Journal of Chromatography, 579 (1992) 355–360
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6445

Short Communication

Determination of trimethoprim and its oxidative metabolites in cell culture media and microsomal incubation mixtures by high-performance liquid chromatography

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(First received February 25th, 1992; revised manuscript received May 7th, 1992)

ABSTRACT

A high-performance liquid chromatographic method is presented for the determination of trimethoprim (TMP), 3′-hydroxy-TMP, 4′-hydroxy-TMP, α-hydroxy-TMP and two TMP N-oxides. The last two metabolites appear to decompose on liquid extraction. TMP and its oxidative metabolites are separated using a C_{18} radial-compression column and quantified by UV detection at 230 nm. Calibration curves are linear from 0.5 to at least 50 μM. The limit of detection is 0.05–0.15 μg/ml. In *in vitro* rat liver metabolism studies, 3′- and 4′-hydroxylation of TMP appear to be important metabolic pathways whereas TMP N-oxides are minor metabolites.

INTRODUCTION

Trimethoprim (TMP, 2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine) is a dihydrofolate reductase inhibitor, used as an antibacterial drug in human and veterinary medicine [1]. TMP may be used alone or in combination with several sulphonamide drugs. The drug is of special interest because of its extensive veterinary use in food-producing animals, resulting in increasing concern about residues in such foods [2]. Upon ad-

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ministration to humans or animals the drug is metabolized, the pathways and elimination rates depending on the species. Several oxidative metabolites, resulting from TMP mono-oxygenation, are known [1] (Fig. 1). The metabolites and the parent compound may be converted into glucuronides or sulphates.

Several high-performance liquid chromatographic (HPLC) methods for the analysis of TMP alone or in sulphonamide combinations have been published [3–10]. In these papers, TMP metabolites were not included. Early papers on the metabolism of TMP commonly describe a thin-layer chromatographic (TLC) separation of [^{14}C]TMP and its metabolites, followed by scintillation counting [11–15]. Other detection methods used after TLC separation were spectrofluorimetry [16] and polarography [17].

Analysis of TMP and some of its metabolites has been performed using normal-phase HPLC [18,19]. Consequently, several interesting reversed-phase HPLC methods [20–22] have been published. These methods led to the separation and quantitation of TMP and its two demethylation products. Other oxidative metabolites were not included.

The aim of the present study was to develop a simple and sensitive methodology for the determination of TMP and its oxidative metabolites, formed in rat liver. The method was applied to the analysis of samples obtained from incubation media for drug metabolism studies *in vitro*, including hepatocyte cultures and incubations of liver microsomes.

EXPERIMENTAL

Reagents, solvents and materials

TMP was obtained from Sigma (St. Louis, MO, USA). TMP metabolites were kindly donated by Drs. W. F. Rehm and T. A. Graser, F. Hoffmann-La Roche (Basle, Switzerland) and diaveridine by Dr. C. Lustig, Coopers Agrovet (Haarlem, Netherlands).

Triethylamine (Gold Label) and limpet acetone powder (LAP, a crude extract with glucuro-

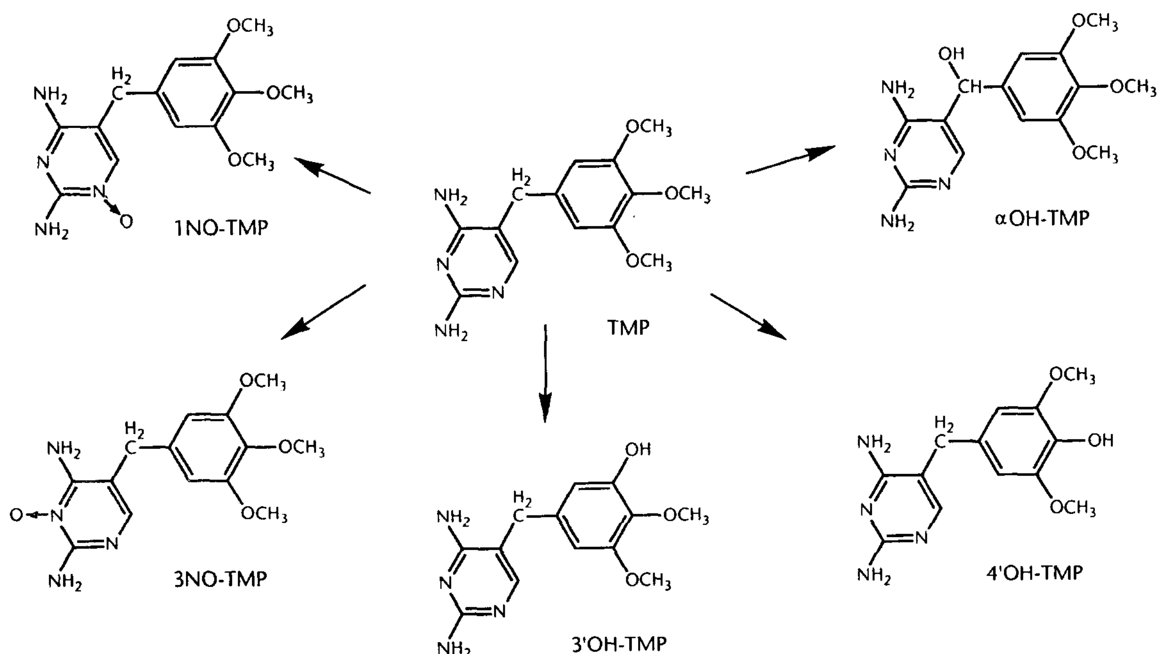

Fig. 1. Oxidative metabolism of trimethoprim (TMP). Demethylation at the 3'- or 4'-position (resulting in 3'-OH- and 4'-OH-TMP, respectively), hydroxylation of the carbon-atom linking the two rings (resulting in α-OH-TMP) and pyrimidine nitrogen oxidation (resulting in either 1-NO-TMP or 3-NO-TMP) may occur.

nidase and sulphatase activity) were purchased from Sigma. NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase (140 U/mg, 1 mg/ml suspension) were from Boehringer (Mannheim, Germany).

HPLC-grade acetonitrile was from Rathburn (Walkerburn, UK). Demineralized water was filtered through a Milli-Q device from Millipore (Etten-Leur, Netherlands). KH_2PO_4, Na_2HPO_4, diethyl ether, dichloromethane and 2-propanol (all p.a. grade) were obtained from Baker (Deventer, Netherlands).

All other chemicals were of the best available grade.

Apparatus

The chromatographic system consisted of a Promis autosampler from Spark Holland (Emmen, Netherlands), a 400S solvent-delivery system and a 783A programmable absorbance detector set at 230 nm from Applied Biosystems (Maarssen, Netherlands), and an SP4290 integrator from Spectra-Physics (San Jose, CA, USA).

The stationary phase was a 100 × 8 mm μBondapak RP C_{18} Radial-Pak cartridge column (particle size 5 μm) equipped with a 4 × 6 mm μBondapak C_{18} guard column (particle size 5 μm) from Waters (Etten-Leur, Netherlands). The mobile phase consisted of 0.05 *M* aqueous potassium phosphate, containing 0.2% (v/v) triethylamine, adjusted to pH 6.0, and acetonitrile in a ratio of 90:10 (v/v). It was filtered over a 0.45-μm filter (Millipore) and degassed under vacuum before use. The flow-rate was 1.8 ml/min at a back-pressure of 80 bar. Analyses were performed at ambient temperature.

Animals and in vitro incubations

Male Wistar rats (Crl:WI(Br), Charles River, Sulzfeld, Germany), aged 12 weeks (350–400 g), were used throughout this study. Microsomes were prepared by differential ultracentrifugation of homogenized rat liver [23]. Microsomes were incubated with 1.0 m*M* TMP during 1 h at 37°C in 2.5 ml of a 0.125 *M* phosphate buffer (pH 7.4) containing 1.0 mg of NADPH, 1.5 mg of glucose-6-phosphate, 1 μl (0.14 U) of glucose-6-phosphate dehydrogenase and 0.6 mg of $MgCl_2 \cdot 6H_2O$.

Rat hepatocytes were isolated essentially as described by Seglen [24], and cultured in Waymouths' MB 752/1 (Gibco, Breda, Netherlands). After preincubation for 2 h, medium was changed and cells were incubated with 0.5 m*M* TMP in serum-free medium for 2 h.

The content of cytochrome P450 and protein in the microsomes and hepatocyte cultures were measured according to Rutten *et al.* [23].

Sample preparation

Microsomal incubation samples (3.0 ml) were mixed with 20 μl of a 1 m*M* solution of diaveridine (internal standard) and 1.0 ml of an aqueous solution of 6.2% (w/v) boric acid and 7.5% (w/v) potassium chloride, adjusted to pH 9.0 with 4 *M* NaOH. After saturation with *ca.* 1 g of ammonium sulphate, samples were extracted twice with 4.0 ml of diethyl ether–dichloromethane–2-propanol (60:40:0.5, v/v/v), previously used for sulphonamide analysis [25]. The combined organic fractions were evaporated to dryness under nitrogen. Dry residues were redissolved in 300 μl of the mobile phase, and 20 μl were injected for analysis.

As an alternative to liquid extraction, microsomal incubation mixtures were deproteinized by the addition of 0.5 ml of 40% (w/v) $ZnSO_4$ and 1 ml of saturated $Ba(OH)_2$ solution. The mixture was centrifuged at 500 *g* for 10 min.

Hepatocyte culture medium samples (2.5 ml) were mixed with 1 ml of 0.5 *M* acetate buffer (pH 4.5) and *ca.* 20 mg of LAP. Samples were incubated for 3 h in a water-bath at 37°C. Further handling was identical with that for microsomal incubation samples.

RESULTS AND DISCUSSION

Chromatography

Analysis of a reference mixture (Fig. 2) shows separation of TMP, its metabolites and diaveridine to be complete. Tailing of TMP and, especially, the two N-oxide peaks could not be fully prevented by addition of the capping agent

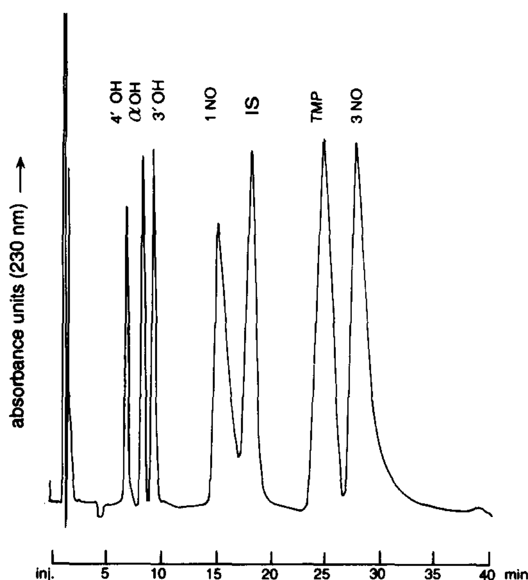

Fig. 2. Chromatogram (a.u.f.s. = 0.016) of a 20-μl injection of a mixture containing from 10 μM (hydroxylated metabolites) to 40 μM (TMP) of reference compounds: IS = internal standard (diaveridine); other peak labels refer to the structures in Fig. 1. The retention of α-OH-TMP is slightly pH-dependent.

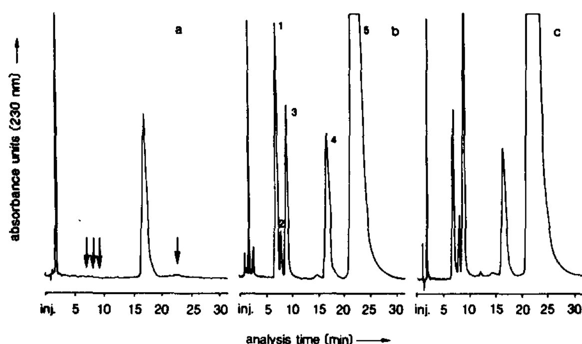

Fig. 3. Representative sample chromatograms (a.u.f.s. = 0.016) from (a) a blank sample, (b) a hepatocyte culture medium (2 h incubation with 0.5 m*M* TMP) and (c) a microsomal incubation mixture (1 h incubation with 1 m*M* TMP). Peaks: 1 = 4'-OH-TMP (in (b), *ca.* 90 ng/20 μl); 2 = α-OH-TMP (20 ng/20 μl); 3 = 3'-OH-TMP (60 ng/20 μl); 4 = diaveridine (internal standard); 5 = TMP.

triethylamine to the eluent. The use of a deactivated column for basic compounds appears to solve these problems and to result in a more rapid elution (Dr. M. Alvinerie, personal communication).

α-OH-TMP is eluted between 4'-OH-TMP and 3'-OH-TMP. Its position in the chromatogram is slightly pH-dependent: a small increase (≤ 0.1 unit) of the pH decreases its retention relative to 4'-OH-TMP and 3'-OH-TMP. The latter two metabolites are not sensitive to similar changes in the pH. This feature was used to optimize the separation of these three polar metabolites.

Blank samples from both cell cultures and microsomal incubations yielded straight baselines with no interfering peaks (Fig. 3a). Analysis of cell culture medium (Fig. 3b) or microsomal incubation mixtures (Fig. 3c) yielded sharp and fully separated peaks of 3'-OH-TMP, 4'-OH-TMP, and α-OH-TMP. No N-oxides were detected.

Diaveridine, a structural analogue of TMP, was chosen as the internal standard. The extraction recovery (see below) is not significantly different from that of TMP or its hydroxylated metabolites. Diaveridine elutes between TMP and 1-N-oxide (Figs. 1 and 2). In our studies, no other peaks occurred in this area.

Sample extraction and recovery

Media from hepatocyte cultures and incubation buffers with microsomes are relatively clean matrices, compared with plasma or meat. For the latter two, liquid extraction is the usual method to isolate the compounds of interest. For *in vitro* media, simple deproteinization preceding analysis is sometimes sufficient. In our method, liquid extraction was included mainly because it allows concentration of the sample and enables more complex matrices to be used. In parallel *in vivo* studies, 300 μl of plasma were extracted and the residue was reconstituted in 150 μl of eluent; injection of 20 μl into the column allowed the quantitation of 3'-OH-TMP, 4'-OH-TMP and α-OH-TMP (results not shown).

Extraction recoveries were determined using cell culture medium and incubation mixtures for microsomes spiked with TMP, its metabolites and diaveridine in concentrations ranging from 1 to 100 μ*M*. Recoveries ($n=6$; mean ± S.D.) were: TMP, 88.1 ± 2.4%; 3'-OH-TMP, 80.4 ± 7.2%; 4'-OH-TMP, 76.3 ± 6.9%; α-OH-TMP; 79.2 ± 6.4%; and diaveridine, 86.0 ± 4.2%.

Recovery TMP N-oxides

TMP N-oxides (Fig. 1) yielded recoveries of less than 30%. N-Oxidation of tertiary amines may result in extremely water-soluble metabolites that resist extraction into non-polar solvents [26]. Nevertheless, Watkins and Gorrod [18] reported the extraction of TMP N-oxides with ethyl acetate. Using the present method, less than 20% of the apparently non-extracted N-oxides could be detected. This observed loss of TMP N-oxides indicates decomposition. Especially in alkaline solutions, chemical decomposition of tertiary N-oxides is known to occur [26]. Simple deproteinization instead of liquid extraction is an alternative for *in vitro* studies. Decreasing the extraction pH was not considered because it lowers the recovery of TMP and its hydroxylated metabolites (all bases, $pK_a = 7.2$). The latter metabolites are of far greater importance than TMP N-oxides, which appear to be minor metabolites *in vitro*, when assessed in non-extracted samples. These results (not shown) are in accordance with *in vivo* studies in rats by Meshi and Sato [12].

Linearity and limit of detection

For TMP, diaveridine, 3′-OH-, 4′-OH- and α-OH-TMP, calibration graphs were constructed using spiked blank samples for analysis. Linear correlations ($r = 0.991$–0.999) were found for concentrations ranging from 0.5 μ*M* to 50 μ*M* (0.15–15 μg/ml). For concentrations exceeding 1 μ*M*, the inter-assay precision (four consecutive assays) was better than 5% (coefficient of variation, C.V.). The accuracy (C.V.) was better than 6% (corrected for internal standard).

In the analytical system described, the limit of detection was 0.05 μg/ml for the three hydroxylated TMP metabolites and, in part owing to tailing, only 0.15 μg/ml for the parent compound, TMP.

TABLE I

TRIMETHOPRIM METABOLISM IN RAT HEPATOCYTES AND RAT LIVER MICROSOMES

Hepatocytes were incubated with 0.5 m*M* TMP for 2 h; microsomes were incubated with 1.0 m*M* TMP for 1 h. Experiments were performed in triplicate, using livers from three different animals. Results are expressed as means of three individual rats ± S.D. N-Oxides could not be detected.

	Hepatocytes	Microsomes
Cytochrome P450 (pmol-mg protein)	154.0 ± 21.2	841.8 ± 88.4
Metabolite formation (pmol/pmol P450 per h)		
3′-OH-TMP	156.0 ± 16.0	27.1 ± 1.6
4′-OH-TMP	174.7 ± 19.5	20.0 ± 3.7
α-OH-TMP	22.1 ± 4.2	4.0 ± 0.6

Application in TMP metabolism studies

The biotransformation of TMP was studied using rat liver microsomes and cultured rat hepatocytes. Results are shown in Table I. A remarkable difference in the ratio of 3′-OH-TMP to 4′-OH-TMP between hepatocytes and microsomes is found. These results show that TMP biotransformation in microsomes is different, qualitatively as well as quantitatively, from that in intact hepatocytes. In microsomal incubations a high substrate concentration was chosen to achieve maximal enzymatic activity conditions, whereas such high concentrations give rise to cytotoxicity in hepatocytes (results not shown). Furthermore, in intact cells, processes such as uptake, intracellular distribution, cofactor supply, and the arrangement of biotransformation enzyme systems will all contribute to the metabolic fate of compounds.

For both *in vitro* systems, hydroxylated TMP metabolites can easily be quantified by the HPLC method described here. Since the extraction method is also appropriate for blood plasma, urine and other complex matrices, the method has applicability for TMP studies *in vivo* as well.

ACKNOWLEDGEMENT

The authors thank Dr. M. Alvinerie (INRA, Toulouse, France) for valuable discussions.

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