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DETERMINATION OF METHOTREXATE AND ITS METABOLITES 7-HYDROXYMETHOTREXATE AND 2,4-DIAMINO-N^{10}-METHYLPTEROIC ACID IN BIOLOGICAL FLUIDS BY LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

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SUMMARY

A high-performance liquid chromatographic method involving post-column cleavage and fluorimetric detection has been developed for the determination of methotrexate and its metabolites in biological fluids. The cleavage is based on photooxidative reaction of methotrexate and its metabolites to highly fluorescent products. The photoreaction occurs during the flow of eluate containing a certain amount of hydrogen peroxide through a PTFE capillary irradiated by UV light. The method allows the determination of methotrexate, 7-hydroxymethotrexate and 2,4-diamino-N^{10}-methylpteroic acid in plasma, urine and ascitic fluid samples at concentrations as low as $2 \cdot 10^{-8}$ *M*.

INTRODUCTION

Methotrexate (MTX; 2,4-diamino-N^{10}-methylpteroylglutamic acid) is a widely used cytostatic. Its effect on cell growth can be explained by the inhibition of dihydrofolate reductase, one of the key enzymes of the nucleic acid biosynthetic pathway.

A number of high-performance liquid chromatographic (HPLC) methods with UV detection have been published for MTX assay in biological samples [1—9], with a detection limit of ca. $1 \cdot 10^{-7}$ *M*. The sensitivity of the assay can be improved about ten-fold using on-column concentration techniques [10, 11], extraction [12—14] or Sep-Pak C_{18} cartridges [15]. The most sensitive methods (detection limit of ca. $1 \cdot 10^{-8}$ *M*) involve pre-column derivatization and fluorimetric detection [16, 17]. Their disadvantage is a time-consuming preparation of samples and also a decrease in selectivity of the assay. For the

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transformation of MTX and its metabolites to fluorescent products, Nelson et al. [16] used oxidation by permanganate (limit of detection $2 \cdot 10^{-8}$ *M*) and Deen et al. [17] reduced MTX by dithionite (limit of detection $5 \cdot 10^{-9}$ *M*). In this paper, a new detection method for the assay of MTX and its metabolites is described, which is based on simple post-column photooxidative cleavage of MTX and its metabolites to fluorescent products and their detection.

EXPERIMENTAL

Chemicals

MTX (99.5% purity by HPLC), citrovorum factor (93%), 2,4-diamino-N^{10}-methylpteroic acid (APA, 95%), 7-hydroxymethotrexate (7-OH-MTX, 80%), aminopterin (95%) and methopterin (95%) were prepared in the Research Institute of Pure Chemicals (Lachema, Brno, Czechoslovakia). Folic acid (96.6% purity by HPLC) was supplied by Fluka (Buchs, Switzerland) and acetonitrile LiChrosolv by Merck (Darmstadt, F.R.G.). All other chemicals were of analytical grade.

Equipment

The basic liquid chromatograph consisted of a Spectra-Physics Model SP 8100 liquid chromatograph with a Model SP 8110 autosampler (Spectra-Physics, Darmstadt, F.R.G.) fitted with a 10-μl or 80-μl loop, and a Schoeffel Instruments Model FS 970 fluorescence detector (Kratos, Trappenkamp, F.R.G.) with 5-μl flow cell. Excitation and emission wavelengths were set at 282 nm (or 360 nm) and 417 nm, respectively. For the separation, a stainless-

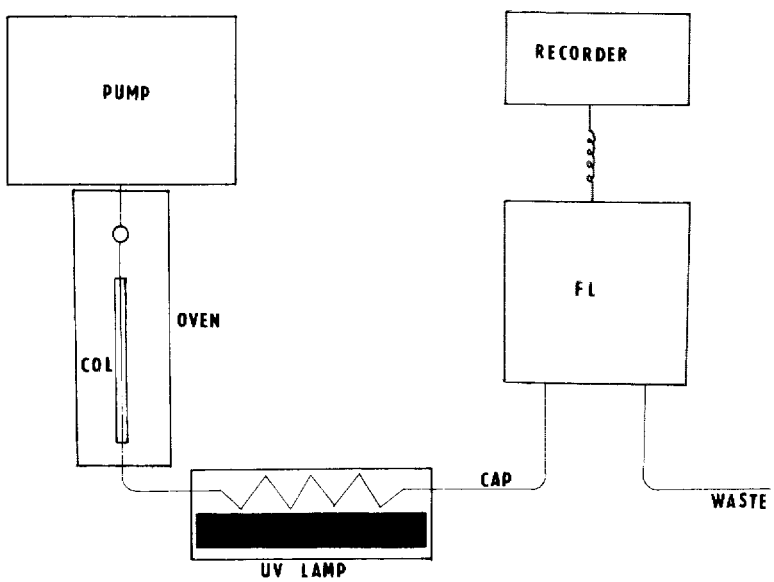

Fig. 1. Scheme of the chromatographic system for the analysis of MTX and its metabolites with post-column cleavage and fluorimetric detection. Abbreviations: COL = column, CAP = PTFE capillary tubing, FL = fluorescence detector. Individual parts of the system are described in Experimental.

steel column (25 × 0.46 cm I.D.) packed with Silasorb C_{18}, particle size 10 μm (Lachema), was used, which contained (on the top) a 3-mm thick layer of Silipor C_{18} (coarse silica derivative, Lachema) separated from the microparticulate sorbent by a stainless-steel net. The separation was tested on a stainless-steel column (25 × 0.46 cm I.D.) packed with Silasorb Amine (silica gel modified with aminopropyl groups, Lachema), particle diameter 10 μm.

The photooxidation was accomplished in a PTFE capillary (0.25 mm I.D., 1.59 mm O.D.), which was inserted between column and detector. The capillary was irradiated by a Sylvania G8T5 germicidal lamp (254 nm), the length of irradiation being 90 cm. A scheme of the apparatus is shown in Fig. 1.

The course of photolytic reaction was checked using a system with an additional column attached to the outlet of the irradiated capillary. The second column (6.8 × 0.46 cm I.D., packed with Silasorb C_{18}, particle size 7.5 μm) was connected with a Spectra-Physics Model SP 8400 variable-wavelength detector set at 307 nm.

Mobile phase

The mobile phase was 0.05 *M* phosphate buffer (pH 6.2)—acetonitrile—30% hydrogen peroxide (10:1:0.015). After degassing by helium purging, it was pumped through the column and the irradiated capillary at a flow-rate of 1 ml/min. Both the chromatographic separation and photooxidation were carried out at 45°C.

Samples

Heparinized blood was centrifuged for 12 min at 12 000 *g* before analysis. The volume of plasma samples injected into the column was 80 μl. Samples of urine and of ascitic fluid were diluted to obtain the concentration of MTX within the range 10^{-6}—10^{-5} *M* and the volume injected was 10 μl. Samples which could not be chromatographed immediately were stored frozen at −24°C.

Standard containing $5 \cdot 10^{-7}$ *M* MTX, 7-OH-MTX and APA, respectively, was prepared fresh before its application to the column by mixing 0.9 ml of blank plasma with 0.1 ml of a $5 \cdot 10^{-6}$ *M* aqueous solution of MTX, 7-OH-MTX and APA. Standards for analysis of urine and ascitic fluid were prepared in a similar way.

Quantification of MTX, 7-OH-MTX and APA was based on peak heights from the standard solution.

RESULTS AND DISCUSSION

First of all, the separation of MTX and its metabolites was tested on Silasorb Amine and Silasorb C_{18} columns. Better results were obtained with reversed-phase sorbent, which was then used for all subsequent separations. The analytical column was prevented from clogging by a 3 mm thick layer of coarse C_{18} silica derivative placed on the top of the analytical packing and separated by a stainless-steel net. Such an adapted column could be used for about 25 injections of 80-μl plasma samples before the protecting sorbent was renewed. Need of this change was indicated by an increase of back-pressure to 80 bar, while standard working pressure was ca. 30 bar.

The optimum composition of the mobile phase was found to be a mixture of 10 vol. of 0.05 *M* phosphate buffer (pH 6.2), 1 vol. of acetonitrile and 0.015 vol. of 30% hydrogen peroxide. Using this mobile phase and the column described above, the resolution (R_s) of MTX and 7-OH-MTX was 1 and the retention time of APA was 8.5 min. The amount of sample injected into the column was 10 or 80 μl, according to the MTX level: for concentrations higher than $5 \cdot 10^{-7}$ *M*, a sample volume of 10 μl was satisfactory; some samples of urine or ascitic fluid had to be diluted. In this case, the separation efficiency of the column was ca. 3500 theoretical plates for MTX and the injection of 80-μl samples resulted in about a 10% decrease in efficiency.

According to the literature [18], folic acid can be photolytically cleaved to 2-amino-4-hydroxypteridine-6-carboxaldehyde. On further irradiation, it is oxidized to carboxylic acid which is then decarboxylated to 2-amino-4-hydroxypteridine. All products of photolytic decomposition when irradiated by UV light at wavelengths of 350—360 nm show a strong fluorescence with maximum emission at 450 nm. A higher response is observed for aldehyde and carboxylic acid derivatives than for the decarboxylated product.

A similar course of photooxidation and high reaction rate were presumed for MTX, 7-OH-MTX and APA. The photolytic cleavage of these compounds in the eluate from the column was carried out in the presence of hydrogen peroxide by passing this solution through the capillary irradiated by a UV light source at 254 nm. The advantage of hydrogen peroxide in comparison with other oxidants (e.g. potassium permanganate or potassium dichromate) is its transparency; it does not absorb light at wavelengths near to the emission maximum of the fluorescent products of MTX cleavage. The dependence of the

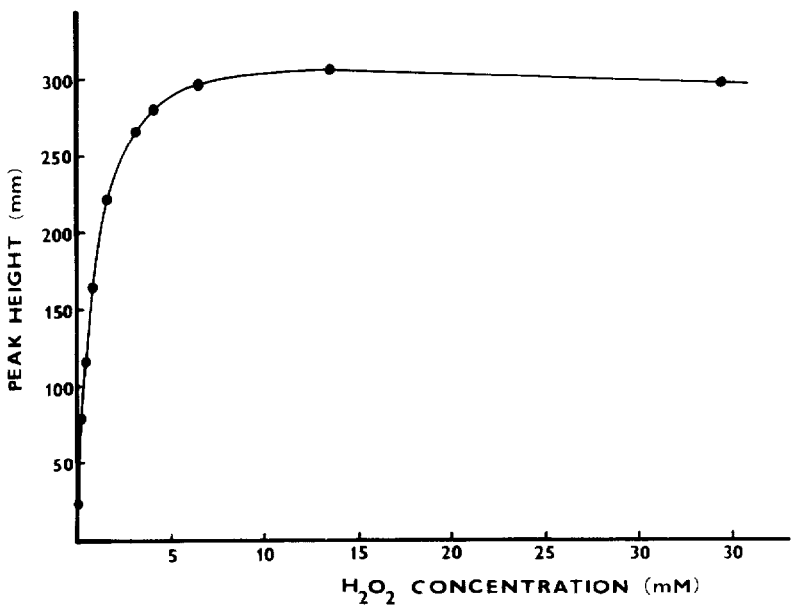

Fig. 2. The dependence of the fluorescence detector's response on the concentration of hydrogen peroxide in the mobile phase for MTX determination. The irradiated part of the PTFE capillary was 110 cm long.

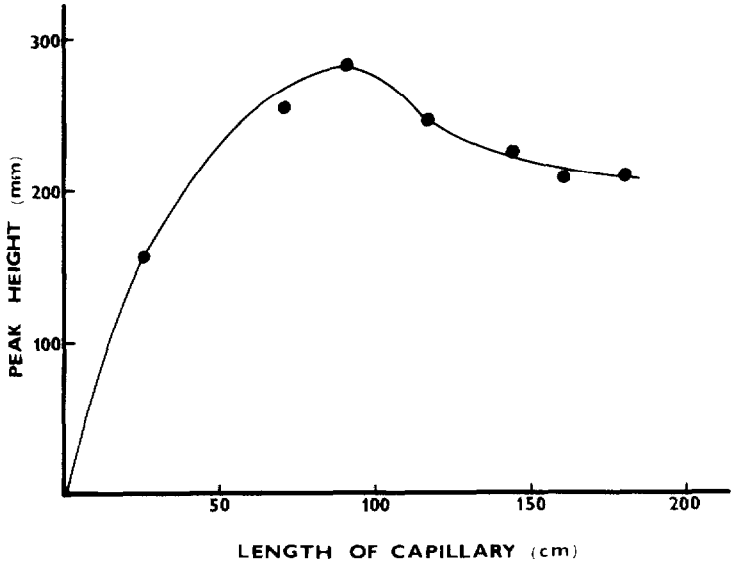

Fig. 3. The dependence of the fluorescence detector's response on the length of the irradiated part of the PTFE capillary. The concentration of hydrogen peroxide in the mobile phase was 12 m*M*.

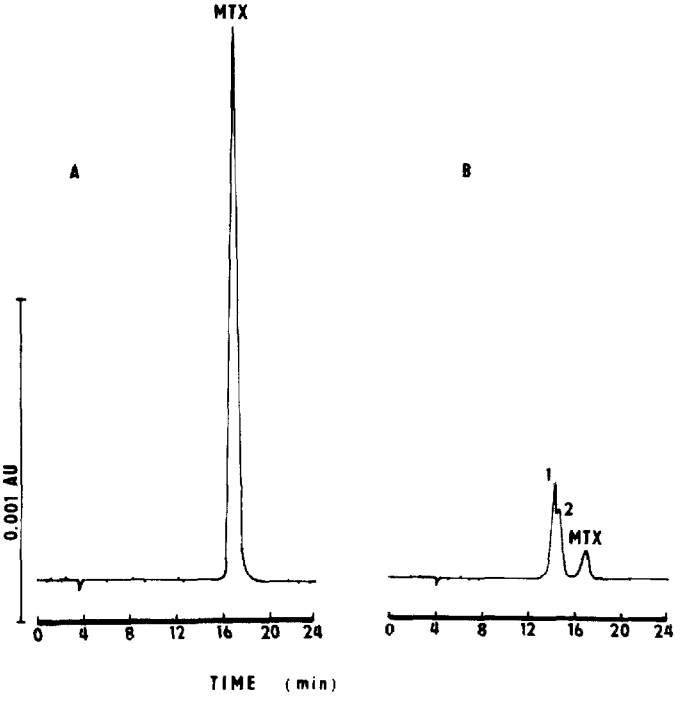

Fig. 4. Chromatograms used for calculation of the degree of conversion of MTX to products of its photolytic cleavage. Aliquots of 80 μl of a $5 \cdot 10^{-5}$ *M* aqueous solution of MTX were injected into the system of two columns described in Experimental. (A) Chromatogram of MTX (without irradiation); (B) chromatogram obtained after irradiation of the eluate from the first column.

fluorescence detector's response on the hydrogen peroxide concentration is shown in Fig. 2. The maximum response of the detector was found for a 12 m*M* concentration of hydrogen peroxide; larger amount of hydrogen peroxide causes a response decrease. Such a concentration of hydrogen peroxide needed for photooxidation does not influence the retention behaviour of MTX and its metabolites.

Fig. 3 shows the dependence of the detector response for MTX on the length of capillary irradiated by UV light, i.e. on reaction time. At a flow-rate of 1 ml/min, 0.25 mm I.D. and 45°C, the best results were obtained for a 90-cm

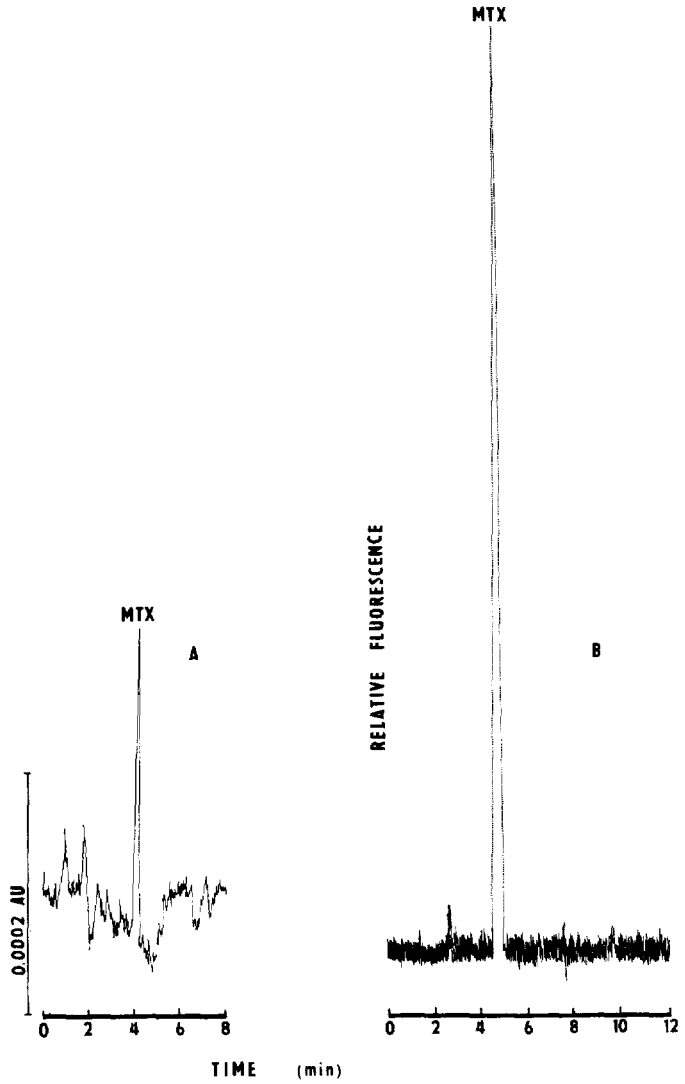

Fig. 5. Comparison of the sensitivity of the post-column cleavage method with fluorimetric detection and with UV detection. Sample: 10 μl of 10^{-6} *M* MTX; (A) UV detection at 307 nm; (B) fluorimetric detection (excitation wavelenght 282 nm, emission cut-off filter 417 nm). Column, packing, mobile phase and conditions of analysis are described in Experimental.

capillary. In shorter capillaries, the optimum conversion of MTX to fluorescent products was not achieved and in longer ones a subsequent reaction took place yielding less fluorescent products.

During the optimization study, the course of photolytic reaction was checked using a system with an additional column and UV detector (see *Equipment*). Chromatographic separation of MTX and its photolytic products in this system at optimum reaction conditions is shown in Fig. 4B. If the eluate from the first column was not irradiated, only a single peak of MTX was obtained (Fig. 4A). The heights of MTX peaks from both experiments was used for the calculation of the degree of conversion; a value of ca. 98% was obtained. With the fluorescence detector, only one fluorescent product (peak 1) was detected, which could be attributed to 2,4-diaminopteridine-6-carboxaldehyde or to the corresponding carboxylic acid. A second photolytic product

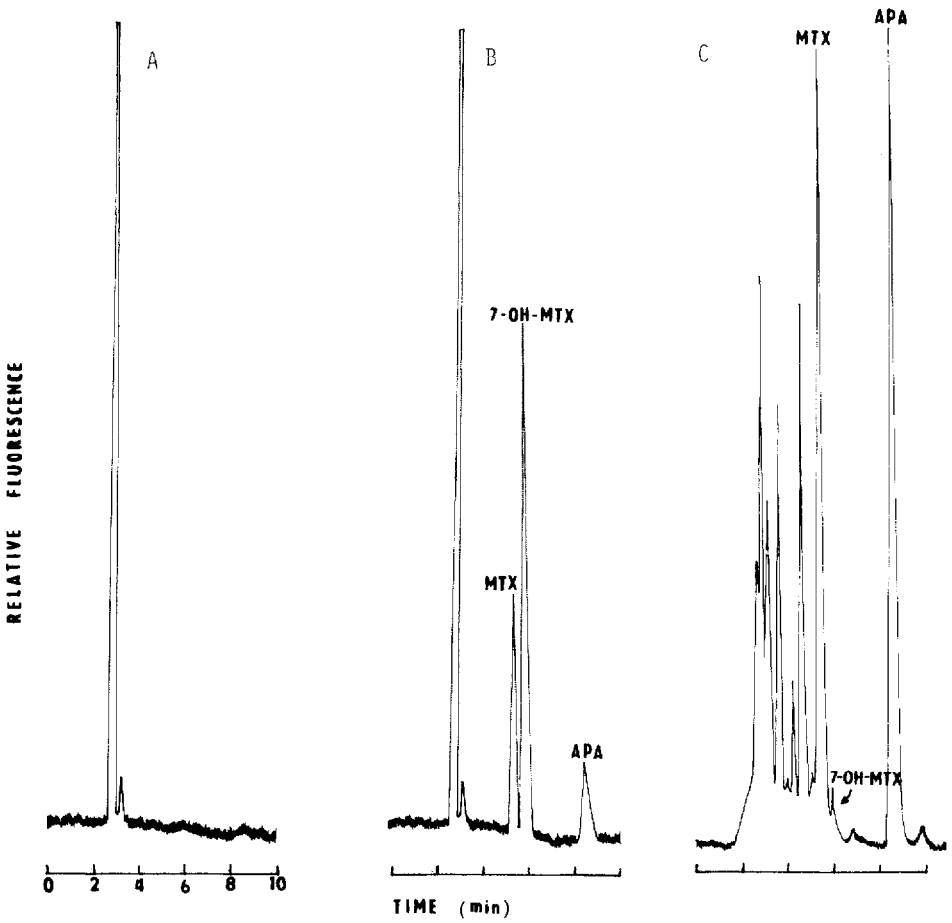

Fig. 6. Chromatograms of plasma and urine samples of a patient with an ascitic tumour treated with MTX. (A) Chromatogram of plasma before MTX administration; (B) plasma 24 h after administration of 50 mg of MTX into the ascitic tumour (concentrations of MTX and its metabolites found in the sample: MTX, 192 ng/ml; 7-OH-MTX, 417 ng/ml; APA, 95 ng/ml); (C) chromatogram of urine of the same patient 4 h after MTX administration (concentrations found: MTX, 11 μg/ml; 7-OH-MTX, 0.3 μg/ml; APA, 22 μg/ml).

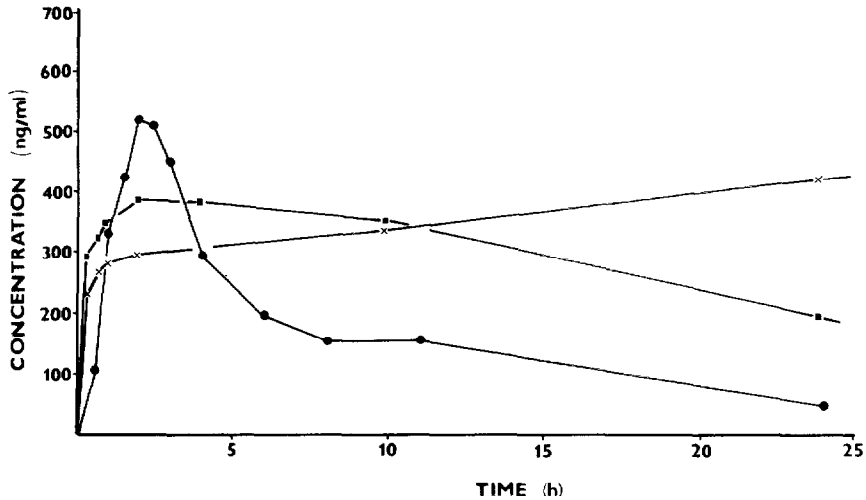

Fig. 7. Time dependence of the MTX (▪) and 7-OH-MTX (×) levels in the plasma of a patient after a single dose of 50 mg of MTX into the ascitic tumour, and time dependence of the MTX (•) level in plasma of a patient after oral administration of 15 mg of MTX.

(peak 2) detected by the UV detector could be *p*-aminobenzoylglutamic acid.

As follows from the chromatograms in Fig. 5, post-column photooxidative cleavage with fluorescence detection is five- to ten-fold more sensitive than UV detection. Minimum concentrations of MTX and its metabolites in plasma determined by the proposed cleavage method (80-μl sample) were $2 \cdot 10^{-8}$ *M* for MTX, $3 \cdot 10^{-8}$ *M* for 7-OH-MTX and $3 \cdot 10^{-8}$ *M* for APA. Folic acid and citrovorum factor, which could be present in some samples, did not interfere with the determination of MTX and its metabolites.

A linear relationship between peak height and MTX concentration up to $5 \cdot 10^{-5}$ *M* was observed. A coefficient of variation of $< 2.5\%$ was found for concentrations ranging from $5 \cdot 10^{-8}$ to $1 \cdot 10^{-6}$ *M*.

The method was used for monitoring the concentrations of MTX, 7-OH-MTX and APA in plasma, urine and ascitic fluid. The time of analysis was 10 min. Fig. 6 shows chromatograms for blank plasma (A), for a sample of plasma collected 24 h after the administration of a single dose of 50 mg of MTX into a human ascitic tumour (B) and for a sample of urine excreted 4 h after the same application (C).

In Fig. 7, the time dependence of MTX and 7-OH-MTX in plasma after a single dose of 50 mg of MTX into a human ascitic tumour and the time dependence of the MTX plasma level after oral application of 15 mg of MTX is presented.

CONCLUSIONS

The main advantage of the described method is the use of sensitive and selective detection, enabling samples to be analysed without concentrating MTX and its metabolites. The sensitivity of the method allows the monitoring of MTX and its metabolites in biological fluids even after the application of low doses of the cytostatic. According to the results of preliminary experiments,

the method described in this paper could be applied also to the detection of folic acid, methopterin and aminopterin.

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