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DETERMINATION OF TRIMETHOPRIM METABOLITES INCLUDING CONJUGATES IN URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH COMBINED ULTRAVIOLET AND ELECTROCHEMICAL DETECTION

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SUMMARY

A high-performance liquid chromatographic method for the determination of trimethoprim metabolites in pig urine was developed. The metabolites — glucuronic acid and sulphuric acid conjugates of phenolic metabolites formed by demethylation of trimethoprim — were quantitated after treatment of urine with β-glucuronidase (*Escherichia coli*). The sulphuric acid conjugate was not susceptible to enzymatic hydrolysis and was therefore assayed as the conjugate by use of ion-pair chromatography on the reversed-phase column. In order to find suitable conditions for enzymatic hydrolysis of the glucuronides, the conjugates were obtained by gel chromatography of urine from a [^{14}C]trimethoprim-treated pig

INTRODUCTION

Conjugated drug metabolites are often determined indirectly after enzymatic hydrolysis or after cleavage by acid because of difficulties in obtaining standards of the native conjugates. Furthermore, the deconjugated metabolites formed by hydrolysis may be more convenient for a chromatographic analysis, because these products as a rule are less hydrophilic and thus more easy to retain on, for instance, a reversed-phase high-performance liquid-chromatographic (HPLC) column. Hydrolysis of the conjugates may give rise to erroneous results if the hydrolysis is not completed or if some of the conjugates are not substrates of the chosen enzyme.

Trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] (TMP), a synthetic antibacterial, has been reported to be metabolized to six different compounds [1—3] (Fig. 1) in various species. M_1 and M_4 are partly conjugated

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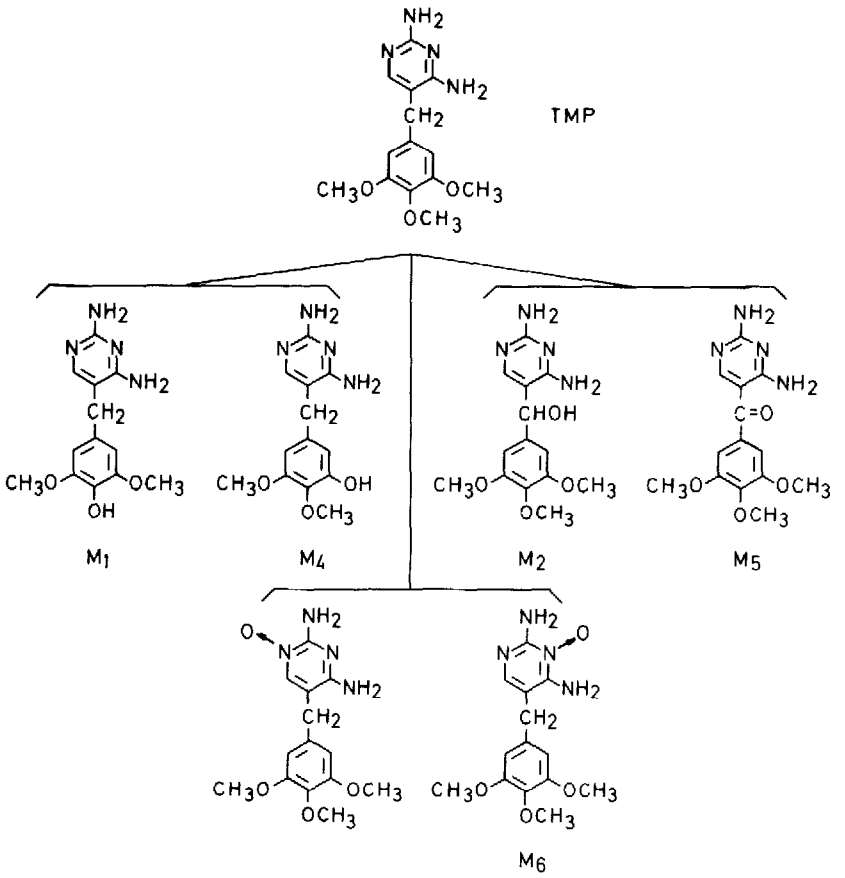

Fig. 1. Structures of trimethoprim and metabolites formed by bio-oxidation.

with glucuronic acid or sulphuric acid. Nielsen and Dalgaard [4] have isolated an M_4-sulphate from pig and goat urine. These authors have determined the structure and reported a unique resistance to various arylsulphatases.

Several analytical methods for the quantitation of TMP in body fluids have been used, including microbiological assay [5], spectrofluorimetry [6, 7], autoradiography [1], differential pulse polarography [8, 9], thin-layer chromatography with densitometry [10—12], gas—liquid chromatography [13], and HPLC [14—25]. Only a few methods include quantitation of TMP metabolites [1,8,10,11], and in all cases the determinations were done on phase I and/or deconjugated metabolites. Methods concerning the quantitation of the sulphuric acid conjugate have not previously been published.

The aim of this investigation was to develop an HPLC method which allowed the simultaneous determination of bio-oxidation products (phase I metabolites) and conjugated metabolites of TMP in urine.

In order to optimize the conditions necessary for complete hydrolysis of conjugates, fractions containing M_1- and M_4-glucuronides were isolated from urine from a pig after intravenous administration of [^{14}C]TMP. These conjugates were in turn added to blind pig urine for recovery studies.

MATERIALS AND METHODS

Chemicals

TMP was a gift from Syntex, Grindsted, Denmark. M_1 and M_4 were synthesized according to an earlier described procedure [26]. [^{14}C]TMP and urine from a pig which had been given 400 mg of [^{14}C]TMP (0.5 μCi/mg) intravenously were donated by the Department of Pharmacology and Toxicology, Royal Veterinary and Agricultural University, Copenhagen, Denmark. Glucuronides of M_1 and M_4 and the sulphate conjugate of M_4 were isolated in fractions as described below. Standardization of M_4-sulphate was done by liquid scintillation counting of a chromatographically (HPLC) pure fraction obtained by the procedure described below. A standard of M_4-sulphate was furthermore kindly donated by the Department of Pharmacology and Toxicology, Royal Veterinary and Agricultural University. β-Glucuronidase (*Escherichia coli*) was obtained in preweighed vials of 1000 sigma units from Sigma (St. Louis, MO, U.S.A.) and diluted with 1 *M* potassium dihydrogen phosphate, pH 6.8. Arylsulphatase (crude solution from *Helix pomatia*) was also obtained from Sigma. All other chemicals were of analytical grade.

High-performance liquid chromatography

HPLC was performed with (I) a Waters Model 6000 solvent delivery system equipped with a Rheodyne injection valve (100 μl loop), and a Waters Model 440 absorbance detector (254 nm), and (II) Micromeritics equipment consisting of a Model 750 solvent delivery system equipped with a Model 725 autoinjector, and a Model 786 variable-wavelength detector operated at 254 nm. Both systems were equipped with an electrochemical detector consisting of a Model 656 detector cell and a Model 641 VA detector operated in the oxidative mode (Metrohm, Switzerland). Working electrode: glassy carbon. Reference electrode: silver/silver chloride. Auxiliary electrode: glassy carbon. The detectors were connected in series with the electrochemical detector downstream from the ultraviolet (UV) detector. An Omniscribe dual-channel recorder (Houston Instruments, Houston, TX, U.S.A.) was employed. A stainless-steel HPLC column (Knauer, 250 × 4.6 mm I.D.) packed with LiChrosorb RP-18 (5 μm) particles (Merck, Darmstadt, F.R.G.) was used. The mobile phase consisted of 85 parts of 0.1 *M* potassium dihydrogen phosphate adjusted to pH 7.5 with potassium hydroxide and 15 parts of acetonitrile. Tetrabutylammonium hydrogensulphate (TBA) was added to the mobile phase giving a concentration of $0.7 \cdot 10^{-3}$ *M*. The procedure was carried out at a flow-rate of 1.0 ml/min (250 bars).

Isolation of conjugated TMP metabolites

Gel chromatographic separation was mainly done according to the procedure of Nielsen and Dalgaard [4]. Radioactivities of the urine fractions were determined by mixing 0.5 ml of urine and 3 ml of Ria-Luma (Lumac Systems, Basel, Switzerland) and counting to 1% standard deviation on a Tricarb scintillation counter (Packard Instruments).

The urine sample was evaporated in vacuo at 40°C to a volume of 30 ml. The fraction was centrifuged for 1—2 min at ca. 1200 *g* and the supernatant

applied on a Sephadex G-25 column (100 × 2.6 cm I.D.) (Pharmacia Fine Chemicals, Uppsala, Sweden), eluted with water by use of a Bifok, FIA-08, peristaltic pump. Fractions of 25 ml were collected and 0.5 ml from each fraction was taken for liquid scintillation counting. The column was further eluted with 0.1 *M* ammonium acetate followed by 1 *M* ammonium acetate. Radioactive fractions (almost exclusively from the water eluates) were analysed by HPLC system I using detector settings at (+) 700 mV vs. Ag/AgCl and 254 nm and peaks were collected for liquid scintillation counting. Radioactivity eluted from Sepahdex G-25 was grouped into two parts, the last eluted group containing TMP and phase I metabolites (almost exclusively TMP). The other was applied to a Sephadex G-10 column (33 × 2.6 cm I.D.) and eluted with water. Fractions of 10 ml were collected for liquid scintillation counting giving two groups of fractions containing radioactivity. Radioactive fractions were again analysed by the HPLC system and UV-absorbing compounds were collected for liquid scintillation counting. The last eluted group of fractions from Sephadex G-10 consisted solely of M_4-sulphate. The other group of fractions showed on the HPLC UV trace two compounds which by liquid scintillation appeared to be radioactive.

Hydrolysis of conjugated trimethoprim metabolites

One millilitre of the first eluted group of fractions from Sephadex G-10 containing radioactivity and 100 μl of β-glucuronidase (*E. coli*, 200 sigma units) were incubated at 38°C. The hydrolysis was followed by HPLC using UV detection at 254 nm and electrochemical detection (ED) at (+) 700 mV vs Ag/AgCl.

One millilitre of the second eluted group from Sephadex G-10 containing radioactivity (M_4-sulphate), 100 μl of arylsulphatase (*Helix pomatia*, 400 sigma units), 500 μl of 0.2 *M* potassium dihydrogen phosphate, pH 5, and 100 μl of water, 0.01 *M* barium chloride, or 0.1 *M* barium chloride were incubated at 38° C. The progress of hydrolysis was monitored by HPLC.

Enzymatic hydrolysis of the glucuronides was investigated in the pH range 5.5—7.8 with a diluted urine sample rich in M_1-and M_4-glucuronides as substrates. Conditions were as described below.

Quantitation of metabolites

Urine was diluted ten times with distilled water and 10 μl of diluted urine were injected into HPLC system II for quantitation of M_1, M_4, and M_4-sulphate. Quantitation of M_1-glucuronide and M_4-glucuronide was done by incubating 1 ml of diluted urine and 100 μl of glucuronidase (*E. coli*, 100 sigma units) at 38°C overnight. The hydrolysate was injected into the HPLC system and the amounts of M_1-glucuronide and M_4-glucuronide were calculated by subtraction of the M_1 or M_4 concentration, respectively, before and after enzymatic hydrolysis corrected for differences in dilutions. Concentrations of the metabolites were determined by comparing peak heights of aqueous standards with peak heights of the urine samples. Recovery studies were done by adding standards of M_1- and M_4-glucuronides obtained from the gel chromatographic separation to urine and subjecting these samples to the described assay.

RESULTS AND DISCUSSION

Introduction of an ion-pairing agent into the chromatographic system resulted in a decrease of the capacity factor for TMP, M_1 and M_4. When a new column was employed the TBA concentration had to be adjusted with respect to both the M_4-sulphate and TMP, M_1 and M_4 (a well known problem for reversed-phase columns; see, for example, ref. 27). The relationship between the ion-pairing agent and the capacity factors for TMP, M_1 and M_4 are outlined in Fig. 2. This behaviour of non-ionic components in reveresed-phase ion-pair HPLC has recently been described thermodynamically by Stranahan and Deming [28] who ascribed the phenomenon to an alteration in the interfacial tension between the mobile phase and an adsorbed phase consisting of a monolayer that is adsorbed on the stationary phase.

Hydrolysis of the radioactive Sephadex G-10 fraction containing M_4-sulphate did not take place within 24 h. This was in agreement with earlier findings [4]. A chromatogram of the other radioactive fraction showed two peaks on the UV trace, which disappeared within 4 h of enzymatic hydrolysis giving M_1 and M_4. The compounds corresponding to the two peaks on the UV trace were not detectable by ED, indicating that a free phenol group or other easily oxidized group was not present. However, a chromatogram of the hydrolysate showed two peaks with the same retention volume and UV/ED ratio as M_1 and M_4 indicating that the peaks probably were due to M_1-glucuronide and M_4-glucuronide, respectively (Fig. 3). In the investigations of a suitable pH for hydrolysis of the glucuronides, total hydrolysis appeared to take place within 1 h in the pH interval 5.5—7.0. Above pH 7 the hydrolysis appeared to be complete with 1 h, but the yield of products was lower than for samples treated at pH $\leqslant$ 7.0. So far no explanation can be given for this behaviour. Recoveries of M_1 and M_4 after hydrolysis of their glucuronides are outlined in Table I.

Investigations of the optimal ED setting were done. The detector response as

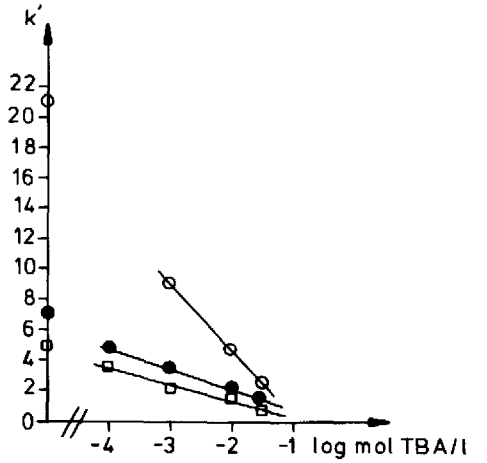

Fig. 2. Reversed-phase ion-pair liquid chromatography of TMP (○); M_1 (□); and M_4 (●). Column: 25 cm LiChrosorb RP-18 (5 μm). Mobile phase: TBA in acetonitrile—0.1 *M* potassium dihydrogen phosphate adjusted to pH 7.5 with potassium hydroxide (15:85).

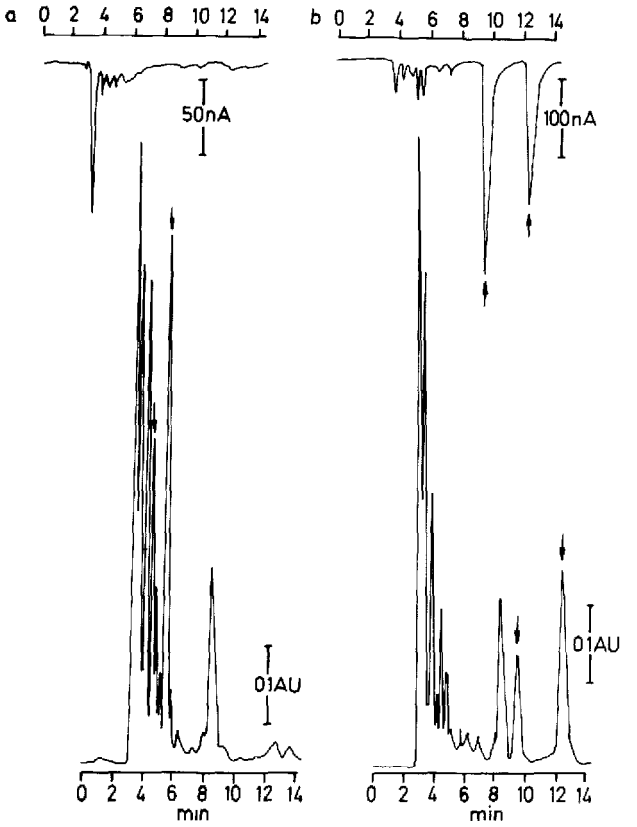

Fig. 3. Chromatograms of a Sephadex G-10 fraction containing M_1- and M_4-glucuronides before (a) and after (b) hydrolysis with β-glucuronidase (*E coli*). Upper trace: ED at (+) 700 mV vs. Ag/AgCl. Lower trace· UV detection at 254 nm. Peaks corresponding to radioactive compounds are indicated with arrows.

TABLE I

RECOVERY OF M_1 AND M_4 AFTER ENZYMATIC HYDROLYSIS OF THEIR GLUCURONIDES IN URINE

Concentrations of M_1 and M_4 added as glucuronides are determined by liquid scintillation counting. Recovery data represent mean ± S.D for five determinations of samples at each concentration level obtained by HPLC after enzymatic hydrolysis

Concentration of M_1 as glucuronide (ppm)	Recovery (%)	Concentration of M_4 as glucuronide (ppm)	Recovery (%)
0.3	108 ± 7	0.5	105 ± 6
1.2	96 ± 4	1.9	104 ± 2
6.0	98 ± 4	9.4	104 ± 2

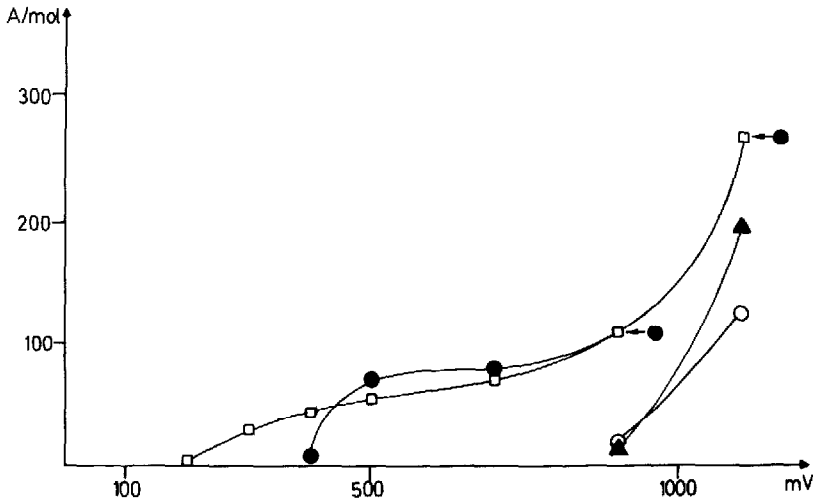

Fig. 4. Applied potential (positive vs. Ag/AgCl) vs. output current. Output current is determined by injecting the same sample of M_1 (□); M_4 (●); M_4-sulphate (▲); and TMP (○) at varying potentials.

a function of applied voltage is shown in Fig. 4. It is seen that in the range of approximately (+) 500—800 mV vs. Ag/AgCl the sensitivity to M_1 and M_4 is not enhanced. It is therefore most convenient to use (+) 500 mV to obtain maximum selectivity. A minor drawback of selecting (+) 500 mV is that the current yield for M_4 is strongly dependent on the applied potential in the range (+) 400—500 mV. In some instances the detector response of M_4 appeared to be rather low (compare Figs. 4 and 5). Alteration in the M_4 response was only seen when the column and the composition of the mobile phase were changed.

In this work standards were injected for every 5—10 samples in order to check the detector response. Within-day variations of the ED response were usually in the order of 5%.

At (+) 500 mV and 254 nm a linear relationship was found between the concentration of metabolites and their peak heights in the concentration range studied (5—250 ng on-column sample weight). The detection limit in diluted urine defined as twice the baseline noise, was approx. 0.1 ppm using UV detection (254 nm) for M_1, M_4, and M_4-sulphate, and approx. 0.2 ppm for TMP. In the case of ED at (+) 500 mV, the detection limits for M_1 and M_4 were approx. 0.1 ppm and approx. 0.1—0.2 ppm, respectively, while conjugates and TMP were not detected at (+) 500 mV. The detector response could be drastically improved by using a higher oxidation potential (Fig. 4). This however, caused unacceptable interference from other substances in urine making extraction of the metabolites necessary.

Extraction of M_1 and M_4 could be performed with ethyl acetate at pH 8 but simultaneous quantitation of M_1, M_4, and M_4-sulphate was not possible by this procedure due to the ionic property of M_4-sulphate. In this study ED was therefore mainly included for qualitative detection, but was a valuable supplement to the specificity of the UV detector in cases of interfering peaks on the UV trace. Chromatograms of several blind urine samples from different pigs were obtained. In some cases peaks interfering with M_1 and M_4 were seen

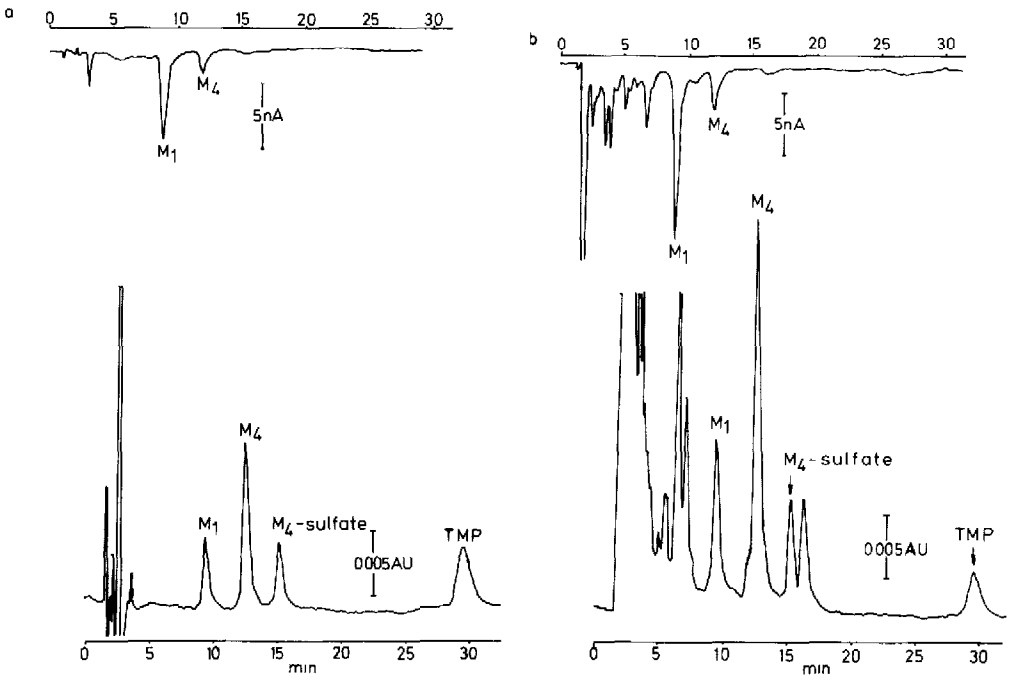

Fig. 5 (a) Standard chromatogram of M_1 (44 ng), M_4 (99 ng), M_4-sulphate (50 ng) and TMP (79 ng). (b) Chromatogram of a hydrolysed urine sample from a 2-month-old pig 2 h after administration of TMP. Upper trace: ED at (+) 500 mV vs. Ag/AgCl. Lower trace: UV detection at 254 nm.

on the UV trace. In such cases ED could always be used for quantitation. A typical chromatogram of urine from a pig after administration of TMP is shown in Fig. 5.

Although this assay includes the possibility of quantitating TMP, this compound would, because of the relatively large retention volume, be better analysed in another system [25]. On gel chromatographic separation only two metabolites were detected (M_1 and M_4); these metabolites were in turn conjugated to glucuronic acid and sulphuric acid in the case of M_4, and to glucuronic acid in the case of M_1. These findings were in agreement with earlier published results [4].

A detailed study of the metabolism of TMP in pigs during the first weeks after birth will be reported elsewhere [29].

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