to 27 min after the injection, eluent A was changed linearly to 7, 9, 9 and 1%, respectively. Over the next 6 min the column was allowed to come to equilibrium (1% eluent A) and the next sample was injected. The detection wavelength was 257 nm.

Human Liver Microsomes

Microsomes were prepared from eight human liver samples as described previously (9). The samples were provided by the Department of Clinical Pathology of the University of Erlangen-Nürnberg (Prof. Dr. C. Wittekind). The samples were from patients undergoing liver resection for various clinical reasons. The protein and cytochrome P450 content were estimated using standard procedures (10–11).

Validation of the Method

Standard curves were obtained by injecting the extracts of microsomes from one liver (without NADPH generating system, immediate extraction, extraction procedure see below) spiked with several concentrations of dipyrone metabolites (see table I). Peak area ratios relative to the internal standard were plotted versus concentrations of the standards. Inter-day variability was determined with control samples that were extracted and injected daily (six-fold) on three successive days. Similarly within-day variability was assessed with six samples injected on the same day.

Recovery values were determined by comparing extracted spiked samples with unextracted standard solutions.

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Table 1. Intra-Day (Day 1) and Inter-Day Precision of Dipyrone Metabolites (MAA, AA, AAA, FAA) from Human Liver Microsomes (C.V., Coefficient of variation)

MAA (μg ml^{-1})	Day 1 mean (C.V. %), n = 6	Day 2 mean (C.V. %), n = 6	Day 3 mean (C.V. %), n = 6
0.05	0.051 (6.8)	0.052 (10.3)	0.051 (7.1)
0.1	0.105 (3.5)	0.100 (6.1)	0.101 (5.7)
0.5	0.500 (9.0)	0.491 (2.9)	0.496 (8.4)
1.0	0.990 (11.7)	1.00 (3.4)	0.991 (9.6)
5.0	4.44 (13.8)	4.35 (9.7)	5.15 (12.6)
10.0	10.48 (10.3)	10.31 (8.0)	11.29 (13.1)
50.0	52.64 (9.8)	51.39 (4.2)	48.90 (14.7)
100.0	97.44 (10.3)	98.95 (5.8)	98.86 (10.9)
AA (μg ml^{-1})	Day 1 mean (C.V. %), n = 6	Day 2 mean (C.V. %), n = 6	Day 3 mean (C.V. %), n = 6
0.05	0.048 (2.1)	0.048 (3.6)	0.052 (14.0)
0.1	0.104 (5.3)	0.105 (6.5)	0.099 (8.3)
0.5	0.497 (3.3)	0.497 (7.3)	0.497 (7.4)
1.0	1.14 (5.2)	1.13 (9.0)	1.15 (6.6)
5.0	4.87 (11.5)	4.91 (8.6)	4.85 (6.8)
10.0	9.98 (7.1)	9.96 (8.0)	10.00 (7.6)
AAA (μg ml^{-1})	Day 1 mean (C.V. %), n = 6	Day 2 mean (C.V. %), n = 6	Day 3 mean (C.V. %), n = 6
0.05	0.049 (5.2)	0.049 (13.0)	0.050 (14.1)
0.1	0.106 (6.8)	0.102 (7.8)	0.102 (14.2)
0.5	0.496 (8.0)	0.498 (5.8)	0.497 (7.1)
1.0	1.10 (8.7)	1.02 (9.9)	1.14 (13.4)
5.0	5.00 (12.6)	5.01 (4.7)	4.88 (10.0)
10.0	9.89 (8.6)	9.96 (5.1)	9.92 (10.6)
FAA (μg ml^{-1})	Day 1 mean (C.V. %), n = 6	Day 2 mean (C.V. %), n = 6	Day 3 mean (C.V. %), n = 6
0.05	0.050 (4.7)	0.048 (11.7)	0.048 (5.5)
0.1	0.106 (7.6)	0.104 (7.3)	0.105 (9.0)
0.5	0.493 (11.2)	0.497 (6.3)	0.495 (8.5)
1.0	1.06 (8.8)	1.09 (8.2)	1.09 (6.4)
5.0	5.00 (8.3)	5.04 (8.7)	4.98 (7.5)
10.0	9.93 (7.0)	9.87 (10.2)	9.92 (8.8)

Application of the Method

MAA (100 μM) was incubated at 37°C in 0.1 M potassium phosphate buffer (pH 7.4) with human liver microsomes (containing 100 pmol of CYP and a NADPH-generating system consisting of 13.7 mM glucose 6-phosphate, 0.66 mM $NADP^+$ and 2.8 I.U. of glucose 6-phosphate dehydrogenase) in a total volume of 500 μl. After 30 min the reaction was stopped by adding 50 μl of a solution containing sodium carbonate (1 M) and sodium chloride (2 M), and 50 μl of internal standard (20 μg ml^{-1}) was added followed by a double extraction with chloroform (2.0 ml). The combined organic layers were evaporated using a gentle stream of nitrogen. The residue was redissolved in 500 μl of sodium acetate buffer (pH 2.2) and 100 μl were injected. Time dependency was investigated with microsomes from one human liver by incubating MAA (100 μM) for 5, 10, 20, 30 and 60 min. Inhibition experiments were done by coincubation with ketoconazole, an inhibitor of CYP3A4 at 10 μM.

RESULTS AND DISCUSSION

The complex metabolism of dipyrone has been the subject to many in vivo studies (see 1, for review). However, the specific CYP enzymes catalyzing the fomation of the also active metabolite AA (12) is still not known. The aim of the present study, therefore, was (i) to validate a HPLC method suitable to study the formation of dipyrone metabolites in human liver microsomes and (ii) to get first insights into the CYP enzymes involved.

Figure 2 presents typical chromatograms of microsomes spiked with a standard solution containing MAA, AA, FAA, AAA and I.S., and a chromatogram of human liver microsomes obtained 10 min after incubation with MAA (100 μM). The separation was completed within 27 min. To get an optimal resolution of MAA (used as substrate) and AA a gradient system had to be employed. The relative order of peak retention and retention times were AA, ~6.0 min, MAA, ~7.5 min, FAA, ~13.7 min, AAA, ~15.7 min and I.S., ~23.7 min. To obtain good analytical recovery for all of the four metabolites double extraction with chloroform had to be performed. It mainly improved the recovery of MAA and AA as described previously (6). The mean analytical recovery rates over the given concentration ranges (table I) for MAA, AA, FAA and AAA were 89, 93, 88, and 91%, respectively. The peak area ratios of the four substances of interest were always linearly related ($r > 0.999$) to the amount of metabolites added to human liver microsomes in the concentration ranges given in table I. The inter-day and intra-day precisions in human liver microsomes over three days are summarazied in table I. The quantification limit (less than 15% deviation of precision and/or accuracy values) was found to be 50 ng ml^{-1} for all analytes (13). The method proved to be sufficiently sensitive for *in vitro* investigations of the metabolism of dipyrone in human liver microsomes.

Incubations of MAA (100 μM) in human liver microsomes from seven different livers showed that AA is formed rather quickly (figure 2, table 2). FAA was not detected under these incubation conditions. Since it is known that CYP3A4 catalyzes the N-demethylation of a large number of drugs and CYP3A4 may account for up to 80% of the liver cytochrome P450 content it was reasonable to test human liver microsomes with different CYP3A4 content. It becomes evident that formation of AA goes along with the content of CYP3A4 in the microsomes (table II). First inhibition experiments with ketoconazole (inhibitor of CYP3A4 (14)) are in favour of the hypothesis that CYP3A4 is most likely one enzyme responsible for the formation of AA from MAA. These preliminary data, however, also suggest that most likely other enzymes are also involved in this metabolic step. Detailed experiments using specific CYP inhibitors, immunoinhibition techniques and cDNA-expressed CYP are in progress to study the complex metabolism of dipyrone. The analytical method described above is simple, sensitive

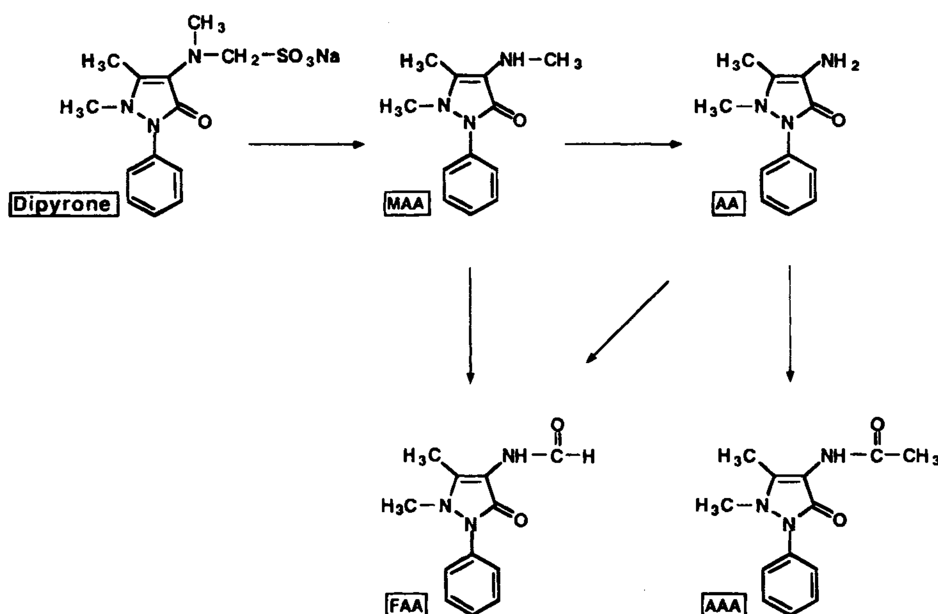

Fig. 1. Structure and biotransformation of dipyrone and its main metabolites in man. MAA = 4-methylaminoantipyrine; AA = 4-aminoantipyrine; AAA = 4-acetylaminoantipyrine; FAA = 4-formylaminoantipyrine (1).

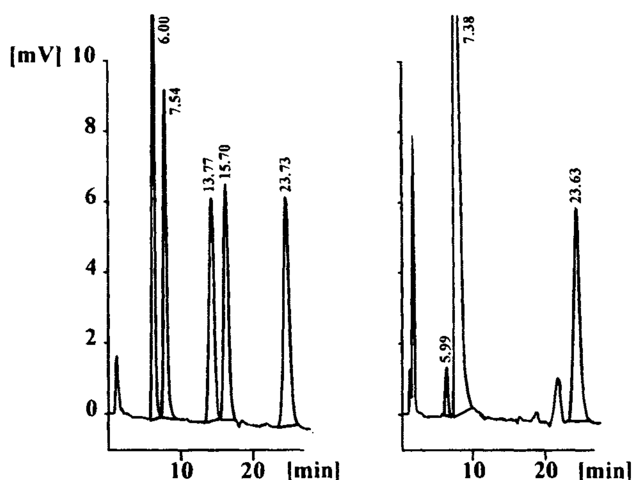

Fig. 2. Typical chromatograms of the main metabolites of dipyrone: (Left) Extract from human liver microsomes spiked with 1 μg ml^{-1} each of AA, MAA, FAA, AAA and I.S. without NADPH-generating system. (Right) Extract from human liver microsomes obtained 10 min after incubation with MAA (100 μM; incubation conditions see materials and methods). Peaks: AA (~6 min), MAA (~7.5 min), FAA (~13.7 min), AAA (~15.7 min), I.S. (~23.7 min).

Table II. Formation of Aminoantipyrine (AA) Without and with Coincubation with Ketoconazole (10 μM) in Human Liver Microsomes from Different Livers. Activities Are Expressed as nmol/min/nmol CYP. CYP3A4 Activity Is Given in nmol/min/nmol CYP Using a Denitro Derivative of Nifedipine as Specific Substrate (15)

Liver	CYP3A4 activity	AA	with ketoconazole
CH48	1.4	0.38	0.25
CH51	2.4	0.73	0.36
CH55	2.5	0.89	0.56
CH57	1.7	0.63	0.43
CH58	2.2	0.64	0.45
CH59	1.3	0.56	0.39
CH60	0.8	0.71	0.52

and reliable for the detailed investigation of the metabolism of dipyrone *in vitro*.

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