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Determination of methotrexate in human urine at nanomolar levels by high-performance liquid chromatography with column switching

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ABSTRACT

An high-performance liquid chromatographic method with column switching for the detection of less than 4 ng of methotrexate in the urine of oncologic nurses is described. Urine samples were purified by solid-phase extraction on silica-bonded phenyl columns, eluting impurities with ethyl acetate. After elution from the column, the analyte was concentrated ten-fold, evaporating the solvent. On a strong anion-exchange column (Nucleosil 100 SB), methotrexate was separated from the remaining interfering substances, was then switched to a reversed-phase column (LiChrospher 100 RP-18e), and finally eluted by a linear gradient in a solvent system consisting of ammonium formate buffer (pH 2.7) and acetonitrile. Absorbance was monitored at 310 nm. This method has proved to be suitable for detecting traces of methotrexate in urine in order to individualize risks and to reduce further the occupational safety hazard for hospital personnel.

INTRODUCTION

The toxicity of therapeutic regimens involving antineoplastic agents is well documented. Some of the symptoms occurring in patients have also been observed in hospital personnel involved in patient care as well as in drug handling. This hazard was first proven by Falck *et al.* [1], who described an elevated frequency of mutagenicity in the urine of oncologic nurses. In toxicological studies, irreversible fibrosis of the liver and higher rates of foetal loss in nurses handling alkylating cytostatics have been reported [2,3].

Evaluation of occupational exposure has several difficulties. Analytical methods for the direct determination of drugs often lack the necessary sensitivity to detect anti-cancer agents in biological fluids after occupational contact, and the analyst is confronted with a wide spectrum of drugs [4,5]. The significance of mutagenicity tests has been a matter of discussion [6–8], since a variety of interferences are known and not all anti-cancer agents are mutagenic, partly explaining the contradictory results of some investigations [9–12]. Sensitive radioimmunoassays for methotrexate are susceptible to interfering substances such as the metabolite 7-hydroxymethotrexate and co-administered other drugs. Published methods suitable for the trace analysis of metho-

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trexate are generally appropriate for serum samples and not validated for urine samples at low concentrations [13,14]. A sensitive method for urine samples uses an electrochemical detector, which is not available in all laboratories and is unable to detect the barely oxidizable metabolite 7-hydroxymethotrexate at low potential values [15]. The recovery of this analytical method is high in serum, but only 80% in urine.

Thus, we developed an HPLC method for the detection of traces of methotrexate in urine in order to evaluate the exposure of oncologic nurses to methotrexate during the preparation and handling of drug infusions and the care of patients receiving 20 g of methotrexate for the treatment of osteosarcoma.

EXPERIMENTAL

Equipment

A Model 1090M liquid chromatograph from Hewlett Packard equipped with a diode-array detector (Waldbronn, Germany) was used in combination with two Model 7010 six-port switching valves from Rheodyne (Berkeley, CA, USA). The strong anion-exchange column contained Nucleosil 100 SB (10-μm particles, 250 mm × 4 mm I.D.; Macherey-Nagel, Düren, Germany), and the reversed-phase column utilized was a LiChrospher 100 RP-18e (10-μm particles, 250 mm × 4 mm I.D.; Merck, Darmstadt, Germany).

Reagents

Methotrexate for the preparation of infusions was obtained from Lederle Cyanamid (Wolfratshausen, Germany) and from Sigma (St. Louis, MO, USA) as a reference substance for chromatography. Water (Rathburn, Walkerburn, UK) and acetonitrile (Promochem, Wesel, Germany) were of HPLC grade. All other reagents were purchased from Merck and were of analytical grade.

Solvent A was 314 m*M* ammonium formate buffer, pH 2.7, prepared by titration of a solution of 314 m*M* formic acid in water with ammonia (a solution of 25% in water). Solvent B was acetonitrile.

Samples

As the urinary excretion of methotrexate in patients receiving low-dose regimens is completed within 48 h, the collection of urine samples from staff was initiated after an absence from the hospital of at least three days [16,17]. These samples were used as urine blanks to ascertain the absence of interfering substances. In the 36 h after the first possible contact with the drug, urine was collected over three time periods of 12 h. Manipulation of methotrexate included the preparation of drug infusions and intensive care of patients suffering from osteosarcoma. An aliquot of 4 ml from each urine sample was stored at −20°C until analysis within two weeks after collection. In our experience, samples are stable under these conditions for at least 23 days, when positive urine samples were re-examined. In the literature methotrexate is reported to be stable in urine for one month at −20°C [18] and for years in aqueous solution when kept in the dark at room temperature [19].

Sample clean-up procedure

Frozen samples were thawed, vortex-mixed and centrifuged at 1000 *g*. A 1-ml aliquot of the clear supernatant was adjusted to pH 5.0 ± 0.1, first with 2 *M* trichloroacetic acid, then with 0.6 *M* trichloroacetic acid. The entire sample was applied to a silica-bonded phenyl column conditioned with 1 ml of methanol and 1 ml of water (Bond Elut Phenyl, 100 mg of sorbent, Analytichem, Harbor City, CA, USA). The column was washed with 2 ml of water and 1 ml of ethyl acetate. The analyte was eluted with 2 ml of methanol and evaporated to dryness under a gentle stream of nitrogen in a water bath at 37°C. The sample was reconstituted to 0.1 ml with distilled water and injected onto the HPLC system after centrifugation.

HPLC method

After injecting 10–50 μl of sample onto the HPLC system, methotrexate was eluted isocratically with 1% solvent B (flow-rate = 1 ml/min) from the anion-exchange column at a typical retention time of 11.2 min and switched onto the

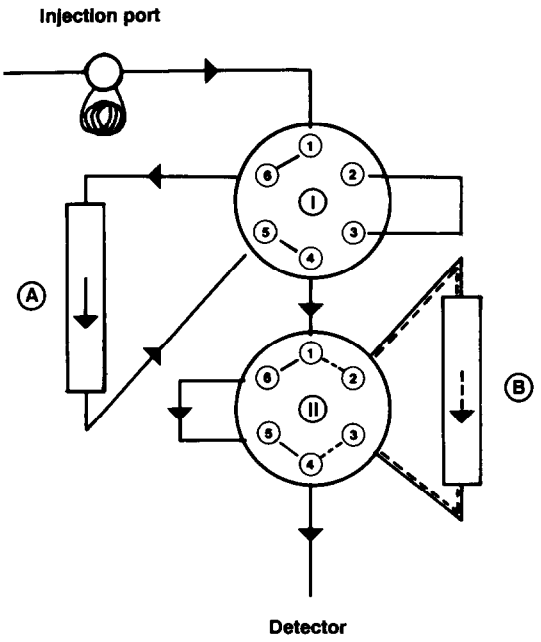

Fig. 1. Column-switching diagram: ——→, direction of flow before column switching [sample passes through column A (anion-exchange) only]; – – – →, direction of flow after switching valve II [sample passes through column A (anion-exchange) and B (reversed-phase)].

analytical column (Fig. 1). The switching time was 9.5 min for valve II and 14.5 min for valve I.

Acetonitrile was raised at 14.5 min to 25% by a linear gradient (1% per min) to elute the analyte. Before the next injection both columns were conditioned with 1% solvent B for 15 min. The absorbance was measured at 310 nm with a bandwidth of 10 nm. The column temperature of the anion-exchange column was maintained at 25 ± 1°C. Both columns were separately washed with 100% acetonitrile at the end of each working day.

RESULTS

Method description

The separation of methotrexate from the vast majority of urine impurities and the concentration of the sample by a factor of 10 was achieved by solid-phase extraction on silica-bonded phenyl columns. Ethyl acetate removes the major part of the urine impurities without reducing the recovery of methotrexate. The mechanism of this phenomenon remains unclear, since one would expect some interaction between this solvent and methotrexate.

Using 314 m*M* ammonium formate buffer and acetonitrile, the combination of an anion-exchange column (Nucleosil 10 SB) and LiChrospher 100 RP-18e was found to result in the most effective separation of methotrexate from endogenous compounds in plasma. At a pH of 2.7, a reasonable retention time for methotrexate (between 11 and 12 min with 1% acetonitrile in the buffer) was achieved. At this pH, the ionization of the carboxylic groups of methotrexate is suppressed and the analyte is concentrated on the head of the reversed-phase column, eluting as a sharp peak in a gradient of acetonitrile (peak width = 0.2 min).

The strict control of the temperature (25 ± 1°C) on the anion-exchange column is important: a deviation of a few degrees centigrade has a dramatic impact on the retention time of methotrexate.

Quality control

The recovery and the precision of the method are documented in Tables I and II. Calculations are based on the injection of 50 ng of methotrexate in aqueous solution used as external standard calibrator. The calibration curve shows excellent linearity in the range 4–1000 ng of methotrexate (coefficient of correlation > 0.999 in linear regression analysis).

TABLE I

RECOVERY OF METHOTREXATE FROM URINE AND INTRA-ASSAY VARIANCE

A 1-ml aliquot of urine blank spiked with methotrexate was purified and assayed as described in the Experimental section five times on the same day.

Amount (ng)	Recovery (%)	C.V. (%)
4	97.3	4.1
20	99.5	2.2
40	98.8	2.2

TABLE II

RECOVERY OF METHOTREXATE FROM URINE AND INTER-ASSAY VARIANCE

A 1-ml aliquot of a frozen urine stock solution was thawed, purified and assayed on five consecutive days as described in the Experimental section.

Amount (ng)	Recovery (%)	C.V. (%)
4	101.5	4.8
20	101.9	4.6
40	98.3	3.5

For the determination of the intra-assay variance, a urine blank was spiked with methotrexate at three different concentration levels. Aliquots of 5 ml of this sample were purified as described in the Experimental section and injected onto the HPLC system five times on the same day after calibration of the chromatographic system with 50 ng of methotrexate. To determine the inter-assay variance a urine blank was spiked with methotrexate at three different concentrations and the stock solutions were stored at −20°C in aliquots of 1 ml. On five consecutive days, 1 ml of urine was thawed, purified as described in the Experimental section and injected onto the chromatograph. The chromatographic system was calibrated daily with an external standard calibration (mean detector response: 70.3 mAU per 50 ng of methotrexate; day-to-day C.V. = 2.5%). The retention time of methotrexate on the anion-exchange column as well as the baseline were controlled daily by injecting 40 μl of distilled water and switching the columns.

The high recovery in intra- and inter-assay variances (97–102% in the range 4–40 ng of methotrexate) and a C.V. below 5% emphasize that the method is a precise and accurate tool to evaluate low concentrations of methotrexate in urine.

The stability of the frozen urine samples was ascertained by repeating the analysis of selected samples. In accordance with the literature, methotrexate was found to be stable in urine for at least 23 days at −20°C [18].

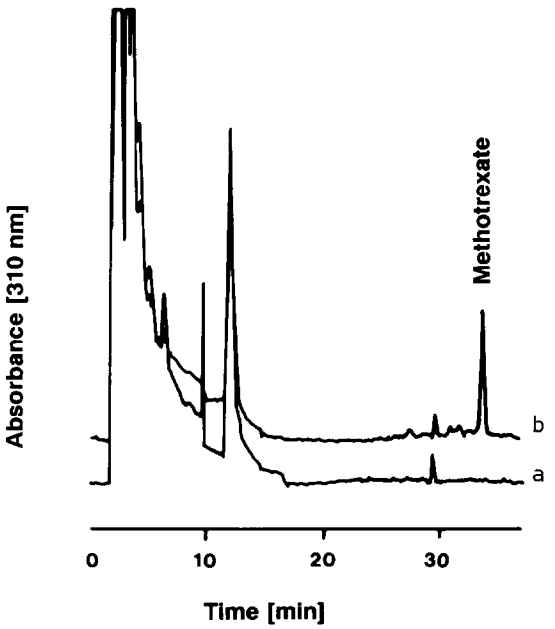

Fig. 2. Chromatograms of collected urine samples (range of FS: 20 mAU): (a) urine blank (before handling) and (b) sample containing 143 ng of methotrexate per ml of urine from an oncologic nurse (absolute amount: 57 ng of methotrexate).

Urine samples

The highest cumulative urinary excretion was observed in nurses preparing the methotrexate infusions, but traces of the substance were also detected in the urine of nurses engaged exclusively in the care of patients receiving 20 g of methotrexate. A chromatogram from a urine sample collected from a nurse is shown in Fig. 2. In some urine extracts an unidentified peak elutes close to methotrexate in post-exposure urine but not in urine blanks collected prior to exposure. This could be the metabolite 7-hydroxymethotrexate.

DISCUSSION

Several methods for the therapeutic drug monitoring of methotrexate in blood samples have been published [17,20,21]. Being used also as an anti-inflammatory agent in the treatment of severe steroid-dependent asthma, an HPLC method for the determination of methotrexate in plasma after low-dose administration of 10 mg of

methotrexate per m^2 body surface area was developed recently and emphasizes the need for highly sensitive analytical methods for the determination of some anti-cancer agents [22]. Clinically, the dose of the rescue agent leucovorin after high-dose administration of methotrexate depends on the serum concentrations of methotrexate 24, 48 and 72 h after its administration [23]. The pharmacokinetics of methotrexate is therefore well known. The renal elimination amounts to approximately 70% of the parent drug within 24 h after administration [17]. The bioavailability ranges from 50 to 100% when comparing subcutaneous and oral doses with intravenous administration [24]. The excretion in urine of the nephrotoxic metabolite, 7-hydroxymethotrexate, is 10–15% of that of the parent drug; metabolism is probably dose-dependent. The coincidence of favourable pharmacokinetics and frequent high-dose administration in the treatment of patients suffering from osteosarcoma makes methotrexate a useful pilot drug for the monitoring of occupational safety in orthopaedics.

Exposure to methotrexate was found particularly in the group preparing the dosing solutions, even after using all possible laboratory precautions (thick latex gloves, a coat used excusively during the handling of the drug and a dust-proof mask). This indicates that inhalation and resorption of aerosols through mucous membranes is the primary route of intoxication. Since resorption through the skin can be ruled out by wearing thick latex gloves [25], the exposure of caretaker nurses is probably due to the manipulation of patient urine and incidental contact with the patient.

Although great efforts were made to eliminate the exposure of oncologic staff to anti-cancer agents, the problem has not been completely solved. Sensitive and reliable analytical methods are a prerequisite to identify risks and to enhance the occupational safety of hospital personnel.

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