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Determination of methotrexate in serum by high-performance liquid chromatography

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Abstract

A sensitive high-performance liquid chromatographic method with ultraviolet detection was developed to quantitate methotrexate in serum-based calibrators, controls and patient samples. Sample clean-up was achieved with C_{18} Sep-Pak Classic cartridges. The chromatographic separation was accomplished on a 5-μm Ultrasphere ODS Beckman column. 8-Chlorotheophylline was used as an internal standard. The method was validated by recovery, linearity, accuracy and precision studies. Two standard curves were constructed to cover the high and the low ends of the calibrator range (0.05–1.0 μmol/l). Response was found linear over the whole range of the calibrator set with a correlation coefficient of 0.999 and 1.00 for the low-level and the high-level curves, respectively. Accuracy varied from 12% at the lowest level to 1.2% at the highest level. The precision study showed a C.V. of 14.4% at the lowest level and 3.3% at the highest level.

Keywords: Methotrexate

1. Introduction

In cancer treatment, the effectiveness of high-dose methotrexate therapy followed by leucovorin rescue was greatly enhanced by the observation that patients at high risk of serious toxicity might be detected by monitoring serum methotrexate concentrations [1–3]. Low doses of methotrexate have also been used to treat rheumatoid arthritis [4,5], steroid-dependent asthma [6,7] and psoriasis [8,9]. To insure appropriate therapy, an immunoassay reagent system was developed by Abbott Laboratories for the quantitative measurement of methotrexate in serum and plasma. To control the quality of the serum calibrators and controls, an HPLC method was developed. The method was validated by recovery, linearity, accuracy and precision studies. To accommodate the range of the immunoassay calibrators (0.05–1.0 μmol/l), two standard curves were constructed; one to cover the high and one to cover the low end of the calibrator set.

Many HPLC methods have been published in the literature; however, none were found to be applicable to our requirements. Some of the methods have one or more disadvantages, such as a lack of sensitivity [10,11,16,18], a large sample volume [12,14,15, 17,18], incomplete precision data over the range of interest [13,15,17,19,22,23], the need for special equipment [20–22,24], or a derivatization step in the sample preparation [25]. Also, none of the reported methods for the quantitation of methotrexate in clinical samples has been accurately validated to test

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calibrators with definite label claim concentrations. This study reports a validated, simple, sensitive and accurate extraction and chromatographic procedure for controling the quality of serum methotrexate calibrators and controls. This method was also successfully used to analyze clinical samples.

2. Experimental

2.1. Reagents and materials

Methotrexate (L-amethopterin trihydrate) and tetramethylammonium chloride (TMAC) were purchased from Aldrich (Milwaukee, WI, USA). 8-Chlorotheophylline was obtained from Sigma (St. Louis, MO, USA). KH_2PO_4, K_2HPO_4, KOH, H_3PO_4 and methanol (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Acetonitrile (HPLC grade) was purchased from EM Science (Gibbstown, NJ, USA). Normal human serum (NHS) (List No. 99800), TDx/TDxFLx methotrexate calibrators (List No. 9522-01) and controls (List No. 9522-10) were obtained from Abbott Laboratories (Abbott Park, IL, USA).

2.2. Apparatus

The Waters HPLC system (Millipore, Waters Division, Milford, MA, USA) consisted of a 600E solvent delivery system, a 994 programmable photodiode-array detector set at 313 nm and a WISP 710B autoinjector. The column, operated at ambient temperature, was a Beckman Ultrasphere ODS, 5 μm particle size, 150×4.6 mm I.D. (Beckman Instruments, San Ramon, CA, USA). Data was recorded with a Hewlett-Packard 3394A integrator (Hewlett-Packard, Avondale, PA, USA). The mobile phase was acetonitrile–buffer (0.01 *M* KH_2PO_4–0.02 *M* TMAC) at pH 2.5 (10:90, v/v), delivered at a flow-rate of 1 ml/min. The C_{18} Sep-Pak Classic extraction cartridges (Millipore, Milford, MA, USA) were conditioned, before applying the serum samples, with 0.2 *M* phosphate buffer at pH 6. An Alltech vacuum manifold (Alltech Associates, Deerfield, IL, USA) was used for the extraction.

2.3. Preparation of stock, working and internal standards

A stock solution of methotrexate in NHS was accurately prepared. The stock solution was serially diluted to prepare a series of working standards ranging from 0.03 to 1.0 μmol/l. The prepared working standards were used for the daily standardization of the HPLC. The internal standard, 8-chlorotheophylline, was prepared at a concentration of 4 μg/ml in the extraction cartridge conditioning buffer (0.2 *M* phosphate buffer, pH 6.0).

2.4. Sample preparation

Methotrexate and the internal standard were extracted using Sep-Pak Classic extraction cartridges. The cartridges were mounted on the vacuum manifold, then connected to 8–10 ml sample preparation reservoirs. The cartridges were prepared with 20 ml methanol followed by 3 ml 0.2 *M* phosphate buffer (pH 6.0).

An aliquot of the serum sample (500 μl for low levels and 200 μl for high levels) was vortex-mixed gently with 1 ml of the internal standard. The sample/internal standard mixture was pipeted into the reservoir and allowed to drain under gravity. A slight vacuum was applied, if necessary, to start the flow. Cartridges were washed with 1 ml water and dried by applying vacuum for five min. Methotrexate and the internal standard were eluted with 2 ml methanol. The eluate was dried under nitrogen at 60°C. The residue was reconstituted in 0.005 *M* HCl (200 μl for low levels and 300 μl for high levels). A 100-μl aliquot of the reconstituted sample was injected onto the HPLC column.

2.5. Quantitation

Quantitation was based on peak-area ratios referenced to the standard curve provided with each analytical run.

3. Results and discussion

3.1. Chromatography

The chromatographic reproducibility was ensured by daily injection of a system suitability solution made of a mixture of 0.8 μmol/l methotrexate and 20 ng/ml 8-chlorotheophylline in water. The flow-rate, the percentage acetonitrile in the mobile phase or both were slightly adjusted to maintain at least a 3–3.5 min difference in the retention time between methotrexate and 8-chlorotheophylline. Fig. 1 shows typical chromatograms of system suitability solution (Fig. 1A), extracted blank serum (Fig. 1B) and extracted spiked serum at 0.05 and 1.0 μmol/l (Fig. 1C and Fig. 1D), respectively.

3.2. Method validation

The method was validated by absolute recovery, linearity, accuracy and precision studies.

3.3. Absolute recovery

Absolute recoveries were determined by comparing the peak-height ratio of extracted controls (low, medium and high) to those of unextracted standards prepared in water. The mean (n=3) percentage absolute recoveries were 88.6±1.4, 88.0±1.5 and 91.2±2.0%, respectively.

3.4. Linearity

The range of the methotrexate immunoassay serum calibrators is 0.05 to 1.0 μmol/l. This range covers all the calibrators containing methotrexate (B–F), the low, medium and high controls as well as the much higher X, Y and Z controls, after dilutions of 1:10, 1:100 and 1:1000, respectively.

As shown in Table 1, the methotrexate calibrators were divided into two concentration ranges (0.03–0.30 and 0.15–1.0 μmol/l). The statistical data

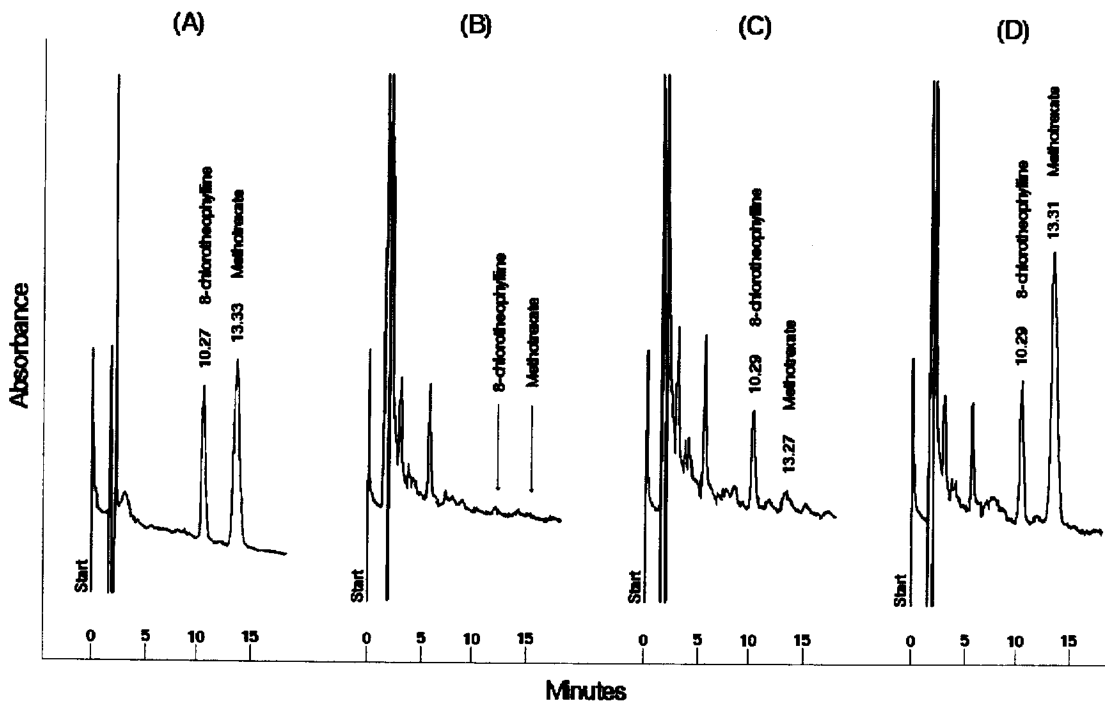

Fig. 1. Chromatograms of (A) system suitalbility solution, (B) blank serum, (C) serum spiked with 0.05 μmol/l and (D) serum spiked with 1.0 μmol/l methotrexate.

Table 1
Statistical data for methotrexate calibration curves in serum

Concentration range (μmol/l)[a]	Correlation coefficient	Slope	Intercept
0.03–0.30 (low level)	$0.9998 \pm 2.00 \times 10^{-4}$	4.01 ± 1.034	0.05 ± 0.0397
0.15–1.0 (high level)	$0.9976 \pm 1.76 \times 10^{-3}$	1.75 ± 0.479	0.003 ± 0.0644

[a]Each range consisted of four points.

reported represents the average correlation coefficient, slope and intercept for three separate standard curves constructed for each range. Each point on the standard curves is an average of two determinations. Covering the calibration range by the construction of two standard curves produced a better fit to points at the low end of the range than could be achieved by the use of a weighting factor ($1/x$, where x equals concentration) for linear regression analysis of a single curve. Splitting the curve into two parts provided a more accurate presentation of the concentrations determined. It was also helpful in meeting the concensus acceptance criteria ($\leq 15\%$ error for precision and accuracy) for quantitative determination of an analyte in a biological matrix [26]. Limit of detection was found to be 0.02 μmol/l (about 9 ng/ml). Limit of quantitation was defined as the lowest point on the low curve (0.03 μmol/l) that can be repeatedly determined with a reproducibility of 15% or less.

3.5. Accuracy

The accuracy of the method was tested by preparing two independent sets of standards and controls in serum. The two sets were extracted in triplicate by two analysts on two different days. The standard curves were constructed, using the data obtained from one set, and were used to determine the concentrations, at all levels, of the second set. The accuracy of the method was determined from the mean ($n=6$) percent deviation, for each level of the second set, from target concentrations. A detailed accuracy study is presented in Table 2.

3.6. Precision

Table 3 presents the precision data collected at all levels of TDx/TDxFLx methotrexate calibrators and controls. Manufacturing calibrators and controls

Table 2
Accuracy of methotrexate determination at all levels of the serum calibrators and controls

Concentration target (μmol/l)	Mean concentration determined (μmol/l, $n=6$)	Accuracy (%)
Calibrators		
0.05 (22.7[a])	0.056	12.0
0.15 (68.1[a])	0.169	12.6
0.30 (136.2[a])	0.316	5.3
0.60 (272.4[a])	0.609	1.5
1.00 (454.0[a])	1.012	1.2
Controls		
0.07 (31.8[a])	0.075	7.1
0.40 (181.6[a])	0.405	1.3
0.80 (363.2[a])	0.790	−1.4

[a]Target concentrations in ng/ml are given in parentheses.

Table 3
Precision data on methotrexate determination at all levels of the serum calibrators and controls

Concentration target (μmol/l)	Mean concentration determined (μmol/l, $n=9$)	C.V. (%)
Calibrators		
0.05 B	0.049	14.4
0.15 C	0.129	8.1
0.30 D	0.299	3.3
0.60 E	0.586	4.0
1.00 F	0.976	3.3
Controls		
0.07 L	0.061	13.2
0.40 M	0.387	4.4
0.80 H	0.766	5.4
5 X[a]	4.77	7.0
50 Y[b]	49.1	4.3
500 Z[c]	479.8	2.7

[a] Dilution 1:10.
[b] Dilution 1:100.
[c] Dilution 1:1000.

were selected. Several vials for each level were pooled into larger bottles, kept refrigerated and used to generate the precision points on three different days by three different analysts.

3.7. Samples

Because this HPLC method is capable of separating 7-hydroxymethotrexate (a methotrexate major metabolite with undesirable cross-reactivity in some methotrexate immunoassays [13,24,27]), the method was used to verify the concentration of methotrexate in clinical samples obtained from subjects receiving methotrexate therapy. A chromatogram of a clinical sample is presented in Fig. 2. A total of 95 clinical samples were analyzed by this HPLC method and by the TDx/TDxFLx Methotrexate II immunoassay. The correlation between the two methods tested over the range 0.03–1000 μmol/l yielded a slope of 1.01, a y-intercept of −0.02 and a correlation coefficient of 0.999 [28].

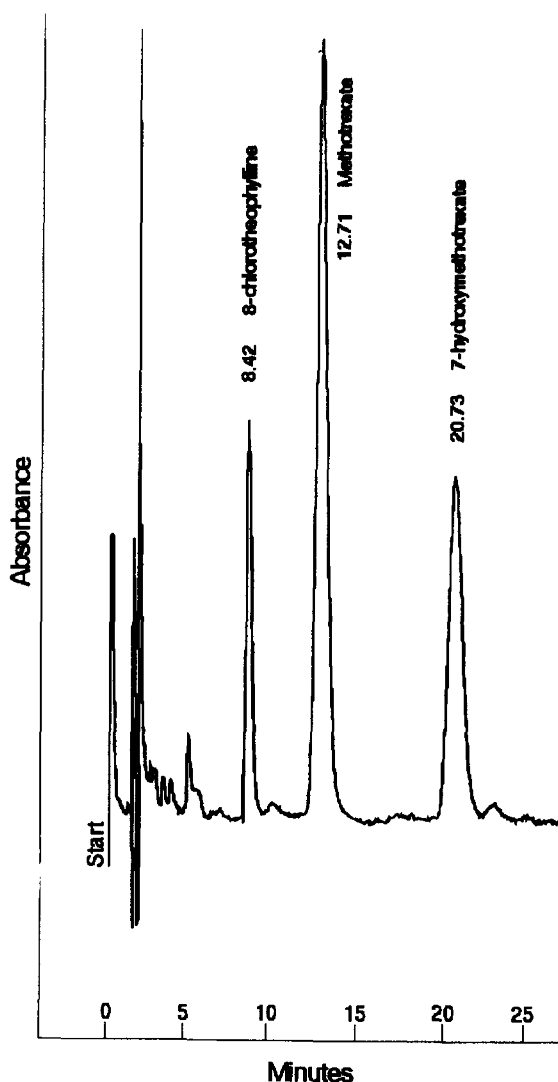

Fig. 2. Representative chromatogram of patient serum extract containing 8.87 μmol/l methotrexate and 8.41 μmol/l 7-hydroxymethotrexate.

4. Conclusion

A simple and reliable HPLC method has been developed for the analysis of methotrexate in serum based calibrators and controls. This method is free from serum matrix interferences and has the necessary sensitivity to quantitate methotrexate accurately at the lowest level of the immunoassay calibrator range. The utility of the method was further demonstrated by measuring methotrexate levels in patient samples.

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