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Determination of methotrexate and 7-hydroxymethotrexate by liquid chromatography for routine monitoring of plasma levels

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Abstract

A high-performance liquid chromatographic (HPLC) method was designed to meet analytical and metrological requirements for routine blood level monitoring of methotrexate (MTX) and its main metabolite 7-hydroxymethotrexate (7OMTX). The metabolite, unavailable as a pure substance, was measured by reference to MTX calibration according to their respective ultraviolet absorbances. Acetonitrile deproteinization and chloroform clean-up provided plasma samples devoid of long-retained contaminants. The precision of the HPLC measurements, reproducibility of clean-up recovery, matrix effects and linearity were assessed by analysis of variance and linear regression in an appropriate experimental design, within a range from 0.205 to 16.7 mg/l of MTX and from 0.084 to 6.83 mg/l of 7OMTX. The clean-up recovery from plasma was 88% for MTX and 72% for 7OMTX, owing to retention on the protein precipitate. The assay was linear, the measurement precision was 3.3% for MTX and 6.2% for 7OMTX and the clean-up reproducibility was 4% for MTX and 3.6% for 7OMTX. By reference to automated fluorescence polarization immunoassay, the HPLC method resulted in plasma MTX values 10% lower, probably owing to the higher specificity of HPLC. Unsystematically sequenced plasma samples from 35 children following 24-h MTX infusions provided estimated half-decay times of 16 and 19 h for MTX and 7OMTX, respectively, and 7OMTX:MTX concentration ratios of 7 at 48 h and of 5 at 72 h from starting infusions.

1. Introduction

Methotrexate (MTX) and its main metabolite 7-hydroxymethotrexate (7OMTX) are currently measured in blood by HPLC [1–4] at oncological doses. Methods have been described to measure "trace amounts", e.g., rheumatological or dermatological lower levels. The most sensitive was published by Beck et al. [5]; however, it involves a lengthy and costly liquid–solid extraction step and a post-column chemo-activation into fluorescent products with a special photochemical reactor. In addition, the metabolite 7OMTX is not yet commercially available, and the substances exchanged as gifts between university laboratories are often of undocumented purity. This work was an attempt at defining analytical and metrological conditions accessible to routine thera-

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peutic drug monitoring of both substances in oncological cures.

2. Experimental

2.1. Reagents

Solutions of MTX were prepared from Ledertrexate (Lederle, Rungis, France), containing 25 mg of MTX in 1 ml. Our source of 7OMTX was ca. 1 mg of a partially purified preparation generously given us by Dr. D.G. Johns (National Cancer Institute, NIH, Bethesda, MD, USA). The stock solution used for this work contained 50 mg/l of both MTX and 7OMTX preparation. They were appropriately diluted in water or incorporated in human plasma in order to obtained the nominal concentrations described in the validation design (true 7OMTX concentrations were calculated as described later.).

2.2. Analytical apparatus

A Model SP8810 pump and a Model Spectra 100 variable-wavelength UV–Vis detector from Spectra-Physics (now Thermo Separation Products, Les Ulis, France) were used. A Model 655A-40 autosampler and D2000 Chromato-integrator were obtained from Merck (Darmstadt, Germany). The column was a LiChroCART cartridge (250 × 4 mm I.D.) filled with LiChrospher 100RP8, particle size 5 μm, from Merck. A TDx automat (Laboratoires Abbott, Rungis, France) was also used, which measures MTX by immunocompetition against a fluorescence polarization marker (FPIA method) with reference to provided calibrators.

2.3. Sample clean-up for chromatography

One volume (e.g., 500 μl) of heparinized plasma and two volumes of acetonitrile were thoroughly mixed. After brief centrifugation (3 min, 2000 *g*), the deproteinized supernatant mixture was transferred into a glass tube and four volumes of chloroform were added. After brief vortex mixing (to obtain stable volume partitioning, which is easy to judge), the aqueous supernatant, which will be referred to as "extract" in the following, was transferred to a sampler vial and injected as such. In the method evaluation procedure, these operations were applied to spiked plasma samples and to aqueous solutions of MTX and 7OMTX.

2.4. Chromatographic quantification

The isocratic mobile phase was acetonitrile–0.025 *M* sodium acetate buffer (pH 3.9) (12:88, v/v). The flow-rate was 1.5 ml/min, the detection wavelength was 307 nm and the whole apparatus was at room temperature. Volumes of 30 μl of "extracts" were injected, which was taken as a basis of sensitivity calculations. No internal standard was added; the accuracy of the autosampler was continually checked by duplicating each measurement and by measuring the same spiked plasma every ten unknown samples. The integrator was programmed to record both heights (H) and areas (S) of peaks; areas were used for quantification and heights for a quick check of the column efficiency from the area-to-height ratio.

2.5. Validation design and analysis of variance (ANOVA)

Peak areas S were measured in chromatograms of "extracts" prepared from aqueous solutions and also from human plasma spiked with methotrexate at five concentrations (0.205, 0.620, 1.85, 5.55 and 16.7 mg/l) or with 7OMTX at the same nominal concentrations, as well as from the same aqueous solutions injected as such in duplicate. The recovery from aqueous extracts and from plasma extracts was calculated as the average ratio of peak areas measured in extracts to the mean peak area measured in duplicates of the corresponding aqueous solutions.

The precision and linearity of the method were assessed on peak areas with our usual mixed (factorial and nested) experimental design [6]. The design factors were drug concentration (five levels) and sample matrix (two levels: water and

human plasma). Each sample was extracted twice and each extract was injected twice according to the nested within-cell pattern, in order to calculate separately the variances of chromatographic measurement and of the clean-up step. All ANOVA calculations were made from napierian logarithms of peak areas ($\ln S$) in a spreadsheet program, as follows (logarithmic transformation will be discussed).

(1) Homoscedasticity was checked though Bartlett's test [7] of all measurement and ln(measurement) duplicates. In the case of duplicates, Bartlett's B term simplifies to $k \cdot \ln[\Sigma(\Delta_i^2)] - \Sigma[\ln(\Delta_i^2)] - k \cdot \ln k$, Δ_i being the difference between the two measurements, or logarithmic measurements, from the same ith extract, and k is the number of such duplicates. Subsequent ANOVA was performed on napierian logarithms of measurements $Y = \ln S$. The critical probability level required to admit non-significance of the subsequent tests was set at $p = 0.1$.

(2) Following factorial analysis, F-tests were applied to [between two matrices, one degree of freedom (df)] extraction difference between water and plasma and to (between concentrations, four df) linear regression common to both matrices, and common non-linearity; the concentration–matrix interaction term (four times one df) was split into non-parallelism and opposite non-linearity of regressions. The denominator of F-tests was the within-cell mean square. The regression coefficient b ("slope" of the bilogarithmic regression line) was compared with the theoretical value of 1 with Student's t-test of $(b-1)/s_b$, where s_b^2 is the within-cell mean square divided by the sum of squares of $\ln C$ as usual.

(3) As already pointed out [8], napierian logarithmic standard deviations directly provide approximations to less than 10% of the corresponding coefficients of variation (C.V.), provided that the C.V. is smaller than 20% (for a thorough justification, see Ref. [9]). The within-cell mean square was split into the measurement (within-extract) variance estimate $\mathrm{Var_m}$ and the variance of extract pairs $\mathrm{Var_{2x}}$; the extraction (between-extracts) variance estimate $\mathrm{Var_x}$ was $\mathrm{Var_{2x}}/2$. C.V.s of the chromatographic measurement and of the sample clean-up step were calculated as the respective square roots.

(4) The inter-patient variability of clean-up loss was assessed on plasma samples randomly obtained from twelve patients under anticonvulsant drug monitoring and spiked with ca. 1 mg/l of the 7OMTX preparation.

2.6. Calculating concentrations

Assuming proportionality between peak area and analyte concentration (i.e., analytical linearity, ascertained by the present work), the bilogarithmic calibration relationship of MTX is (logarithmic transformation will be discussed):

$$Y - Y_m = b(X - X_m)$$

where X is ln[MTX, mg/l], Y is ln(MTX peak area, integrator units) and X_m and Y_m are the mean coordinates. To determine unknown MTX concentrations, the following reciprocal relationship was applied:

$$X = (Y - Y_m)/b + X_m$$

then

$$[\mathrm{MTX, mg/l}] = \exp(X)$$

True concentrations of 7OMTX working solutions were calculated from peak areas (S) measured on the chromatograms of directly injected solutions as follows:

$$\text{mean [7OMTX, mg/l]} = \text{mean [MTX, mg/l]} \cdot [\text{mean 7OMTX } (S)/\text{mean MTX (S)}] \cdot M \cdot A$$

where M is the molecular mass ratio 470/455 of 7OMTX to MTX and A is the UV absorbance ratio 21 600/19 055 of MTX to 7OMTX at 307 nm and acidic pH, reported by Farquhar et al. [10] and Chamberlin et al. [11].

In the same way, concentrations in plasma samples from patients were calculated, not from the unreliable solutions of the crude extract, but from the MTX calibration parameters b and X_m, as follows:

$$X' = (Y' - Y_m)/b + X_m$$

where X' is ln[7OMTX] and Y' ln(7OMTX peak area), with the same units as for MTX,

$$[7OMTX, mg/l] = \exp(X') \cdot M \cdot A \cdot R$$

where R is the clean-up recovery ratio of MTX to 7OMTX. It will be shown that in plasma extracts, $R = 88.5/74.6$ so that $M \cdot A \cdot R = 1.39$.

2.7. Comparison of HPLC MTX determinations with automated FPIA

To check the accuracy, 50 plasma samples from patients were concurrently measured for MTX with the TDx method and with the present HPLC method. Samples were taken from 35 children, 24, 36, 48 and 72 h following the beginning of a 24-h MTX infusion of a dose ranging from 200 to 400 mg/kg, as part of a routine monitoring protocol. TDx assays were made on heparinized plasma within 24 h after venipuncture. The remainder of each sample was then frozen (−20°C) for subsequent HPLC measurements, not more than 5 months later.

All concentrations of MTX and of 7OMTX measured by HPLC in the patients were plotted as logarithms versus time (see Fig. 3) and the regression line was calculated.

3. Results

Fig. 1 shows chromatograms of a spiked plasma from a preliminary study and of plasma samples taken during two successive treatments of the same patient.

The Bartlett test led to the same conclusion for MTX and for 7OMTX: the quasi-chi squared estimator was lower when applied to logarithmic than to arithmetic values of peak areas (49.6 vs. 94.5 for MTX, 43.3 vs. 76.3 for 7OMTX). In other words, the logarithmic transformation generates values closer to homoscedasticity than are the untransformed measurements (the random variability is closer to constant C.V. than to constant S.D. in the concentration range investigated).

3.1. Evaluation of the chromatographic method (Table 1)

Methotrexate

The limit of detection, defined as three times the detector noise, was 0.012 mg/l and the limit

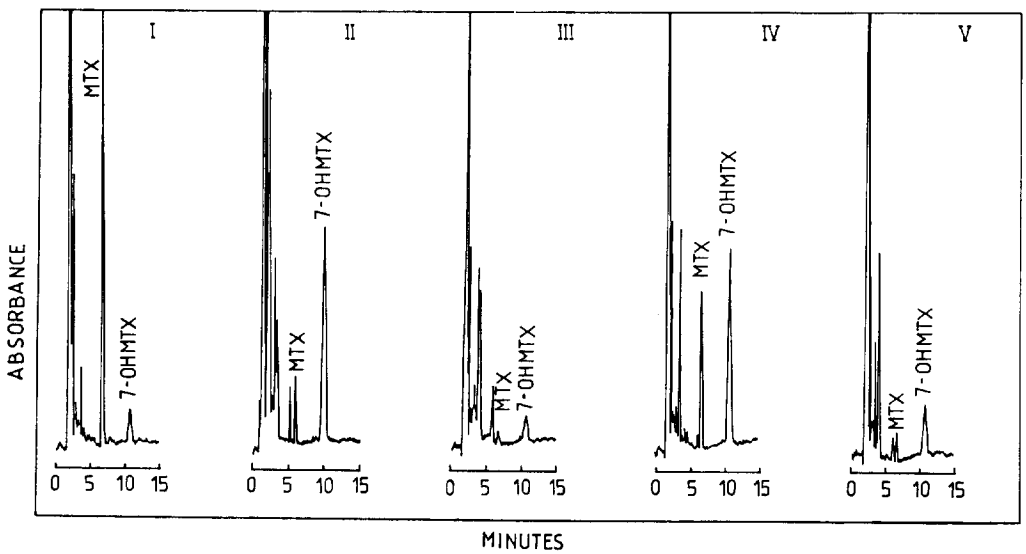

Fig. 1. Chromatograms of methotrexate (MTX) and hydroxymethotrexate (7-OMTX). (I) Plasma spiked with 1.04 and 0.15 mg/l, respectively, from a preliminary trial; (II–III) plasma from a 3-year-old patient, sampled 48 and 72 h following the start of 3.5 g per (24-h infusion): (IV–V) plasma from the same patient, same timing, second treatment.

Table 1
Main results from ANOVA of MTX and 7OMTX validation designs (peak area, napierian logarithms)

ANOVA components	MTX				7OMTX			
	Parameters	$S.D._{ln}$ (C.V.)	Tests	P	Parameters	$S.D._{ln}$ (C.V.)	Tests	P
Between matrices	$\Delta = -8\%$		$F(1/30) = 33$	***	$\Delta = -23\%$		$F(1/30) > 100$	****
Linear regression	$b = 1.0089$	$s_b = 0.0129$	$t_{30} = 0.53$	0.7	$b = 0.9827$	$s_b = 0.0239$	$t_{30} = 0.72$	0.4
1st curvature			$F(1/30) = 2.95$	0.2			$F(1/30) = 3.8$	0.2
Sigmoidicity			$F(1/30) = 1$				$F(1/30) = 1.8$	0.2
Non-parallelism			$F(1/30) = 9.7$	0.005			$F(1/30) < 1$	
Opposite curvature			$F(1/30) < 1$				$F(1/30) = 1.5$	0.2
Between measures		0.03417				0.06237		
Extraction		0.04282				0.03585		

Δ = percentage mean difference (plasma extracts − water extracts)/water extracts.

of quantification, defined as ten times the detector noise, was 0.04 mg/l, corresponding to 1 and 3 pmol in 30-μl injections, respectively. The mean recovery of methotrexate was 96.5% from water samples and 88.5% from plasma samples.

The overall C.V.s were 3.3% for measurements (3.0% in water extracts, 3.6% in plasma extracts) and 4.2% for extraction. The common bilogarithmic regression slope of 1.009 did not differ significantly from the target value of unity, nor were curvature terms significant; the term of non-parallelism was significant.

7-Hydroxymethotrexate

The actual median concentration of 7OMTX, calculated on the whole design by reference to the MTX calibration relationship as described under Experimental, was found to be 0.758 mg/l instead of the nominal value of 1.85 mg/l. Thus, the nominal concentration of our stock solution of 7OMTX was overestimated by a factor 2.44. Mean recoveries of 7OMTX from water and plasma were 97.0% and 74.6%, respectively.

The C.V. of measurements was 6.2% and that of extraction was 3.6%. The one extract–one measurement inter-patient C.V. determined in spiked plasma samples from twelve different patients was found to be 10.0%, to be compared with the intra-patient C.V. of 7.2% expected from the validation design. The common regression slope of 0.983 did not differ significantly from the target value of unity, and no term of higher degree was significant.

3.2. Method comparison

The two regression equations corresponding to the correlation diagram between MTX concentrations (mg/l) found by the two analytical methods (Fig. 2) were $[\text{MTX, LC}] = 0.875 \cdot [\text{MTX, TDx}] - 0.00926$ and $[\text{MTX, TDx}] = 1.1384 \cdot [\text{MTX, LC}] + 0.01215$.

3.3. Application to clinical samples

Fig. 3 shows the plasma levels of MTX and of 7OMTX plotted on a logarithmic scale as a function of time T spent since the beginning of infusions. The regression lines fitted to the points had the following equations:

$$\text{Ln[MTX, LC]} = -0.04166 \cdot \text{T} + 0.2746$$

$$\text{Ln[MTX, TDx]} = -0.04120 \cdot \text{T} + 0.5721$$

$$\text{Ln[7OMTX]} = -0.03556 \cdot \text{T} + 0.99085$$

with [MTX] and [7OMTX] drug levels in mg/l and T in hours. The mean [MTX]/[7OMTX] ratios are given in Table 2.

4. Discussion

The validation experiment was designed from preliminary results in order to cover about a

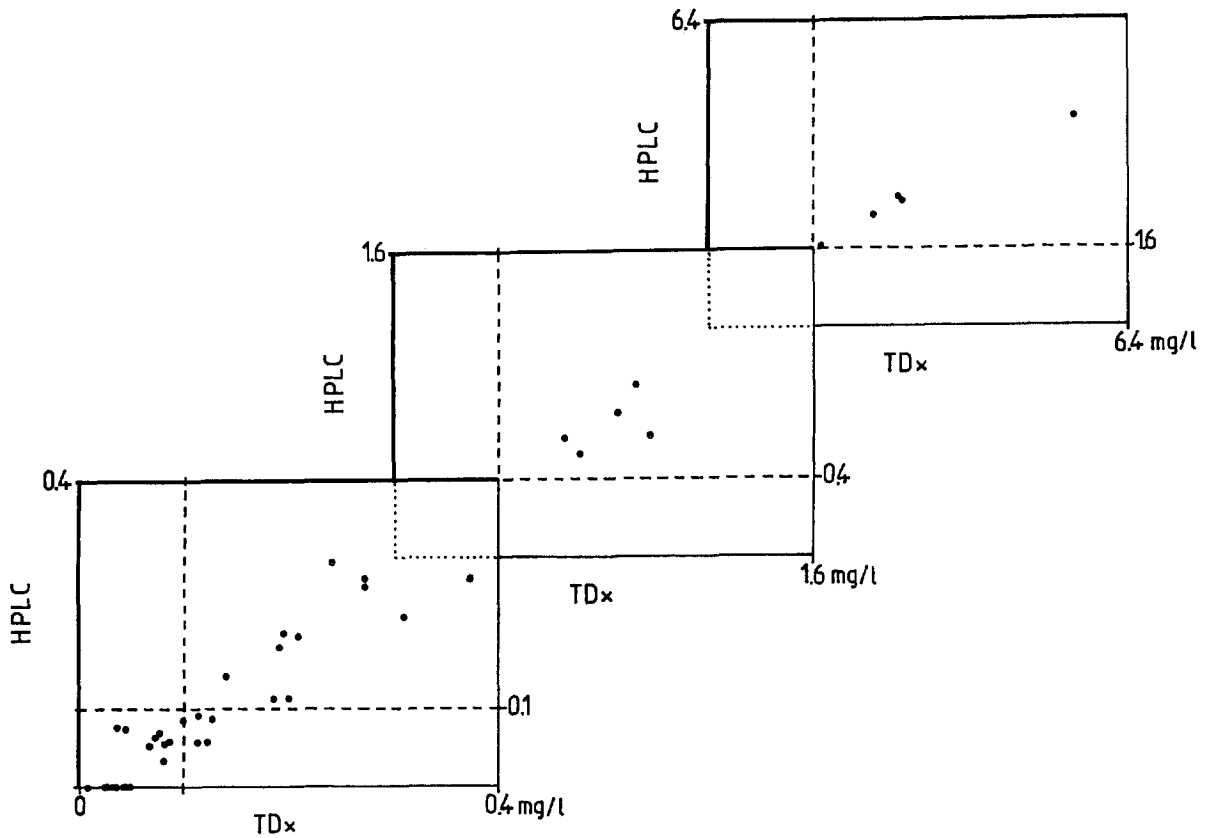

Fig. 2. Correlation diagram comparing HPLC (ordinate) with FPIA (abscissa) measurements of MTX levels in 50 plasma samples. The scale is reduced from left to right so that each rectangle overlaps its own representation on the next one.

100-fold range of concentrations, which is actually obtained with five concentrations in geometrical progression of ratio (dilution factor) 3.

Linearity, precision and matrix effects were assessed in the validation design after logarithmic transformation of HPLC measurements. Logarithmic transforms appear more suitable than arithmetic values for several reasons: the often neglected requirement of homoscedasticity for ANOVA is currently better fulfilled; additive–subtractive statistical comparisons are replaced by the more familiar scale-free relative ("percent") comparisons, e.g., standard deviations by coefficients of variation; finally, analytical non-linearity (which means departure from direct proportionality) can be assessed fairly simply by testing non-linearity of the logarithmic regression line together with departure of the logarithmic slope from the theoretical value of unity.

Plasma samples were stored at −20°C for not longer than 5 months because we had previous experience [12] that delayed HPLC measurements of MTX, compared with immediate FPIA measurements, decreased at a rate of about 1% per month.

Following acetonitrile deproteinization, extraction of acetonitrile into a water-immiscible solvent provides samples virtually undiluted, having a lower elution strength than the mobile phase, and cleaned up from lipophilic (i.e., having long retention times) compounds. The partial co-extraction of analytes appears negligible since the recovery loss from water is less than 3%. Therefore, most of the recovery loss from plasma

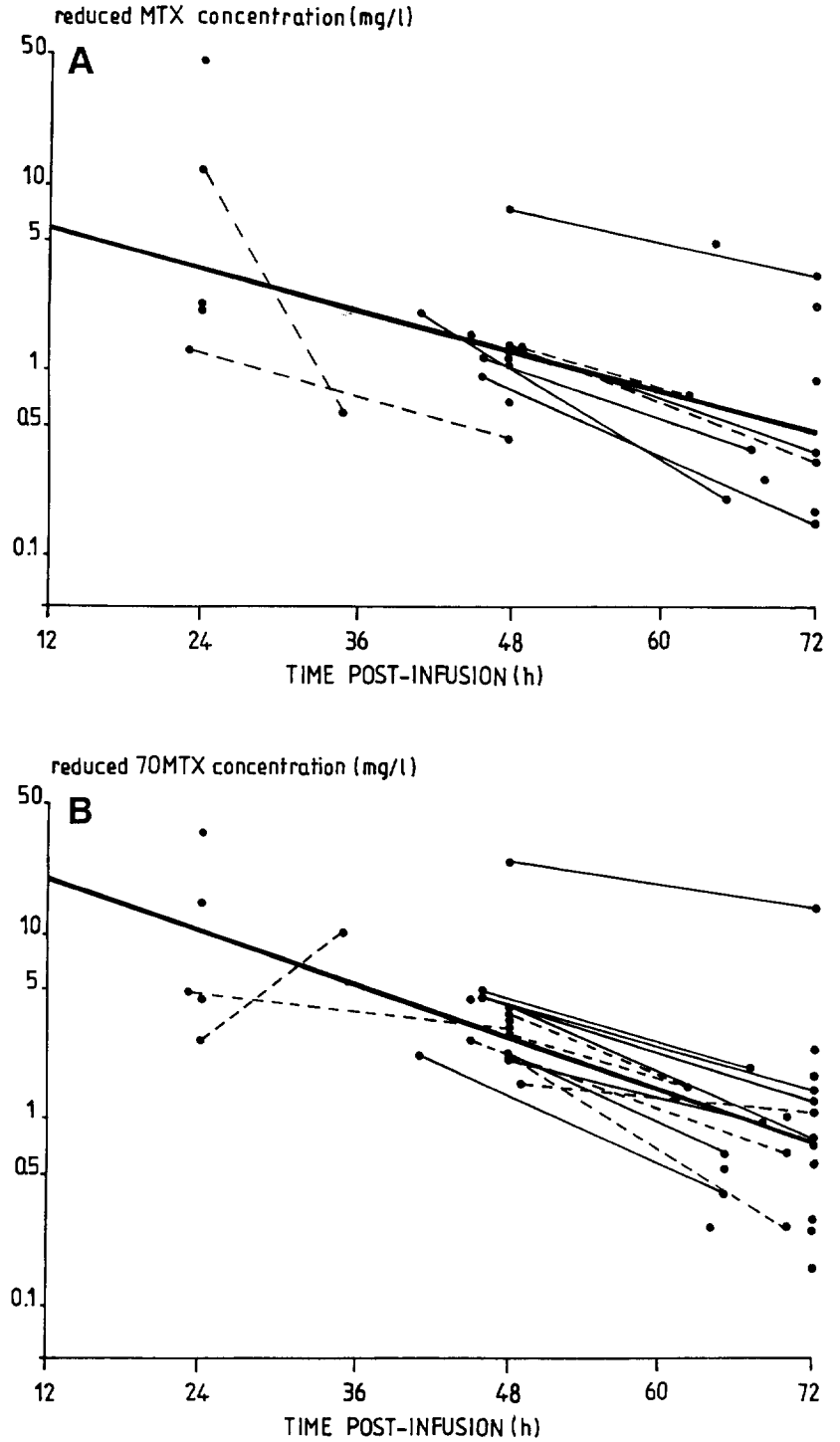

Fig. 3. Time relationship of dose-standardized ("reduced") plasma concentrations (mg/l per g/kg). Timing starts from the beginning of a 24-h infusion of the dose. A, MTX; B, metabolic 7OMTX.

Table 2
Ratio of 7OMTX to MTX concentrations during and after MTX infusions

Time post-infusion (h)	7OMTX/MTX concentration ratio			n	Wolfrom et al.'s mean [15]
	Mean	S.D.	Range		
24	3.9	3.7	0.33–9.41	5	0.31
48	7.2	6.6	1.6–24.5	15	7.8
72	4.9	5.2	0–20.3	30	6.2

samples is ascribable to retention of analytes on the protein precipitate during acetonitrile deproteinization, and 7OMTX is much more retained than is MTX (25.4% compared with 11.5%), either because of the lower molecular concentrations, or for a similar physical reason to why it shows a longer retention time than MTX in reversed-phase chromatography. Chloroform extraction of acetonitrile was preferred to separation by cooling [13] because it provided cleaner samples, because it was quicker than separating phases by cooling and centrifugation and especially because the aqueous layer to be injected was in the upper, instead of lower, location.

With a 30-μl injection volume, the detection limit of MTX was 12 μg/l (30 nmol/l) and quantification limit was 40 μg/l (about 100 nmol/l). The sensitivity can be increased at least threefold by injecting 100 instead of 30 μl, since the resultant broadening of peaks is small owing to the high polarity of the extracts (the area/height ratio increased by only 8%). The TDx method claims a quantification limit of 10 nmol/l for MTX, but given with a fairly large uncertainty since it is read as only digit on the display (7OMTX is not determined). The present detection limit is comparable to the undefined "sensitivity" of So et al. [1], who concentrated the sample tenfold, presumably owing to better performance of later UV detectors. It compares well also with the "lower limit of detection" of Slordal et al. [2] and with the "detection limits" of Nuernberg et al. [3], and it is lower than the results of Assadullahi et al. [4]. Beck et al. [5] recorded detection limits of 0.2 nmol/l for MTX and 1 nmol/l for 7OMTX following injection of the extract from 200 μl of plasma: the present method would thus be about 50 times less sensitive as such, and is expected to be about 20 times less on increasing the injection volume to 100 μl.

The sum of measurement and of clean-up variances results in a calculated C.V. of 5.3% for any single measurement of MTX from an extract and 4.8% for the mean of duplicate measurements. Since the clean-up loss of 7OMTX appeared much larger, there was a suspicion of a larger inter-patient variability between samples from different individuals according to their protein content and/or quality. When this possibility was tested, the one extract–one measurement inter-patient C.V. was found to be 10.0% among twelve patients, not much larger than the intra-patient 7.2% expected from the validation design.

4.1. Linearity

For both MTX and 7OMTX, the calibration relationship of the assay did not differ from $\ln S = \ln a + \ln C$, or $S = a \cdot C^1$: peak-area measurements may be considered proportional to concentrations, which agrees with a linear model without an intercept. It can be seen in Fig. 3 that the range of concentrations validated matches values usually observed in oncological therapeutic drug monitoring.

4.2. Accuracy

Our HPLC method was compared with FPIA, which was taken as a reference method, by linear regression of 50 plasma single measurements by the first versus the second. As indicated in

Section 3.2, the regression of [MTX, LC] versus [MTX, TDx] was

$$[\text{MTX, LC}] = 0.875 \cdot [\text{MTX, TDx}] - 0.00926$$

In fact, the reciprocal regression line of FPIA versus HPLC was essentially the same. On recalculating its equation in the same frame, the regression of [MTX, TDx] vs. [MTX, LC] becomes:

$$[\text{MTX, LC}] = 0.8785 \cdot [\text{MTX, TDx}] - 0.0167$$

Therefore, owing to the small scatter of the analytical data ($r = 0.998$), the regression comes close to a functional relationship, and it is not necessary to seek other statistical means of method comparison [14]. Thus the chromatographic MTX assay results were found to be ca. 12% lower than TDx. In a linear method, one possible source of inaccuracy lies in calibration. In the present case, either the nominal concentration of HPLC calibrators would have been less than their real concentration (HPLC calibrators would have been overconcentrated), or TDx calibrators would have been underconcentrated, or both. French regulations limit to ±5% the maximum deviation of drug content from the nominal amount of preparations; our HPLC calibrators can thus incorporate such a deviation. TDx control samples are valid from ±10% (two highest points) to ±14% (lowest point); it can be assumed that individual measurements are affected by the same uncertainty, but the mean of 50 measurements must vary seven times less. When assaying plasma samples, another potential source of discrepancy between methods lies in the analytical specificity, in particular with regard to the metabolite 7OMTX. The present comparison was made with first-generation TDx reagents, which cross-reacted with 7OMTX. Since the mean 7OMTX/MTX ratio was around 5 (Table 2), a 2% cross-reactivity would be sufficient to result in FPIA measurements 10% higher than HPLC measurements.

4.3. Application to patients' data

The "half-lives" calculated on the regressions illustrated in Fig. 3 were 16.5 h for MTX by HPLC, 15.7 h for MTX by FPIA and 19.5 h for 7OMTX, to be compared to the respective terminal 13 and 11 h found by Wolfrom et al. [15]. Estimating pharmacokinetic parameters from a set of blood levels coming from several individuals [the "naive" approach of Steimer et al. [16] is not considered good pharmacokinetics, but the parallelism of most multiple intra-patient measurements with the global slope (Fig. 3) probably eliminates much of this objection when the half-life is estimated]. The weakest feature of our observational data is probably the fact that doses were occasionally given to individual patients for unequal durations, less than the usual 24 h infusion, particularly those sampled at 24 h. This results in 7OMTX/MTX mean level ratios 1 in five such patients (Table 3); however, the ratios measured at 48 and 72 h are in accordance with those of Wolfrom et al. [15]. This is evidence that the proposed method of calibrating 7OMTX with MTX standards is effective and usable.

5. Conclusion

The HPLC method presented allows the rapid quantification of MTX and of 7OMTX with standard equipment, even when the metabolite is not available for calibration. Acetonitrile deproteinization and clean-up with chloroform are quickly done and provide injection samples devoid of delayed peaks with reproducible recovery. The precision and sensitivity appear acceptable for oncological drug monitoring.

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