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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF DIPYRONE AND ITS ACTIVE METABOLITE IN BIOLOGICAL FLUIDS

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SUMMARY

New high-performance liquid chromatography methods were developed to measure dipyrone and its active metabolite 4-monomethylaminoantipyrine (MAA) in biological fluids. While no detectable level of the unchanged dipyrone was found in plasma of four subjects taking 1000-mg oral doses of the drug, values ranging from 1.25 to 14.99 μg/ml of MAA were observed. Twenty-four hour urinary excretion of MAA varied from 9.56 to 21.21 mg. Concentrations as low as 0.25 μg/ml of MAA can be measured with acceptable coefficients of variation. The method, therefore, is suitable for microquantification of MAA in biological fluid which enables convenient and rapid assessment of the drug disposition in body.

INTRODUCTION

Dipyrone (metamizol, sulpyrine), sodium N-(1,5-demethyl-3-oxo-2-phenyl-pyrazolin-4-yl)-N-methylaminomethanesulfonate, is an effective analgesic and antipyretic agent. Although administration of this drug is associated with severe agranulocytosis [1, 2] and prolongation of bleeding time [3, 4], it is being widely used in many countries in daily doses of as high as 4 g [4]. The analgesic potency of dipyrone seems to be largely due to its rapid biotransformation to its active metabolite, 4-monomethylaminoantipyrine (MAA) [5]. Limited information is available as to the pharmacokinetics of this analgesic [6]. To study the time-course of dipyrone in the body a spectrophotometric method is used [6]. Furthermore, a 0.09—0.70 mg/g range of dipyrone and its major metabolite has been measured in rat tissue hemogenate using a high-performance liquid chromatography (HPLC) method [7].

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The purpose of this paper is to report a highly sensitive and specific HPLC method suitable for micro determination of MAA in plasma and urine following administration of single doses of dipyrone to human subjects.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Model 224 U instrument (Waters Assoc., Milford, MA, U.S.A.) equipped with a dual-channel fixed-wavelength (254 and 280 nm) ultraviolet detector.

For determination of dipyrone a column, 61 cm × 2 mm I.D. with 35—50 μm particle size anion-exchanger packing (Bondapak AX/Corasil, Waters Assoc.), was used. Mobile phase was phosphate buffer, pH 5.6—methanol (85:15) which was pumped at a flow-rate of 0.5 ml/min and initial pressure of 0.7 MPa.

A reversed-phase column, 30 cm × 3.9 mm I.D. with 10-μm particle size packing (μBondapak C_{18}, Waters Assoc.), was used to quantify MAA. The mobile phase was water—methanol—acetic acid (75:20:5) which was pumped at a constant flow-rate of 1 ml/min and an initial pressure of 3.5 MPa.

Chemicals

All organic solvents were of analytical grade. Water was distilled and deionized. Mobile phasss were filtered through 0.45-μm pore size filters (Millipore, Bedford, MA, U.S.A.) before utilization. Antipyrine (Aldrich, Milwaukee, WI, U.S.A.) and sodium salicylate (E. Merck, Darmstadt, F.R.G.) were used as internal standards (IS) in MAA and dipyrone assays, respectively. Powder of dipyrone was a gift from Hoechst Laboratories of Iran.

The metabolite (MAA) was prepared by hydrolysis of dipyrone at 50°C, extraction with chloroform in alkaline environment, separation by means of thick-layer chromatography on silica gel using ethyl acetate—methanol—chloroform (20:20:60) as solvent, re-extraction with chloroform and then crystallization in isopropyl alcohol and diethyl ether. The chemical structure of the metabolite was confirmed using nuclear magnetic resonance (NMR) spectroscopy in accordance with Yoshioka et al. [8, 9] and mass spectroscopy. Melting point of MAA was 170—172°C under the hot-stage condition.

Sample preparation

Dipyrone in plasma. To create two separate layers and direct dipyrone into an alcoholic layer, excessive amount of potassium carbonate powder (600—1000 mg) was added to tubes containing 1—2 ml plasma and 1 ml IS solution (40 μg sodium salicylate in methanol). Tubes were shaken for 1 min and centrifuged. Methanol layers were separated and evaporated under flow of nitrogen and reconstituted in 0.5 ml mobile phase and aliquots of 25—50 μl were injected into the chromatograph.

For the purpose of quantification on three occasions, 100 μl of freshly prepared solutions of dipyrone in water containing 0, 1, 10, 20, 40, 100, 200 and 400 μg of the drug were added to 0.9 ml blank plasma and extracted using the mentioned method. These standard samples correspond to plasma dipyrone

concentrations of 0 to 40 μg/ml. Peak height (dipyrone at 254 nm, IS at 280 nm) ratio method was used and the relationship between the observed ratios and corresponding dipyrone concentrations was examined.

The percent intact dipyrone in tablets was also measured using the Bondapak AX/Corasil column. Twenty tablets of 500 mg dipyrone were weighed, ground to powder and quantities equal to 5 mg of drug were dissolved in 100 ml water. To three 1-ml volumes of this solution was added 1 ml solution of IS containing 40 μg sodium salicylate. Ten μl of the final preparations were injected into the instrument and their dipyrone contents were quantified by examining the observed peak height ratios against those of solutions of equal strenth prepared from pure powder.

MAA in plasma and urine. To 1 ml of plasma or urine samples were added 0.5 ml IS solution (40 μg antipyrine), 0.5 ml of 0.1 *N* sodium hydroxide and 10 ml benzene. After vigorous shaking for 20 min, the benzene layer was separated, transferred into 15-ml centrifuge tubes, evaporated under flow of nitrogen and the residual was dissolved in 0.25 ml methanol. Aliquots of 25 μl were injected into the chromatograph.

To quantify MAA in plasma, four series of standard solutions were prepared by adding 0, 0.25, 0.50, 1, 2, 5, 10, 20 and 40 μg MAA to 1 ml blank plasma and extracting according to the above mentioned method. Standard solutions for determination of MAA in urine contained 0, 10, 20, 50, 100 and 200 μg of the latter in 1 ml urine. Four series of such solutions were examined. Peak height ratios of MAA (at 254 nm) and IS (at 280 nm) were measured and a standard curve was prepared by plotting ratios versus amount of MAA. Statistical parameters were computed using a programmable calculator (Model 41C, Hewlett-Packard, Corvallis, OR, U.S.A.).

Analysis of both dipyrone and MAA in biological samples was always accompanied by preparation of freshly prepared standard solutions.

Recovery of MAA

The efficiency of the MAA extraction method was examined by preparing two series of solutions similar to those of standard solutions but MAA (in methanol) was added to the separated benzene layer after the extraction.

Subjects

Four healthy male subjects volunteered for this experiment. They ranged in age and weight between 20—30 years and 54—65 kg, respectively. Two tablets of 500 mg dipyrone (Novalgin, Hoechst, Iran) were ingested with a glass of water after an overnight fast and at least 1.5 h before breakfast. Venous blood samples were taken from forearms by heparinized disposable syringes at 0, 0.5, 1, 2, 4, 6 and 8 h post-dosing and plasma portions were separated. Following administration, total urine outputs at 0, 1.5, 3, 5, 7, 11, 12.5, 14 and 24 h were collected. Plasma and urine samples were kept frozen until the time of analysis. For determination of intact dipyrone, however, fresh plasma samples up to 4 h post-dosing were analyzed immediately after the collection.

Data treatment

The AUTOAN [10] and NONLIN [11] programs were used to estimate the

best-fit line through the data points and calculate the half-life ($t_{½}$) of the terminal phases of MAA curves.

RESULTS AND DISCUSSION

Fig. 1 depicts peaks representing intact dipyrone and IS added to blank plasma. The drug and IS appeared 6.3 and 11.2 min after injection into the HPLC system. No interfering peaks were observed. An excellent linear relationship (correlation coefficient of 0.996) was found between peak height ratios and the amount of added dipyrone. The best-fit line through the points was described by $y = 0.0429x - 0.0021$. The observed coefficients of variation (C.V.) ranged from 2 to 7% except for solutions containing 0.1 μg dipyrone per ml of plasma which showed a C.V. of 30%. The minimum quantifiable concentration (MQC) was, therefore, set at 1 μg/ml. Analysis of a set of standard solutions after 12 h referigeration showed no significant reduction in response. This indicates the stability of dipyrone under the utilized condition at least for 12 h.

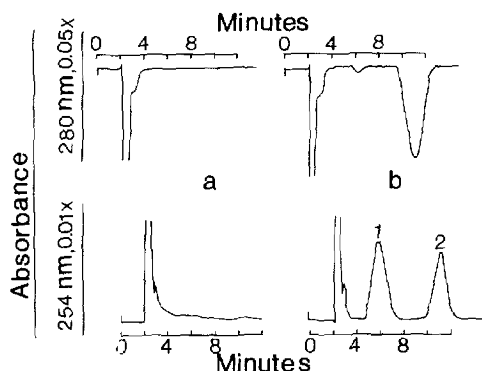

Fig. 1. Chromatograms of (a) blank plasma (b) dipyrone spiked plasma (4 μg/ml). Peaks: 1 = dipyrone, 2 = sodium salicylate.

A chromatogram of a plasma sample taken from subjects during the first 4 h after post-administration of 1 g dipyrone showed no peak with specifications of dipyrone. This observation indicates that the concentration of dipyrone in plasma of subjects taking 1 g dipyrone is substantially less than our MQC of 1 μg/ml.

Chromatograms of plasma and urine samples extracted with benzene and analyzed using a reversed-phase column are shown in Figs. 2 and 3, respectively. Peaks representing MAA and antipyrine were observed 5.2 and 8.0 min after injection into the chromatograph, respectively. Blank plasma and urine contained no interfering compounds. The selectivity of the assay was assured by routine examination of UV absorbance ratios at 280 over 254 nm for MAA (0.33) and antipyrine (0.31). An excellent linear relationship was found between the peak height ratios (MAA at 254/IS at 280) and the amount of MAA in samples. For the pooled data, the best-fit lines through the observed points were described by $y = 0.0161x - 0.0018$ and $y = 0.0200x - 0.0278$ for plasma and urine solutions with correlation coefficients of 0.998 and 0.999.

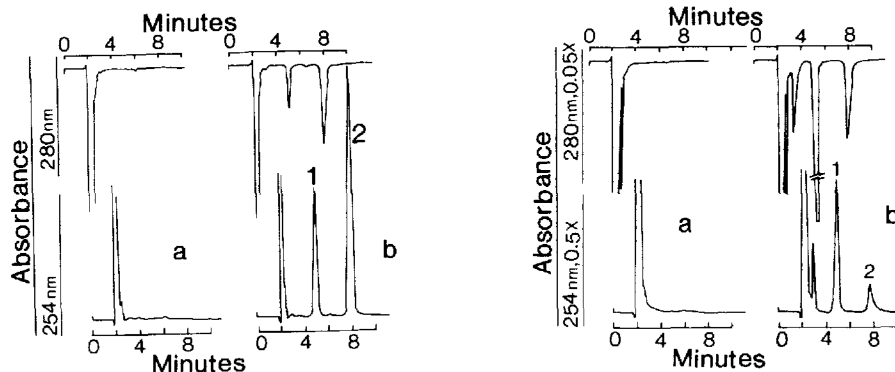

Fig. 2. Chromatograms of (a) blank plasma and (b) plasma of subject 1, 1 h after administration of 1000 mg dipyrone. Peaks: 1 = MAA, 2 = antipyrine. a.u.f.s., 0.05.

Fig. 3. Chromatograms of (a) blank urine and (b) urine of subject 1, 1.5 h following administration of 1000 mg dipyrone. Peaks: 1 = MAA, 2 = antipyrine.

respectively. Coefficient of variation varied from 2 to 9% within the examined ranges except for plasma samples containing 0.25 μg MAA, which was 11%. Averages of 78.1 and 86.1% with standard deviations of 3.8 and 4.7 were found to be extractable from plasma and urine, respectively.

After administration of dipyrone an extra peak was consistently present in urine. The latter which appeared 3.3 min after injection of the urine extracts into the instrument (Fig. 3) was, presumably, another metabolite of dipyrone. No attempt was made to identify the structure of this peak.

Table I depicts plasma MAA concentrations and the elimination half-lives ($t_{1/2}$) in four subjects following administration of single 1000-mg doses of dipyrone. Maximum plasma MAA concentration (C_{max}) varied from 7.52 to 14.99 μg/ml and attained (T_{max}) within the first 2 h post-dosing. At 8—9 h after administration, the concentration was between 1.65 and 2.82 μg/ml. The sensitivity of the method was found adequate to follow plasma and urine concentrations of MAA following administration of a single 1000-mg oral dose of dipyrone since concentrations as low as 0.25 μg/ml were quantifiable with acceptable C.V. values. However, the observed MQC can be further increased by utilization of larger plasma volumes.

Disposition kinetics of MAA conferred the characteristics of a one-compart-

TABLE I

PLASMA MAA CONCENTRATIONS (μg/ml) AND ELIMINATION HALF-LIVES ($t_{1/2}$) AFTER ORAL ADMINISTRATION OF 1000 mg DIPORONE TO HEALTHY SUBJECTS

Subject No.	Time (h)							$t_{1/2}$ (h)
	0.5	1	2	4	6	8	9	
1	9.85	14.99	12.98	6.04	2.86	1.57	—	1.87
2	8.52	14.74	13.67	8.74	6.29	2.82	—	2.22
3	5.91	9.45	9.27	6.86	3.44	1.90	—	2.21
4	1.14	4.88	7.52	4.02	2.54	1.65	1.25	2.46

TABLE II

CUMULATIVE URINARY MAA EXCRETION (mg) AND HALF-LIVES ($t_{1/2}$) OF EXCRETION-RATE PLOTS FOLLOWING ORAL ADMINISTRATION OF 1000 mg DIPYRONE TO HEALTHY SUBJECTS

Subject No.	Time (h)								$t_{1/2}$ (h)
	1.5	3	5	7	11	12.5	14	24	
1	4.43	9.94	15.07	16.67	17.74	17.83	17.95	17.99	1.60
2	5.81	12.64	14.01	17.21	19.75	20.29	21.16	21.21	2.44
3	—	4.77	8.67	11.33	—	14.50	—	15.28	2.96
4	—	2.65	4.01	6.05	6.83	—	8.78	9.56	—

ment model with a first-order input kinetic. The observed $t_{1/2}$ of MAA disappearance from plasma ranged from 1.87 to 2.46 h with a mean and standard deviation (S.D.) of 2.19 h and 0.23, respectively. The observed MAA plasma concentration—time curves seem to agree with those reported earlier [6] using a spectrophotometric assay. These authors reported a mean C_{max} value of approximately 8 ± 4 (S.D.) μg/ml. The amount of MAA excreted in urine and $t_{1/2}$ values of excretion-rate plots as a result of a 1000-mg dose of dipyrone are shown in Table II. A total of 9.56—21.21 mg of the metabolite was recovered in urine during the 24-h collection period. Excretion rate—time plots had comparable $t_{1/2}$ values to those of plasma MAA concentration—time curves (1.60—2.96 h). Due to a substantial fluctuation in the urinary excretion of MAA in subject 4, the $t_{1/2}$ value of the excretion rate—time plot was not considered.

Upon administration, dipyrone is rapidly, and to a great extent, biotransformed to its major metabolite, MAA [6] which seems to be more effective than the parent compound [7]. In our laboratory, using an anion exchange system, we were able to quantify dipyrone in concentrations as low as 1 μg/ml when added to blank plasma. Following oral administration of 1000 mg dipyrone, however, we failed to detect even traces of the unchanged drug in plasma. This phenomenon is not likely to be caused by a lack of dipyrone chemical stability. The administered tablets contained 102—109% of the claimed dipyrone content. Furthermore, it has been found by others that chemical degradation of dipyrone is minimal in methanol [7]. In this work, we extracted dipyrone into methanol by adding excessive sodium carbonate to saturate the aqueous layer. The absence of significant changes in the peak height ratios of dipyrone-spiked plasma solutions (standards) after 12 h refrigeration supports this suggestion. It, therefore, seems reasonable to suggest that the absence of intact dipyrone in plasma of subjects taking this drug may be due to its rapid and extensive metabolism to MAA upon the first-pass through the gut and/or liver. This metabolic characteristic of dipyrone is similar to that of acetylsalicylic acid which upon administration is rapidly biotransformed to salicylic acid [12].

This method is superior to that of Tamura et al. [7] in sensitivity and peak resolution. Their method was applied to measure dipyrone and its metabolites in muscle in 0.09—0.70 mg/g range and failed to achieve a complete separation

of the resultant peaks. Dipyrone is administered orally, intravenously or intramuscularly as single doses of 500—2500 mg to relieve pain. The method outlined here seems to be quite suitable to follow the time course of its major metabolite, MAA, in human urine and plasma.

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