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Determination of methotrexate and its main metabolite 7-hydroxymethotrexate in human urine by high-performance liquid chromatography with normal solid-phase extraction

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Abstract

A practical and sensitive high-performance liquid chromatographic method using normal solid-phase extraction has been developed for the determination of methotrexate (MTX) and its main metabolite 7-hydroxymethotrexate (7-OH-MTX) in human urine. A urine specimen followed by the addition of pH 5.0 acetate buffer was purified by solid-phase extraction on a Sep-Pak silica cartridge. The analyte was chromatographed on a reversed-phase Inertsil ODS-2 column using phosphate buffer–acetonitrile at pH 5.3 as the mobile phase, and the effluent from the column was monitored at 303 nm. A good linear relationship between peak height and concentration was found for both of MTX and 7-OH-MTX in the range 5 to 1000 ng/ml of human urine. The inter-day coefficients of variation for the assay ($n=5$) were 8.8% (5 ng/ml), 3.4% (50 ng/ml) and 2.0% (500 ng/ml) for MTX, and 7.2, 2.7 and 2.3% for 7-OH-MTX in urine, respectively. The present method should prove useful for the evaluation of urinary drug excretion in patients undergoing MTX low-dose therapy.

Keywords: Methotrexate; 7-Hydroxymethotrexate

1. Introduction

Methotrexate (MTX; 2,4-diamino-N^{10}-methylpteroylglutamic acid) is a potent folic acid antagonist which inhibits reduction from folic acid to tetrahydrofolic acid, thereby leading to the suppression of the nucleic acid biosynthetic pathway, and cell death. It has been widely used in the treatment of neoplastic disorders since the late 1940s [1,2].

Recently, on the other hand, MTX low-dose therapy (5–25 mg/m^2 per week) has been used increasingly for the treatment of patients with rheumatoid arthritis (RA) or psoriasis [3–5]. Therapeutic drug monitoring is of importance in order to improve MTX–RA therapy because of the severe side-effects of MTX, e.g., marrow suppression, gastro-intestinal lesion, renal insufficiency, hepatic failure, hypoalbuminemia, and pancytopenia [5–7]. The major metabolite 7-hydroxymethotrexate (7-OH-MTX) may also contribute to renal toxicity because of its poor solubility [8]. Furthermore, concomitant administration with non-steroidal anti-inflammatory drugs (NSAIDs) and/or other drugs may lead to delayed elimination of MTX and 7-OH-MTX, and subsequently increase the risk of toxicity [6,9–12].

Hence a high-performance liquid chromatographic (HPLC) method which is able to determine both

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MTX and 7-OH-MTX seemed to be useful as a clinical tool of MTX low-dose therapy, but only a few published papers dealing with high sensitivity are applicable to human urine analysis [13,14]. Recently, Albertioni et al. [15] accomplished a high sensitivity of 10 n*M* (ca. 4.5 ng/ml) as the detection limit for both of MTX and 7-OH-MTX in human urine by using a fluorescence post-labelling technique with photolytic oxidation and reversed solid-phase extraction (C_8 Isolute). Mader et al. [16] also reported a high sensitivity of 4 ng/ml as the detection limit for MTX in human urine by means of column-switching HPLC and a reversed Bond-Elut phenyl column for the solid-phase extraction. However, these methods need costly instrumentation, i.e., post-labelling or column switching.

A method combining simple manipulation and widely used instrumentation with no special requirements would seem to be very useful in routine clinical use. In this study, the authors developed a favourable sample clean-up procedure using normal solid-phase extraction for the determination of MTX and 7-OH-MTX in human urine by isocratic HPLC with UV detection.

2. Experimental

2.1. Materials and reagents

MTX (99.8% purity by HPLC) was obtained from Lederle Laboratories (Pearl River, NY, USA) and 7-OH-MTX (98%) was synthesized from MTX according to the procedure of Dawson et al. [17]. The Sep-Pak silica cartridge used for sample treatment was purchased from Waters (Milford, MA, USA). Acetonitrile was HPLC grade and other chemicals used in this study were of analytical grade, and they were all from Wako (Osaka, Japan) or Kanto (Tokyo, Japan).

The 1 mg/100 ml stock solutions of each of MTX and 7-OH-MTX were prepared in distilled water. These solutions were stable for at least 1 month when stored at −20°C in a freezer. The dilutions for the working standards were made with distilled water and freshly prepared each day.

2.2. Instrumentation and chromatography

The chromatographic system consisted of a Waters 600E solvent delivery pump (Waters, Milford, MA, USA) equipped with a Rheodyne 7125 loop injector (Berkeley, CA, USA), a Shimadzu SPD-6AV variable-wavelength detector and a Shimadzu C-R6A integrator (Kyoto, Japan).

Chromatography was performed on a reversed-phase Inertsil ODS-2 column (5 μm particle size, 150×4.6 mm I.D.; GL Science, Tokyo, Japan) using 50 m*M* phosphate buffer–acetonitrile (90.5:9.5, v/v) at pH 5.3 as the mobile phase at a flow-rate of 1.1 ml/min under ambient temperature. The effluent from the column was monitored at 303 nm. The capacity factor (k') was calculated as $(t_r - t_o)/t_o$, where t_r is the retention time of the analyte and t_o is the retention time of the void elution determined by the injection of methanol.

2.3. Sample preparation

A 250-μl volume of 1 *M* acetate buffer pH 5.0 was added to a 1.0-ml urine specimen and vortex mixed. A 250-μl aliquot of the resulting mixture was directly loaded onto the Sep-Pak silica cartridge, and dried by aspiration of air. The cartridge was successively washed with 5 ml of ethyl acetate and 5 ml of ethanol–methanol (6:4, v/v), and dried by aspiration of air. The cartridge was then eluted with 3 ml of a 1% ammonia–methanol solution, and the eluate was evaporated to dryness under a stream of nitrogen in a water bath at 37°C. The dried residue was reconstituted with 100 μl of mobile phase, and a 20-μl aliquot was injected onto the HPLC system.

Drug-free urine used in this study was obtained from the healthy investigators. Standard samples were prepared by spiking drug-free urine with known amounts of MTX and 7-OH-MTX working standard solutions, and used for construction of calibration curves and method validation.

2.4. Quantitation

The concentrations of MTX and 7-OH-MTX in urine specimens were calculated by comparison of their respective peak heights with those of calibration

samples prepared by spiking drug-free human urine with MTX and 7-OH-MTX in the range 5–1000 ng/ml as the final concentrations.

3. Results

3.1. Sample purification and chromatography

As shown in Fig. 1, the k' value of 7-OH-MTX rapidly decreased in response to increase in the pH of the mobile phase, whereas it slowly decreased in MTX under the chromatographic conditions described in Section 2.

When ethanol was used as the washing solvent instead of ethanol–methanol (6:4, v/v) as described in sample preparation (Section 2.3), recoveries of MTX and 7-OH-MTX from urine were excellent, but many interfering peaks were observed in the chromatogram. Increasing the methanol contents up to 50%, favourably removed the urinary interferences but recoveries of both compounds were lowered. Ethanol–methanol (6:4, v/v) was eventually the most appropriate, although the recovery of 7-OH-MTX was slightly lowered compared to when only ethanol was used.

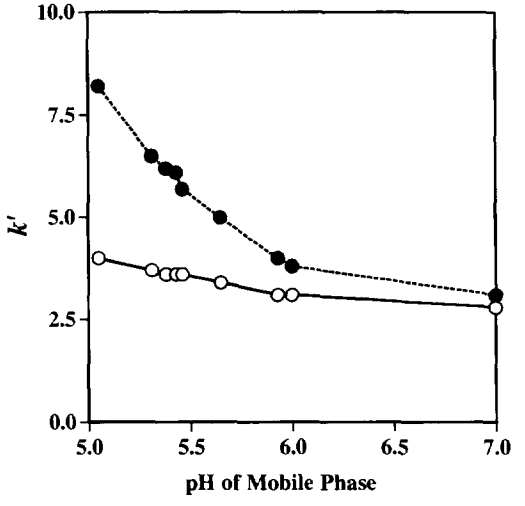

Fig. 1. Relation between pH of mobile phase and the capacity factor (k') of MTX (○) and 7-OH-MTX (●). Column: Inertsil ODS-2, 5 μm, 150×4.6 mm I.D. Mobile phase: 50 m*M* phosphate buffer–acetonitrile (90.5:9.5, v/v), 1.0 ml/min, room temperature. Monitor: UV 303 nm.

Two appropriate chromatographic conditions (pH of mobile phase at 7.0 and 5.3) were obtained following the above results as shown in Fig. 2. This method adopted the latter one (pH 5.3). Fig. 3 shows the typical chromatograms of standard, drug-free, spiked urine and a 0–24 h post-dose urine specimen obtained according to the method described in Section 2. No interfering peaks due to endogenous substances were observed, and chromatographic separation was favourably done within 20 min.

3.2. Linearity and reproducibility

For linear assessment study, drug-free human urine was spiked with each of MTX and 7-OH-MTX in the range 5–1000 ng/ml as the final concentrations, and samples were assayed in duplicate according to the method described in Section 2. A good linear relationship between peak height and drug concentration was found for both MTX and 7-OH-MTX in the above ranges (correlation coefficients of $r>0.9998$ for the two compounds). The detection limit at a signal-to-noise ratio of 2.5 was 2.5 ng/ml urine for the two compounds.

The absolute recoveries of MTX and 7-OH-MTX from human urine were examined at two different concentrations: 500 and 1000 ng/ml, each assayed in triplicate. The absolute peak heights of MTX and 7-OH-MTX obtained for the extracted samples were compared with those of known amounts of fresh standards prepared in mobile phase. As shown in Table 1, ca. 80% of MTX and 70% of 7-OH-MTX were constantly recovered from urine.

For the determination of the intra-assay accuracy and precision, drug-free human urine was prepared containing four different concentration levels: 5, 50, 500 and 1000 ng/ml each of MTX and 7-OH-MTX, and samples were assayed with 5 replicates. The coefficients of variation were less than 8.4 and 7.8%, and the bias ranged from −3.6 to 2.6 and from −2.8 to 2.6% for MTX and 7-OH-MTX, respectively.

To determine the inter-assay accuracy and precision, drug-free human urine was spiked with each analyte at three different concentrations: 5, 50 and 500 ng/ml, and samples were then frozen in 1-ml

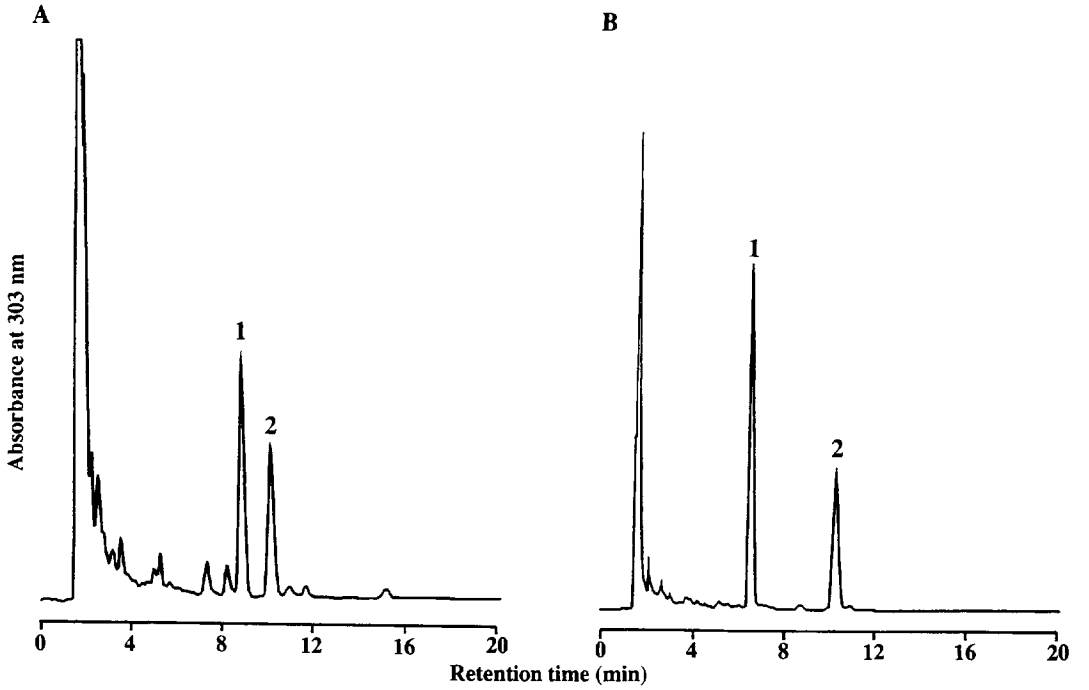

Fig. 2. Chromatograms from analysis of human urine containing 1 μg/ml (final concentration) each of MTX (1) and 7-OH-MTX (2). (A) Mobile phase, 50 m*M* phosphate buffer–acetonitrile (92.4:7.6, v/v) at pH 7.0; flow-rate, 1.0 ml/min. (B) Mobile phase, 50 m*M* phosphate buffer–acetonitrile (90.5:9.5, v/v) at pH 5.3; flow-rate, 1.1 ml/min. The other chromatographic conditions are the same as in Fig. 1.

aliquots at −20°C. The reproducibility was evaluated over five consecutive days. The coefficients of variation were less than 8.8 and 7.2%, and the bias ranged from −0.4 to 4.0 and from −3.6 to 7.6% for MTX and 7-OH-MTX, respectively. The results are summarized in Tables 2 and 3.

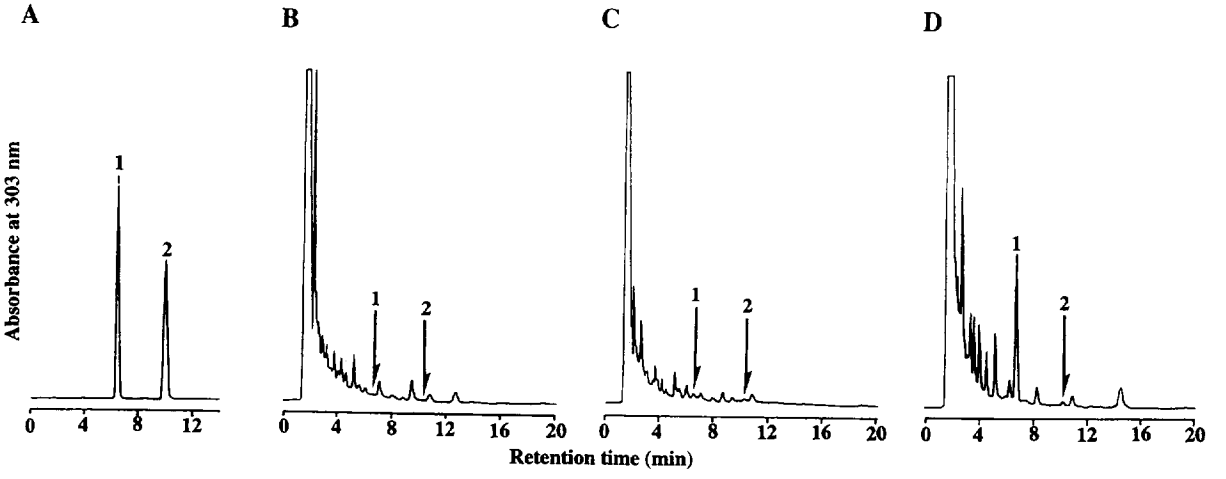

Fig. 3. Chromatograms of (A) standard, (B) drug-free human urine, (C) urine spiked with 5 ng/ml MTX and 7-OH-MTX, and (D) urine specimen 0–24 h post-dose (MTX, 2 mg orally; concentration found, 299 ng/ml of MTX and 13.5 ng/ml of 7-OH-MTX). Peaks: 1=MTX; 2=7-OH-MTX. Chromatographic conditions are the same as in Fig. 2B. Detector sensitivity: B, C, 8 mV/full scale and D, 16 mV/full scale.

Table 1
Recoveries of MTX and 7-OH-MTX from human urine

Spiked concentration (ng/ml)	MTX		7-OH-MTX	
	Recovery (%)	n	Recovery (%)	n
500	81.3±0.8	3	71.2±0.6	3
1000	79.1±1.4	3	69.7±0.9	3

Table 2
Intra-assay and inter-assay accuracy and precision for the determination of MTX in human urine ($n=5$)

Amount added (ng/ml)	Amount found (mean±S.D.) (ng/ml)	C.V. (%)	Bias (%)
Intra-assay			
5	5.13±0.43	8.4	2.6
50	48.2±1.1	2.3	−3.6
500	495.4±13.4	2.7	−0.9
1000	1019.2±28.7	2.8	1.9
Inter-assay			
5	5.20±0.46	8.8	4.0
50	49.8±1.7	3.4	−0.4
500	500.0±9.9	2.0	0.0

3.3. Interference by NSAIDs

Since several NSAIDs are frequently co-administered to MTX therapy with RA patients, we investigated possible analytical interferences of the following substances. A small amount of methanol solution containing sulindac, piroxicam, loxoprofen, naproxen, felbinac, fenbufen, diclofenac and indomethacin was evaporated to dryness in a water bath at 37°C, 1.0 ml of human urine containing MTX and 7-OH-MTX was added, and vortex-mixed. Prepared samples were assayed according to the method described in Section 2, then the fractions of ethyl acetate and ethanol–methanol (6:4) washing were also concentrated, reconstituted with mobile phase, and analyzed by HPLC. Chromatographic separation of NSAIDs was performed on the same analytical column as MTX analysis (Inertsil ODS-2) using 50 m*M* phosphate buffer–acetonitrile (58:42, v/v) at pH 5.0 as the mobile phase. The flow-rate was 1.0 ml/min, and the UV absorbance of the column effluent was monitored at 230 nm.

Table 3
Intra-assay and inter-assay accuracy and precision for the determination of 7-OH-MTX in human urine ($n=5$)

Amount added (ng/ml)	Amount found (mean±S.D.) (ng/ml)	C.V. (%)	Bias (%)
Intra-assay			
5	4.86±0.34	7.0	−2.8
50	51.3±0.9	1.8	2.6
500	508.6±11.0	2.2	1.7
1000	1015.0±29.5	2.9	1.5
Inter-assay			
5	5.38±0.39	7.2	7.6
50	48.3±1.3	2.7	−3.4
500	500.2±11.3	2.3	0.0

The results are shown in Fig. 4 and Table 4. Under the above chromatographic conditions, NSAIDs used in this study were well separated, whereas MTX and 7-OH-MTX overlapped at the position of almost void volume. Over 95.5% of the NSAIDs were recovered from the sum of the ethyl acetate and ethanol–methanol (6:4) fractions. Almost all of them were recovered from the ethyl acetate fraction except fenbufen (70.9%). The recoveries of MTX (80.7%) and 7-OH-MTX (70.7%) obtained from the 1% ammonia–methanol fraction were consistent with the results obtained in the recovery study (Table 1).

4. Discussion

Solid-phase extraction techniques are often used for the sample purification of MTX analysis by HPLC. These include reversed-phase [14–16,18–20] or anion-exchange resin columns [21]. The authors [22] also previously established a high sensitivity of 2.5 ng/ml as the quantitation limit for both MTX and 7-OH-MTX in human serum by HPLC with UV detection at 303 nm, using a Sep-Pak C_{18} cartridge. For urine analysis, however, no available results adapted to isocratic HPLC with UV detection were found although we tested several reversed-phase or anion-exchange resin columns for the sample purification.

Normal solid-phase extraction with direct loading of small amounts of a urine specimen was examined next. In the results, urinary interferences could be favourably removed by the sample preparation described in Section 2, thereby leading to the high sensitivity of 5 ng/ml as the quantitation limit for both MTX and 7-OH-MTX in human urine by means of HPLC with UV detection at 303 nm.

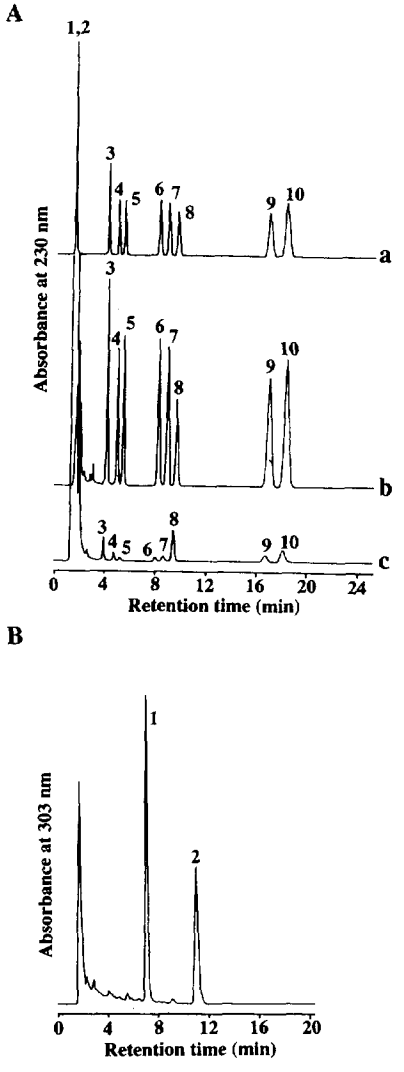

Fig. 4. Chromatograms obtained in the analysis of standard mixture (A–a), and human urine spiked with MTX, 7-OH-MTX, and several NSAIDs (A–b, c, and B). Human urine containing 1 μg/ml of MTX (1) and 7-OH-MTX (2), 5 μg/ml of naproxen (6), 20 μg/ml of sulindac (3), 25 μg/ml of piroxicam (4), and 50 μg/ml of loxoprofen (5), felbinac (7), fenbufen (8), diclofenac (9) and indomethacin (10) was assayed according to the method described in Section 2. Then the fractions of ethyl acetate (A–b) and ethanol–methanol (6:4) washing (A–c) as well as the 1% ammonia–methanol eluate (B) were analyzed by HPLC. Chromatographic separation of the NSAIDs (A) was carried out on the same analytical column as MTX analysis (Inertsil ODS-2) using 50 m*M* phosphate buffer–acetonitrile (58:42, v/v) at pH 5.0 as the mobile phase. The flow-rate was 1.0 ml/min, and the column effluent was monitored at 230 nm. Chromatographic conditions for MTX and 7-OH-MTX analysis (B) are the same as in Fig. 2B.

Since patients with RA often receive NSAIDs together with MTX, we investigated possible analytical interferences of the following eight typical kinds of NSAIDs used for RA therapy: sulindac, piroxicam, loxoprofen, naproxen, felbinac, fenbufen, diclofenac and indomethacin. In the results, no interferences, including a change in the recoveries of MTX and 7-OH-MTX from urine, were found. Furthermore, more than 70% of the NSAIDs used in this study were recovered from the fraction of ethyl acetate washing. The results indicate that these NSAIDs can be analyzed from the same specimen, and can be recovered during the sample purification process.

A high inter-individual variability for both plasma concentrations and urinary excretion rate of MTX and 7-OH-MTX has been confirmed during MTX low-dose therapy [23,24]. A number of factors may contribute to this variability, e.g., influence of the combined drugs on renal clearance of MTX, inter-individual differences in bioavailability due to the existence of active transport through drug absorption phase, gastro-intestinal lesion, renal insufficiency, and the participation of other physical conditions. Recently, on the other hand, Seideman [25] reported that inter-individual differences in dose–effect response curves were observed but a clear dose–effect relationship was obtained in individual RA patients on 5–20 mg oral MTX given once weekly. Hence MTX doses should be adjusted individually for each patient in order to improve efficacy and decrease dose-dependent side effects.

Therefore, it is considered that drug monitoring not only in blood but also in urine is of importance in order to improve the MTX low-dose therapy in RA patients.

5. Conclusions

The present method could favourably remove the urinary interferences by using a normal solid-phase extraction, thereby enabling simple manipulation and reducing analytical time to less than 20 min as well as achieving a high sensitivity of 5 ng/ml as the quantitation limit for both of MTX and 7-OH-MTX in human urine. The method may be suitable for

Table 4
Recoveries of MTX, 7-OH-MTX and several NSAIDs from human urine

Compound	Spiked concentration (μg/ml)	Recovery (%)			
		Ethyl acetate fraction	EtOH–MeOH (6:4) fraction	1% NH_3–MeOH fraction	Total
MTX	1.0	-	-	80.7	-
7-OH-MTX	1.0	-	-	70.7	-
Sulindac	20	87.1	10.7	-	97.8
Piroxicam	25	91.4	6.1	-	97.5
Ioxoprofen	50	101.9	2.8	-	104.7
Naproxen	5	100.5	2.9	-	103.4
Felbinac	50	98.6	4.0	-	102.6
Fenbufen	50	70.9	26.7	-	97.6
Diclofenac	50	92.4	6.2	-	98.6
Indomethacin	50	87.0	8.5	-	95.5

Data are expressed as mean ($n=2$).

routine monitoring of urine levels of MTX and 7-OH-MTX during low-dose MTX–RA therapy.

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