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## DETERMINATION OF METHOTREXATE AND ITS MAJOR METABOLITE, 7-HYDROXYMETHOTREXATE, USING CAPILLARY ZONE ELECTROPHORESIS AND LASER-INDUCED FLUORESCENCE DETECTION

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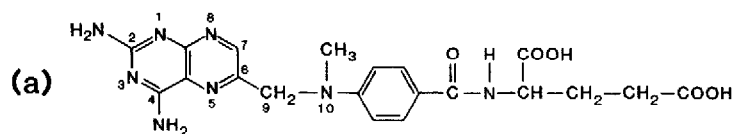
### SUMMARY

High-dosage methotrexate therapy requires careful monitoring of the drug in serum to ensure minimal toxic effects. A simple, rapid and sensitive method for the separation and quantitation of methotrexate and its major metabolite, 7-hydroxymethotrexate, using high-voltage capillary zone electrophoresis combined with laser-induced fluorescence detection is described. The detection limit for methotrexate is as low as  $5 \cdot 10^{-10}$  M (signal-to-noise ratio = 3), while that for 7-hydroxymethotrexate is  $2 \cdot 10^{-9}$  M. The linearity of the system extends over nearly four orders of magnitude for both methotrexate and 7-hydroxymethotrexate. The extraction efficiency for the drug and its metabolite from serum is 80-85% using a Sep-Pak C<sub>18</sub> cartridge. Quantitation of methotrexate in serum was possible in the  $10^{-10}$  M range, nearly two orders of magnitude lower than that currently obtainable by existing methods. Good correlation ( $r=0.99$ ) for serum methotrexate concentrations was obtained with an enzyme-multiplied immunoassay technique. Comparison with an enzyme inhibition assay also provided similar results

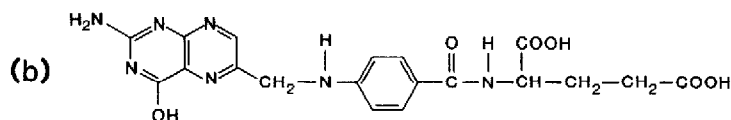
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### INTRODUCTION

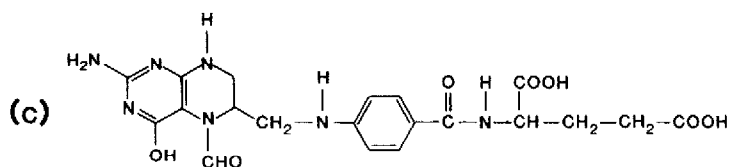
The antifolate methotrexate (MTX, amethopterin, 2,4-diamino-N<sup>10</sup>-methylpteroylglutamic acid, L-(+)-N-[p-[(2,4-diamino-6-pteridiny]methyl]-methylamino]benzoyl] glutamic acid), is one of the most widely used anticancer drugs [1,2]. It has been shown to be highly effective in the treatment of acute leukemia, osteosarcoma, choriocarcinoma and non-Hodgkin's lymphoma. The molecular structure of MTX, as shown in Fig. 1a, is very similar to that of folic acid (Fig. 1b); it differs only in that folic acid has a hydroxyl group in place of the 4-amino group on the pteridine ring and has no methyl group at the N<sup>10</sup> position. It is well known that the production of reduced folates from folic acid by the enzyme dihydrofolate reductase (DHFR) is necessary for the metabolic



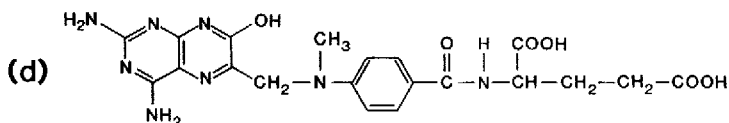
METHOTREXATE



FOLIC ACID



LEUCOVORIN



7-HYDROXYMETHOTREXATE

Fig. 1. Chemical structures of (a) methotrexate, (b) folic acid, (c) leucovorin and (d) 7-hydroxymethotrexate.

transfer of 1-carbon units in a variety of biochemical reactions [3]. One of these reactions, which is essential in cellular proliferation, is the biosynthesis of thymidylic acid, the nucleotide specific to DNA. Reduced folates are also important as cofactors in the conversions of glycine to serine and of homocysteine to methionine. The mechanism of action of MTX is by inhibition of DHFR due to a much greater affinity of the drug for the enzyme than the closely related folic acid. Therefore, in presence of MTX, key reactions in the multiplication of cells are shut down. Since the introduction, in 1948, of folic acid antagonists by Farber et al. [4] the dose of MTX administered to patients has steadily increased. A major step occurred in 1975, when Djerassi [5] undertook the study of high-dose MTX therapy, up to 20 g/m<sup>2</sup> body surface, followed with leucovorin (citrovorum factor, folinic acid) rescue. The supply of leucovorin, a tetrahydrofolate whose structure is shown in Fig. 1c, bypasses the reduced folates biosynthesis with DHFR, and consequently rescues normal, as well as tumor cells. High-dose MTX therapy is generally more successful than the conventional low-dose therapy (25–50 mg/m<sup>2</sup>),

without leucovorin rescue. MTX elimination varies widely from patient to patient due to different metabolism routes and organ excretions. Also, the much lower solubility of the major metabolite, 7-hydroxymethotrexate (7-OHMTX) (Fig. 1d), has been associated with deposition in kidney nodules and consequent nephrotoxicity. Therefore, it becomes imperative to quantify rapidly and accurately MTX serum levels to coordinate effectively the citrovorum factor rescue.

Several assay methods for the determination of MTX in biological fluids have been reported. These include enzymatic assay [6], enzyme-multiplied immunoassay techniques (EMIT) [7], radiochemical competitive protein binding assay [8], radioimmunoassay [9,10], radiometric assay [11] and fluorescence polarization immunoassay [12]. These microbiological assays, however rapid and sensitive (in the  $10^{-8}$  M range), lack specificity, and cross-reactions with 7-OHMTX [13] often result in erroneous values for MTX concentrations [14]. In parallel to these assays, more specific methods have been developed, e.g. mass spectrometry [15] and isotachopheresis [16]. However, mass spectrometry is not suitable for quantitation and isotachopheresis is too insensitive for practical clinical applications. The recent development of high-performance liquid chromatography (HPLC), coupled with UV [17], fluorescence [18,19], coulometric [20] or amperometric [21] detection, has considerably improved MTX monitoring, since HPLC methods have the potential to measure both the parent drug and its metabolites. Detection limits for MTX as low as  $2.2 \cdot 10^{-9}$  M have been reported [21]. On the other hand, such HPLC techniques require considerable hardware, expensive and easily-damaged separation columns and sometimes long sample preparation and analysis times.

Capillary zone electrophoresis (CZE), first introduced by Mikkers et al. [22] and then by Jorgenson and De Arman Lukacs [23], has been increasingly used in the analysis of a variety of substances, such as amino acids [24], proteins [25], nucleotides [26], nucleosides [27], drugs [28,29], etc. The effective heat dissipation from the small-bore tubing makes it possible to apply high voltages, resulting in high separation efficiencies in short analysis times [30]. In this paper, we present the application of CZE to the simultaneous determination of MTX and its major metabolite, 7-OHMTX, in serum of patients under high-dose MTX therapy. Sensitivity and linearity of the method will be discussed, and the results will be compared with assays routinely used in clinical laboratories.

## EXPERIMENTAL

### *Apparatus*

The experimental set-up of the capillary zone electrophoresis-laser-induced fluorescence (CZE-LIF) detection system is similar to that used by Gassmann et al. [31] and is shown in Fig. 2. A 100 cm length (75 cm to detector) of  $75 \mu\text{m}$  I.D. fused-silica capillary (Scientific Glass Engineering, Austin, TX, U.S.A.) was used as the conduit for the electrophoretic separation. Each end of the capillary was dipped in a 4-ml glass vial containing approximately 2 ml of buffer-electrolyte solution. Connection to the high-voltage power supply was provided by a strip of platinum foil dipping in the same solution as the end of the capillary. The current

through the capillary was measured as a voltage across a 1-k $\Omega$  resistor in the grounded end of the circuit. The capillary was encased in a plexiglass box, which when opened automatically shut off the high voltage. The capillary was exposed to ambient air, i.e., the capillary was air-cooled. Another set-up, similar to the one described above, utilized a 60 cm length (45 cm to the detector) of 200  $\mu\text{m}$  I.D. fused-silica capillary (Hewlett-Packard, Santa Clara, CA, U.S.A.). The capillary was coiled on a plastic perforated strip and inserted into the cavity of an aluminum block. Water from a temperature-controlled bath circulated in the cavity allowing for thermostated conditions. The 325-nm radiation ( $\sim 17$  mW) of a Model 4050B He-Cd laser (Liconix, Sunnyvale, CA, U.S.A.) was directed into the capillary (a 1-cm region of the capillary was stripped of the polyimide coating to render it transparent to the UV radiation) using a 66  $\mu\text{m}$  core diameter fused-silica optical fiber (Galileo Electro-Optics, Sturbridge, MA, U.S.A.) as shown in the Fig. 2 inset. A 200  $\mu\text{m}$  core diameter fused-silica fiber (Diaguide, Fort Lee, NJ, U.S.A.) was used with the 200- $\mu\text{m}$  capillary. Fluorescence was collected at right angles using a 1000  $\mu\text{m}$  core diameter fused-silica optical fiber (Math Assoc., Westbury, NY, U.S.A.). The fluorescence emission from the exit end of the 1000- $\mu\text{m}$  fiber was passed through a monochromator (ISA Model H10) or sodium nitrite-saturated solution filter to minimize background radiation and through a 450-nm interference filter (35-nm FWHM bandpass) to maximize fluorescence signal collected. The radiation was then directed onto a Centronix 4249B photomultiplier tube (PMT). The PMT signal was conditioned using a current-to-voltage converter and a low-pass filter (rise time = 100 ms) before being fed into a strip-chart recorder.

### Reagents

MTX, 2-(N-morpholino)ethanesulfonic acid (MES), N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and tris(hydroxymethyl)-ami-

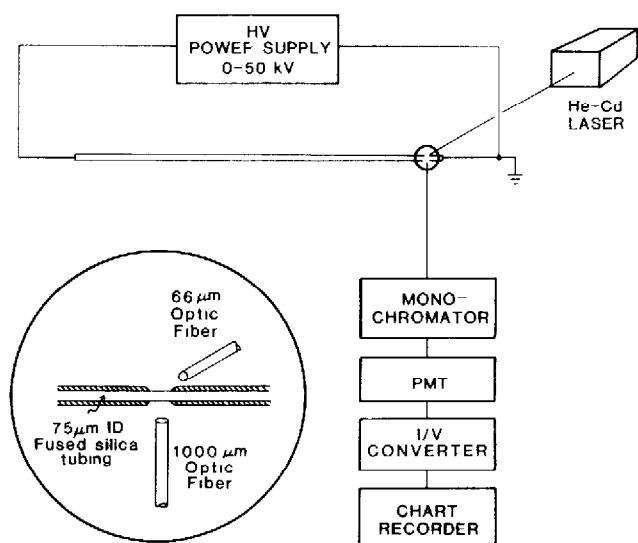


Fig. 2. Experimental set-up for capillary zone electrophoresis with laser-induced fluorescence detection.

noethane (Tris) were purchased from Sigma (St. Louis, MO, U.S.A.) and used without further purification. 7-OHMTX was a gift from Dr. David Johns (National Cancer Institute, Bethesda, MD, U.S.A.). Reagent-grade sodium chloride, sodium sulphate, sodium acetate and sodium hydroxide were used for all experiments. Deionized, distilled water was used throughout. HPLC-grade methanol was used for the experiments requiring methanol. Solvents were degassed before use. A stock solution of MTX ( $1 \cdot 10^{-3} M$ ) was prepared by dissolving the appropriate amount of MTX in 10 mM sodium chloride. A stock solution of 7-OHMTX ( $2 \cdot 10^{-5} M$ ) was prepared in water. The solutions were stored in the freezer at  $-4^{\circ}C$  and were found to be stable for more than a month. Preparation of standard solutions from the stock solution was achieved by dilution with water.

#### *Electrophoretic conditions*

For the analysis with MTX alone in the sample, the following buffer-electrolyte was used: 5 mM MES, 5 mM Tris and 1 mM sodium chloride; the pH of the solution was adjusted to 6.7 with 1 M sodium hydroxide. A 20-kV potential gradient was used for the analysis with either the 75- or 200- $\mu m$  capillary.

When analyzing samples containing MTX and 7-OHMTX, a buffer mixture of 5 mM MES and 16 mM sodium sulphate was prepared and the pH adjusted to 6.7 with 1 M sodium chloride. The solution was then made 30% (v/v) in methanol. Separations were performed in a 75  $\mu m$  I.D. capillary with an applied potential of 25 kV. Electrokinetic injection was chosen because it is easy to perform and the concentrations of analytes were low in comparison to the buffer-electrolyte [32]. Injection of sample was effected by placing one end of the capillary in a vial containing the analyte and applying a voltage of 10 kV for a period of 5–10 s.

#### *Sample treatment*

Two methods were considered for recovery studies of MTX and 7-OHMTX from serum samples.

*Method A.* To 200  $\mu l$  of serum, 200  $\mu l$  of 20% (w/v) trichloroacetic acid (TCA) were added. The sample was heated at  $100^{\circ}C$  for 5 min, cooled and then centrifuged at 12 600  $g$  for 10 min to remove precipitated proteins. An aliquot of the supernatant was used for the oxidation.

*Method B.* A Sep-Pak  $C_{18}$  cartridge (Waters Assoc., Milford, MA, U.S.A.) was conditioned using 10 ml of methanol followed by 10 ml of 0.2 M sodium acetate (pH 5.10). A 500- $\mu l$  volume of serum was mixed with 1500  $\mu l$  of 0.2 M sodium acetate (pH 5.10) and loaded on the prepared Sep-Pak cartridge. The cartridge was then washed with 10 ml of water to remove proteins. MTX was eluted from the cartridge with 2 ml of methanol. The eluate was evaporated to dryness under vacuum at  $60^{\circ}C$ . The residue was reconstituted in 1 M MES and an aliquot used for oxidation.

#### *Oxidation of MTX and 7-OHMTX*

An aliquot of MTX and/or 7-OHMTX standard solution or serum extract was mixed with 100  $\mu l$  of 1 M MES (pH 4.00). A 75- $\mu l$  volume of 0.35% potassium permanganate was added and the mixture vortexed. After 1 min, the excess po-

tassium permanganate was reduced using 20  $\mu\text{l}$  of 3% hydrogen peroxide. The solution was then diluted with the buffer-electrolyte used for the electrophoresis before injection.

## RESULTS AND DISCUSSION

MTX is oxidized by potassium permanganate in acidic solution to 2,4-diaminopteridine-6-carboxylic acid, as shown in Fig. 3. This provides the basis for a sensitive method of monitoring MTX [18,19,33,34] because the adduct is more highly fluorescent than MTX. In acidic solutions, the formation of the 2-amino-4-hydroxypteridine-6-carboxylic acid is prevented [35]. The concentration of the potassium permanganate solution used here is a factor of 10 lower than that used in earlier reports [33,34]. Maximum signal was obtained after a 1-min reaction time and was used for all our analyses. The lower concentration of potassium permanganate and the short reaction time minimizes possible side-reactions.

Fig. 4 shows an electropherogram of MTX using the conditions shown in the caption of the figure. This buffer-electrolyte combination was found by optim-

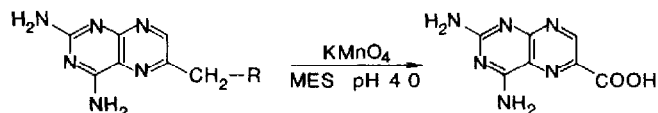


Fig. 3. Oxidation scheme of MTX by potassium permanganate in acidic solution, leading to the formation of 2,4-diaminopteridine-6-carboxylic acid, a highly fluorescent compound.

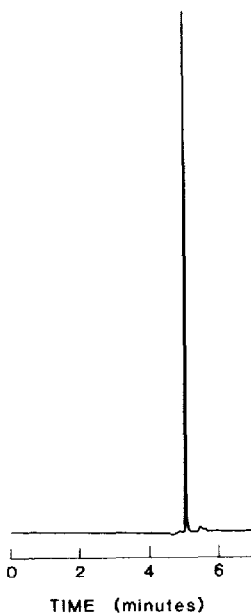


Fig. 4. Electropherogram of MTX, using a 5 mM MES, 5 mM sodium chloride pH 6.7 buffer-electrolyte solution. Capillary, 100 cm  $\times$  75  $\mu\text{m}$  I.D.; applied voltage, 25 kV; current,  $\approx 20 \mu\text{A}$ . The MTX ( $1 \cdot 10^{-6} \text{ M}$ ) sample solution was electrokinetically injected at 10 kV for 10 s.

izing the pH and the type of buffer used. Use of a lower pH gave a broader peak for MTX. Also, Freeman [33] has shown that MTX fluorescence in aqueous solution is optimum at a pH between 5 and 6. In our optimization procedure the pH was not increased above 8 because of possible precipitation of manganese hydroxide and solubilization of silica at higher pH values. Changing the buffer from MES to HEPES or Tris also yielded peaks which were broader. The calculated plate number for the peak obtained in Fig. 4 is greater than 400 000. The high sensitivity of the system is due to the extremely sharp peaks obtainable with our system and the intense radiation ( $\sim 17$  mW) provided by the laser source. The detection limit based on a signal-to-noise ratio of 3 was  $3 \cdot 10^{-9}$  M with the 75  $\mu\text{m}$  I.D. capillary. Further enhancement of sensitivity was obtained by using a 200  $\mu\text{m}$  I.D. capillary. The detection limit for MTX using the 200  $\mu\text{m}$  I.D. capillary is  $5 \cdot 10^{-10}$  M signal-to-noise ratio. This reduction in detection limit using the larger-size capillary is due to the increased volume of sample injected and the increased path length and volume of the detection cell. However, the 200  $\mu\text{m}$  I.D. capillary needed to be thermostated to avoid heating of the electrolyte in the capillary which would lead to peak broadening and loss of resolution. The linearity of the detection system extends over nearly four orders of magnitude ( $1 \cdot 10^{-5}$ – $5 \cdot 10^{-9}$  M) for the 75- $\mu\text{m}$  capillary. Linearity was extended at the lower end to less than  $1 \cdot 10^{-9}$  M when the 200- $\mu\text{m}$  capillary was used.

A broad peak was obtained for 7-OHMTX under the conditions used above. The peak was also partially overlapped with the MTX peak. Since the charge on the MTX and 7-OHMTX is expected to be similar at the pH used (pH 6.7), broadening of the 7-OHMTX peak is probably due to the interaction of the hydroxyl group of the 7-OHMTX with the wall silanol groups and/or the double layer formed. To minimize the interactions with the wall, the ionic strength of the buffer–electrolyte was increased by increasing the sodium chloride concentration from 1 mM up to 25 mM. Only marginal improvement of the broad 7-OHMTX peak was seen. There was also little or no improvement in resolution of the MTX and 7-OHMTX peaks. However, when sodium chloride was replaced by sodium fluoride, the 7-OHMTX peak became quite sharp, whereas there was some broadening of the MTX peak. MTX and 7-OHMTX were baseline-resolved but another small peak partially overlapping the 7-OHMTX peaks was seen. This impurity was found to occur when pure 7-OHMTX was injected. Attempts to resolve the impurity from the 7-OHMTX peak by increasing the salt concentration were unsuccessful. The use of sodium sulphate (16 mM) gave results similar to that obtained with 25 mM sodium fluoride. Accordingly sodium sulphate was chosen as the electrolyte for all subsequent analyses because of the etching property of fluoride on silica in aqueous solutions. The retention times were increased from 4 min for MTX using 1 mM sodium chloride as the electrolyte to 10 min when 16 mM sodium sulphate was used. This increase in retention time is explained by the fact that the electroosmotic flow is inversely related to the concentration of the electrolyte [36].

Further resolution of MTX, the unknown peak, and 7-OHMTX was attempted by manipulating their small differences of interaction with an organic modifier. A 25% (v/v) solution of acetonitrile in the electrolyte did not result in any fur-

ther resolution of the unknown peak and 7-OHMTX. Replacing acetonitrile with methanol resulted in almost baseline resolution of the two peaks. The best results in terms of resolution and plate numbers were obtained with 30% (v/v) methanol in the electrolyte solution. Fig. 5 shows an electropherogram of the separation of a standard mixture of MTX and 7-OHMTX. Higher concentrations of methanol resulted in peak broadening, some loss in resolution and longer migration times. Jorgensen and De Arman Lukacs [37] have shown that the efficiency of separation in CZE is independent of length and directly proportional to the applied voltage under conditions when diffusion is the major source of peak broadening. However, we found that by increasing the capillary length to the detector, there was an increase in the separation of the three peaks. These results are in accordance with the findings of Zhu and Liu [38]. The small unknown peak present in the 7-OHMTX sample was not characterized because the small amount of sample available did not allow for separation and determination of the impurity by mass spectrometry or other methods of analysis. However, attempts were made to determine if the small peak corresponded to one of the commercially available pteridines. No match was found and it was concluded that the synthetic sample had degraded with time to form some impurity. Another peak occurring around 10 min in Fig. 5 was also not characterized and was found to be present in both synthetic samples of MTX and 7-OHMTX and also in the clinical samples. This may be a side-product of the oxidation process or an impurity in the commercial sample of MTX [39]. A calibration curve constructed for 7-OHMTX extends over nearly three orders of magnitude ( $1 \cdot 10^{-6}$ – $5 \cdot 10^{-9}$  M). Higher concentrations could not be studied because of the relative insolubility of 7-OHMTX in

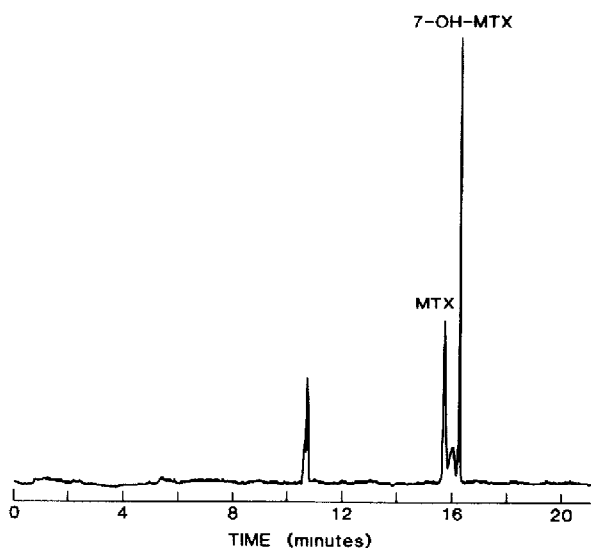


Fig. 5. Electropherogram of MTX and its major metabolite, 7-OHMTX, using a 5 mM MES, 16 mM sodium sulphate, 30% (v/v) methanol, pH 6.7 buffer-electrolyte solution. Same capillary as in Fig. 4. Applied voltage, 25 kV; current,  $\approx 40 \mu\text{A}$ . The MTX and 7-OHMTX ( $1 \cdot 10^{-6}$  M in each) sample solution was electrokinetically injected at 10 kV for 10 s.



aqueous solutions. The peak heights for MTX and 7-OHMTX at any given concentration are reproducible to within 10%. Each injection required preparation of fresh sample because 7-OHMTX shows a substantial decrease in peak heights (10–20%) over a period of 1 h. This reduction may be due to degradation of the oxidation product with time in the presence of hydrogen peroxide and/or the buffer–electrolyte solutions.

Clinical samples were initially deproteinized using method A. Recovery was found to be >95% by comparing with a standard solution of MTX spiked with TCA. However, the response obtained for a sample spiked with TCA was only 65% of that obtained without TCA. This suggests that TCA is causing fluorescence quenching of MTX. Method B gave recoveries of 80–85% for both MTX and 7-OHMTX as shown in Table I. This compares favorably with other reports using the same procedure [14,17,21,40]. By using method B concentration of dilute samples by a factor of 5–10 is possible allowing  $10^{-10}$  M levels of MTX in serum to be monitored. This is one to two orders of magnitude lower than existing methods for the quantitation of MTX in serum [18,19,21,41]. The electropherogram of a clinical sample, deproteinized using method B is shown in Fig. 6. The absence of any extraneous peaks overlapping the analytes of interest shows the effectiveness of the clean-up procedure and the selectivity of the separation–detection system. MTX in clinical samples was extracted using method B and quantitated using CZE–LIF. The values obtained were found to correlate well with an EMIT [7] performed in another laboratory (double-blind study). Fig. 7 shows a good correlation between the two procedures ( $r=0.99$ ) for a wide range of concentrations. In addition to the information about MTX in serum, our procedure can simultaneously provide the concentration of the major metabolite, 7-OHMTX, in serum. A comparison of our method with an enzyme inhibition assay procedure [6] for another set of clinical samples showed good correlation between the two methods ( $r=0.99$ ). However, MTX concentrations were lower by as much as 50% for the CZE–LIF method. The higher value of the enzyme inhibition assay may possibly be due to cross-reactivity of metabolites and related compounds with the enzyme.

TABLE I

## RECOVERY OF MTX AND 7-OHMTX FROM SERUM USING METHOD B

Analyte	Concentration ( $\mu$ M)	Recovery (mean $\pm$ S.D., $n=3$ ) (%)
MTX	0.5	87.0 $\pm$ 2.5
MTX	0.05	80.0 $\pm$ 5.5
7-OHMTX	0.5	82.2 $\pm$ 6.2
7-OHMTX	0.05	79.9 $\pm$ 7.5

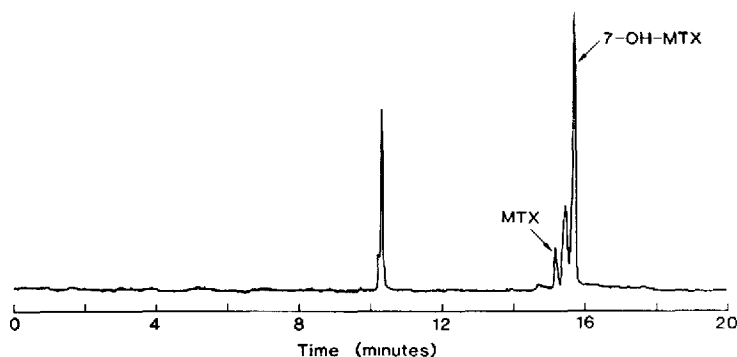


Fig. 6. Electropherogram of a clinical sample from a patient undergoing high-dose MTX therapy. The experimental conditions are the same as in Fig. 5.

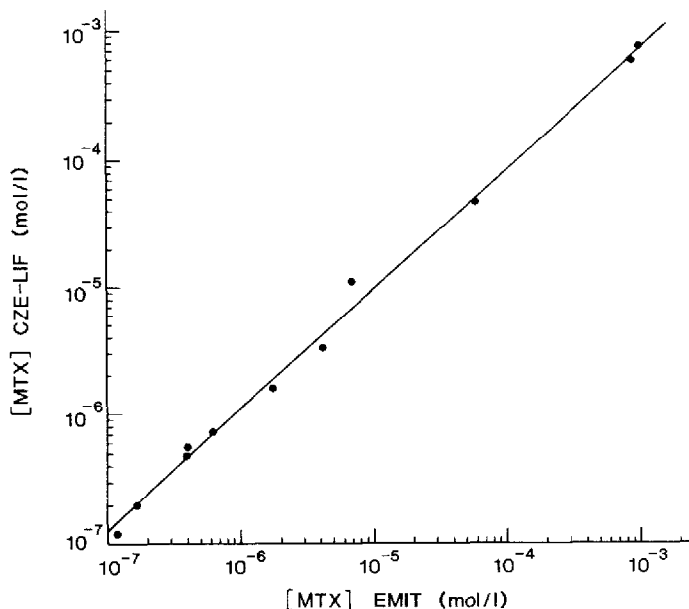


Fig. 7. Comparison of MTX concentrations obtained by CZE-LIF, and an EMIT [7].

## CONCLUSION

We have developed a method for the simultaneous monitoring of MTX and its major metabolite, 7-OHMTX, in serum using the separation power of CZE and the detection power of LIF detection. The sensitivity of our method is one to two orders of magnitude higher than existing methods, caused in part by the extremely sharp peaks characteristic of CZE and in part by the use of LIF detection. The method is quite rapid and an analysis of a clinical specimen can be accomplished in 35–40 min. If only MTX needs to be monitored, the analysis time can be reduced to 15–20 min. The rapid analysis time of the method allows for its use in pharmacokinetic studies. The system is relatively simple to use, and the cap-

illaries are extremely cheap when compared to HPLC columns. The detection system can be made quite inexpensive by using a pen-ray Hg lamp source instead of the laser source. However, this will result in a concomitant loss in sensitivity. Both MTX and its major metabolite, 7-OHMTX, can be quantitated simultaneously in serum samples. The selectivity provided by the separation process and the detection system allow for clean well resolved electropherograms. The system can be automated to handle large numbers of samples on a routine basis, subsequent to the deproteinization step.

#### ACKNOWLEDGEMENTS

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