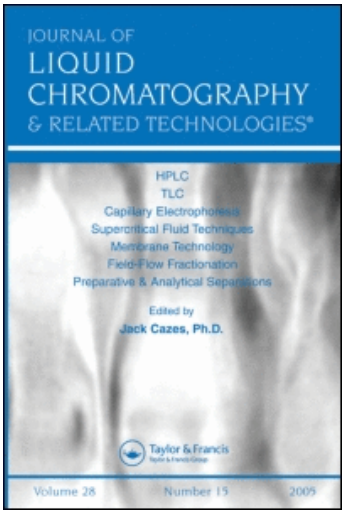

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Rapid Determination of Methotrexate and 7-Hydroxymethotrexate in Serum and Cerebrospinal Fluid by Radial Compression Liquid Chromatography

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To cite this Article El-yazigi, Adnan and Martin, Cazemiro R.(1984) 'Rapid Determination of Methotrexate and 7-Hydroxymethotrexate in Serum and Cerebrospinal Fluid by Radial Compression Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 7: 8, 1579 — 1591

To link to this Article: DOI: 10.1080/01483918408074068

URL: http://dx.doi.org/10.1080/01483918408074068

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(8), 1579-1591 (1984)

RAPID DETERMINATION OF METHOTREXATE AND 7-HYDROXYMETHOTREXATE IN SERUM AND CEREBROSPINAL FLUID BY RADIAL COMPRESSION LIQUID CHROMATOGRAPHY

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ABSTRACT

A rapid, specific and sensitive radial compression reverse phase liquid chromatographic method for the analysis of methotrexate and 7-hydroxymethotrexate in serum and cerebrospinal fluid is reported. A mobile phase consisting of acetonitrile-methanol-pH 3 phosphate (8:15:77) at 6 ml/min flow rate was employed. The U.V. detector was set at 317 nm, and folic acid was used as an internal standard. A rapid extraction of methotrexate and 7-hydroxymethotrexate was performed using Sep-Pak cartridges with high extraction efficiency for both compounds. Patients serum and cerebrospinal fluid samples were analyzed by the described method and the concentrations of methotrexate were compared to those obtained by an enzyme immunoassay. No interference from other metabolites or anticancer drugs in the described assay was observed.

INTRODUCTION

Methotrexate, L-(+)-N-[p-[[(2,5-Diamino-6- pteridinyl) methyl] methyl-amino] benzoyl] glutamic acid, is a folic acid

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 0148-3919/84/0708-1579$3.50/0

antimetabolite, highly effective in treatment of acute leukemia (1), non-Hodgkin's lymphoma (2), and trophoblastic tumors such as choriocarcinoma (3). In recent years, high doses of methotrexate with leucovorin rescue have been utilized for the treatment of various neoplasmic diseases. High plasma concentrations of 7-OH methotrexate are normally produced following this treatment. These concentrations are usually associated with nephrotoxicity due to low solubility in normal pH urine. This has generated a considerable interest in monitoring both methotrexate and its 7-OH metabolite in serum and other biological fluids. Numerous techniques have been utilized for the analysis of methotrexate in biological fluids. These include fluorometric methods (4,5), radioimmunoassays (6,9), competitive protein binding assay (10), enzyme immunoassay (11), radiochemical-ligand binding assay (12), isotachphoresis (13), enzyme assay (14) and high performance liquid chromatographic (HPLC) methods (15-22).

The use of HPLC methods eliminates the potential cross-reactivity of methotrexate with its metabolites, and makes the simultaneous analysis of this drug and its 7-OH metabolite feasible. Various modes of chromatography including anion-exchange, reverse-phase, and paired-ion have been employed for the analysis of methotrexate and other pteroylglutamate related compounds. The use of radial compression separation system for methotrexate and its 7-OH metabolite has not been previously reported. The utilization of such a system, coupled with a rapid C18 Sep-Pak sample cleanup as described in this report, results in higher sensitivity and chromatographic efficiency, and shorter analysis time.

MATERIALS

Reagents and Solvent

Methotrexate, U.S.P. grade (American Cyanamid Co.) was used as received. The purity of methotrexate was 80±4% as determined

by the U.V. absorbance of 0.01% (w/v) in neutral solution at 315 nm assuming molar absorptivity of 23,000 (23). An analytical sample of 7-OH methotrexate was kindly provided by Dr. David Johns (National Cancer Institute, Bathesda, MD). Folic acid (Eastman Kodak), leucovorin (American Cyanamid Co.), vincristine-sulfate (Eli Lilly Co.), adriamycin (Adria Lab.), bleomycin-sulfate (Bristol Lab.), etoposide (Mead Johnson Co.), cytarabine (Upjohn Co.), and prednisone (Philips Roxane Lab.) were either reagent or pharmaceutical grade. Sodium monobasic phosphate, 85% phosphoric acid, methanol, and acetonitrile, all from Fisher Scientific Co., were HPLC grade, and were employed without further purification. The HPLC purified water used was obtained by passing reverse osmosis water through a Norganic (TM) trace organic removal cartridge and 25-venting membrane filter (Millipore Co.).

Apparatus

The chromatography was performed using an HPLC system (Waters Associates) consisting of a system controller (Model 720), a ternary solvent delivery system (Models 6000 A and 45), a sample injection module (WISP), a data module (Model 730), and a radial compression separation system (Z-Module). A radial Pak uBondapak C18, 10 μ, 8 mm x 10 cm cartridge (Waters Associates) was employed.

Chromatographic Conditions

A mobile phase consisting of 0.08 M sodium monobasic phosphate-acetonitrile-methanol (77:8:15) adjusted to pH 3-3.1 with 85% phosphoric acid and filtered before use was employed. The flow rate used was 6 ml/min (pressure = 1500-1800 psi). The detector was set at 317 nm. The chromatography was performed isocratically on a Model 45 solvent delivery system after the mobile phase composition was established.

Calibration Curves Preparation

Separate stock solutions of methotrexate, 7-OH methotrexate, and folic acid in 0.01 N sodium hydroxide solution were prepared and employed for the construction of the calibration curves. Three ranges of calibration curves for methotrexate (viz. 0.0064-0.4, 0.4-8, and 8-80 µg/ml) or 7-OH methotrexate (viz. 0.01-0.5, 0.5-10, and 10-50 µg/ml) were constructed by transferring appropriate aliquots of the methotraxete or 7-OH methotrexate stock solutions to 1 ml of serum or CSF to yield serial concentrations in the above ranges. Aliquots of the folic acid stock solution equivalent to 0.14, 5, or 50 µg of folic acid were then added and the volume was brought to 1.75 ml with 0.01 N sodium hydroxide. The solution was then submitted to sample cleanup procedure.

Sample Cleanup

Sep-Pak C18 cartridges (Waters Associates) were utilized for sample cleanup and concentration. The cartridge was pretreated with 10 ml of methanol-acetonitrile (50:50) followed by 10 ml of methanol, 10 ml of sodium phosphate buffer (pH 3), and 10 ml of HPLC water, consecutively. The diluted sample was then passed slowly through the cartridge which was then treated with 15 ml of the phosphate buffer and 25 ml of HPLC water. The cartridge was vacuum dried and the compounds were eluted with 0.5 ml of methanol-water (95:5). The eluate was evaporated to dryness under gentle stream of nitrogen. The residue was then reconstituted with 75 µl of the mobile phase by sonication for 2 minutes, and transferred to an autosampler microvial. The tube was rinsed with additional 75 µl of the mobile phase (or mobile phase adjusted to pH 10 when a complete dissolution was not obained) and the rinsate was added to the microvial. The solution was then analyzed in duplicate under the above conditions. The automatic sample

injection processor was programmed to inject 40-75 µl of each microvial in duplicate. The cartridge was reused once, after washing and pretreatment as described above.

Patient Samples Analysis

Serum samples were collected at preselected time intervals from patients treated with different doses of methotrexate (Table 4). Cerebrospinal fluid specimens were also obtained from one of these patients at different intervals. To one ml of each of these samples, an aliquot of the internal standard stock solution equivalent to that used for the calibration curve was added, and the resulting solution was analyzed as described above. The samples were also analyzed for methotrexate by an enzyme immunoassay method (24) and the results of both techniques were compared.

RESULTS AND DISCUSSION

Representative chromatograms of blank, calibration, and patients serum and CSF samples are shown in Figures 1 and 2. As demonstrated in these figures, the peaks were sharp and symmetrical and all three compounds were eluted rapidly (viz. less than 6 min). High capacity factors were also preserved.

The specificity of the described assay was examined by determining the retention times of other anticancer drugs commonly used with methotrexate in combined chemotherapy (Table 1). In high dose methotrexate treatment, no other anticancer is utilized, and leucovorin rescue is usually initiated 24 hours after infusion of methotrexate. No interference in the assay from leucoverin was observed (retention time = 1.4 min). The serum endogenous folic acid was hardly detectable even at low AU detection setting (viz. 0.005). A full resolution between methotrexate and its 7-OH metabolite was obtained.

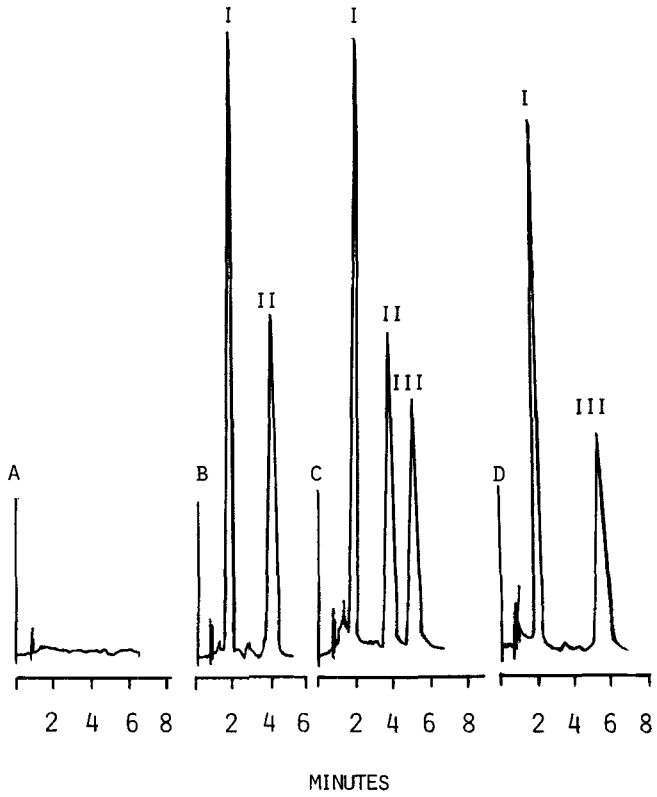

FIGURE 1
Representative chromatograms of a blank serum sample (A), a calibration serum sample containing 5 µg/ml of I and 1.6 µg/ml of II (B), a patient's serum sample collected 18 hours following the initiation of 6-hour infusion of 100 mg/kg of II and contains 5 µg/ml of I (C), and a calibration serum sample containing 5 µg/ml of I and 2.5 µg/ml of III (D),. Key internal standard (I); methotrexate (II); 7-OH methotrexate (III).

The determination of concentrations of methotrexate and 7-OH methotrexate in patient samples was performed using peak height ratio (methotrexate or 7-OH methotrexate/internal standard) vs. concentration calibration curves. Since the concentrations of methotrexate and its 7-OH metabolite following high dose methotrexate infusion extend over a wide scope, three calibration

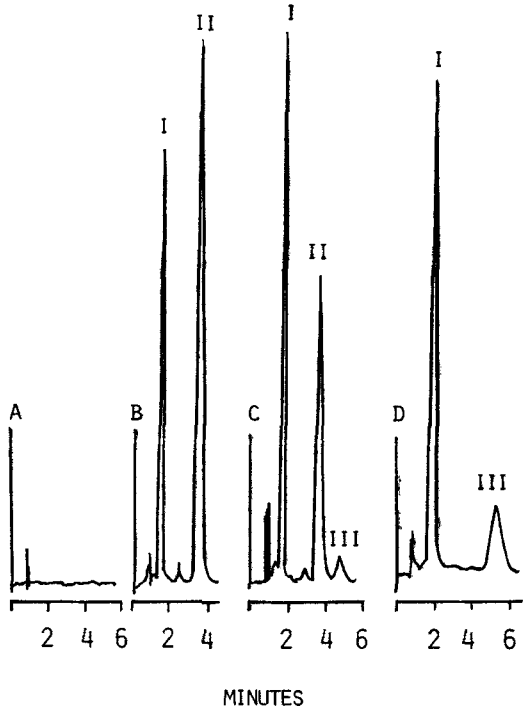

FIGURE 2
Representative chromatograms of a blank CSF sample (A), a calibration CSF sample containing 5 µg/ml of I and 4.8 µg/ml of II (B), a patient's CSF sample collected 24 hours following the initiation of 6-hour intravenous infusion of 100 mg/kg of II and contains 5 µg/ml of I (C), and a calibration CSF sample containing 5 µg/ml of I and 1 µg/ml of III (D). Key: internal standard (I); methotrexate (II); 7-OH methotrexate (III).

curves for methotrexate or 7-OH methotrexate had to be constructed to cover this range. Excellent linearity was obtained for these curves. Table 2 presents the ranges and correlation coefficients obtained for these curves.

A rapid extraction of methotrexate, 7-OH methotrexate, and folic acid from serum or CSF samples was obtained using Sep-Pak C18 cartridges under the conditions utilized. The recovery of

TABLE 1

RETENTION TIMES OF METHOTREXATE AND OTHER RELATED COMPOUNDS UNDER THE CONDITIONS EMPLOYED

Name of Drug	Retention Time (min.)
Methotrexate	3.79
7-Hydroxymethotrexate	5.06
Folic Acid	1.83
Leucovorin	1.41
Adriamycin	ND*
Vincristine	ND
Prednisone	ND
Etoposide	0.75
Bleomycin	ND
Cyclophosphamide	ND
Cytarabine	0.64

* Non-detectable in 10 minutes after injection.

methotrexate exceeded 91% at concentrations equal to 6.4 ng/ml (the sensitivity limit of the described method for methotrexate). Equally high recovery was also obtained for 7-OH methotrexate (viz. >0.95) at the sensitivity limit of the assay for this metabolite viz. 10 ng/ml. Various solvents and cartridges treatments were investigated, and the use of a small fraction (0.5 ml) of methanol-water (95:5) for elution of the compouds yielded cleaner chromatograms and substantially reduced the evaporation time.

TABLE 2

STANDARD CURVE DATA FOR METHOTREXATE AND 7-HYDROXYMETHOTREXATE IN PLASMA AND CEREBROSPINAL FLUID

Compound	Concentration Range (μg/ml)	No. of Experiments	Specimen	Correlation Coefficient
Methotrexate	0.0064-0.4	6	plasma	0.996 * (0.00693)
	0.4-8.0	6	plasma	0.9961 (0.00515)
	0.4-8.0	2	CSF	0.9998 (0.000033)
	8-80	3	plasma	0.9949 (0.011)
7-Hydroxymethotrexate	0.01-0.5	5	plasma	0.9949 (0.00311)
	0.5-10.0	5	plasma	0.9947 (0.00286)
	0.1-10.0	2	CSF	0.9952 (0.000778)
	10.0-50.0	2	plasma	0.9898 (0.0129)

* standard deviation

The accuracy of the described assay was tested by spiking blank serum samples with different amounts of methotrexate or 7-OH methotrexate and performing the analysis in duplicate as described earlier. Table 3 lists the amounts added and found of both compounds and the percent error for the samples analyzed. As illustrated in Table 3, the percent error for no sample did it exceed 7.8%, indicating excellent assay accuracy and reproducibility.

Table 4 presents the plasma and CSF concentrations of methotrexate and 7-OH methotrexate at different intervals in

TABLE 3

ACCURACY OF THE DESCRIBED HPLC METHOD

Compound	Amount Added (μg)	Amount Found (μg)	Percent Error
Methotrexate	0.0064	0.0059	-7.81
	0.032	0.0302	-5.75
	0.064	0.0673	+5.16
	0.160	0.157	-1.88
	0.320	0.317	-0.94
	0.400	0.403	+0.75
	0.800	0.804	+0.5
	2.0	2.065	+3.25
	4.0	3.862	-3.45
	6.0	5.835	-2.75
	8.0	7.721	-3.49
	20.0	19.656	-1.72
	40.0	39.735	-0.66
	60.0	58.995	-1.68
	80.0	76.810	-3.99
7-Hydroxymethotrexate	0.01	0.0102	+2.0
	0.05	0.0534	+6.8
	0.125	0.118	-5.6
	0.500	0.492	-1.6
	2.5	2.489	-0.44
	5.0	5.003	+0.06
	10.0	10.587	+5.87
	20.0	20.996	+4.98
	30.0	28.584	-4.72

patients treated with different doses of methotrexate. As demonstrated in this table, the methotrexate concentrations obtained by the described method were generally smaller (= 29% less) but well correlated (r = 0.996) with those obtained according to a commonly used immunoassay (EMIT) (24). Similar differences were found between another HPLC method (18) and those acquired by a protein binding assay. These variations appear to

TABLE 4

METHOTREXATE AND 7-HYDROXYMETHOTREXATE PLASMA CONCENTRATIONS IN PATIENTS TREATED WITH METHOTREXATE

Patient No.	Time after Initiation of Infusion (hr)	specimen	Methotrexate Concentrations (μg/ml) Described Method	EMIT Method	7-Hydroxy-methotrexate Concentrations (μg/ml)
1[a]	3.0	serum	49.91	68.6	25.982
	6.0	serum	81.362	105.3	27.467
	12.0	serum	8.775	11.4	18.943
	18.0	serum	1.119	1.6	3.416
	27.0	serum	0.367	0.27	2.009
	39.0	serum	0.172	0.14	1.299
	51.0	serum	0.0933	<0.100	0.947
	63.0	serum	0.0756	<0.100	0.862
	3.0	CSF	2.352	1.63	ND
	6.0	CSF	4.500	5.86	ND
	12.0	CSF	2.065	3.00	0.097
	24.0	CSF	0.451	0.54	0.263
	48.0	CSF	0.251	0.14	0.0114
2[b]	0.5	serum	4.131	4.90	0.259
	8.5	serum	2.068	2.18	0.285
	22.5	serum	0.695	1.09	0.300
3[c]	3.0	serum	28.091	38.2	3.709
	6.0	serum	33.127	33.0	7.336
	12.0	serum	13.930	16.7	10.810
	18.0	serum	6.160	5.5	3.405

[a] Dose = 100 mg/kg infused over 6 hours.

[b] Dose = 5.9 mg/kg I.V. bolus followed by 11.8 mg/kg infused over 24 hours.

[c] Dose = 38.8 mg/kg infused over 6 hours.

be the result of the difference in specificity between the two techniques. While the metabolites and other structurally related compounds are completely separated from methotrexate by the described HPLC method, a 30% quantitation error is expected by EMIT due to interferences of these compounds at concentration > 1 μM (24).

In conclusion, the described HPLC assay is an accurate and specific method for the simultaneous analysis of methotrexate and

7-OH methotrexate in serum and cerebrospinal fluid. The high rapidity and sensitivity of this method make it an excellent tool for routine monitoring of this drug and its 7-OH metabolite in biological samples.

ACKNOWLEDGMENT

The authors wish to thank Drs. Farouk Al-Watban and Nicholas Gretz for their encouragement and support. Thanks are also extended to Dr. Magid Amer for providing patients' specimens, and to Mr. Warren Skoog for performing the enzyme immunoassay.

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