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ANALYSIS OF METHOTREXATE BY ISOTACHOPHORESIS

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SUMMARY

This paper shows that the anion of methotrexate (MTX) can be readily separated and quantified by isotachophoresis. An extraction method for MTX is also presented, appropriate for isotachophoretic studies. The extraction of MTX is based on the complexation and precipitation of MTX with metal ions. The recovery of MTX from plasma is about 75%.

INTRODUCTION

Isotachophoresis is a technique appropriate for analysing ionic compounds such as drugs and drug metabolites. This paper describes a method to determine methotrexate (MTX): the isotachophoretic analysis of this compound is compared with an already existing analytical method based on an enzyme reaction.

The pK values of MTX are 3.36 (α-carboxyl); 4.70 (γ-carboxyl) and 5.71 (N-1) [1]; hence at pH 8.4 MTX has a negative charge.

The compound is only slightly soluble in water at pH 7, and more soluble at lower or higher pH. MTX is almost insoluble in many organic solvents, pyridine and dimethylformamide being the exceptions.

In isotachophoresis the separation time depends among other things on the concentrations of the ionic species; ions with both high concentrations and mobilities require an increase in the time needed for separation. Thus the chloride ion prevents a rapid analysis for MTX in plasma. Plasma samples can be pretreated by various methods to eliminate chloride, several procedures having been tried by ourselves. Neither the use of ion-exchange resins nor the use of electro-dialysis proved to be useful, because of inadequate recovery or too great a complexity. In the course of our experiments it was found MTX precipitates with certain metal ions, and an isolation procedure was based on this finding.

MATERIALS AND METHODS

Apparatus

Isotachophoretic experiments were performed in an apparatus provided with both UV absorption and conductivity detection, as described by Everaerts et al. [2]*. The separation capillary was approx. 200 mm × 0.2 mm I.D. The electric current was stabilized at 17.5 μA. The electrolyte system used in the isotachophoretic experiments is specified in Table I.

TABLE I

OPERATIONAL SYSTEM FOR MTX ANALYSIS

Parameter	Electrolyte	
	Leading	Terminator
Anion	Cl^-	histidine
Concentration	0.008 *M*	0.008 *M*
Counter ion	Tris	Tris
pH	8.4	9.5
Additive	0.05% Mowiol	
Solvent	water	water

The enzymatic assay of MTX was carried out according to the method of Overdijk et al. [3]. Spectrophotometric determinations were performed at 305 nm, using a Cecil CE 505 double beam instrument.

Radioactivity was measured in an Isocap 300 (Searle). As counting solution, Dimilume (Packard) was used.

Reagents

Reagents used were of analytical grade. Water was purified by Millipore ultrafiltration or was of double distilled quality. Mowiol 8-88 was donated by Hoechst-Holland (Amsterdam, The Netherlands) and purified by ion-exchange (Merck V, Merck, Darmstadt, G.F.R.) chromatography. A commercial preparation of methotrexate (82% MTX) from Lederle (Haarlem, The Netherlands) and a 95% pure preparation kindly supplied by them, were used. [3′-5′,9(*n*)-^{3}H]Methotrexate (spec.act. 250 mCi/mmol) was obtained from The Radiochemical Center (Amersham, Great Britain).

Isolation procedure

An isolation procedure consisting of three steps was used.

(1) Plasma (0.4 ml) and 0.1 ml dimethylformamide were pipetted into a centrifuge tube. After mixing, 0.4 ml trichloroacetic acid (10%, w/v) and 0.4 ml 1 *N* $AgNO_3$ were added. The contents of the tube were mixed in an ultrasonic bath and thereafter centrifuged for 10 min at 1500 *g*. The supernatant was poured off into a second tube, and the extraction was repeated twice, using 0.2 ml dimethylformamide and 0.3 ml trichloroacetic acid.

*The instrument was assembled by the technical staff of the laboratory, in collaboration with the Department of Analytical Instrumentation, Technische Hogeschool Eindhoven. Information about this aspect can be obtained from the authors.

(2) To the combined supernatants 3 ml 1 *N* mercaptoethanesulfonic acid—Tris mixture (pH 6.4) was added and after centrifuging (10 min, 1500 *g*) the supernatant was discarded.

(3) The precipitate was washed with 3 ml water, dried under nitrogen at room temperature and dissolved in 80 μl 1 *N* Tris. A 1-μl aliquot was used for isotachophoresis.

RESULTS AND DISCUSSION

Figs. 1 and 2 show isotachopherograms of 95% pure MTX and the commercial product (82% MTX). The differences between the two are due to impurities and decomposition products in the commercial preparation.

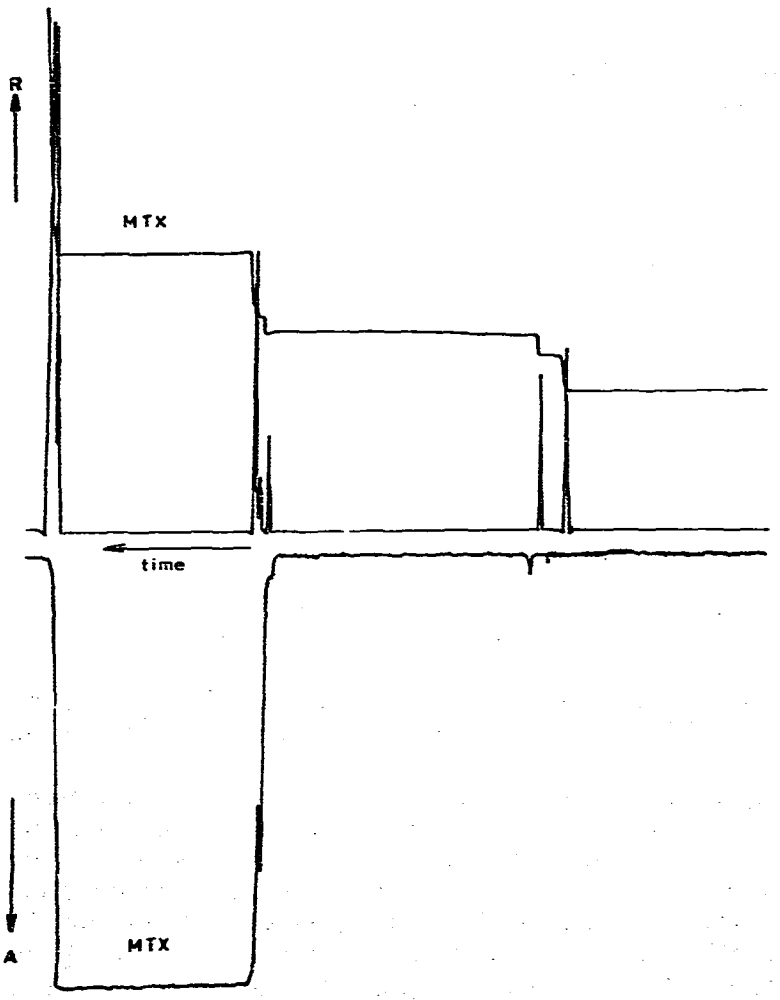

Fig. 1. Isotachopherogram of 1 μg 95% pure MTX. The nature of the anions in the zones is indicated by their resistance level (R) or UV absorption (A). The zone length indicates the quantity of anion passing the detector. Also shown is the differential signal of the conductometer, facilitating the determination of zone length.

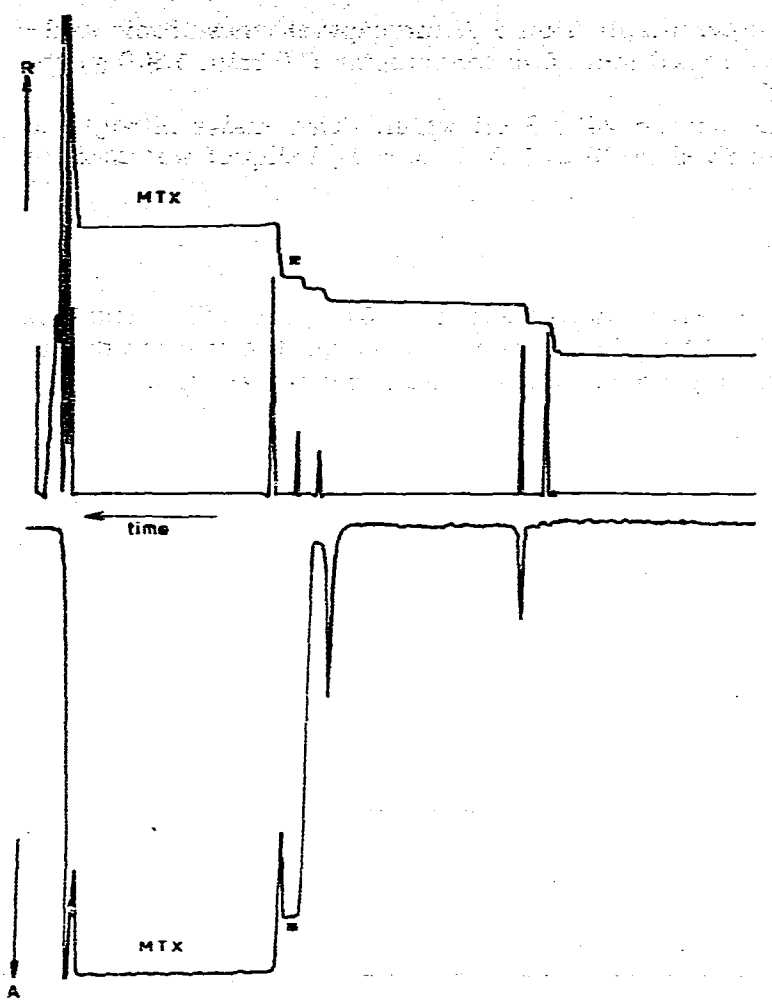

Fig. 2. Isotachopherogram of 1 μg MTX (82%) of commercial grade, containing impurities. The impurity marked * has isotachophoretic properties almost the same as those of folic acid. Recording speed 100 mm/min. Analysis time approx. 18 min.

A calibration curve for the commercial MTX preparation is obtained by injecting known amounts of MTX onto the column. The results are shown in Fig. 3.

To analyse plasma samples containing MTX, it is necessary to obtain protein- and chloride-free extracts. In the first isolation step, proteins are eliminated by the addition of trichloroacetic acid, and chloride ions are precipitated by $AgNO_3$ at pH 2. MTX forms complexes with Co^{3+}, Al^{3+}, Hg^{+}, Ti^{+} and Ag^{+}, and other metal ions. The solubility of the Ag—MTX complex depends on the pH. Addition of $AgNO_3$ at a pH of about 2 results in a soluble Ag—MTX complex. In the second step this Ag—MTX complex is precipitated by elevating the pH to about 6. The supernatant then contains a negligible quantity of MTX (Fig. 4).

In the third step the Ag—MTX precipitate is rinsed, dried and redissolved in Tris buffer; the isotachopherogram of 1 μl of such an extract of human plasma is shown in Fig. 5.

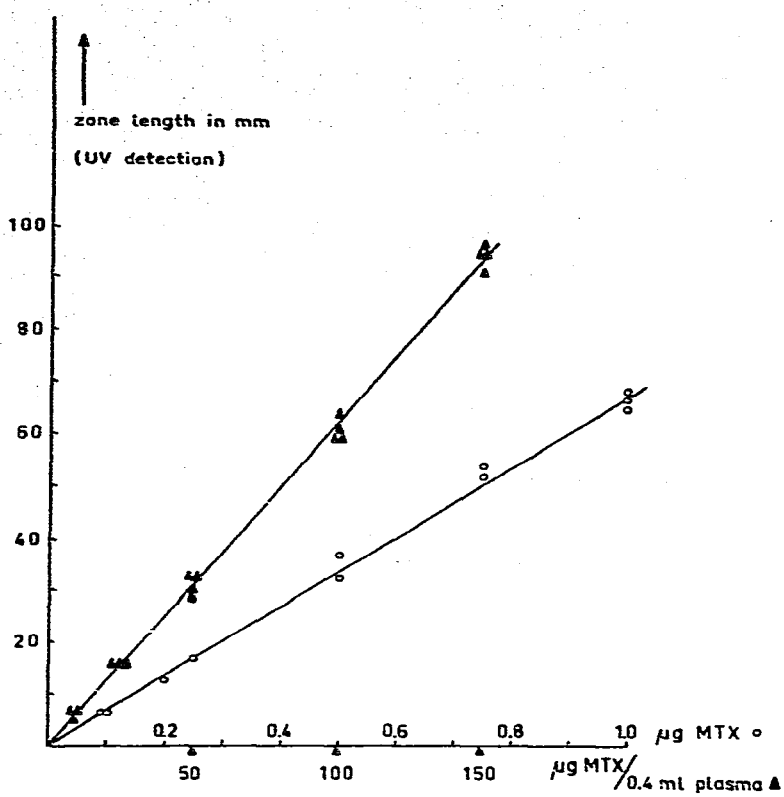

Fig. 3. Standard curves of commercial grade MTX, measured directly (○) and added to plasma and measured after isolation (▲).

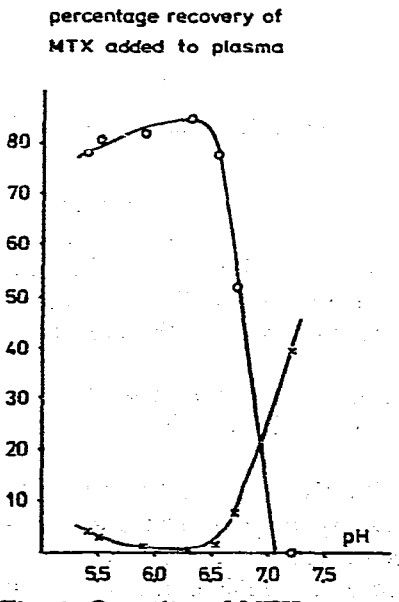

Fig. 4. Quantity of MTX, recovered in the supernatant of step 2 of the isolation procedure (×) and in the final extract of step 3 (○) at different pH values, as measured by spectrophotometry.

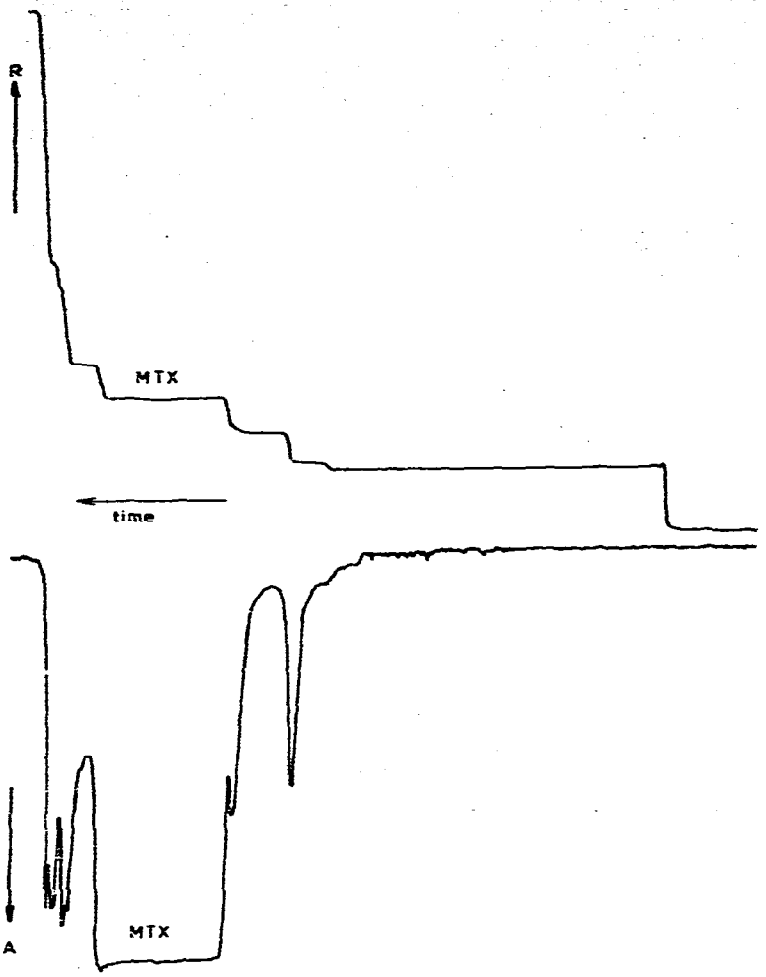

Fig. 5. Isotachopherogram of 1 μl of an extract of patient plasma. Recording speed 100 mm/min. Analysis time approx. 20 min.

A calibration curve of MTX added to plasma and isolated as described is depicted in Fig. 3. The recovery of these samples was 75% with a standard deviation of 3.2. The lower limit in this assay is approx. 10 μg per 0.4 ml plasma. The isolation procedure for MTX was also checked by adding quantities of labeled MTX to plasma. Table II shows the losses in the different steps of the procedure.

The overall recovery is in agreement with the recoveries shown in Fig. 4, measured spectrophotometrically. The recovery is twice as high as in a previously described isolation method of MTX [4].

The isotachophoretic method was compared with the enzymatic assay of MTX. Table III shows the results. In these runs the isotachophoretic assay is more reproducible. At the moment, the minimum quantity of MTX which is detectable does not cover all the requirements of clinical practice. However, by means of coupled columns [5] and by mixed zone isotachophoresis [6], now under study, the detection limits will greatly diminish.

TABLE II

RECOVERY OF ^{3}H-MTX FROM PLASMA

	Radioactivity (%)	S.D. (%)	n
Recovery in extraction step 1	93	3.2	6
Waste in extraction step 2; MES—Tris buffer	10	0.5	4
Waste in extraction step 3; 3 ml wash water	1	0.3	6
Final recovery	84	2.0	4

TABLE III

COMPARISON OF THE ISOTACHOPHORETIC AND ENZYMATIC ASSAY METHODS

Patients were administerd high doses of MTX. In both methods duplos were obtained on differend days using different standard curves.

Patient No.	MTX concentration in plasma (μg/ml)			
	Isotachophoresis		Assay with folic acid reductase	
1	40	43	24	20
2	124	121	242	139
3	514	506	424	345
4	462	473	282	311
5	68	80	65	80
6	276	244	247	276

Analysis by isotachophoresis is based on well defined physico-chemical parameters for separation and detection, and thus has a high degree of specificity. This is illustrated by Fig. 6. An MTX extract was dried under nitrogen at 70° in an acidic medium. The isotachopherogram shows evidence of significant decomposition.

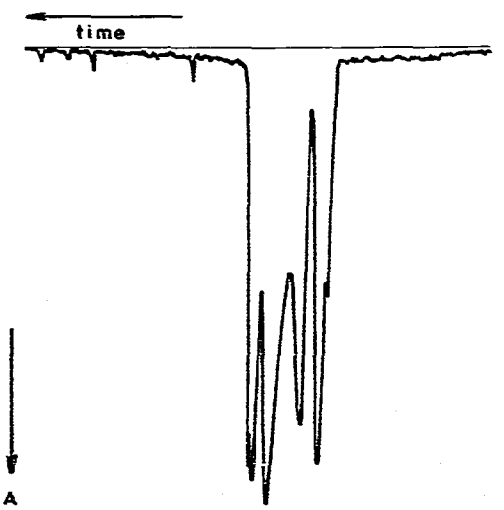

Fig. 6. Isotachopherogram of an extract of MTX, dried under nitrogen at 70° in an acidic medium. The 1-μl sample injected should have contained about 1 μg MTX. UV signal only is shown.

The advantages of isotachophoresis over many of the other analytical techniques are the simplicity of the instrumentation and instrument handling; great versatility (columns can be used immediately after filling with leading electrolyte; no column packing or equilibration) and the absence of band-broadening during analysis. We observed that several cytostatic drugs could be readily separated in an isotachophoretic system. Hence, it can be anticipated that isotachophoretic assay procedures for other drugs and metabolites will follow.

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