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DETERMINATION OF THE ANTINEOPLASTIC AGENT METHOTREXATE IN BODY FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

Some preliminary data concerning the anodic behaviour of the antineoplastic agent methotrexate are reported, and a high-performance liquid chromatographic method with electrochemical detection for its determination in human serum and urine is described. The method uses a reversed-phase C_{18} column and isocratic elution with amperometric detection on glassy carbon at +0.95 V vs. Ag/AgCl reference electrode. Sample treatment consists of a simple deproteinization step (serum) or an extraction procedure on Sep-Pak C_{18} cartridges (serum) or Amberlite column (urine). The detection limit for methotrexate in serum is 2.2 nmol/l. The method should prove useful for the evaluation of the drug pharmacokinetics in patients undergoing anticancer therapy.

INTRODUCTION

Methotrexate (MTX) combined with citrovorum factor rescue is widely used for the treatment of several human neoplasms. Currently, high doses of MTX (up to 200 mg/kg) are used [1] in the treatment of osteogenic sarcoma, acute lymphocytic leukaemia, cancer of the head and neck and other malignancies. Although the effectiveness of MTX appears to increase with higher-dosage regimens, the risk of haematologic and renal toxicity, often unpredictable and life-threatening, has also increased [2]. Citrovorum factor (folinic acid) is used to protect patients from overdosage toxicity effects associated with high concentrations of MTX in plasma and/or with a delay in MTX elimination. Because of the inherent risk of toxicity in high-dose therapy, the management of patients requires plasma or serum MTX analysis to allow a reliable monitoring of the therapy itself.

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Several types of assay are currently used, including fluorimetry [3,4], radioimmunoassay [5—7], enzyme-multiplied immunoassay technique (EMIT) [8] and high-performance liquid chromatography (HPLC) with UV detection [9—15]. Immunoassay methods, although relatively fast, present the risk of cross-reactivity with 7-hydroxymethotrexate (7-OH-MTX, the major metabolite of MTX in humans) and possess, with respect to HPLC methods, a lower sensitivity and a reduced linear range. For these reasons a growing interest has been devoted recently towards liquid chromatographic (LC) methods.

HPLC with anodic electrochemical detection (ED) is particularly attractive for the analysis of complex matrices such as body fluids, because of its high sensitivity and selectivity. Anodic electrochemistry of MTX is, however, unknown, although several efforts [16] have been made to elucidate its reductive behaviour.

Recently an LC—ED procedure for MTX determination in serum has been reported [17]. The method proposed, however, has been specifically optimized only for a dual coulometric detector operating in an oxidative screen mode. Moreover, the solvent extraction procedure gave only a 38% recovery and none of the drugs generally co-administered with MTX has been tested for interference effects.

In this paper some preliminary data are presented concerning the anodic behaviour of MTX, and an LC—ED (amperometric detection at a single electrode) method is reported for its determination in human serum and urine. For serum, in particular, two different sample pretreatments (i.e. simple deproteinization and microcolumn extraction) were evaluated; both gave a recovery exceeding 93%. The microcolumn extraction method coupled to the use of a 3-μm C_{18} HPLC analytical column gave detection limits at least one order of magnitude lower than other HPLC methods already reported.

EXPERIMENTAL

Reagents

MTX was obtained from Sigma (St. Louis, MO, U.S.A.) and used without further purification. A stock solution (1 mg/ml) was prepared by dissolving MTX in 0.2% sodium bicarbonate and stored in the dark at 4°C (no decomposition has been observed during a six-month period). More diluted solutions were prepared, when necessary, by dilution with mobile phase.

A stock solution (15 ng/μl) of theophylline (Sigma) was prepared in distilled water.

Sep-Pak C_{18} cartridges used for sample treatment were obtained from Waters Assoc. (Milford, MA, U.S.A.).

The anion-exchange resin was Amberlite (Rohm and Haas, Philadelphia, PA, U.S.A.) in the chloride form obtained from Sigma.

All solvents were HPLC grade (J.T. Baker, Deventer, Holland); the other chemicals were analytical-reagent grade (Carlo Erba, Milan, Italy).

Buffers used in the mobile phase were previously filtered and degassed through a 0.45-μm membrane (Gelman Sciences, Ann Arbor, MI, U.S.A.).

Apparatus

A Perkin-Elmer (Norwalk, CT, U.S.A.) Model 3B pump module equipped with a Rheodyne 7125 injector and a reversed-phase column (Brownlee RP 18, 10 μm, 250 × 4.6 mm I.D., Brownlee Labs, Santa Clara, CA, U.S.A.) was used as the chromatographic system. A Brownlee RP-GU guard cartridge (30 × 4.6 mm I.D.) fitted into a Brownlee MPLC holder was used to protect the analytical column unless otherwise specified. In some experiments a Perkin-Elmer HS-3 C_{18} column (100 × 4.6 mm I.D., 3-μm packing) was used.

A thin-layer amperometric cell (TL-5A, Bioanalytical Systems, West Lafayette, IN, U.S.A.) with a 3-mm diameter glassy carbon working electrode and a 0.13-mm thick PTFE gasket was used as electrochemical transducer. PTFE tubing (7 cm × 0.25 mm I.D.) was used to connect the detector flow cell to the column.

A PAR 174 A polarographic analyzer (EG&G Princeton Applied Research, Princeton, NJ, U.S.A.) was used to control the electrochemical cell. A laboratory made pulse dampener was placed between the pump outlet and the injector in order to ensure a pulseless delivery of the chromatographic eluate to the flow-sensitive electrochemical detector.

Chromatographic conditions

Unless otherwise specified the following conditions were used: mobile phase, 0.05 *M* acetate buffer (pH 5.5)—methanol—acetonitrile (87:6.5:6.5, v/v); flow-rate, 1.5 ml/min (0.8 ml/min for the HS-3 C_{18} column); injection volume, 20 μl; temperature, ambient.

Sample treatment

Serum. The two sample-pretreatment methods adopted differed in terms of execution time and concentration working range. The first (method A) consisted of a simple deproteinization step, and the second (method B) was based on a microcolumn extraction procedure (similar to that reported in ref. 10) and provided extensive sample purification and analyte preconcentration as well.

Method A. To 0.5 ml of serum (or plasma) was added 0.5 ml of 0.8 *M* trichloroacetic acid (TCA); the sample was vortex-mixed for 1 min and then centrifuged at ca. 1800 *g* for 5 min. The supernatant can be directly injected onto the HPLC column. The method can be modified to allow the use of an internal standard simply by adding (before the TCA addition step) 10 μl of theophylline stock solution.

Method B. To 1 ml of serum were added 2 ml of distilled water followed by 2 ml of 0.01 *M* tris buffer; after brief mixing the resulting solution was pushed through a Sep-Pak cartridge previously treated with 10 ml of methanol followed by 10 ml of 0.05 *M* acetate buffer (pH 5.5). The cartridge was washed with 10 ml of acetate buffer followed by 2 ml of 0.1 *M* sodium hydroxide and 3 ml of acetate buffer. MTX was eluted with 2 ml of methanol and evaporated to dryness. The residue was reconstituted with 100 μl of mobile phase and injected.

Urine. To 1 ml of urine was added 1 ml of 0.1 *M* Tris buffer, and the resulting solution was loaded on an Amberlite column slurry-packed in a Pasteur pipette for a 4-cm height. The column was washed with 10 ml of water and

4 ml of methanol, and eluted with 6 ml of acetic acid—methanol mixture (1:3). The eluate was evaporated to dryness, reconstituted with 1 ml of mobile phase and injected. A smaller volume of mobile phase (down to 0.1 ml) can be used if a sample preconcentration is required.

Quantitation

For method A (theophylline as internal standard) the MTX concentration can be calculated by the peak-height ratio of analyte to the internal standard and comparison with the standard curve. In other cases a working standard was prepared daily (by spiking drug-free serum or urine with a known amount of MTX) and processed according to the described procedures. The MTX concentration in the unknown sample was then obtained by direct comparison of peak heights in the sample and in the working standard. Repetitive injections of the working standard at regular intervals of time permits also to monitor the "calibration status" of the detector.

RESULTS AND DISCUSSION

Anodic electrochemistry

Anodic behaviour of MTX has not yet been explored, although several attempts have been made to elucidate its reductive behaviour. The presence of a pteridine moiety in its molecule should render MTX anodically electroactive; Fig. 1 shows a hydrodynamic voltammogram relevant to 200 ng of MTX dissolved in the mobile phase. As can be seen, the first, well defined, oxidation step is followed by further oxidation processes. The same behaviour is observed in classical DP voltammetry (glassy carbon electrode) where a well defined peak [E_p = +0.8 V vs. saturated calomel electrode (SCE)] is followed by a shoulder on the background discharge.

Some preliminary massive electrolysis experiments performed in 1 *M* acetic acid at a potential of +0.95 V (e.g. on the plateau of the first wave) on a

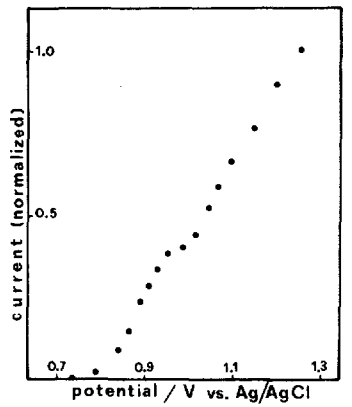

Fig. 1. Hydrodynamic voltammogram relevant to 200 ng of MTX dissolved in the mobile phase. Column, Brownlee RP C_{18} 10 μm (250 × 4.6 mm I.D.); flow-rate, 1.5 ml/min; mobile phase, 0.05 *M* acetate buffer (pH 5.5)—methanol—acetonitrile (87:6.5:6.5, v/v); current values normalized to the value recorded at +1.25 V vs. Ag/AgCl.

glassy carbon large anode showed that two electrons are involved in the overall process. The electrolysis product was cathodically electroactive on mercury, showing two waves with half-wave potentials ($E_{1/2}$) equal to −0.38 V and −0.85 V vs. SCE respectively (1 *M* acetic acid as supporting electrolyte).

The $E_{1/2}$ value of the first wave is practically coincident with the one relevant to the reduction of the carbon—nitrogen double bond in the pyrazine ring of 7-hydroxypteridine (—0.38 V), whereas the $E_{1/2}$ value relevant to the second wave is in good agreement with the $E_{1/2}$ value of the second reduction wave of MTX ($E_{1/2}$ = —0.9 V), ascribed [16] to the reductive cleavage of the C-9—N-10 bond.

These findings could thus indicate the overall process shown in Fig. 2 as highly probable, even if at the present stage of work a more direct confirmation of the nature of the oxidation product of MTX has not been undertaken mainly because of the lack of a standard of 7-OH-MTX. The process occurring at potential values more anodic than +1 V could involve, as for other pteridines [18], oxidation to a diimine species that very rapidly hydrates to a diol.

A potential value in the range 0.95—1.0 V vs. Ag/AgCl, i.e. on the plateau of the first wave, offered the best compromise between sensitivity and selectivity and was thoroughly used for LC—ED purposes. At this potential value MTX should be selectively detected in presence of its major metabolite 7-OH-MTX, which would become detectable only at higher potential values.

Calibration, linearity and reproducibility of the detector

Calibration curves of MTX dissolved in mobile phase were linear (typical correlation coefficient 0.9996 and intercept not significantly different from zero at 95% confidence level) in the range 5—2000 ng injected. The detection limits based on a signal-to-noise ratio of 5 were 2 ng (4.4 pmol) for a 250 × 4.6 mm I.D. column with 10-μm packing and 150 pg (0.33 pmol) for a 100 × 4.6 mm I.D. column with 3-μm packing.

The within-day relative standard deviation (R.S.D.) for repetitive determinations of 100 ng of MTX dissolved in the mobile phase was 1.2% (n=10), and the day-to-day R.S.D. (n=6 over three days) was 4.6%.

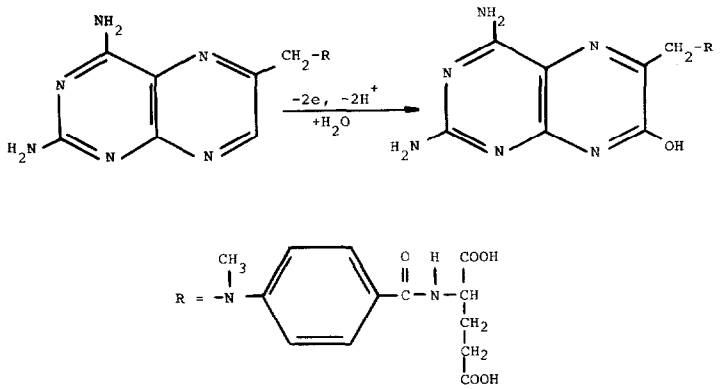

Fig. 2. Proposed overall electrode reaction for the oxidation of MTX at a vitreous carbon electrode.

An external standard quantitation procedure was preferred because no suitable internal standard has yet been found with MTX comparable extractability (method B) and suitable electrochemical characteristics. However, for method A, theophylline (retention time 12 min in the chromatographic conditions of Fig. 7) can be used as internal standard. A calibration plot (MTX-to-theophylline peak-height ratio versus amount of MTX injected) generated in the range 5—1600 ng of MTX on column (150 ng of internal standard) was linear with an intercept not significantly different from zero.

Recovery

Serum method A. A simple deproteinization of serum is highly desirable because it is very simple to perform and reduces considerably the analysis time, which is an important parameter in the monitoring of MTX therapy. For recovery assessment, working samples were prepared by spiking drug-free serum with known amounts of standard in order to cover about two decades in MTX concentration. Direct comparison of MTX peak heights with those relevant to the injection of untreated standards, prepared by dissolving MTX in the mobile phase, gave the recoveries reported in Table I.

Serum method B. Treatment according to method B is more time-consuming but provides extensive sample purification coupled with a ten-fold preconcentration factor. In this way detection limits can be considerably lowered (see later) so that this sample treatment is particularly useful in monitoring low concentrations and/or for extended pharmacokinetics studies. The recovery is also reported in Table I.

Urine. Direct injection of urine was not feasible owing to some interfering compounds eluting with MTX (at least when the chromatographic conditions optimized for serum are maintained). A proper selection of the various chromatographic parameters would solve this problem but could be impractical because of the time loss necessary to re-equilibrate the system each time the analysed matrix is altered. A sample pretreatment could then represent a convenient alternative choice. The method presented here is sufficiently fast because the eluent used gives an azeotropic mixture with a marked reduction in the time required for the evaporation step. The recovery of the procedure was 80 ± 4% (n = 5).

TABLE I

RECOVERY OF MTX FROM SERUM SAMPLES TREATED ACCORDING TO METHODS A AND B

Method	MTX (mol/l)	Percentage recovery (mean ± S.D.)	n
A	$3 \cdot 10^{-4}$	95.7 ± 2.2	3
	$5 \cdot 10^{-5}$	96.4 ± 2.4	3
	$8 \cdot 10^{-6}$	95.3 ± 2.5	3
B	$1 \cdot 10^{-5}$	94.8 ± 3.1	5
	$1 \cdot 10^{-7}$	93.6 ± 3.5	5

Analytical applications

Figs. 3—7 give some examples of chromatograms obtained under various conditions on different serum and urine samples. Method A gave detection limits of 0.5 μg/ml (or $1.1 \cdot 10^{-6}$ *M*) for a conventional 250 ×4.6 mm I.D. column with 10-μm packing, and 50 ng/ml (or $1.1 \cdot 10^{-7}$ *M*) for the Perkin-Elmer HS-3 C_{18} column with 3-μm packing. The procedure is fast and sufficiently sensitive for the pharmacokinetics of MTX (associated to the most commonly used high-dosage regimens) to be followed for at least 24 h after drug administration. The within-day R.S.D. at $5 \cdot 10^{-5}$ *M* was ca. 3% (n=5), and the day-to-day R.S.D. was ca. 7%.

Method B gave a detection limit of 20 ng/ml for the conventional 250×4.6 mm I.D. column with 10-μm packing and 1 ng/ml (or $2.2 \cdot 10^{-9}$ *M*) for the column with 3-μm packing. The within-day and the day-to-day R.S.D. (n=5) are 4 and 9%, respectively, at $5 \cdot 10^{-7}$ *M*.

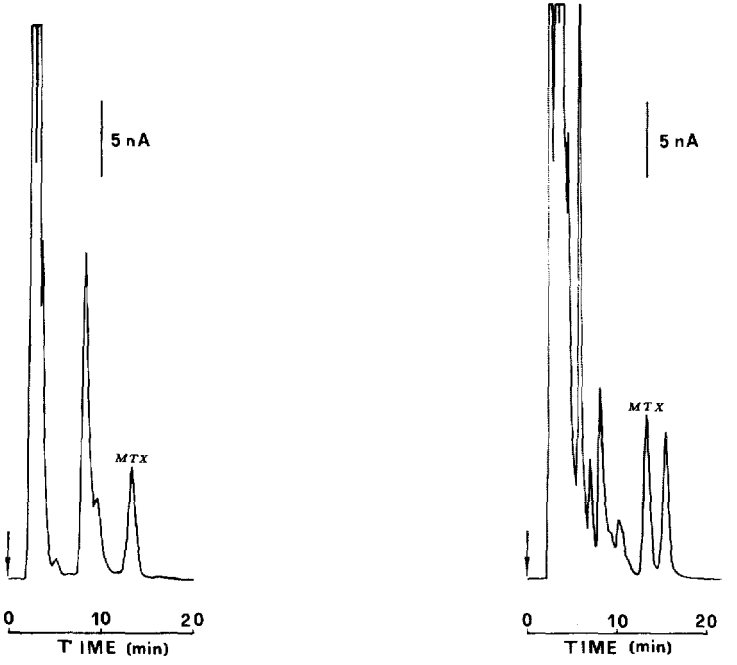

Fig. 3. Chromatogram relevant to a serum sample from a patient under MTX therapy (1.8 g of MTX by intravenous infusion over 8 h). Sample collected 8 h after administration and treated according to method A. Applied potential, +0.95 V vs. Ag/AgCl. Other chromatographic conditions as in Fig. 1. The MTX concentration in serum was estimated at $1.1 \cdot 10^{-5}$ *M*.

Fig. 4. Chromatogram relevant to a urine sample spiked with MTX and treated according to the experimental section. Chromatographic conditions as in Fig. 3. The peak shown corresponds to 80 ng of MTX on column.

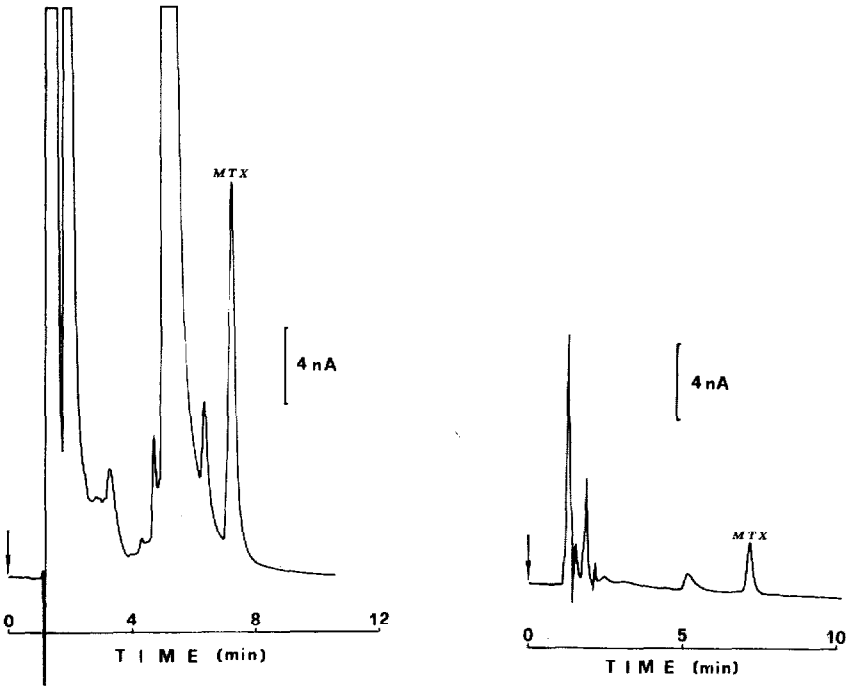

Fig. 5. Chromatogram of a patient serum sample collected 16 h after MTX administration (see caption for Fig. 3) and treated according to method A. Column, Perkin-Elmer HS-3 C_{18} (100 × 4.6 mm I.D., 3-μm packing); mobile phase, 0.05 *M* acetate buffer (pH 5.5)—methanol—acetonitrile (85:8:7, v/v); flow-rate 0.8 ml/min; applied potential +0.95 V.

Fig. 6. Chromatogram of serum sample collected from a patient 24 h after MTX administration and treated according to method B. Chromatographic conditions as in Fig. 5. The MTX serum concentration has been evaluated at $3.3 \cdot 10^{-8}$ *M*.

Interferences

Several lots of drug-free plasma have been checked for potential endogenous interferences. No interferent has been found coeluting with MTX. None of the following substances which could be coadministered with MTX interferes in the assay previously described: adriamycine, 5-fluorouracil (anticancer agents), folinic acid (antidote), paracetamol, diazepam, caffeine and morphine. Interferences from folic acid can also be excluded.

CONCLUSIONS

The method described in this paper represents the first liquid chromatography—amperometric detection approach for specific and rapid MTX determination in body fluids. Electrochemical detection coupled to the microcolumn extraction procedure based on the use of a Sep-Pak C_{18} cartridge and the use of a short HPLC analytical column with 3-μm packing (Perkin Elmer HS-3 C_{18} or equivalent) gives detection limits about one order of magnitude lower than those offered by previously reported HPLC procedures employing UV detection. The main drawback of the proposed method is that it is im-

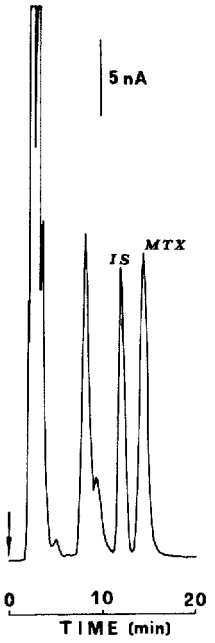

Fig. 7. Chromatogram relevant to a serum sample spiked with MTX, with theophylline as internal standard (IS) and treated according to method A. Mobile phase, 0.05 *M* acetate buffer (pH 5.5)—methanol—acetonitrile (88:6:6, v/v); applied potential, +0.95 V vs. Ag/AgCl; column and flow-rate as in Fig. 1. The MTX and IS peaks are relevant to 140 and 150 ng on column, respectively.

possibile to detect simultaneously 7-OH-MTX and 4-deoxy-4-amino-N^{10}-methylpteroic acid, which are the major metabolites of MTX. However, methods [13] that are capable of this multideterminations make use of gradient elutions at the expense of a considerable increase in total analysis time due to gradient return and column re-equilibration. The procedure proposed may thus be of advantage when the main task is to investigate individual MTX pharmacokinetics in order to provide the most reliable management of patients undergoing anticancer therapy.

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