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High-performance liquid chromatographic determination of methotrexate, 7-hydroxymethotrexate, 5-methyltetrahydrofolic acid and folinic acid in serum and cerebrospinal fluid

Susanne Belz*, Christine Frickel, Christiane Wolfrom, Heinz Nau[1], Günter Henze

Universitätsklinikum Rudolf Virchow Kinderklinik, Abteilung Hämatologie/Onkologie, Freie Universität Berlin, Reinickendorfer Strasse 61, 13347 Berlin, Germany

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Abstract

A method for the simultaneous determination of the antifolates methotrexate and 7-hydroxymethotrexate as well as the folates 5-methyltetrahydrofolic acid and folinic acid (5-formyltetrahydrofolic acid) in serum and cerebrospinal fluid (CSF) is described. High-performance liquid chromatography with gradient elution and dual detection (ultraviolet absorption and fluorescence) was used to separate and quantitate the analytes. Serum samples containing high levels of the substances of interest and CSF samples were injected directly onto the HPLC column. For determination of low concentrations, serum samples were subjected to a solid-phase extraction method for clean-up and concentration purposes. The determination limits were 10 ng/ml for both antifolates, 100 ng/ml for folinic acid, and 0.1 ng/ml for the physiologically occurring methylated folate which is about 1/100 the serum concentration in healthy children. The suitability of the method for pharmacokinetic monitoring of high-dose methotrexate therapy combined with leucovorin rescue administered to children with acute lymphoblastic leukemia was demonstrated. Minimum values of the serum folate during treatment ranged from 0.2 to 3.1 ng/ml. Even those very low concentrations could be reliably measured.

1. Introduction

The cytostatic drug methotrexate (MTX, amethopterin, 4-amino-10-methylpteroyl-L-glutamic acid, see Fig. 1) acts as an antimetabolite and is widely used in the therapy of human malignancies. As an analog of folic acid (pteroylglutamic acid, PteGlu) MTX inhibits the enzyme dihydrofolate reductase causing a lack of reduced folates which are donors of one-carbon units in the biosynthesis of nucleic acids and some amino acids. Like the folates, MTX is intracellularly converted to polyglutamates, which inhibit further enzymes of the folate metabolism, and thus take part in the cytostatic action of the drug [1,2].

MTX is metabolized in the liver to 7-hydroxymethotrexate (7-OH-MTX), which is about 200-fold less effective as inhibitor of dihydrofolate reductase than the parent compound [3]. On the

* Corresponding author. Present address see [1].

[1] Freie Universität Berlin, Universitätsklinikum Rudolf Virchow, Institut für Toxikologie und Embryopharmakologie, Garystrasse 5, 14195 Berlin, Germany.

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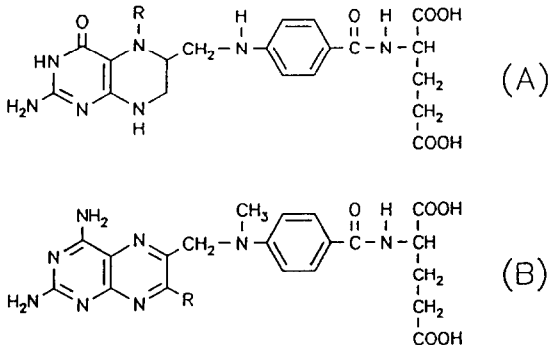

Fig. 1. Chemical structures of (A) 5-CH_3-H_4PteGlu (R = CH_3), 5-CHO-H_4PteGlu (R = CHO), (B) MTX (R = H), 7-OH-MTX (R = OH).

other hand, polyglutamates of the metabolite are highly cytotoxic [4,5].

High-dose MTX therapy must be combined with administration of reduced folates to prevent severe toxicity in the patients. For this purpose, leucovorin, the calcium salt of folinic acid ([6*R*,*S*]-5-formyltetrahydrofolic acid, 5-CHO-H_4PteGlu), is used. Its 6*S*-isomer is rapidly converted to [6*S*]-5-methyltetrahydrofolic acid (5-CH_3-H_4PteGlu) which is the transport form of the folates and occurs physiologically in serum and cerebrospinal fluid (CSF). The serum levels of healthy individuals are 5.8 ± 2.8 ng/ml for adults and 2–20 ng/ml for children (1–15 years), respectively [6–8]. The concentration in CSF is two to four times that of blood serum [6,9]. The 6*R*-isomer of 5-CHO-H_4PteGlu is eliminated in a non-metabolized form by the kidneys [10].

Although the pharmacokinetics of MTX has been extensively studied [5,11], neither the optimal MTX dose nor the best leucovorin regimen have yet been established [12]. With better knowledge about the metabolization of the drug and the behaviour of folates during MTX and leucovorin administration, we expect to get better insight in the mechanisms of MTX and rescue efficacy as well as of toxicity. For this purpose, a method for the simultaneous monitoring of MTX, 7-OH-MTX, 5-CHO-H_4PteGlu and 5-CH_3-H_4PteGlu serum levels was required.

In the literature a number of methods are described that either analyze MTX (HPLC [13–15], immunoassays [16,17]) or folates (HPLC [18–20], microbiological [21,22] or radiometric assays [23]). Only few methods allow the simultaneous determination of folates and antifolates [12,24–26]. However, all of them show disadvantages: either determination of 7-OH-MTX is not possible [25,26], or the detection of 5-CH_3-H_4PteGlu is not sensitive enough to measure the expected low serum concentrations during therapy and even physiological levels cannot be determined [12,24]. To overcome these problems a sensitive HPLC method was developed with ultraviolet and fluorescence detection following a sample clean-up and concentration procedure using solid-phase extraction (SPE).

2. Experimental

2.1. Chemicals and reagents

The calcium salt of [6*R*,*S*]-5-methyl-5,6,7,8-tetrahydrofolic acid was obtained from Merck (Darmstadt, Germany), leucovorin ([6*R*,*S*]-5-formyl-5,6,7,8-tetrahydrofolic acid calcium salt) and methotrexate were supplied by Lederle (Wolfratshausen, Germany). Acetonitrile came from Baker (Gross-Gerau, Germany) and was of HPLC quality. All other chemicals obtained from Merck were of analytical grade.

In order to obtain standardized conditions for blank and spiked samples we used a commercially available serum protein solution (Serumar, Armour Pharma, Eschwege, Germany). The chromatogram of Serumar showed no interference with the peaks of interest and 5-CH_3-H_4PteGlu could not be detected.

7-OH-MTX standard solutions were prepared in 0.05 *M* NaOH. For other standards and for sample solutions we used a buffer with pH 7.6 made of 0.1 *M* Tris-HCl with 1% L-ascorbic acid and flushed with nitrogen to remove the oxygen (in the following referred to as Tris-HCl buffer). Stored at −20°C in the dark the standard solution of 5-CH_3-H_4PteGlu remained stable for about 6 months; all other standard solutions were stable for a period of 2 years.

2.2. Preparation of 7-OH-MTX

The MTX metabolite 7-OH-MTX was prepared enzymatically by the method described by Cairnes and Evans [27], in which a rabbit liver homogenate was incubated with MTX. After 3 h the incubation mixture yielded 72% of 7-OH-MTX and 28% of MTX as measured by HPLC. The metabolite was subsequently recrystallized four times from acid solution and dried in a desiccator above phosphorus pentoxide. The resulting orange coloured powder contained 97% of 7-OH-MTX (as determined by HPLC).

2.3. Sample preparation

Blood samples were collected in non-heparinized tubes. After clotting at room temperature and centrifugation (5 min at 3000 *g*), the serum was separated and frozen until analysis. Samples of cerebrospinal fluid were taken by lumbar puncture and frozen. Samples were stored at −20°C for up to 7 days, thereafter at −80°C. No degradation was observed for spiked serum samples during a test period of 6 months. Before thawing, 5 mg of ascorbic acid was added per milliliter and the samples were thawed slowly at a temperature of about 4°C.

Storage and sample preparation were performed under protection from light. Serum samples containing more than 5 μg/ml of MTX or 7-OH-MTX were diluted five-fold with Tris-HCl buffer before direct injection. Other serum samples were cleaned up and concentrated with a solid-phase extraction method [12]. For this purpose two different buffers were used: Buffer A with a pH of 5.0 consisted of 0.1 *M* citric acid and 0.2 *M* disodium hydrogenphosphate, buffer B was a two-fold dilution of buffer A with distilled water. A 500-μl aliquot of each serum sample was mixed with 2.5 ml of buffer A and extracted on a C_{18} SPE column (200 mg Bond Elut, ICT, Frankfurt, Germany) that was pretreated with 5 ml of methanol and 3 ml of buffer B. After transferring the sample solution to the SPE column, the column was washed three times with 1 ml of buffer B, 0.5 ml of 20 m*M* NaOH and again with 0.5 ml of buffer B. The analytes were extracted with 1.0 ml of methanol. Then the methanol was evaporated at 60°C under a stream of nitrogen. The residue was redissolved in 100 μl of Tris-HCl buffer and the solution was mixed thoroughly. After membrane filtration (pore size 0.2 μm, Chromafil AO-20/3, Macherey-Nagel, Düren, Germany) the solutions were injected onto the HPLC column.

Cerebrospinal fluid samples were injected directly onto the HPLC column after membrane filtration.

2.4. High-performance liquid chromatography

The system consisted of a gradient pump (Model 480G, Gynkotek, Germering, Germany) with a degassing device (Shodex Degas, Showa Denko, Japan) and an automatic injector (GINA, Gynkotek). The injection volume was 20 μl. In order to obtain a better stability of the injected solutions the sample racks were cooled to 10°C using a cryostat (RMT 6, Lauda, Lauda-Königshofen, Germany). Two detectors were employed in series: a fluorescence spectrophotometer (F-1050, Merck/Hitachi, Darmstadt, Germany) with excitation and emission wavelengths of 295 and 355 nm, respectively, was used to detect low levels of 5-CH_3-H_4PteGlu (< 100 ng/ml in the injected solution). For the determination of MTX, 7-OH-MTX, folinic acid and high levels of 5-CH_3-H_4PteGlu, we used an ultraviolet detector (UVD 320, Gynkotek) operated at 310 nm. Data were processed by an interface (PE Nelson 900, Perkin Elmer, Überlingen, Germany) and a computing system. The separation was performed at room temperature on a Supelcosil LC-18-DB column (150 × 4.6 mm I.D., 3 μm), in connection with a precolumn (20 × 4.6 mm I.D.) packed with Supelcosil LC-18-DB, 5 μm. The mobile phase was a modification of that described by Hahn et al. [25]. It consisted of a gradient system using acetonitrile and phosphate buffer (5 m*M* KH_2PO_4, adjusted to pH 2.3 with phosphoric acid and filtered using a membrane with 0.45-μm pore size). The mobile phase components were mixed as mentioned in Table 1. The last step of the gradient was

Table 1
Mobile phase composition

Time (min)	Solvent A[a] (%)	Solvent B[a] (%)
0	7	93
5	7	93
20	13	87
26	21	79
27	21	79
29	7	93

[a] A = acetonitrile, B = 5 m*M* KH_2PO_4 buffer, pH 2.3.

added to reequilibrate the column with the initial mobile phase. The flow-rate was 0.9 ml/min.

2.5. Calibration and recovery

For quantification, peak area integration with the external standard method was used. To determine the linear range and the detection limits, standard solutions with various concentration levels were chromatographed. The resulting calibration curves were used to quantitate the directly injected samples. For calibration of the samples subjected to the SPE we used the following method: a series of serum protein solutions spiked with standard mixtures of different concentrations was analyzed according to the method described here with SPE and HPLC. The resulting curves were then used to calculate the sample contents. The recovery was calculated by comparing the peak areas of the standard solutions and the spiked serum protein solutions.

3. Results

3.1. High-performance liquid chromatography

Fig. 2 shows the fluorescence and ultraviolet chromatograms of a standard mixture. In the fluorescence detector signal 5-CH_3-H_4PteGlu and 5-CHO-H_4PteGlu are visible, while the ultraviolet chromatograms show MTX, 7-OH-MTX and 5-CHO-H_4PteGlu. Methylated folate can also be detected with the ultraviolet detector

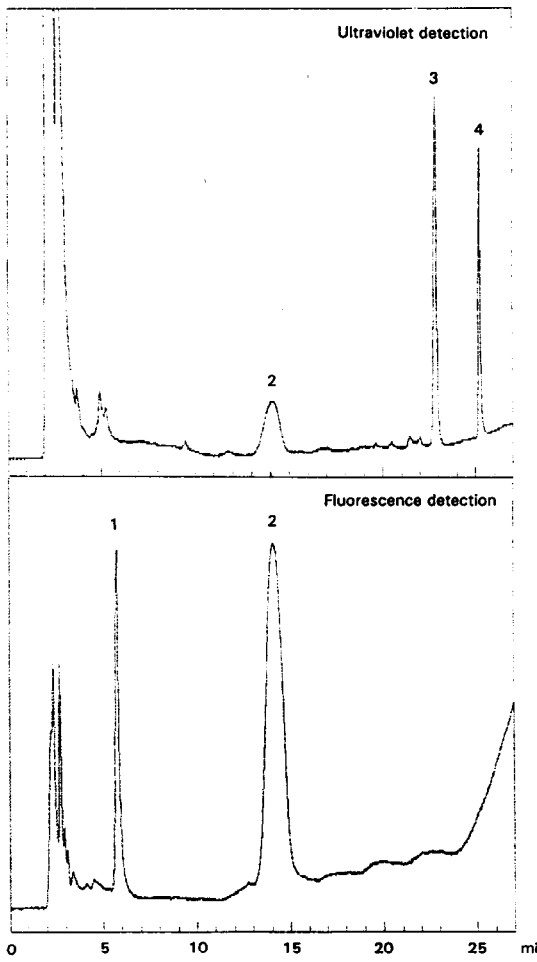

Fig. 2. Chromatograms of a standard mixture. Peak identification: 1 = 5-CH_3-H_4PteGlu, 2 = 5-CHO-H_4PteGlu, 3 = MTX, 4 = 7-OH-MTX. The absolute amounts injected were 0.4 ng of 5-CH_3-H_4PteGlu and 40 ng of each of the other substances. Chromatographic conditions see text.

when more than 2 ng are injected (see Fig. 4). HPLC of 5-CHO-H_4PteGlu gives an unusual broad but symmetric peak as can also be seen from chromatograms in other papers [12,19,24–26,28–30]. The separation is finished within 27 min and after an additional 5-min reequilibration time the next injection can be made.

The relative absorption maxima of the substances dissolved in mobile phase were at 295 nm for 5-CHO-H_4PteGlu and 5-CH_3-H_4PteGlu and at 310 nm for MTX and 7-OH-MTX, respectively. Because sensitive detection of the methylfo-

late was performed using the fluorescence signal and the antifolates were of greater relevance than the formylfolate (see Discussion), 310 nm was chosen as detection wavelength.

Chromatograms of two serum samples and a CSF sample are shown in Figs. 3, 4 and 5, respectively. With the described gradient system the four substances of interest, MTX, 7-OH-MTX, 5-CH_3-H_4PteGlu and 5-CHO-H_4PteGlu, are well separated from endogenous peaks, at least in one of the detector signals. Analyzing samples taken from acute lymphoblastic leukemia (ALL) patients at different time points during therapy, we observed some additional

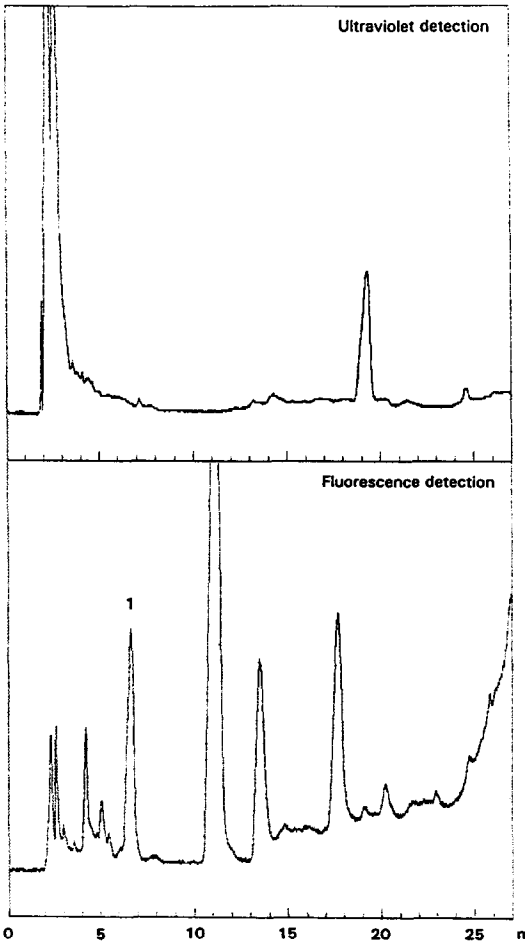

Fig. 3. Chromatograms of a serum sample from an ALL patient taken before start of therapy. Sample was analyzed using SPE. Chromatographic conditions see text, peak identification see Fig. 2. 5-CH_3-H_4PteGlu concentration was 6.1 ng/ml.

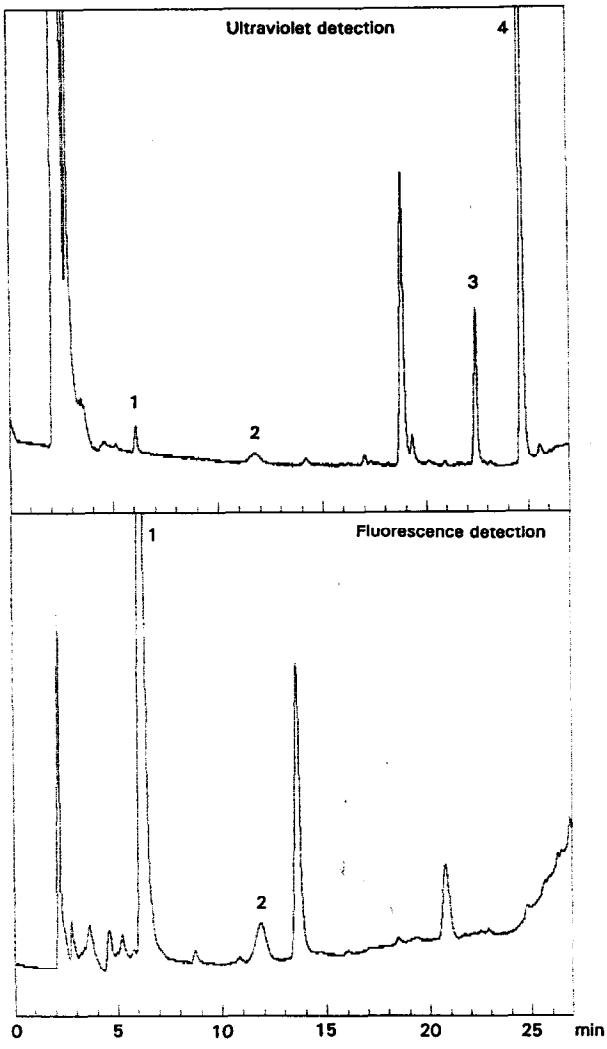

Fig. 4. Chromatograms of a serum sample from an ALL patient taken after MTX and leucovorin treatment. Sample was analyzed using SPE. Chromatographic conditions see text, peak identification see Fig. 2. Serum concentrations were 56.7 ng/ml of 5-CH_3-H_4PteGlu, 0.38 μg/ml of 5-CHO-H_4PteGlu, 0.19 μg/ml of MTX and 2.6 μg/ml of 7-OH-MTX.

peaks appearing in the chromatogram (not shown). That was why the chromatographic run-time could not be shortened without loss of separation. Since there was an unidentified peak with the same retention time as 5-CHO-H_4PteGlu, appearing in the fluorescence but not in the UV signal (see Figs. 3 and 5), we determined the formyl folate from the UV chromatogram.

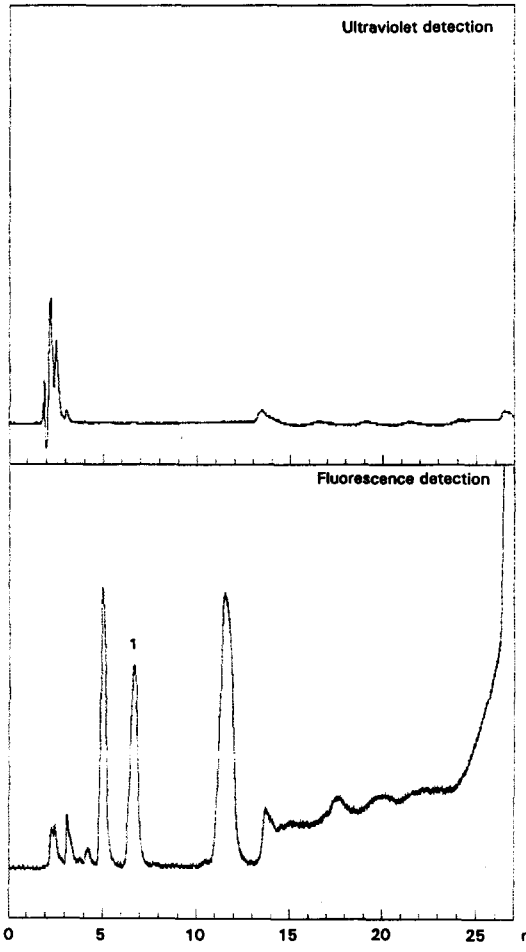

Fig. 5. Chromatograms of a CSF sample taken from an ALL patient before the intrathecal MTX application. Sample was directly injected onto the HPLC column. It contained 26.0 ng/ml of 5-CH_3-H_4PteGlu. Chromatographic conditions see text, peak identification see Fig. 2

3.2. Sample preparation

Because folates are labile substances, we performed recovery experiments with spiked serum protein solutions. It was shown that the thawing temperature influences the stability of 5-CH_3-H_4PteGlu and 5-CHO-H_4PteGlu. Maintaining a temperature of about 4°C while thawing the samples and addition of L-ascorbic acid were found to prevent loss of folates.

It is known that deproteinization of serum samples by heat treatment or trichloroacetic acid results in decomposition of the folates [19,30]. We observed that precipitation with perchloric acid even in the presence of ascorbic acid also caused high losses. Therefore, we used a SPE method to remove proteins and other interfering substances. Furthermore, there is also a concentrating effect. By using a 500-μl volume of serum or CSF and dissolving the residue of the SPE eluate in 100 μl of buffer we achieved a five-fold concentration.

At a temperature of 10°C in the dark, sufficient stability of the sample solutions during storage in the automated injector was achieved. Over a time period of 24 h we observed a loss of methyl-folate of ca. 5% and a less than 2% loss of the other substances.

3.3. Calibration and recovery

The curves of 5-CH_3-H_4PteGlu derived from analysis of the spiked serum protein solutions were linear over a concentration range of 0.1–100 ng/ml using fluorimetric detection and of 20–5000 ng/ml using the ultraviolet detector. 5-CHO-H_4PteGlu gave a linear range of 100–5000 ng/ml, MTX and 7-OH-MTX of 10-5000 ng/ml. The correlation coefficients were between 0.9942 and 0.9995 for MTX, 7-OH-MTX and 5-CH_3-H_4PteGlu, respectively; for 5-CHO-H_4PteGlu the correlation coefficient was 0.9889. The limits of determination are presented in Table 2. The precision of the sample preparation method was calculated by analyzing a spiked serum protein solution (1 μg/ml of each substance) five times in series on one day to get the intra-day variation (v_{id}) and on different days to get the day-to-day variation (v_{dd}), respectively. We found v_{id} values of 5% for 5-CH_3-H_4PteGlu and MTX, 7% for 7-OH-MTX and 23% for 5-CHO-H_4PteGlu. The v_{dd} values were 8% for 5-CH_3-H_4PteGlu, 2% for MTX and 7-OH-MTX, 34% for 5-CHO-H_4PteGlu.

The results of the recovery experiments are shown in Table 3. Using human serum we obtained similar results as with the commercial serum protein solution.

Table 2
Limits of determination (calculated with a signal-to-noise ratio of 5)

Compound	Directly injected aqueous standard solutions		Spiked serum protein solutions injected after sample preparation	
	ng	pmol	ng/ml	μmol/l
5-CH_3-H_4PteGlu (FL)[a]	0.01	0.02	0.1	0.0002
5-CH_3-H_4PteGlu (UV)[a]	2	4.4	20	0.04
5-CHO-H_4PteGlu	2	4.2	100	0.2
MTX	1	2.2	10	0.04
7-OH-MTX	1	2.1	10	0.04

[a] Detection method: ultraviolet (UV) or fluorescence (FL).

3.4. Analyzed samples

A total of 220 serum samples was analyzed. Serum was taken from five children with ALL, each treated with four successive cycles consisting of high-dose MTX combined with leucovorin rescue. The observed concentration ranges are listed in Table 4. Before the start of the therapy the concentration of 5-CH_3-H_4PteGlu was between 2.2 ng/ml and 25.6 ng/ml (median 10.1 ng/ml). During the first phase of each therapy cycle we observed a decrease to 0.2–3.1 ng/ml (median 1.1 ng/ml).

We also analyzed 14 CSF samples from ALL patients that were taken before the first MTX dose was applied. In all cases an enrichment step was not necessary to determine 5-CH_3-H_4PteGlu because its concentration was high enough for the direct injection method. The levels ranged from 8.0 to 25.8 ng/ml (median 13.1 ng/ml).

4. Discussion

In our laboratory some already existing HPLC methods [12,24–26] were tested. Using the method of Giulidori et al. [26] we could not achieve satisfactory separation of 5-CHO-H_4PteGlu and 5-CH_3-H_4PteGlu. In two cases [12,24] the sensitivity of the 5-CH_3-H_4PteGlu detection (28 and 0.4 ng per injection, respectively) was not sufficient for our purposes. Hahn et al. [25] attained a sensitive determination of 5-CH_3-H_4PteGlu (0.007 ng per injection) using fluorescence detection. For the simultaneous detection of MTX,

Table 3
Recoveries

Compound	Recovery (%)				
	0.1 ng/ml[a]	1 ng/ml[a]	10 ng/ml[a]	100 ng/ml[a]	1000 ng/ml[a]
5-CH_3-H_4PteGlu	94	95	95	96	–[b]
5-CHO-H_4PteGlu	–	–	–	50	47
MTX	–	–	100	103	102
7-OH-MTX	–	–	93	98	94

[a] Concentration level in serum.
[b] – = Not determined.

Table 4
Concentration range of serum samples taken from children with ALL during treatment

Compound	Concentration range
5-CH_3-H_4PteGlu	0.2–230 ng/ml
5-CHO-H_4PteGlu	0.2–13.7 μg/ml
MTX	0.01–59.4 μg/ml
7-OH-MTX	0.15–54.4 μg/ml

which they used as internal standard, an on-line oxidation procedure was necessary, which however complicated the method. Furthermore, 7-OH-MTX was not analyzed, as is also the case in the paper published by Giulidori et al. [26].

Our method allows the simultaneous determination of MTX, 7-OH-MTX and folinic acid as well as of very low levels of the serum folate 5-CH_3-H_4PteGlu (limit 0.02 ng per injection). This was achieved using HPLC with fluorimetric and ultraviolet detection. In combination with the SPE concentration step, we attained a limit of determination of 0.1 ng/ml for the methyl compound, which is about 1/100 the normal serum concentration in healthy children [7,8].

The lability of the substances and in particular of 5-CH_3-H_4PteGlu is a problem during handling and preparation of the samples. As oxidation of the folates is the main problem, we used L-ascorbic acid as an antioxidant. Several authors used heat treatment [26] or precipitation with perchloric acid [20,24] as deproteinization methods, but we observed high losses of the folates with these procedures, even in the presence of ascorbic acid. Therefore we used SPE to remove proteins, lipids and inorganic ions from the samples. With this mild procedure together with the addition of ascorbic acid to the samples, handling them in the darkened laboratory and thawing them slowly at 4°C, we achieved good stability of the analytes. SPE also results in enrichment of the analytes. The five-fold enrichment we achieved was found to be sufficient for the samples analyzed. However, if necessary a ten-fold concentration effect can be obtained, by subjecting 1 ml of sample to the SPE.

To obtain standardized conditions for the recovery experiments and the calibration curves, we used Serumar, a commercially available serum protein solution. This artificial matrix contains all proteins and ions of human serum [31] but it is folate-free as proved by HPLC analysis. Since recovery experiments with both real serum and serum protein solution showed similar results, Serumar was used in this study. Another advantage is the possibility to calibrate 5-CH_3-H_4PteGlu in the lower concentration range.

With 5-CH_3-H_4PteGlu, MTX and 7-OH-MTX we achieved good recovery values with the SPE procedure at different levels, as can be seen from Table 3. However, 5-CHO-H_4PteGlu showed a poor recovery (about 50%). Because this 5-formyl compound is known to be the most stable folate [6,32], decomposition is an unlikely cause of the high loss. We suspect that on the SPE column, the substance has a broad elution profile, similar to that seen on the HPLC column (see Fig. 2), and a considerable amount passes the SPE column before the final elution step. Experiments with some modifications of the SPE method did not lead to an improvement. Probably a larger amount of SPE material (e.g. 500 mg) or another kind of solid phase, e.g. C_8 or phenyl instead of C_{18}, would give better results. To overcome the problem, we calculated the processed samples using the curve resulting from the analysis of spiked serum protein solutions. Although the recovery of 5-CHO-H_4PteGlu is low we obtained satisfactory linearity of the calibration curve, as indicated by a correlation coefficient of 0.9889. But due to the poor recovery a relatively high limit of determination (100 ng/ml) compared to the other compounds and a high variation during sample preparation (v_{id} = 23% and v_{dd} = 34%) were found.

It has to be emphasized that the described HPLC method does not distinguish between the 6*R*- and 6*S*-isomers of the tetrahydrofolates. Therapeutically, leucovorin is administered as a mixture of the stereoisomers of 5-CHO-H_4PteGlu. However, only the 6*S*-isomer is rapidly converted to [6*S*]-5-CH_3-H_4PteGlu. Therefore, the obtained values for the formyl compound represent mainly the 6*R*-isomer which is of minor interest for clinical purposes because

it has no biological effect. For therapeutic monitoring, 5-CH_3-H_4PteGlu levels are much more relevant since they reflect the extent of metabolization of the administered [6*S*]-5-CHO-H_4PteGlu. Therefore, the less exact quantification of the formyl folate is acceptable.

In our laboratory the described method was used for pharmacokinetic analysis. One aim of the study was to investigate the influence of MTX therapy on the serum folate content. The measured 5-CH_3-H_4PteGlu levels before start of the therapy (2.2–25.6 ng/ml) are in good agreement with data published for healthy children [7,8]. During the infusion of MTX we observed a decrease to about 10% of the initial level. Even those very low serum concentrations (0.2–3.1 ng/ml) could be reliably measured.

Clinical results of the pharmacokinetic study will be published elsewhere [33].

The method also allowed the analysis of CSF samples. The 14 tested samples were injected directly onto the HPLC column because of their high content. In order to determine low 5-CH_3-H_4PteGlu levels in CSF, the samples can also be subjected to the described sample preparation method.

5. Conclusions

The described method is capable of measuring simultaneously MTX, 7-OH-MTX, 5-CH_3-H_4PteGlu and 5-CHO-H_4PteGlu, substances which are important during high-dose MTX therapy for various malignant diseases. Although there are some problems in determining 5-CHO-H_4PteGlu, the technique is a reliable and valuable tool for therapeutic monitoring and will contribute to more insight in MTX and folate metabolism and to early detection of clinical toxicity.

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