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Determination of brodimoprim and its hydroxy metabolite in human plasma, blood and urine by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method was developed to measure the concentration of brodimoprim and its metabolite, hydroxybrodimoprim, in small volumes of blood, plasma and urine. The procedure involved a simple extraction step with chloroform, followed by chromatographic separation on a short reversed-phase column deactivated for the analysis of basic compounds. The column effluent was monitored by fluorescence (excitation wavelength 290 nm, emission wavelength 340 nm). The recoveries of both compounds were similar in all three biological fluids, and averaged 84 and 72%, respectively. The detection limit for both compounds reached 5 ng/ml. No endogenous compound interfered in the assay. The linearity of the method and its within- and between-day precision were analytically satisfactory.

INTRODUCTION

Brodimoprim (BDP), a trimethoprim analogue diaminopyrimidine, 2,4-diamino-5-(4′-bromo-3′,5′-dimethoxybenzyl)pyrimidine, is a new inhibitor of bacterial dihydrofolate reductases (DHFRs), active against a broad spectrum of Gram-positive and -negative bacteria [1]. Clinically it has been proven effective in the management of infection of the upper and lower respiratory tract and bacterial gastroenteritis [2].

After oral administration, peak plasma concentrations are reached within 3–4 h. BDP extensively distributes into the extravascular and tissutal compartments, with a volume of distribution of 1.52 ± 0.18 l/kg, and it is mainly eliminated by hepatic metabolism with a rather low systemic clearance (0.58 ± 0.20 ml/min/kg) [3,4]. These data are compatible with the good lipophilicity of the compound (octanol/water partition coefficient 66.2) and with the high plasma protein binding (92.9 ± 0.27%) [5,6].

About 7% of the administered dose is excreted in the urine as intact drug and more than 50% as the O-desmethyl derivative (hydroxybrodimoprim, OH-BDP), N-1 and N-3 oxides, and α-hydroxy derivative, either in their free from or as glucuronide/sulphate conjugates [7].

About 10% of the administered dose is excreted in faeces, mainly as unmodified BDP and less than 2% as OH-BDP, both compounds being found in their free and conjugated form. Among these metabolites, only OH-BDP shows some inhibitory activity on bacterial DHFRs, although

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ca. ten times less effective than BDP [8].

In order to extend pharmacokinetic investigations to humans, a method of analysis of the drug in biological fluids was called for. Since no chromatographic procedures have been published so far, we developed a rapid, sensitive and reproducible high-performance liquid chromatographic (HPLC) assay for measuring the concentrations of BDP and its major metabolite, OH-BDP, in blood, plasma and urine. The procedure has been successfully used to analyse more than 1000 samples in pharmacokinetic studies in humans.

EXPERIMENTAL

Chemicals

BDP, OH-BDP and the internal standard, Ro-11-3926, were generous gifts from Helsinn (Chiasso, Switzerland). The molecular structures are shown in Fig. 1. Stock solutions containing 1, 2, 5, 10 and 100 μg/ml of each compound were appropriately prepared in methanol. The standard solutions were stored at 4°C, and were stable for at least two months (determined by HPLC). Acetonitrile and methanol (Farmitalia Carlo Erba, Milan, Italy) were HPLC grade; chloroform stabilized with ethanol (Code No. 2445) was supplied by Merck (Darmstadt, Germany). All other chemicals were analytical grade.

Extraction procedure

BDP, OH-BDP and the internal standard (50 μl of a methanolic solution of 5 μg/ml) were extracted with chloroform from 200 μl of plasma, blood, and urine (diluted 1:10 with twice-distilled water) buffered at pH 9 with 500 μl of 0.4 *M* borate buffer. The samples were vortex-mixed for a few seconds and then centrifuged at 1700 *g* for 10 min at 4°C. The organic layer was then separated and taken to dryness under a gentle nitrogen stream in a water-bath at 37°C, and the residue was dissolved in 200 μl of the mobile phase. The samples were allowed to stand for at least 1 h at room temperature to improve the solubility, and then 20–50 μl aliquots were injected into the liquid chromatograph.

Internal calibration curves for BDP and OH-BDP were prepared for each set of samples. The methanolic solution containing the required amount of the drug and the metabolite was dried under a gentle nitrogen stream before addition of the necessary amounts of plasma, blood or urine.

Deconjugation procedure for BDP and OH-BDP

Aliquots (200 μl) of plasma or diluted urine were pipetted into 10-ml centrifuge tubes containing 100 μl of β-glucuronidase (68 U/ml) (Sigma, St. Louis, MO, USA) in 0.5 *M* sodium acetate buffer at pH 5.0. After incubation at 37°C for 4 h, 100 μl of 0.4 *M* borate buffer (pH 10) and 50 μl of internal standard solution were added. The mixture was extracted with chloroform as previously described.

Chromatographic equipment

A Model 342 liquid chromatograph (Beckman, Fullerton, CA, USA) equipped with a programmable fluorescence detector (Chrompack, Middelburg, Netherlands) with a 150-W xenon lamp (excitation wavelength 290 nm, slit 18 nm; emission wavelength 340 nm, slit 18 nm) and a 6.5-μl flow-cell was used. A Chromatopac C-R3A integrator (Shimadzu, Kyoto, Japan) was used for the detector signal output.

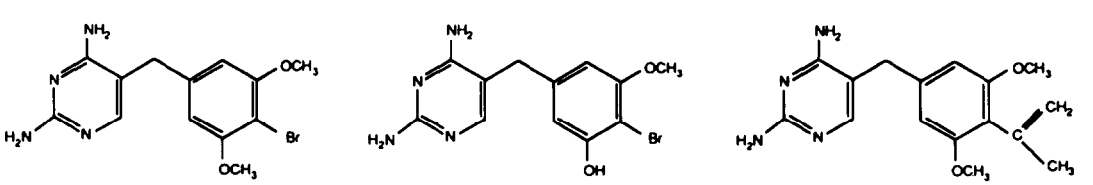

Fig. 1. Structures of brodimoprim, hydroxybrodimoprim and the internal standard, Ro-11-3926.

The samples were injected using a Promis II autoinjector (Spark Holland, Emmen, Netherlands) and chromatographed on a reversed-phase guard column (Chrompack, particle size 30 μm, 10 mm × 3 mm I.D.) connected to a reversed-phase column, with reduced activity for analysis of basic compounds (Pecosphere-3 × 3 CR C_{18}, particle size 3 μm, 33 mm × 4.6 mm I.D., Perkin-Elmer, Norwalk, CT, USA). An old RP-18 column was connected between the pump and the autoinjector as a mobile-phase silica-saturating column.

Solvent system

The mobile phase was acetonitrile–25 m*M* phosphate buffer (23:77) plus 0.1% triethylamine (brought to pH 7.5 with 10 *M* KOH) at a flow-rate of 1.0 ml/min. The eluent was prepared every day before analysis, filtered and degassed under vacuum using a polycarbonate 0.4-μm membrane (Nucleopore, Cambridge, MA, USA).

Calibration, recovery, precision and accuracy

The linearity was evaluated by constructing a calibration curve with BDP and/or OH-BDP concentrations ranging from 100 to 2500 ng/ml in the various biological media. The linearity was tested by linear regression of the peak-height ratios of the drug or the metabolite/internal standard *versus* the concentration of each compound. The recovery was calculated by comparing the peak-height ratios of spiked samples of each medium with those obtained by direct injections of the same amount of both compounds. The intra-assay precision was verified at two concentrations (500 and 2000 ng/ml) for ten analyses. The inter-day precision was determined at the same two concentrations in five replicates. The accuracy was calculated as the percentage error from the true value by processing ten samples containing known amounts of both compounds.

RESULTS AND DISCUSSION

Fig. 2 illustrates typical chromatograms obtained in the assay of plasma, whole blood and urine. The retention times for BDP, OH-BDP and the internal standard were 1.1, 3.3 and 4.2 min, respectively. The best chromatographic conditions were obtained by adjusting the pH of the

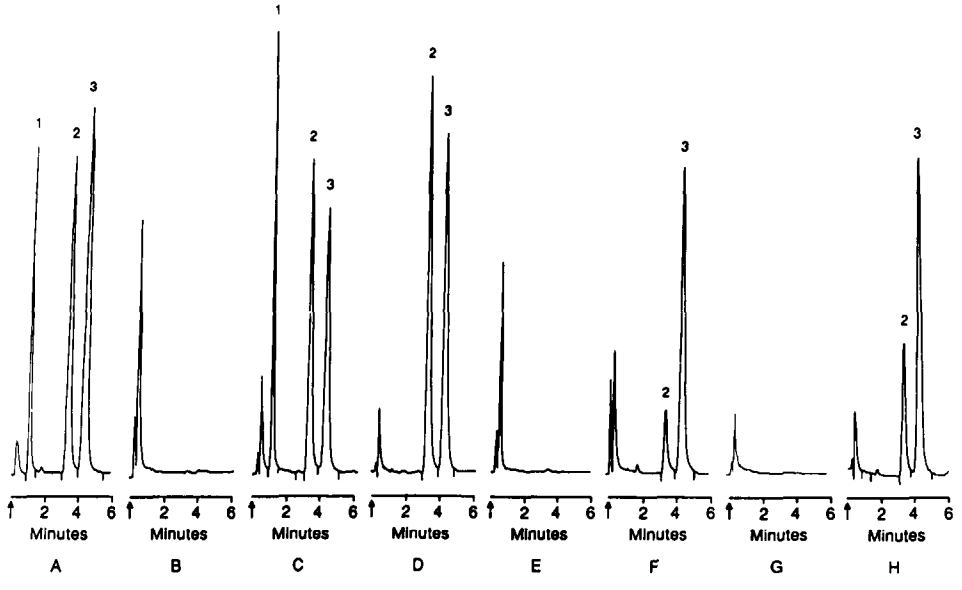

Fig. 2. Chromatograms of (A) standard mixture, (B) blank plasma, (C) plasma spiked with 200 ng each of BDP and OH-BDP, (D) hepatopathic patient's plasma 12 h after an oral dose of BDP (200 mg), (E) blank blood, (F) child's blood 1 h after an oral dose of BDP (10 mg/kg), (G) blank urine, (H) patient's urine collected between 6 and 12 h after an oral dose of BDP (200 mg). Peaks: 1 = OH-BDP; 2 = BDP; 3 = I.S.

mobile phase to exactly 7.5 and the acetonitrile–buffer ratio to 23:77, precisely. The pH of the mobile phase did not affect the retention time of the compounds in practice, but it was found to have a marked effect on the fluorescence: the higher the pH, the lower the quenching of the signal; below pH 3 the compounds were almost undetectable. The fluorescence titration of BDP (Fig. 3) in 25 m*M* phosphate buffer showed that its pK_a value is 7.13, indicating that the ionized molecule (existing in solution in a greater proportion when the pH is lower than 7.13) has a low intrinsic fluorescence. Titrations performed in an eluent buffered at different pH values gave similar results (pK_a 7.14). The theoretical curves were obtained as the best fit of the experimental points by the least-squares method [9]. Thus, the optimal pH of mobile phase was established to be 7.5, the highest compatible with a reversed-phase column.

The problem of possible dissolution of the stationary phase in the eluent, owing to the pH value, was successfully overcome by using a silica-saturating column.

The extraction recoveries obtained with this simple and rapid liquid–liquid extraction for BDP and the internal standard were 84 ± 3%, for OH-BDP 72 ± 4%. These values were similar for plasma, blood and urine samples.

However, the recovery was found to be dependent on the kind of chloroform used in the extraction. The chloroform stabilized with ethanol (Merck Code No. 2445) gave the highest recovery, but when chloroform stabilized with 2-methyl-2-butene (Merck Code No. 2444) was used the recovery was lower than 10%. This finding could be due to the possible adsorption of BDP and related compounds on the glassware, which can be overcome by use of extraction solvents containing small amounts (0.1–2%) of alcohols [10].

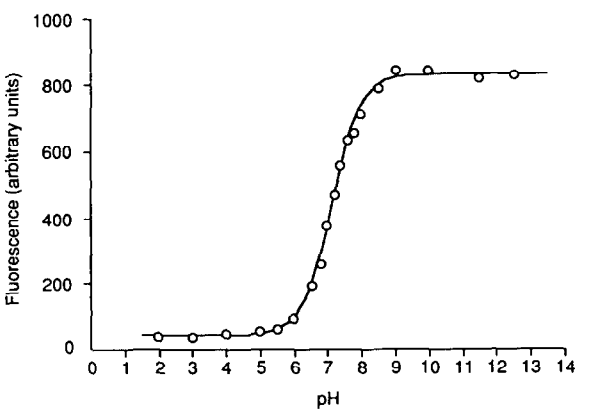

Fig. 3. Fluorescence titration of BDP. The flex point of the curve represents the pK_a value of the compound.

The within-day and day-to-day coefficients of variation for BDP and its metabolite in the three biological media are reported in Table I. These values were always below 6.5% for BDP and 9% for OH-BDP, thus demonstrating the good reproducibility of the method. The accuracy of the method was similar in the three media: for BDP 3.39, 4.08 and 3.21% and for OH-BDP 5.86, 7.31 and 8.12%, in plasma, blood and urine, respectively.

The standard curves of the drug and its metabolite in each biological fluid exhibited excellent linearity over the concentration range 100–2500 ng/ml; the coefficient of correlation was never less than 0.996.

The limit of detection, defined at a signal-to-

TABLE I

REPRODUCIBILITY OF THE HPLC METHOD

Concentration (ng/ml)	Biological fluid	Coefficient of variation (%)	
		BDP	OH-BDP
Within-day (n = 10)			
500	Plasma	3.15	6.90
	Blood	4.28	8.19
	Urine	3.46	8.05
2000	Plasma	1.32	4.09
	Blood	2.86	4.33
	Urine	3.08	5.03
Between-day (n = 5)			
500	Plasma	6.03	7.82
	Blood	5.75	8.87
	Urine	6.20	6.88
2000	Plasma	4.22	5.03
	Blood	4.90	6.18
	Urine	4.19	7.02

noise ratio of 3:1, was at least 5 ng/ml for both BDP and OH-BDP.

Total hydrolysis of glucuronides took place within 3 h at 37°C and at *ca.* pH 5.5, a pH value that is reached by adding the acetate buffer to the sample. A hydrolysis time of 4 h was chosen to allow complete deconjugation of the compounds. No degradation of either BDP or OH-BDP was observed during the deconjugation step, and both the recovery and the reproducibility of the method were similar to those obtained for non-conjugated compounds (data not shown).

Several blanks of plasma blood and urine, as well as plasma, blood and urine samples taken from either healthy volunteers or patients with mild renal insufficiency and hepatic diseases, were analysed according to the described procedure. No interfering endogenous components were observed. However, a small peak eluting at *ca.* 5.5 min was present in some plasma specimens, and a sharp peak eluting at 1.5 min was occasionally observed in plasma and blood samples that had been repeatedly thawed and frozen.

Fluorescence detection markedly increased the specificity and the sensitivity of the assay compared with UV detection at 290 nm (the absorbance maximum of BDP) thus allowing the use of small amounts of either plasma or blood. This step could be important, especially for pharmacokinetic studies in which many samples are needed and when ethical reasons (*e.g.* studies in pediatric patients) call for a reduced sample volume.

If an even higher detection limit is required, one should consider the use of chemically stable columns at high pH in order to obtain the maximum of the fluorescence, using an eluent buffered at pH 9–11.

On the other hand, when the sample volume is not a problem and a limit of detection of 80–100 ng/ml is adequate, UV detection could be used. In this case, a more easily available internal standard (*e.g.* clonazepam, pinazepam, nimesulide) is also suitable. However, care must be taken to test for possible interference from other drugs.

Drugs with physicochemical and fluorescence properties that could interfere with the assay were tested by injecting a stock solution of these compounds onto the column. Only β-blockers were detectable, though at very high concentrations, and they were either eluted in the solvent front or appeared markedly later than the BDP peak as very broad and tailed peaks. Furthermore, trimethoprim was eluted in the solvent front and flecainide gave a relatively good peak at *ca.* 15 min.

The validity of the assay was assessed by determining the BDP plasma concentration–time profile after a single oral dose (200 mg) of the drug to a hepatopathic patient (Fig. 4). The method showed adequate sensitivity and precision for monitoring BDP and its pharmacologically active metabolite, OH-BDP.

The use of a short column (3.3 cm), deactivated for basic compounds, allowed a quick analysis and resulted in sharper peaks with almost no tailing. Their shapes were further improved by adding triethylamine (0.1%) to the eluent. A similar performance was not obtained using standard C_{18} columns, even by increasing the molarity of the buffer and/or the percentage of the amine modifier.

Dissolving the extracted dry samples in eluent proved to be a very important step for the overall performance of the assay, since BDP and analogous substances have a relatively poor solubility in the eluent. Complete solubilization, and thus

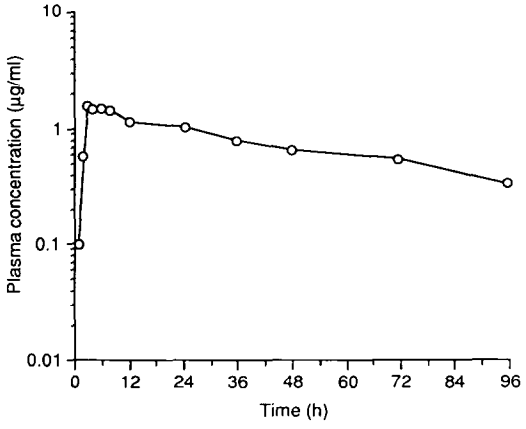

Fig. 4. Plasma BDP concentration–time curve following oral administration of 200 mg to a hepatopathic patient.

reproducible results, were obtained by leaving the dissolved samples for *ca.* 1 h at room temperature.

In conclusion, the ease and rapidity of sample handling and the analytical specificity for BDP and OH-BDP make this procedure useful for monitoring both compounds in small volumes of biological fluids in particular when pharmacokinetic studies involve "at risk" patients.

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