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**Note****Rapid quantitation of methotrexate and its metabolites in human serum, urine and bile, using solid-phase extraction and high-performance liquid chromatography**BERND NUERNBERG<sup>a,\*</sup>*Department of Pharmacology and Toxicology, University of Erlangen-Nuernberg, Universitaetsstrasse 22, 8520 Erlangen (F R G)*

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Since the folate antagonist methotrexate (MTX) was introduced into anti-cancer therapy in 1953 [1], a large number of methods for the quantitation of MTX and its metabolites in human serum and urine have been published [2-10]. Owing to the high dosage regimens utilized (hundreds of milligrams per square metre of body surface [11]), high sensitivity in analysing serum samples was not required. Recently, however, low doses of MTX (5-10 mg/m<sup>2</sup>) have been used to treat rheumatoid patients [12,13]. Therefore, high analytical sensitivity became a major requirement for both pharmacokinetic investigations and drug monitoring. There are few published papers dealing with highly sensitive quantitation of MTX in human serum. Several publications have detailed the analysis of MTX in human serum, but these have used either radioimmunoassay techniques [9,14,15] or costly time-consuming high-performance liquid chromatography.

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graphic (HPLC) procedures [2,3,5,14]. None of them described the determination of MTX and its main metabolites in human bile.

We have developed a simple, sensitive and reliable HPLC-method for the quantitation of MTX, 7-hydroxymethotrexate (7-OH-MTX) and 2,4-diamino-N<sup>10</sup>-methylpteroic acid (APA) in human serum, urine and bile.

## EXPERIMENTAL

### *Materials and reagents*

Methotrexate, 2,4-diamino-N<sup>10</sup>-methylpteroic acid, the internal standard 3-bromomethotrexate (I.S.), 7-hydroxymethotrexate and methotrexate polyglutamates (glutamates  $n = 2-5$ ) were obtained from Lederle Labs. (Pearl River, NY, U.S.A.).

HPLC-grade acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). All other reagents were of analytical grade. Deionized water was further purified in a Milli-Q<sup>TM</sup> water purification system (Millipore, Bedford, MA, U.S.A.). Monosodium and disodium phosphate buffers used in the mobile phase were filtered and degassed through a 0.45- $\mu\text{m}$  membrane (Type FH, Millipore). Bond-Elut cartridges (200 mg of solid phase C<sub>18</sub>, 3-ml reservoir) used for sample treatment were obtained from Analytical International (Harbor City, CA, U.S.A.) and washed with 10 ml of acetonitrile and 10 ml of methanol, followed by 10 ml of phosphate buffer (0.05 M, pH 2.7).

### *Stock solutions*

The stock standards of the compounds were prepared as follows: MTX (595  $\mu\text{g}/\text{ml}$ ) was dissolved in citric buffer (0.1 M, pH 2.0); 7-OH-MTX (23.5  $\mu\text{g}/\text{ml}$ ), APA (56  $\mu\text{g}/\text{ml}$ ) and the I.S. (520  $\mu\text{g}/\text{ml}$ ) were dissolved in Tris buffer (0.01 M, pH 7.0). These stock solutions were kept at  $-20^\circ\text{C}$  for a maximum of eight weeks and used for spiking serum, urine and bile, as well as for preparing calibration curves. The stock solutions were checked weekly and no degradation was observed.

The solutions for external standards were made from mobile phase spiked with various amounts of the stock solutions: MTX, 6.7–2146.8 ng/ml ( $n = 8$ ); 7-OH-MTX, 7.5–470.7 ng/ml ( $n = 6$ ); APA, 7–448 ng/ml ( $n = 7$ ); I.S., 2.1–520  $\mu\text{g}/\text{ml}$  ( $n = 7$ ).

Whole blood and urine samples were obtained from the healthy drug-free investigators. Serum was harvested and used in the preparation of all recovery studies. MTX-free bile was obtained from a patient who underwent cholecystectomy and had a temporarily inserted biliary t-drain. For recovery studies, 1 ml of serum, urine and bile was spiked with MTX, 7-OH-MTX or APA to the following final concentrations: MTX, 2146, 214.7, 107.3 and 6.7 ng/ml; 7-OH-MTX, 470.4, 47.0, 23.5 and 4.7 ng/ml; APA, 448.0, 44.8, 11.2 and 5.6 ng/ml.

### *Chromatography*

An isocratic Waters HPLC system (Millipore, Waters Division, Milford, MA, U.S.A.) was utilized. It consisted of a Model 590 pump, a Model 481 variable-wavelength detector set at 303 nm, and a Wisp<sup>TM</sup> Model 710 B autosampler (in-

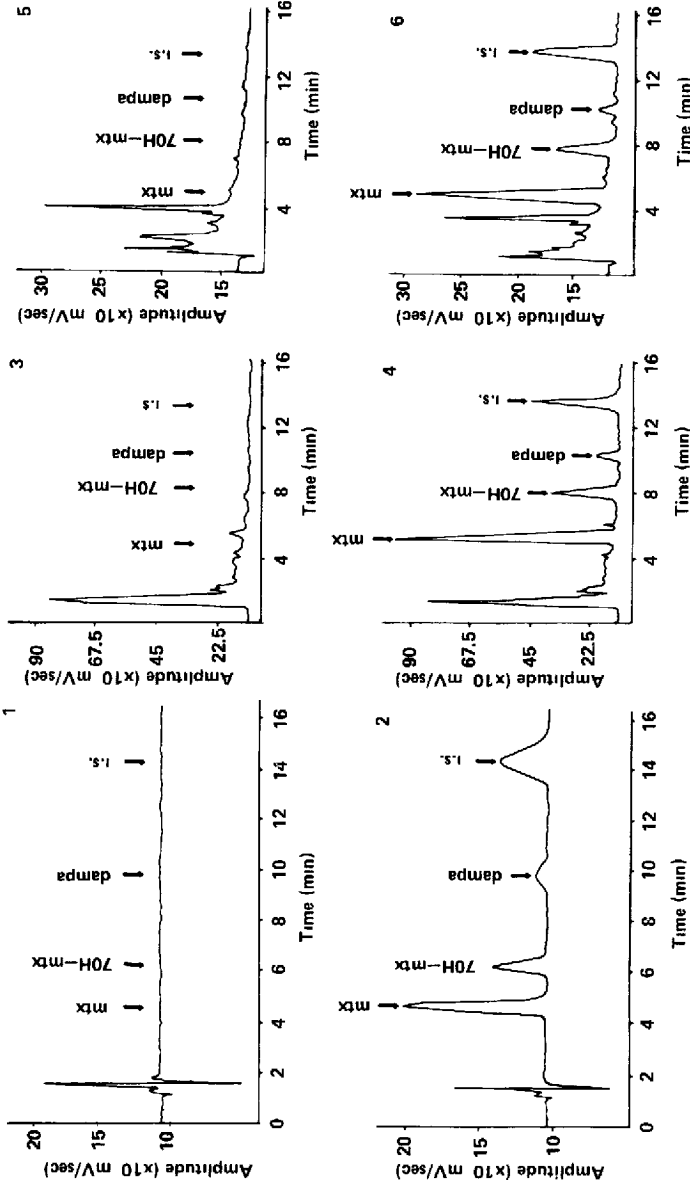


Fig. 1. Typical chromatograms of blank serum (1), spiked serum (2), blank urine (3), spiked urine (4), blank bile (5) and spiked bile (6).

jection volume 50  $\mu$ l) connected to a Model 3357 Hewlett-Packard integrator (Paramus, NJ, U.S.A.). The columns were a 150 mm  $\times$  3.9 mm I.D. Nova Pak<sup>TM</sup> C<sub>18</sub> column with a particle size of 4  $\mu$ m (Millipore, Waters Division) and a Brownlee guard column C<sub>18</sub>, 15 mm  $\times$  3.2 mm I.D., with a particle size of 7  $\mu$ m (Rainen Instruments, Woburn, MA, U.S.A.). The mobile phase was acetonitrile-phosphate buffer, 0.05 M, pH 2.7 (12:88, v/v), delivered at 20°C at a flow-rate of 1.5 ml/min.

#### *Extraction procedure*

The compounds were extracted using a Bond-Elut column containing ODS-modified silica. The columns were placed in a luer that fitted onto the top of the Vac Elut cover, which was loaded with up to ten columns. A mild vacuum was applied to the manifold to carry out the various steps of the extraction procedure. To 1 ml of serum, urine or bile, was added either 0.1 ml (5.2  $\mu$ g/ml) of I.S. solution for serum, or 1 ml for urine and bile, followed by 1–2 ml of Tris buffer (0.01 M, pH 7.0). After brief mixing, the sample was applied to the Bond-Elut column. The cartridge was washed with 10 ml of phosphate buffer (0.05 M, pH 2.7) followed by 2 ml of sodium hydroxide (0.1 M) and another 3 ml of phosphate buffer. The compounds were eluted with 3 ml of methanol and evaporated to dryness. The residue was reconstituted with at least 0.25 ml of mobile phase, and aliquots were injected onto the HPLC column.

#### *Quantitation*

Peaks were identified by using retention times compared with the external standards. Amounts were calculated using both the peak-area ratio of analyte to the I.S. and comparison with standard curves of the various compounds.

## RESULTS AND DISCUSSION

All compounds were well separated within 16 min (Fig. 1) and no interfering peaks were observed. The chromatographic system allowed quantitation in the linear range 6.7–2146.8 ng/ml for MTX ( $r = 0.9998$ ;  $n = 8$ ), 7.5–420.7 ng/ml for 7-OH-MTX ( $r = 0.9998$ ;  $n = 6$ ), 7–448 ng/ml for APA ( $r = 0.997$ ;  $n = 7$ ) and 2–520  $\mu$ g/ml for the I.S. ( $r = 0.999995$ ;  $n = 7$ ).

The results of the recovery experiments are listed in Table I. Between 77.7 and 100.1% of the compounds were recovered. Recovery was constant (Table II) and the coefficient of variation (C.V.) ( $n = 3$ ) over the concentration range studied ranged between 0.82 and 5.68%. Concentration of the samples led to detection limits of ca. 1 ng/ml for all compounds in the biological fluids.

As a group, methotrexate polyglutamates ( $n = 2$ –5) could be well separated from all other peaks (Fig. 2), but separation of the polyglutamates from one another was not possible.

In addition to UV detection, electrochemical detection (ED) with an LC4 detector (Bioanalytical System, West Lafayette, IN, U.S.A.) was examined. For the tested chromatographic conditions, ED was as useful as UV detection (Fig. 2). The instrumental conditions were the same as reported by Palmisano et al.

TABLE I

RECOVERY AND ACCURACY OF METHOTREXATE, 7-HYDROXYMETHOTREXATE AND 2,4-DIAMINO-N<sup>10</sup>-METHYLPTEROIC ACID

Concentration (ng/ml)	Serum		Urine		Bile	
	Mean	C.V. (%) <sup>a</sup>	Mean	C.V. (%) <sup>a</sup>	Mean	C.V. (%) <sup>a</sup>
<i>Methotrexate</i>						
2146.8	93.6	1.23	97.1	1.86	97.7	1.31
214.7	97.0	1.60	100.1	2.64	97.1	1.64
107.3	95.2	2.00	97.8	3.57	96.5	2.47
6.7	96.0	2.53	97.6	3.96	96.8	2.86
Accuracy <sup>b</sup>	95.4	2.00	98.1	2.88	97.2	1.87
<i>7-Hydroxymethotrexate</i>						
470.4	79.2	1.41	81.5	1.07	80.5	0.82
47.0	78.3	1.83	80.6	2.31	78.3	2.72
23.5	80.5	2.41	82.2	1.95	81.6	3.17
4.7	77.7	3.12	79.2	3.01	80.4	3.66
Accuracy <sup>b</sup>	79.1	1.51	80.8	1.73	80.6	1.93
<i>2,4-Diamino-N<sup>10</sup>-methylpterioic acid</i>						
448	87.7	1.78	90.8	2.02	89.8	3.13
44.8	90.7	1.83	91.5	2.43	88.5	3.42
11.2	90.8	2.91	90.2	3.93	92.1	2.12
5.6	91.9	4.61	88.7	4.57	91.2	5.68
Accuracy <sup>b</sup>	90.2	2.85	90.3	2.89	90.7	3.16

<sup>a</sup>n = 3<sup>b</sup>Mean (n = 12) and C.V. of four levels of each compound in body fluids as a percentage of the spiked amount.

TABLE II

## AVERAGE LINEAR REGRESSION (n = 3) (LEAST-SQUARE FIT) DATA FOR CALIBRATION CURVE

Sample	r	Slope (mean ± S.D.)	Intercept <sup>a</sup> (mean ± S.D.)
<i>Methotrexate</i>			
Serum	0.998 467	$8.88 \cdot 10^{-3} \pm 9.45 \cdot 10^{-5}$	$5.07 \cdot 10^{-3} \pm 8.78 \cdot 10^{-3}$
Urine	0.998 933	$9.82 \cdot 10^{-4} \pm 5.02 \cdot 10^{-5}$	$-9.90 \cdot 10^{-5} \pm 5.07 \cdot 10^{-3}$
Bile	0.997 967	$9.84 \cdot 10^{-4} \pm 1.80 \cdot 10^{-5}$	$-9.35 \cdot 10^{-4} \pm 5.58 \cdot 10^{-3}$
<i>7-Hydroxymethotrexate</i>			
Serum	0.993 930	$8.66 \cdot 10^{-3} \pm 2.94 \cdot 10^{-4}$	$-2.00 \cdot 10^{-2} \pm 9.93 \cdot 10^{-3}$
Urine	0.998 533	$8.91 \cdot 10^{-4} \pm 3.57 \cdot 10^{-5}$	$7.94 \cdot 10^{-5} \pm 7.86 \cdot 10^{-4}$
Bile	0.998 901	$9.00 \cdot 10^{-4} \pm 2.11 \cdot 10^{-5}$	$-6.83 \cdot 10^{-4} \pm 9.47 \cdot 10^{-4}$
<i>2,4-Diamino-N<sup>10</sup>-methylpterioic acid</i>			
Serum	0.999 712	$8.32 \cdot 10^{-3} \pm 1.87 \cdot 10^{-4}$	$6.61 \cdot 10^{-4} \pm 4.66 \cdot 10^{-3}$
Urine	0.998 972	$8.23 \cdot 10^{-4} \pm 5.31 \cdot 10^{-5}$	$9.67 \cdot 10^{-5} \pm 1.46 \cdot 10^{-3}$
Bile	0.991 339	$8.52 \cdot 10^{-4} \pm 3.86 \cdot 10^{-5}$	$-3.77 \cdot 10^{-4} \pm 8.15 \cdot 10^{-4}$

<sup>a</sup>None of the intercept values was significantly different from zero (P > 0.05).

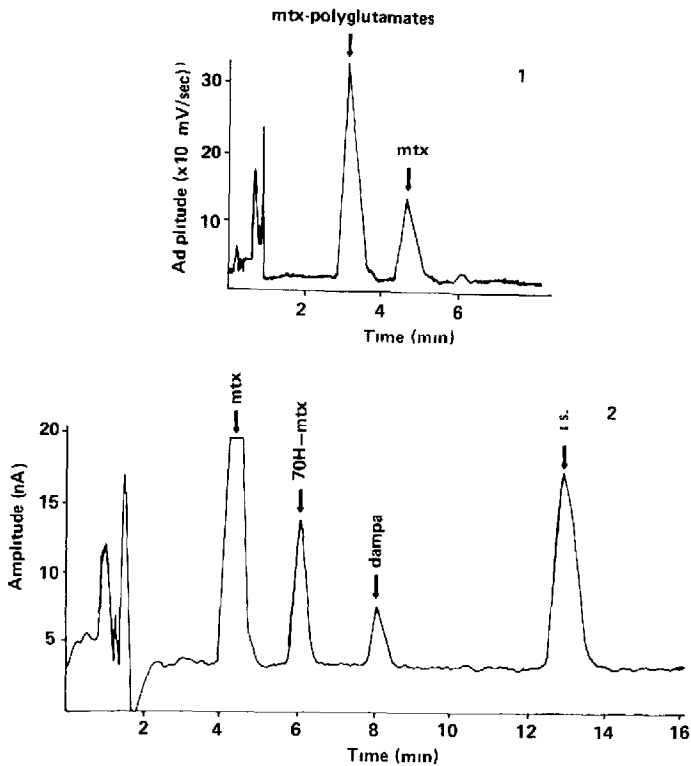


Fig. 2. Separation of methotrexate polyglutamates from MTX (1) and typical chromatogram obtained with ED (2).

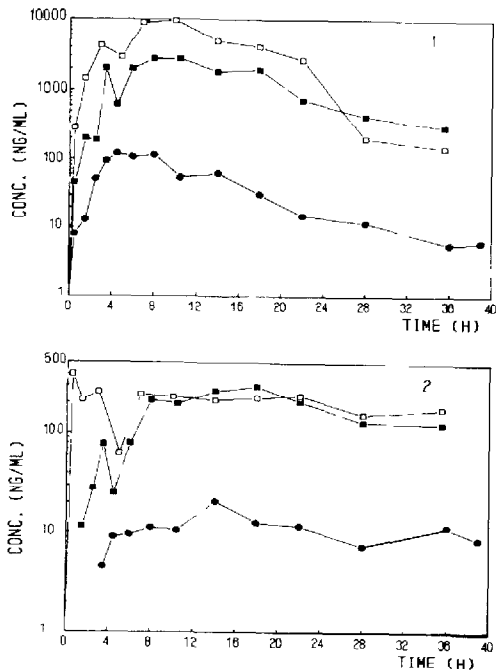


Fig. 3. (1) Concentrations of MTX in patient's serum (●), urine (□) and bile (■) and (2) concentrations of 7-OH-MTX in patient's serum (●), urine (□) and bile (■)

[5]. Unfortunately, use of ED did not improve the detection limit, and equilibration procedures required much more time with ED than with UV detection.

Using the above methodology, serum, bile and urine MTX and 7-OH-MTX concentration-time profiles were successfully obtained from a patient given a 10 mg/m<sup>2</sup> oral dose of MTX (Fig. 3). No APA could be found in any of these biological fluids, although the assay was sensitive to less than 5 ng/ml.

The method described in this paper allows rapid determination of MTX and its major metabolite in serum, bile and urine, even at the very low concentrations found in patients treated for rheumatoid arthritis.

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#### REFERENCES

- 1 W.A. Blayer, *Cancer*, 41 (1978) 36-51
- 2 J. Šalamoun, M. Smrž, F. Kiss and A. Šalamounová, *J. Chromatogr.*, 419 (1987) 213-223.
- 3 J. Šalamoun and J. František, *J. Chromatogr.*, 378 (1986) 173-181.
- 4 B.A. Kamen and N. Winick, *Methods Enzymol.*, 122 (1986) 339-346.
- 5 F. Palmisano, T.R.I. Cataldi and P.G. Zambonin, *J. Chromatogr.*, 344 (1985) 249-258
- 6 C.L. Zimmerman, T.J. Franz and J.T. Slattery, *J. Pharmacol. Exp. Ther.*, 231 (1984) 242-247.
- 7 M.L. Chen, W.P. McGuire, T.E. Lad and W.L. Chiou, *Int. J. Clin. Pharmacol. Ther. Toxicol.* 22 (1984) 1-6
- 8 M.L. Chen and W.L. Chiou, *J. Chromatogr.*, 226 (1981) 125-134.
- 9 G.W. Aherne and M. Quinton, *Cancer Treat. Rep.*, 65 (Suppl. 1) (1981) 55-60.
- 10 N. So, D.P. Chandra, J.S. Alexander, V.J. Webster and D.W. O'Gorman Hughes, *J. Chromatogr.*, 337 (1985) 81-90.
- 11 C.J. Allegra, G.A. Curt, J. Baram, P.W. Sholar, G.C. Yeh and B.A. Chabner, in H.M. Pinedo and B.A. Chabner (Editors), *Cancer Chemotherapy*, Vol. 8, Elsevier, Amsterdam, New York, Oxford, 1986, Ch. 1, pp. 1-8.
- 12 D.E. Furst and J.M. Kremer, *Arthritis Rheum.*, 31 (1988) 305-314
- 13 D.E. Furst, *J. Rheumatol.*, 12 (Suppl. 12) (1985) 11-14.
- 14 T. Anzai, N. Jaffe and Y.-M. Wang, *J. Chromatogr.*, 415 (1987) 445-449.
- 15 A. Schalhorn, W. Wilmanns and G. Stupp-Poutot, *Onkologie*, 4 (1980) 193-196.