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Note

Gas chromatographic determination of trimethoprim in plasma during high-dose trimethoprim–sulfamethoxazole treatment for *Pneumocystis carinii* pneumonia

U. ERNEMANN, D. SCHIRMER, A. WÖBER, P. LANGMANN, K. WARZECHA, R. JOERES, H. KLINKER and E. RICHTER*

Medizinische Universitatsklinik, Josef-Schneider Strasse 2, 8700 Würzburg (F.R.G.)

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The antibiotic combination trimethoprim–sulfamethoxazole (TMP–SMX) is the drug of choice for the treatment of *Pneumocystis carinii* pneumonia in patients with the acquired immunodeficiency syndrome (AIDS) [1]. Intravenous or oral doses of trimethoprim, 15–20 mg/kg body weight per day, and sulfamethoxazole, 75–100 mg/kg body weight per day, are applied every 6 h. Under this high-dose treatment a high frequency of severe side-effects has been reported [2], with leucopenia and thrombocytopenia being the most serious adverse reactions, occurring in up to 30–50% of AIDS patients treated with TMP–SMX [3]. In order to achieve maximal efficacy with the least possible side-effects, Sattler et al. [3] proposed to adjust the dosage of TMP–SMX to maintain serum trimethoprim levels at 5–8 mg/l.

The clinical task of monitoring plasma levels requires the development of a sensitive method for the quantitation of TMP in small amounts of plasma. Reduction of sample size is important in order to save plasma for further investigations, for example the determination of concurrent sulfonamide levels.

Several authors have described methods for the determination of TMP in biological fluids. Assays based on microbiological [4], differential pulse polarographic [5] and spectrofluorimetric [6] methods have been reported. The

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most widely used method in recent years has been high-performance liquid chromatography (HPLC). Van der Steuijt and Sonneveld [7] have published an HPLC method for the simultaneous analysis of TMP and SMX, using plasma samples of 1 ml. Land et al. [8] have proposed a gas chromatographic (GC) analysis using 2-propanol as solvent. This paper describes a sensitive GC assay with nitrogen–phosphorus detection using methanol as solvent. It is capable of determining TMP in small plasma samples of 0.2 ml, even with considerable comedication.

EXPERIMENTAL

Chemicals and drugs

Trimethoprim, 2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine, was obtained from Sigma (St. Louis, MO, U.S.A.). Mepivacaine (Scandicain®) was purchased from Astra Chemicals (Wedel Holstein, F.R.G.). Chloroform (pro analysi) and methanol were purchased from Merck (Darmstadt, F.R.G.).

Gas chromatography

A Hewlett-Packard gas chromatograph 5880A equipped with a nitrogen–phosphorus detector was used. For calculation a GC-Terminal HP 5880 was attached.

Injections were carried out in the splitless mode using a direct on-column glass liner, connected with a 15 m × 0.54 mm I.D. fused-silica capillary column coated with DB5 (0.0015 mm film thickness; Carlo Erba Instruments). The helium gas flow-rates were: carrier, 7.7 ml/min; auxiliary gas, 22.3 ml/min; septum sweep, 1.5 ml/min; split, 60 ml/min. Detector gases flow-rates were: hydrogen, 2.83 ml/min; synthetic air, 52.3 ml/min.

The temperatures were 250 °C at the injection port and 300 °C at the detector. The column temperature was programmed from an initial value of 150 °C to a final value of 280 °C in steps of 10 °C. The overall time was 1 min. The post-value was 300 °C, and the post-time was 2 min.

Methods

TMP-SMX was applied intravenously or orally in doses ranging from 9.3 to 26.8 mg/kg per day divided into four doses (median 19.2 mg/kg per day).

Plasma samples were taken 6 h after the last dose of TMP, from the arm not used for infusion. The plasma was separated by centrifugation and either analysed immediately (prospective measurements) or kept frozen at −20 °C; the TMP level was determined retrospectively within a limit of four months.

Extraction procedure for plasma

Plasma samples (0.2 ml) containing TMP were placed in extraction tubes to which were added 0.05 ml of the internal standard mepivacaine (1 mg per

50 ml of water) and 0.2 ml of carbonic acid buffer (pH 9.4). Subsequently the mixture was extracted twice for 15 s with 5 ml of chloroform on a whirlmixer. The liquid phases were then separated by centrifugation at 1500 *g* for 3 min. The combined organic solvent layers were evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was redissolved in 0.2 ml of methanol and transferred to a microvial. The vial was capped for subsequent GC analysis of 0.001-ml aliquots the same day. Under these conditions sulfamethoxazole will not be extracted.

Preparation of standard solutions

In order to prepare standard solutions of TMP in plasma we used 0.2 ml of pooled patients' plasma, obtained from ca. 100 plasma samples from the central laboratory of the University Clinic. The use of this plasma, containing a variety of drugs (Fig. 1), was meant to prove the present method capable of determining TMP in plasma samples from patients who receive considerable comedication. The chromatogram of pooled plasma spiked with 4 mg/l TMP in Fig. 1 demonstrates that the peaks of mepivacaine with a retention time of

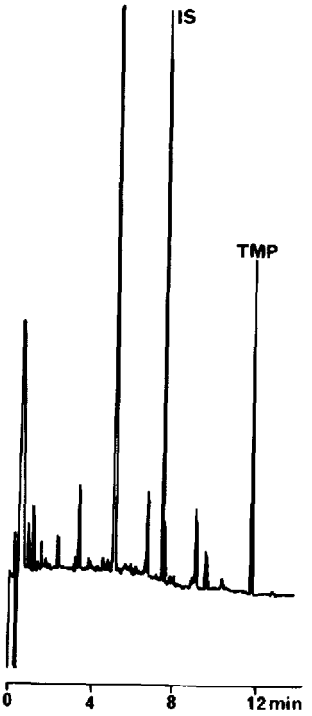

Fig. 1. Pooled plasma spiked with 4 mg/l TMP. Retention times mepivacaine (internal standard, IS), 7.4 min; TMP, 11.8 min

7.4 min and of TMP with a retention time of 11.8 min can be clearly identified without interference from other peaks. The peak at 5.1 min is that of caffeine.

To obtain calibration curves, this plasma was spiked with the appropriate volumes of TMP from two stock solutions of 1.6 mg of TMP in 50 ml of water and 16 mg of TMP in 100 ml of water. The samples were extracted as described above.

RESULTS

Calculation of results and validation of the method

A known amount of internal standard was added to TMP standard solutions in the range 1.6–24 mg/l. A calibration curve was constructed with the concentration of TMP on the abscissa and the ratio of the peak area of TMP to the

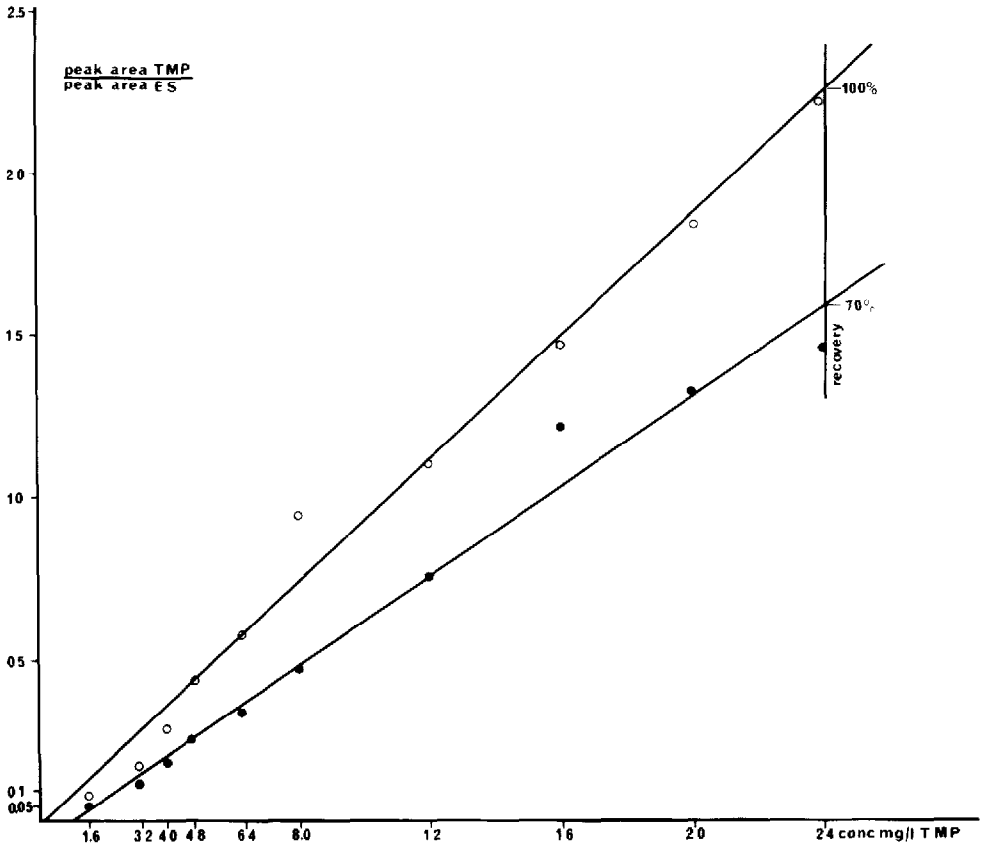

Fig. 2. (●) Ratio of the peak area of TMP to the peak area of external standard (ES) plotted against the TMP concentration. (○) Dilutions of TMP plus external standard dissolved in methanol. The mean values of each set of measured values are marked along the calibration curves.

peak area of the internal standard on the ordinate. The calibration curve has a good linearity and correlation ($y=0.0038+0.0438x$, coefficient of correlation, $r=0.994$, coefficient of variation, C.V. $=11.27\%$). The detection limit for TMP in plasma has been calculated as 0.2 mg/l, which is far below the therapeutic range. A further extraction was carried out with mepivacaine as external standard added to the combined organic layers before evaporation. The ratio of the peak area of TMP to the peak area of the external standard was plotted against the concentration of TMP (Fig. 2). The comparison between the curve of the external standard calibration and the curve of pure dilutions of TMP plus external standard dissolved in methanol shows that TMP was 70% extracted.

Applications

In retrospective and prospective measurements we analysed TMP plasma levels in a total of 115 plasma samples taken from fourteen patients undergoing high-dose TMP–SMX treatment. Fig. 3A, B and C show typical chromatograms of patients with low, medium and high range of TMP concentrations.

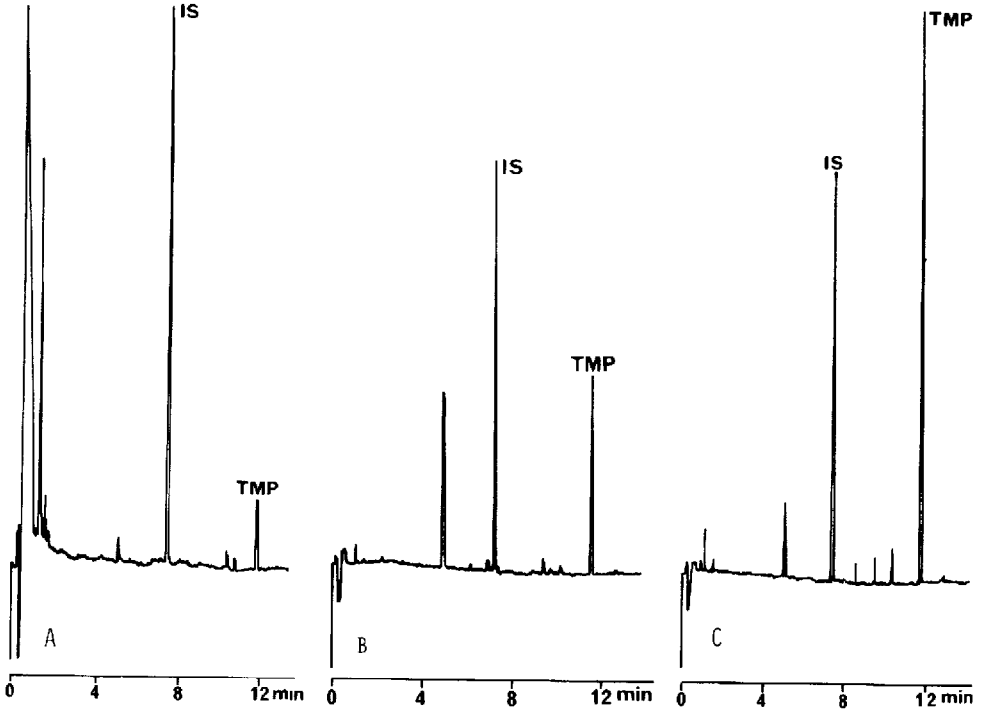

Fig. 3. Chromatograms from patients with TMP levels below (A), within (B) and above (C) the therapeutic range. TMP concentrations are 2.0 mg/l (A), 6.8 mg/l (B) and 22.3 mg/l (C). Retention times: internal standard, IS, ca. 7.5 min; TMP, 11.7 min.

TABLE I

DOSAGE AND TMP PLASMA LEVELS IN NINE PATIENTS UNDERGOING TMP-SMX TREATMENT

Patient[a]	Diagnosis[b]	Dosage[c] (mg/kg)	TMP plasma level[d] (mg/l)
1r	PcP/AIDS	20.6, i.v.	14.1 (4)
1r	PcP/AIDS	15.4, i.v.	10.5 (6), 10.8(12), 9.1(14), 6.9(18)
1p	PcP/AIDS	21.3, p.o.	13.7 (2), 16.3 (6), 7.4(13), 4.5(20)
2p	PcP/AIDS	19 2, i.v.	8 7 (4), 4.8 (6), 7.4 (9), 6.7(13)
2r	PcP/AIDS	15.4, i.v.	6.1(15), 3.4(18), 3.9(20), 4 1(21)
3r	PcP/AIDS	20 3, i.v.	4.3 (5), 4.6 (6)
3r	PcP/AIDS	15.0, i.v.	3.9 (7), 2.7 (9), 2.0(13), 1.5(15)
3r	PcP/AIDS	12 7, i.v.	1.7(17), 0.8(18), 0.7(19)
3r	PcP/AIDS	10.7, i.v.	0 5(21), 0.5(22)
3r	PcP/AIDS	13.7, i.v.	0.9(24), 2.0(30), 4.5(32), 2.5(37)
4r	PcP/AIDS	16.1, i.v.	8.2 (7), 7.2(10), 7.2(12), 9.6(14)
5r	PcP/AIDS	11.8, i.v.	2.9 (1)
5r	PcP/AIDS	23.5, i.v.	10.2 (4), 8.5 (8), 7.2(16), 6.2(23)
6r	PcP/AIDS	18.8, i.v.	9.4 (3), 9.0 (4), 10.8 (5)
6r	PcP/AIDS	14.1, i.v.	6.8(12), 4.1(17), 4.4(23)
7p	PcP/AIDS	19.6, i.v	1.5 (1)
7p	PcP/AIDS	24.5, i.v	12.3 (3), 8.8 (5)
8p	PcP/Cyto	20.2, i.v.	20.8 (3), 15.3 (4), 10.0 (5), 10.5 (6)
9p	PcP/Cyto	20.0, i.v.	16.5 (4)
9p	PcP/Cyto	9.3, i v.	18.2 (5), 10 3 (7)

[a]r = retrospective, p = prospective.
[b]PcP = *Pneumocystis carinii* pneumonia; Cyto = cytostatic treatment.
[c]i.v. = intravenous application; p.o. = oral application.
[d]Values in parentheses are days of therapy.

Table I lists a series of retrospective and prospective determinations of TMP plasma levels of nine patients undergoing high-dose TMP–SMX treatment for *Pneumocystis carinii* pneumonia. Prospective measurements are important in order to ensure that TMP concentrations remain within the therapeutic range. Patient 3, whose plasma samples were analysed retrospectively, underwent intensive care treatment with artificial respiratory support requiring extended sedation with phenobarbital. The doses of TMP, ranging from 10.7 to 20.3 mg/kg per day, were insufficient with regard to the recommended TMP plasma levels, remaining far below the therapeutic range (median 1.6 mg/l; range 0.5–4.9 mg/l). Patient 7, whose plasma levels were determined prospectively, is an example of a necessary adjustment of dosage. The dosage was increased from 19.6 mg/kg per day to 24.5 mg/kg per day in order to achieve plasma concentrations with sufficient antibiotic action. TMP plasma levels rose from 1.5 to 8.8 mg/l.

DISCUSSION

The method described has been developed as an assay for clinical drug monitoring. The applications reported emphasize the important role of a pharmacokinetic follow-up in the evaluation of a patient's clinical situation. The main problem in the development of a routinely applicable method lay in the choice of an adequate solvent for TMP and mepivacaine to ensure reproducible retention times. Benzyl alcohol had excellent solvent properties, but produced unstable peaks following the measurement of a series of plasma samples, probably owing to its high viscosity. 2-Propanol, which was proposed by Land et al. [8], resulted in a peak-area ratio that was not reproducible. We were able to overcome these analytical problems by using methanol, which proved to be an adequate solvent for both TMP and mepivacaine.

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