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High-performance liquid chromatographic assay for the determination of 5-methyltetrahydrofolate in human plasma

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Abstract

An isocratic high-performance liquid chromatographic method for the determination of 5-methyltetrahydrofolate (5-MTHF) in human plasma is described. The method involves solid-phase extraction of 5-MTHF and *p*-aminoacetophenon (an internal standard) using Sep-Pak C_{18} cartridges. Separation was achieved with an ODS column using acetonitrile and phosphate buffer supplemented with octanesulfonic acid (an ion-pairing agent). The pH of the mobile phase (2.5) was optimal with respect to the mode of detection (fluorescence). The method was validated in the range of 5-MTHF concentrations from 0.0625 μmol/l to 4.0 μmol/l. Within-day and inter-day precision expressed by the relative standard deviation was less than 8.1% and inaccuracy did not exceed 8.7%. The method is specific, accurate and sensitive enough to be used in pharmacokinetic studies for the assessment of the systemic availability of 5-MTHF after leucovorin administration to patients as a rescue after high-dose therapy with methotrexate. The limit of detection was 0.17 pmol which corresponds to a plasma concentration of 1.7 nmol/l. Thus, the assay could potentially be used for the measurement of 5-MTHF in the range of physiological concentrations in plasma (5–20 nmol/l). © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

It has been shown that both intravenous and oral administration of 5-formyltetrahydrofolate (leucovorin, LE) to humans causes plasma elevation of the biologically active reduced folates: 5-methyltetrahydrofolate (5-MTHF), tetrahydrofolate, 5,10-methylenetetrahydrofolate and 10-formyltetrahydrofolate [1]. LE is repeatedly administered to patients at doses of 10–20 mg/m^2 to prevent the toxicity of the high-dose methotrexate therapy for leukemia and solid tumors. More recently, it has been used in combination with 5-fluorouracil in the treatment of colorectal carcinoma [1,2]. LE is a mixture of two stereoisomers. The L-stereoisomer of LE is rapidly converted to 5-MTHF, the predominant metabolite in plasma, the other folates accounting for only 10–15% [1]. Thus, determination of 5-MTHF in plasma is an important method for the assessment of the systemic availability of active folates after LE administration.

Published methods of high-performance liquid chromatography (HPLC) analysis of folates in plasma employ UV detection at 280 nm [3–5]. More recently, electrochemical [6,7] and fluorimetric detection systems [8,9] have been used. The latter

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method was adopted in our laboratory also, since it offers excellent sensitivity in comparison with UV detection. A conventional reversed-phase HPLC of 5-MTHF is complicated by the relatively weak interaction between the analyte and the stationary phase, resulting in short retention times and poor separation of different reduced folates. Therefore, gradient methods are frequently used [7,10]. Alternatively, separation using anion-exchange chromatography has been described [11]. Here we report on the development of a highly sensitive isocratic ion-pair reversed-phase method for the determination of 5-MTHF in human plasma with a pH 2.5 mobile phase, which is optimal with respect to the mode of detection [9]. The method has been subjected to a thorough validation procedure.

2. Experimental

2.1. Chemicals

Folinic acid calcium salt, 5-methyltetrahydrofolic acid, dihydrofolic acid disodium salt, tetrahydrofolic acid, *p*-aminoacetophenone (internal standard, I.S.) and 1-octanesulfonic acid were purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol (HPLC grade) were products of Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Instrumentation and chromatographic conditions

The assay was developed using a Model LC10 AD solvent-delivery system, a Model RF10A fluorescence detector and a Model SIL-10A autoinjector and sample cooler (Shimadzu, Kyoto, Japan). Data acquisition was accomplished using an Apex-extra PC-integrator (ECOM, Prague, Czech Republic). Fluorescence was monitored at the excitation and emission wavelengths of 305 nm and 365 nm (bandwidths 15 nm), respectively. Chromatographic separation was achieved at ambient temperature using a Spherisorb ODS2 column (5 μm, 4.6 mm× 250 mm, M. Grom, Herrenberg, Germany). The mobile phase, delivered at a flow-rate of 1.2 ml/min, was comprised of 0.05 *M* potassium dihydrogenphosphate buffer (pH 2.5)–acetonitrile (86.5:13.5, v/v) and contained 15 m*M* octanesulfonic acid.

2.3. Sample preparation procedure

An aliquot of 1.5 ml of 0.02 *M* Tris buffer (pH 7.0) containing 20 μmol/l *p*-aminoacetophenone (I.S.) and 5 μmol/l tetrabutylammomium hydroxide was added to 0.5 ml of plasma in a polypropylene test tube and the tube was briefly vortex-mixed. This mixture was applied on a Sep-Pak C_{18} cartridge (Millipore, Waters Chromatography, Milford, MA, USA). Prior to use, the extraction cartridge was activated by washing successively with 2 ml of methanol, 2 ml of water and 3 ml of 20 mmol/l Tris buffer (pH 7.0). Plasma constituents were desorbed with 1 ml of Tris buffer (pH 7) and 0.25 ml of 30% methanol in Tris buffer (pH 7). Then, the analytes were eluted with 1 ml of 50% methanol in 50 mmol/l phosphate buffer (pH 3.0). The eluates were evaporated under a stream of nitrogen at 37°C. The dried samples were redissolved in 0.5 ml of 1 g/l ascorbic acid and 100-μl aliquots were injected onto the column.

2.4. Preparation of calibration standards and quality control samples

A stock solution of approximately 30 μmol/l 5-methyltetrahydrofolic acid was prepared by dissolving an accurately weighed amount of 5-methyltetrahydrofolic acid (Sigma) in an aqueous solution of ascorbic acid (1 g/l). Three stock solutions were prepared. Each stock solution was diluted 50-fold using a calibrated Brand pipette (Brand, Wertheim, Germany) and a 10-ml glass vial with 50 m*M* phosphate buffer (pH 7). Three dilutions of each stock solution were prepared. The absorption spectra of the dilutions were measured at 285 nm (a Model UV-2101 PC spectophotometer, Shimadzu) against the ascorbic acid solution (1 g/l) diluted with phosphate buffer 50-fold. The average absorbance of the three diluted stock solutions was compared. The maximum difference was 2.6%. The solutions were then stored in 1 ml aliquots at −80°C.

The calibration curve was prepared in the range 0.0625–4.0 μmol/l to encompass the expected concentration of 5-MTHF in samples. To the blank

human plasma, 1 mg/ml of ascorbic acid was added. For validation of the assay, calibration standards of seven levels (including a blank plasma) and sets of quality control (QC) samples (three levels) were prepared gravimetrically by adding 5-MTHF stock solution to blank human plasma containing ascorbic acid (1 g/l) and gravimetric dilutions of 1 ml of the level *N* with 1 ml of the same blank plasma resulting in the level $N+1$.

2.5. Validation procedures

A full validation of the assay was performed consisting of the following experiments: specificity, lower limit of quantitation (LLQ), limit of detection (LOD), recovery of the analytes from plasma, intra- and inter-day accuracy and precision, stability of analytes after sample preparation, stability in plasma at approximately 20°C, freeze–thaw stability, storage stability at −20°C and at −80°C. The long-term stability study was extended up to 3 months. All calculations were performed using the statistical package Number Cruncher Statistical System version 5.X (J.L. Hintze, Kaysville, UT, USA).

3. Results and discussion

3.1. Sample preparation procedure and chromatography

The low concentrations of 5-MTHF in plasma require an assay with correspondingly high selectivity and sensitivity. Therefore we used extraction of the analytes on solid-phase extraction columns (Sep-Pak C_{18}, Waters). In order to improve precision of the extraction method we implemented an internal standard *p*-aminoacetophenone. The recovery of 5-MTHF from human plasma was determined using quality control samples and a phosphate buffer (0.15 *M* NaH_2PO_4, pH 7.4), both spiked with three concentrations of 5-MTHF covering the calibration range. The mean (range) recovery obtained as a peak areas ratio (extracts of plasma samples/corresponding samples in the buffer) achieved 98% (93–101%). The recovery of the I.S. was 88%.

With a mobile phase containing 13.5% acetonitrile and 15 m*M* octanesulfonic acid as an ion-pairing agent we achieved separation of the quantitatively important folates 5-MTHF, leucovorin and THF on an isocratic system within 16 min (Fig. 1). Blank human plasma samples of six individuals showed no substances at the retention times of the I.S. or late eluting peaks. There was a peak at the retention time of 5-MTHF, the area of which corresponded to the physiological concentration of 5-MTHF in plasma of less than 20 nmol/l. Selectivity of the assay was further confirmed using the extracts of plasma samples collected from 24 volunteers before the administration of LE. Chromatograms of drug-free human plasma and of a plasma sample from a volunteer 8 h after administration of 30 mg leucovorin are illustrated in Fig. 2.

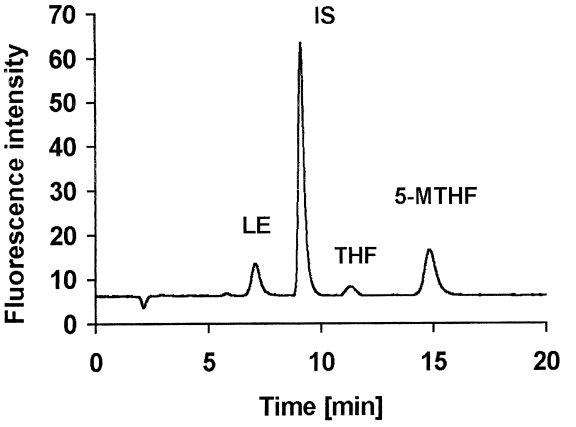

Fig. 1. Separation of leucovorin (LE), *p*-aminoacetophenon (IS), tetrahydrofolate (THF) and 5-methyltetrahydrofolate (5-MTHF). The amounts injected as an aqueous solution were 60 pmol (LE), 1 nmol (I.S.) and 6 pmol (THF, 5-MTHF).

3.2. Linearity, lower limit of quantitation and limit of detection

The calibration curves were constructed using a weighted linear regression (weighing factor $1/\text{concentration}^2$). The peak area ratios of 5-MTHF to the internal standard were plotted vs. the nominal concentrations of the calibration standards. The slopes and intercepts of the regression curves are listed in Table 1. For the assessment of linearity, the relative standard deviation (RSD) of the assayed concentrations and the percentage deviation of the assayed concentrations from the nominal concentration (RE)

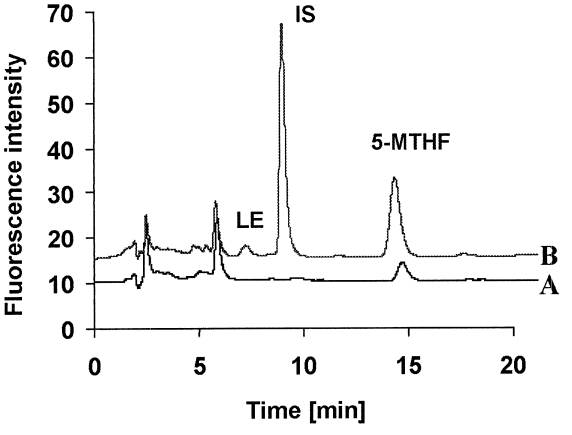

Fig. 2. Chromatograms of (A) drug-free human plasma and of (B) a plasma sample from a volunteer 8 h after administration of 30 mg leucovorin (LE). The respective concentrations of 5-methyltetrahydrofolate (5-MTHF) were 0.092 μmol/l (B) and less than the LLQ of 0.0625 μmol/l (A).

were calculated using data obtained on five consecutive days. The results indicate excellent linearity in the concentration range of 0.0625–4.00 μmol/l (Table 1). The linearity was further examined by the lack-of-fit test [12]. The linear regression model passed the lack-of-fit test at the level of significance $\alpha=0.05$ [$F(5,28)=1.2$ was less than the critical value of 2.6].

The LLQ was determined from the statistical analysis of the back calculated concentrations of the calibration rows measured on five consecutive days. The criteria for precision and accuracy at the LLQ were RSD<15% and RE<15%. The criteria were met by the results of the analysis of the lowest calibration standard (0.0625 μmol/l) where 2.5% and −0.3% were achieved for RSD and RE, respectively. The LOD was estimated using the formula: $LOD=3\cdot SD_N\cdot R/S$, where SD_N is the standard deviation of the blank measurement, R represents the peak area to peak height ratio and S is the slope of the calibration curve. The LOD was 0.17 pmol which corresponds to the plasma concentration of 1.7 nmol/l. Thus, the assay could potentially be used for the measurement of 5-MTHF in the range of physiological concentrations in plasma of 5–20 nmol/l.

3.3. Within-day precision and accuracy

The within-day precision and accuracy of the assay was assessed from the results of five replicate analyses of spiked quality samples (SQCs) prepared at three concentrations using human plasma (one near the LLQ, one in the middle range and one approaching the high end of the calibration range). At these spiked levels, the RSD ranged between 3.4 and 6.4% and the range of the RE was −5.4–3.9% (Table 2).

3.4. Inter-day precision and accuracy

The inter-day precision and accuracy of the assay was determined from the results of the same SQCs as

Table 1
The assayed concentrations of calibration standards of 5-MTHF in human plasma[a]

Nominal concentration (μmol/l)	Assayed concentration (μmol/l)					Mean	RSD (%)	RE (%)
	Day 1	Day 2	Day 3	Day 4	Day 5			
4.0	3.78	4.17	3.86	4.09	4.08	4.0	4.2	−0.1
2.0	2.14	2.09	2.04	2.02	1.97	2.05	3.2	2.6
1.0	1.02	0.98	1.0	0.98	0.96	0.99	2.4	−1.1
0.50	0.512	0.456	0.496	0.512	0.497	0.494	4.6	−1.2
0.25	0.237	0.235	0.262	0.244	0.252	0.246	4.5	−1.5
0.125	0.121	0.140	0.122	0.122	0.131	0.127	6.5	1.6
0.0625	0.0640	0.0604	0.0626	0.0635	0.0610	0.0623	2.5	−0.3
Slope	45 517	49 029	43 548	43 790	42 312			
Intercept (%)[b]	−23.9	−0.8	10.7	−20.8	−3.7			
r	0.998	1.00	1.00	1.00	1.00			

[a] Estimates of the parameters of the regression lines.
[b] In % of the response of the lowest calibration standard.

Table 2
The within-day precision and accuracy of the assay of 5-MTHF in human plasma

	Assayed concentration of 5-MTHF (μmol/l)		
	High SQC	Medium SQC	Low SQC
Day 1	2.61	1.02	0.101
	2.67	1.09	0.0910
	3.01	1.04	0.0933
	2.98	1.00	0.0972
	2.92	1.02	0.0990
Mean	2.84	1.03	0.0963
RSD (%)	6.5	3.3	4.3
Nominal concentration (μmol/l)	3.00	1.00	0.100
RE (%)	−3.5	3.9	−5.4

for the intra-day variability analyzed on five consecutive days. The RSD ranged between 3.9 and 8.1% and the range of the RE was −5.1–8.7% (Table 3). The corresponding inter-day calibration standard data are summarized in Table 1.

3.5. Stability study

3.5.1. Stability in plasma

One set of SQCs at three levels in plasma containing 1 mg/ml ascorbic acid was prepared and kept at room temperature (approximately 22°C) in dark. At time point 0 h, two aliquots of each of the SQC were processed and analyzed. At time point 4 h, the sample preparation was performed again using another two aliquots of each of the SQC. After a HPLC analysis, the mean concentrations of the SQCs were calculated and compared to these at time point 0 h. The concentrations observed in stored samples were on average 3.3% less than in those processed immediately indicating stability of 5-MTHF in plasma over the time interval necessary for processing large batches of approximately 70 samples (Table 4).

3.5.2. Processed sample stability

Six sets of SQC in plasma were prepared and processed at time point 0 h. Two sets were analyzed together with the calibration row. The other two processed sets were kept in the autosampler at approximately 22°C in dark and analyzed 24 h after sample preparation together with the new calibration row. The last two sets were analyzed at time point 48

Table 3
The inter-day precision and accuracy of the assay of 5-MTHF in human plasma

	Assayed concentration of 5-MTHF (μmol/l)		
	High SQC	Medium SQC	Low SQC
Day 1	2.90	1.04	0.0950
Day 2	2.75	1.10	0.102
Day 3	2.70	1.13	0.117
Day 4	3.18	1.13	0.100
Day 5	2.70	1.05	0.101
Mean	2.85	1.09	0.103
RSD (%)	7.2	3.9	8.1
Nominal concentration (μmol/l)	3.00	1.00	0.100
RE (%)	−5.1	8.7	3.0

Table 4
Stability of 5-MTHF in human plasma containing 1 mg/ml ascorbic acid over the time interval of 4 h at 22°C

Nominal concentration (μmol/l)	Assayed concentration (μmol/l)		Ratio (4 h/0 h) (%)
	0 h	4 h	
3.00	2.98	2.83	95
1.00	1.10	1.02	92
0.100	0.0862	0.0888	103
Mean			97
RSD (%)			5.7

h. The entire experiment was repeated with the processed SQC stored at 6 °C. The results are presented in Table 5. The processed samples were stable at 6°C and at 22°C for 2 days.

3.5.3. Freeze and thaw stability

One set of SQC at three levels was prepared and aliquots were pipetted into 2-ml Eppendorf cups. Two aliquots of each of the SQC were processed and analyzed. Another two sets were processed and analyzed after one, two and three freeze–thaw cycles. The concentrations found indicate no significant 5-MTHF loss during repeated thawing and freezing (Table 6).

3.5.4. Long-term stability

One set of SQCs at three levels was prepared in human plasma containing 1 mg/ml ascorbic acid and aliquots were pipetted into 2-ml Eppendorf cups. Two aliquots of each SQC were processed and analyzed. Another two aliquots of each SQC were processed after the different time intervals of the storage at −20°C for up to 3 months. After a HPLC analysis, the mean concentrations of the SQCs were calculated and compared to these at time point 0 h. The entire experiment was repeated with the aliquots stored at −80°C. The results are within the acceptable limits (Table 7). Thus, stability of 5-MTHF in human plasma is confirmed for the studied period.

Table 5
Stability of 5-MTHF (in dark) after sample preparation

Nominal concentration (μmol/l)	Assayed concentration (μmol/l)					Ratio (%)			
	0 h	24 h		48 h		(24 h/0 h)		(48 h/0 h)	
		22°C	6°C	22°C	6°C	22°C	6°C	22°C	6°C
3.00	2.93	3.25	3.11	2.80	2.88	111	106	96	98
1.00	0.993	1.04	1.04	0.960	1.02	105	105	97	102
0.100	0.0948	0.122	0.105	0.108	0.0910	129	111	114	97
Mean						117	108	105	99
RSD (%)						14.5	4.0	11.7	4.1

Table 6
Stability of 5-MTHF in human plasma containing 1 mg/ml ascorbic acid after repeated thawing and freezing

Nominal concentration (μmol/l)	Assayed concentration (μmol/l)				Mean	RSD (%)
	Number of freeze–thaw cycles					
	0	1	2	3		
3.00	2.81	2.75	2.58	2.8	2.74	3.9
1.00	1.05	1.1	0.98	1.06	1.05	4.8
0.100	0.0945	0.0892	0.0941	0.121	0.100	14.4

Table 7
Long-term stability of 5-MTHF in human plasma containing 1 mg/ml ascorbic acid

Nominal concentration (μmol/l)	Assayed concentration (μmol/l)						Mean	RSD (%)
	Day 0	Day 2	Day 3	Day 7	Day 30	Day 60		
Storage temperature −20°C								
3.00	2.81	2.54	2.51	2.86	3.11	2.91	2.790	8.2
1.00	1.05	1.03	0.961	0.974	0.953	0.982	0.992	4.0
0.100	0.0945	0.0835	0.0902	0.124	0.0894	0.0923	0.0957	15.0
Storage temperature −80°C								
3.00	2.81	2.84	2.63	2.85	3.10	2.91	2.857	5.3
1.00	1.05	0.953	0.982	1.08	1.02	0.990	1.013	4.6
0.100	0.0945	0.0861	0.0918	0.123	0.107	0.0970	0.100	13.3

3.6. Application of the assay to a pharmacokinetic study

The assay was applied to the plasma samples from the bioequivalence study. The mean plasma levels of 5-MTHF after 30 mg oral dose of LE were well above the physiological concentrations (Fig. 3). The maximum concentration of LE was on average 15-times higher than the LLQ which indicates a suitability of the analytical method for the above mentioned study.

4. Conclusion

In this paper, an isocratic HPLC method for the determination of 5-MTHF in human plasma is described. The method was validated in the range of 5-MTHF concentrations from 0.0625 μmol/l to 4.0 μmol/l. It is specific, accurate and sensitive enough to be used in pharmacokinetic studies for the assessment of systemic availability of the quantitatively most important folate in plasma after LE at the doses which are administered to patients as a rescue after high-dose therapy with methotrexate.

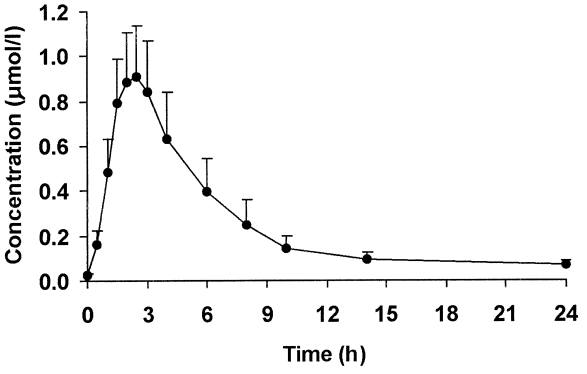

Fig. 3. Mean (SD) plasma concentrations of 5-methyltetrahydrofolate after 30 mg single oral dose of leucovorin (24 healthy volunteers).

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