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Note

Concurrent analysis of methotrexate, trimethoprim, sulphamethoxazole and their major metabolites in plasma by high-performance liquid chromatography

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Methotrexate (MTX), an antifolate cytostatic agent, has considerable activity in the maintenance treatment of acute lymphoblastic leukemia (ALL) and in several combination regimes for solid tumours. During the treatment of ALL, prophylactic antibacterial treatment with co-trimoxazole [trimetoprim (TMP) plus sulphamethoxazole (SMZ)] is administered for the prevention of opportunistic infections during immunosuppression [1]. It has been reported that the proliferation of lymphocytes in vitro is suppressed by co-trimoxazole at concentrations comparable with therapeutic plasma levels [2]. Golde et al. [3] showed that co-trimoxazole can impair human haematopoiesis by inhibition of tetrahydrofolate formation. Thus co-trimoxazole as well as MTX may be responsible for the frequently observed myelosuppression during ALL maintenance therapy. Routine clinical diagnostic procedures to discriminate between these drugs as possible agents involved in myelosuppression in individual patients are not available. However, one may not easily delete any therapy, because of an enhanced risk of either ALL relapse or a higher risk of infections. In these cases it may be highly informative to determine the concentration of MTX as well as the concentrations of TMP and SMZ in plasma of individual patients. Several analytical procedures using high-performance liquid chromatography (HPLC) have been developed for either MTX and its major metabolite 7-hydroxymethotrexate (7-OH-MTX) or TMP/SMZ and the major metabolite of SMZ, N-acetylsulpha-

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metoxazole (N-Ac-SMZ). For the detection of MTX and 7-OH-MTX, UV [4–8], fluorimetric [9] and electrochemical detection (ED) [10] have been applied. ED of TMP [11,12] as well as UV detection [13–16] was used for measurement of TMP, SMZ and N-Ac-SMZ.

We here describe a method for the quantitation of both MTX/7-OH-MTX and TMP/SMZ/N-Ac-SMZ using two extraction methods for either MTX and 7-OH-MTX or TMP, SMZ and N-Ac-SMZ followed by a single HPLC procedure with UV detection.

EXPERIMENTAL

Chemicals

The following chemicals were used: TMP (Multipharma, The Netherlands), pure SMZ (Brunschwig Chemie, The Netherlands), and MTX (Lederle, The Netherlands). 7-OH-MTX was kindly provided by Dr. K. Nooter (Radiobiological Institute TNO, The Netherlands) and pure N-Ac-SMZ was donated by Hoffman-La Roche, The Netherlands. Pure sulphafurazole (SFZ), which was used as an internal standard, was provided by Dr. K. Meilink (University Hospital Dijkzigt, Rotterdam, The Netherlands). All solvents used for chromatography were of HPLC-grade purity.

Extraction of MTX and 7-OH-MTX

The extraction procedure used was adapted from Sonneveld et al. [17]. A plasma sample (0.5 ml) was precipitated with 0.5 ml of 10% trichloroacetic acid in hydrochloric acid (TCA–HCl), containing 1.25 μg of SFZ per 0.5 ml of TCA–HCl. The precipitate was vortexed for 20 s and centrifuged at 8300 *g* for 5 min, and 100-μl aliquots were used for HPLC analysis.

Extraction of TMP, SMZ and N-Ac-SMZ

Plasma (1 ml) was pipetted into a 20-ml glass tube, and 100 μl of the internal standard [SFZ; 5 μg/ml in methanol–water (1:1)], 200 μl of a 1 *M* sodium phosphate buffer (pH 6.8) and 6 ml of ethyl acetate were added. The tube was closed with a glass stopper and shaken for 15 min. After centrifugation at 600 *g* for 10 min, the organic phase was transferred to a 12-ml glass tube and evaporated at 50°C under a gentle stream of nitrogen. The residue was reconstituted in 175 μl of methanol–water (1:4) and vortexed for 1 min. To remove proteins, 25 μl of 40% TCA–HCl were added, and the tube was vortexed again for 30 s. Finally the sample was centrifuged at 8300 *g* for 5 min. Aliquots of 100 μl were directly injected onto the HPLC column.

Apparatus and chromatography

The HPLC separation was adapted from So et al. [7]. A Waters M510 pump was used for solvent delivery (Waters Assoc., The Netherlands). Samples were injected through a Waters U6K injector. Chromatography was performed on a Hibar LiChrosorb RP18 (5 μm) stainless-steel 250 × 4 mm I.D. column (E. Merck, Amsterdam, The Netherlands). The mobile phase consisted of 0.15 *M* ammo-

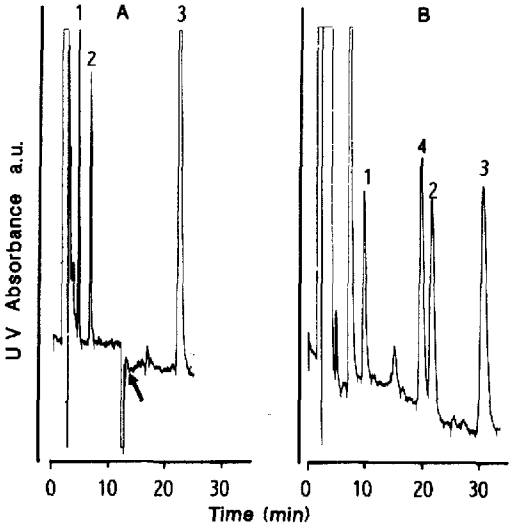

Fig. 1. Chromatogram of a plasma sample spiked with 625 ng/ml MTX (1), 625 ng/ml 7-OH-MTX (2) and 2.5 μg/ml SFZ (3) and precipitated with TCA–HCl. The arrow indicates the switching of the UV detector wavelength from 313 to 254 nm. (B) Chromatogram of a plasma sample spiked with 500 ng/ml TMP (1), SMZ (2), N-Ac-SMZ (3) and SFZ (4) and extracted with ethyl acetate.

nium phosphate buffer (pH 4.85) containing 12.6% acetonitrile at a flow-rate of 1.5 ml/min. Detection was performed using a Schoeffel Instruments spectroflow monitor SF770 (Kratos, The Netherlands) at dual wavelengths of 313 nm (MTX and 7-OH-MTX) and 254 nm (TMP, SFZ, SMZ and N-Ac-SMZ). Peak integration was performed on a Shimadzu CR-3A integrator (United Technologies Packard, The Netherlands).

RESULTS AND DISCUSSION

For the quantification of MTX and 7-OH-MTX, SFZ was used as an internal standard. The recoveries of MTX and 7-OH-MTX after precipitation with TCA–HCl were 64 and 71%, respectively. Because of the different UV absorbance characteristics of SFZ (254 nm) and MTX/7-OH-MTX (313 nm), detection was performed at dual wavelengths (Fig. 1A). Calibration curves showed a linear detection from 40 ng/ml ($8.8 \cdot 10^{-8} M$) (MTX) and 80 ng/ml ($17.8 \cdot 10^{-8} M$) (7-OH-MTX) up to 5 μg/ml for both compounds, with a coefficient of variation (C.V.) of 2–6% at a concentration of 1.25 μg/ml. The HPLC separation of TMP, SMZ and N-Ac-SMZ is shown in Fig. 1B. A linear relationship was found between the peak area's average of concentrations, which were different between TMP (0.16–5.0 μg/ml) and SMZ/N-Ac-SMZ (0.08–40 μg/ml), respectively. The C.V. was 3–4% at 1 μg/ml. The lower limit of detection of TMP and SMZ/N-Ac-SMZ in plasma was 160 ng/ml ($6.5 \cdot 10^{-7} M$) and 80 ng/ml ($3.2 \cdot 10^{-7} M$), respectively. The extraction recovery was calculated by comparing the chromatograms of serial dilutions of TMP, SMZ and N-Ac-SMZ dissolved in methanol–water (1:4) with extracted plasma samples containing the same concentrations. Spreux-Var-

TABLE I

INTRA- AND INTER-ASSAY VARIABILITY OF MTX/7-OH-MTX/TMP/SMZ AND N-Ac-SMZ IN HUMAN PLASMA AT THE LOWER LEVEL OF DETECTION OF EACH COMPOUND

Compound	Lower limit of detection (ng/ml)	Intra-assay variability* (%)	Inter-assay variability** (%)
MTX	40	5 ($n=12$)	13 ($n=7$)
7-OH-MTX	80	4 ($n=11$)	10 ($n=7$)
TMP	160	9 ($n=7$)	13 ($n=10$)
SMZ	80	19 ($n=9$)	6 ($n=10$)
N-Ac-SMZ	80	16 ($n=9$)	5 ($n=10$)

*The intra-variation at the lower limit of detection, as determined by repeated injection of a single sample of the pure compound during a single analysis.

**The inter-variation at the lower limit of detection, as determined by repeated analysis of a single sample during fourteen consecutive working days.

oquaux et al. [15] reported that, when chloroform–ethyl acetate instead of ethyl acetate was used, the extraction recovery of TMP at pH 6.2 increased from 35 to 85%. However, we found equally good results by simple extraction of TMP, SMZ and N-Ac-SMZ with ethyl acetate. Our findings are in agreement with those of Ascalone [16]. The mean recovery values of TMP, SMZ and N-Ac-SMZ were 82, 69 and 72%, respectively.

To assess the reliability and the accuracy of the method we measured the intra-assay variability and the inter-assay variability at the lower limits of detection of TMP/SMZ/N-Ac-SMZ/MTX and 7-OH-MTX. These data are presented in Table I.

An example of a plasma sample of a patient obtained during maintenance treatment of childhood acute lymphoblastic leukemia is depicted in Fig. 2. The sample was obtained 24 h after an oral dose of 30 mg/m^2 MTX and 40 h after an oral dose of co-trimoxazole. For chromatogram A, the plasma sample was precipitated with TCA–HCl for the quantitation of MTX and 7-OH-MTX. For chromatogram B the sample was extracted with ethyl acetate to quantify TMP/SMZ and N-Ac-SMZ.

Fig. 3 shows the plasma disappearance curves of TMP, SMZ and N-Ac-SMZ obtained after administration of a single oral dose of TMP/SMZ (80/400) to two healthy volunteers. The peak plasma concentration and the plasma elimination of either compound as determined with the present assay are in accordance with published data.

It may be concluded that the extraction and analysis procedure described is fast and reliable for the quantification of both MTX and 7-OH-MTX, as well as TMP, SMZ and N-Ac-SMZ. If necessary, one may determine each compound separately in plasma by applying both extraction methods, followed by a single HPLC procedure. Thus, this approach may offer a simple way to estimate the role of TMP, SMZ and MTX in the development of myelosuppression. Recent studies have stressed the role of TMP/SMZ in unexplained leukopaenia as ob-

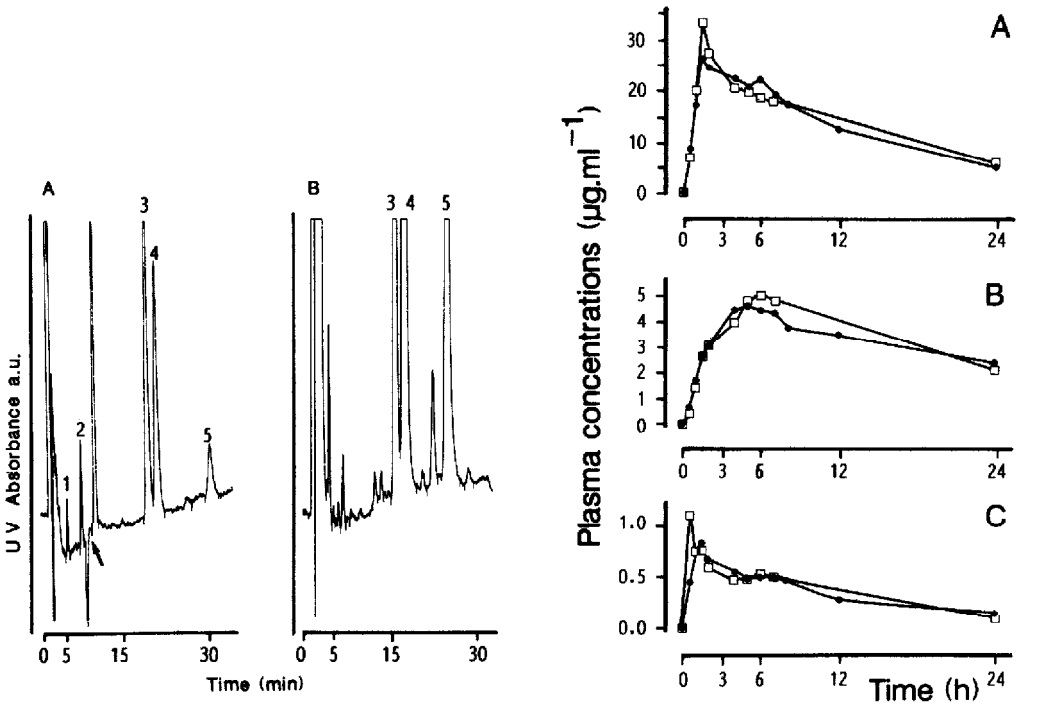

Fig. 2. Chromatogram of a plasma sample obtained from a patient treated with methotrexate and co-trimoxazole for acute lymphoblastic leukaemia. (A) Chromatogram of this sample after precipitation with TCA-HCl; the following concentrations were found: MTX (1), 77 ng/ml; 7-OH-MTX (2), 197 ng/ml; 3 = SFZ. (B) In the same sample after extraction with ethyl acetate, the following concentrations were found: 1.336 µg/ml SMZ (4); 0.46 µg/ml N-Ac-SMZ (5). In this particular patient, no TMP was found in the plasma.

Fig. 3. Plasma concentration-time curves in two healthy volunteers after a single oral dose of 80 mg of TMP and 400 mg of SMZ. (A) SMZ; (B) N-Ac-SMZ; (C) TMP.

served in megaloblastic anaemia, as well as in ALL chemotherapy [18], and following bone marrow transplantation. This assay may thus also help to monitor the plasma concentrations of TMP if the drug is administered simultaneously with other inhibitors of folic acid metabolism.

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REFERENCES

1 Editorial, Lancet, ii (1973) 950.
2 P.M. Gaylarde and I. Sarkany, Br. Med. J., 3 (1972) 144.
3 D.W. Golde, N. Bersch and S.G. Quan, Br. J. Haematol., 40 (1978) 363.
4 C. Canfell and W. Sadee, Cancer Treat. Rep., 64 (1980) 165.
5 H. Iven and H. Bartels, Cancer Treat. Rep., 69 (1985) 825.
6 W.P. Tong, J.L. Wisnicki, J. Horton and D.B. Ludlum, Clin. Chim. Acta, 107 (1980) 67.

7 N. So, D.P. Chandra, I.S. Alexander, V.J. Webster and D.W. O'Gorman Hughes, J. Chromatogr., 337 (1985) 81.
8 M. Stout, Y. Ravindranath and R. Kauffman, J. Chromatogr., 342 (1985) 424.
9 J. Šalamoun and J. František, J. Chromatogr., 378 (1986) 173.
10 F. Palmisano, T.R.I. Cataldi and P.G. Zambonin, J. Chromatogr., 344 (1985) 249.
11 L. Nordholm and L. Dalgaard, J. Chromatogr., 305 (1984) 391.
12 L. Nordholm and L. Dalgaard, J. Chromatogr., 233 (1982) 427.
13 R. Gochin, I. Kanfer and J.M. Haigh, J. Chromatogr., 223 (1981) 139.
14 A. Weber, K.E. Opheim, G.R. Siber, J.F. Ericson and A.L. Smith, J. Chromatogr., 278 (1983) 337.
15 O. Spreux-Varoquaux, J.P. Chapalain, P. Cordonnier, C. Advenier, M. Pays and L. Lamine, J. Chromatogr., 274 (1983) 187.
16 V. Ascalone, J. Chromatogr., 244 (1981) 59.
17 P. Sonneveld, F.W. Schultz, K. Nooter, and K. Hählen, Cancer Chemother. Pharmacol., 18 (1986) 111.
18 P. Sonneveld, K. Hählen, K. van der Steuijt and K. Nooter, Am. J. Pediatr. Hematol. Oncol. (1987) in press.