Methotrexate-induced biochemical alterations of the folate and methyl-transfer pathway in the CNS

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List of abbreviations

a	Intercept
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AICART	5-aminoimidazole-4-carboxamide ribonucleotide transformylase
ALL	Acute lymphoblastic leukaemia
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
b	Slope
BHMT	Betaine-homocysteine methyltransferase
C	Concentration
C1	Day 1 of consolidation
C15	Day 15 of consolidation
CA	Cysteic acid
CAA	Chloroacetaldehyde
Ca-folinate	Calcium folinate
CBS	Cystathionine-ß-synthase
CI	Cranial irradiation
CNS	Central nervous system
CPA	Corrected peak area
CSA	Cysteine sulphinic acid
CSF	Cerebrospinal fluid
CT	Computed tomography
CTC	Common toxicity criteria

C x T	Concentration x time
CV	Coefficient of variation
DHB	Dihydrobiopterin
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DHPR	Dihydropteridine reductase
dTMP	Thymidine monophosphate
dUMP	Uridine monophosphate
ECD	Electrochemical detection
EDD	Equal daily doses
F	Female
FD	Fluorescence detection
FDA	Food and Drug Administration
FGAR	Formyl-glycineamide ribonucleotide
FICAR	5-formamidoimidazole-4-carboxamide ribonucleotide
GAR	Glycineamide ribonucleotide
GART	Glycineamide ribonucleotide transformylase
GTP	Guanosine triphosphate
HCA	Homocysteic acid
HCSA	Homocysteine sulphinic acid
Hcy	Homocysteine
HIAA	5-hydroxyindolacetic acid
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HR	High-risk

HVA	Homovanillic acid
i.cv.	Intracerebroventricular
i.m.	Intramuscular
IMP	Inosine monophosphate
i.th.	Intrathecal
i.v.	Intravenous
IU	International units
LLOQ	Lower limit of quantification
LOD	Limit of detection
LR	Low-risk
max	Maximum
Met	Methionine
5,10-MeTHF	5,10-methylenetetrahydrofolate
MLL	Mixed lineage leukaemia
MR	Middle-risk
MRI	Magnetic resonance imaging
MRD	Minimal residual disease
MS-MS	Tandem mass spectrometry
5-MTHF	5-methyltetrahydrofolate
MTHFR	5,10-methylenetetrahydrofolate reductase
MTX	Methotrexate
MTX-PG	Methotrexate polyglutamates
n.d.	Not detected
NDA	Naphtalene dialdehyde
n.k.	Not known

NMDA	N-methyl-D-aspartate
n.q.	Not quantified
5-OH-Trp	5-hydroxytryptophan
PCNSL	Primary central nervous system lymphoma
Phe	Phenylalanine
PheH	Phenylalanine hydroxylase
P_i	Inorganic phosphate
p.i.	Per infusionem
p.o.	Per os
PRPP	5-phosphoribosyl-1-pyrophosphate
QC	Quality controls
qDHB	Quinoide dihydrobiopterin
r	Coefficient of correlation
R	Recovery
RE	Relative error
R/I	Remission/induction
RSD	Relative standard deviation
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SD	Standard deviation
SR	Standard-risk
THB	Tetrahydrobiopterin
THF	Tetrahydrofolate
TIT	Triple intrathecal therapy
Trp	Tryptophan

TrpH	Tryptophan hydroxylase
TS	Thymidilate synthase
Tyr	Tyrosine
TyrH	Tyrosine hydroxylase
ULOQ	Upper limit of quantification
UV	Ultraviolet
WBC	White blood cell count
$\bar{x}$	Mean value
$\tilde{x}$	Median value

1 INTRODUCTION

1.1 Methotrexate

Methotrexate (MTX, amethopterin) was introduced as a folate antagonist into cancer therapy more than 50 years ago. Since then it has gained widespread use also in the treatment of nonmalignant disorders such as rheumathoid arthritis, psoriasis, asthma and graft-versus-host disease where low doses (5-25 mg/week) have been shown to be beneficial. In the therapy of malignant diseases it is the most widely used antifolate. In conventional doses (< 100 mg/m^2) it still plays an important role in the treatment of breast cancer, choriocarcinoma, squamous cell tumours of the head and neck and bladder cancer. However, progress in the treatment of acute lymphoblastic leukaemia (ALL), lymphoma, medulloblastoma and osteosarcoma was achieved after the introduction of high doses (1 - 33.6 g/m^2) of MTX followed by calcium folinate rescue. Because of the efficacy of MTX, a central nervous system (CNS) directed administration was introduced for prevention of overt ALL relapses. Since then the intrathecal (i.th.) administration of low doses (8-12 mg) of MTX plays an important role in ALL and lymphoma protocols.

1.1.1 Chemical structure

MTX is a folate analogue and consists of a pteridine ring, p-amino benzoic acid and glutamic acid. The pteridine ring and the p-amino benzoic acid together form the pteroic acid. The difference in chemical structure between MTX and the naturally occuring folic acid is the presence of the amino group in position 4 and the methylation of nitrogen in position 10 of the pteroyl rest (see Fig. 1.1).

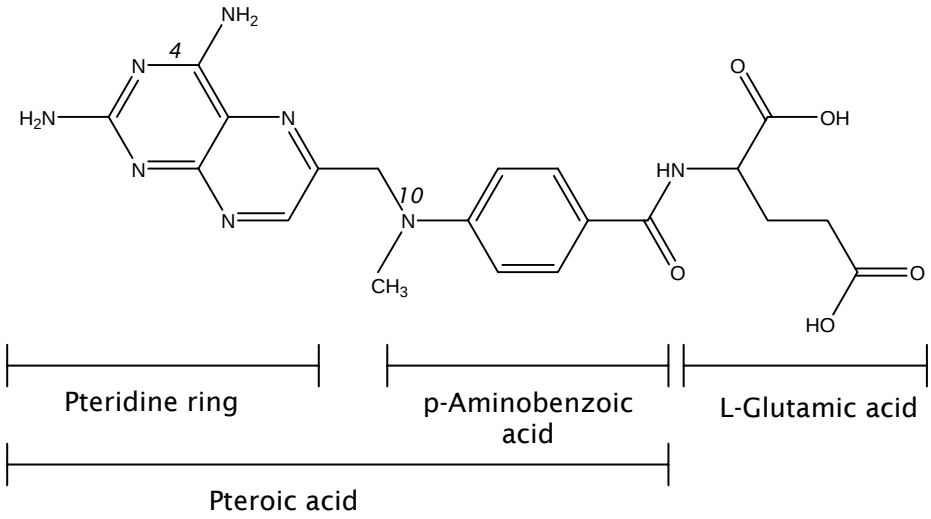

Fig. 1.1: Chemical structure of MTX (4-amino-N^{10}-methyl-pteroylglutamic acid).

1.1.2 Biochemical mechanisms of methotrexate activity

Since MTX is a folate analogue its primary target is the cellular folate pathway. The entrance into a mammalian cell occurs via at least two different transport systems. The first one is the reduced folate carrier which has high affinity for reduced folates and MTX whereas the other utilises a folate receptor which has higher affinity for folic acid compared to reduced folates[1]. At high extracellular concentrations MTX may enter the cell by passive diffusion[2].

In the cell, MTX is, in analogy with cell folates, partly polyglutamated by the enzyme folylpolyglutamate synthetase[3] which adds up to six or seven glutamic acid residues. Both mono- and polyglutamated MTX inhibit the enzyme dihydrofolate reductase (DHFR) with the same affinity[4]. However, MTX-polyglutamates remain bound to the enzyme for a longer period of time[5] and are therefore retained in the cell. As a consequence of the inhibition of DHFR the conversion of dihydrofolates (DHF) into tetrahydrofolates (THF) is disabled leading to a depletion of reduced folates (see Fig. 1.2). In that way MTX indirectly affects the pyrimidine and purine

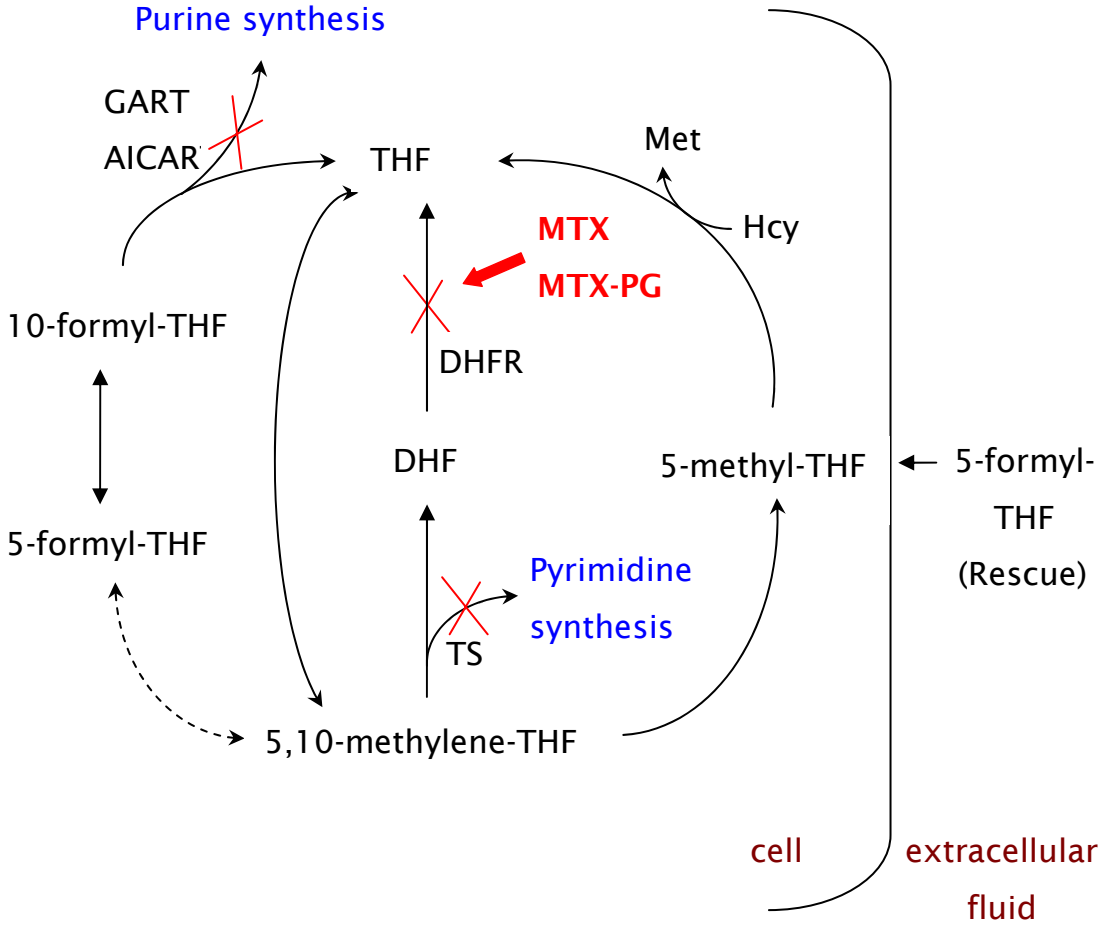

Fig. 1.2: Intracellular folate metabolism.

Abbreviations: AICART: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; DHF: dihydrofolate; DHFR: dihydrofolate reductase; GART: glycineamide ribonucleotide transformylase; Hcy: homocysteine; Met: methionine; MTX-PG: methotrexate polyglutamate; THF: tetrahydrofolate; TS: thymidilate synthase.

Red crosses indicate targets of MTX and MTX-PG whereas the interrupted arrow indicates indirect conversion.

synthesis since 5,10-methylene-THF is required for the thymidylate synthesis while 10-formyl-THF is important for the purine synthesis. Furthermore, MTX indirectly interferes with other metabolic pathways since 5-methyl-THF, the most important transport form of reduced folates, is also the methyl donor for the conversion of homocysteine to methionine. In conjuction with the depletion of reduced folates, dihydrofolates accumulate and are polyglutamated to a higher extent.

MTX polyglutamates and DHF polyglutamates directly affect the pyrimidine synthesis by inhibiting the enzyme thymidilate synthase[6], which is responsible for the conversion of uridine monophosphate (dUMP) to thymidine monophosphate (dTMP). Furthermore, the two enzymes of the purine synthesis glycineamide ribonucleotide (GAR) transformylase and 5-aminoimidazole-carboxamide ribonucleotide (AICAR) transformylase are inactivated by polyglutamates of MTX and DHF[7] (see Fig. 1.2).

1.1.3 High-dose administration of methotrexate

Since its introduction into clinical practice MTX has shown efficacy in the treatment of childhood ALL. However, therapy with conventional doses revealed to be unsuccessful in patients with CNS disease. Therefore, the administration of high-dose MTX (≥ 1 g/m^2) was considered. The theoretical background for this approach was the postulation that more MTX would pass across the blood-brain barrier into the CNS and thus improve therapeutic outcome.

However, the limiting factor for the administration of high doses was toxicity. A breakthrough occured with the pioneering work of Goldin et al.[8] who showed that delayed administration of calcium folinate could rescue nonmalignant cells from MTX toxicity without diminishing the cytotoxic effect of the drug on tumour cells. After that, it was possible to investigate the outcome of elevated doses of MTX in the treatment of ALL.

The aim of high-dose therapy was to provide a prolonged CSF exposure to the MTX concentration of 1 µM[9] which was determined to be cytotoxic in

vitro[10]. High-dose MTX was administered as a continuous infusion often preceeded by a loading dose.

It had already been shown that after doses of 500 mg/m^2 only a low cytotoxic concentration of MTX (0.1 µM) was achieved in the CSF[11]. Therefore, further attempts were made with more intensive dose regimens including 1-33.6 g/m^2. Balis et al.[12] reported concentrations of 10 µM in the CSF after an infusion of 33.6 g/m^2, while Pitman et al.[13] showed that the cytotoxic concentration of 1 µM was achieved after 3-7.5 g/m^2. The introduction of high-dose MTX was accompanied with a notable decrease of systemic relapse rate in children with ALL compared to conventional doses[14]. Furthermore, Evans et al.[15] showed that a relationship existed between the obtained steady-state serum concentration of MTX and the probability of remaining in remission. A serum concentration ≥ 16 µM was associated with a decreased relapse rate. However, the steady-state concentration obtained after 1 g/m^2 was very variable (9.3-25.4 µM) and therefore the optimal dose of MTX in the therapy of childhood ALL still remains to be established.

A benefit of high-dose MTX therapy followed by calcium folinate rescue was also shown in patients with primary central nervous system lymphoma (PCNSL). This is a rare but highly agressive non-Hodgkin's lymphoma which often involves the leptomeninges, the eye and rarely the spinal cord[16]. Initially, PCNSL patients where treated with whole-brain irradiation and the median survival was 12-18 months with a 5-year survival rate of less than 5%. When high-dose MTX-based regimens were applied prior to radiotherapy the median survival increased to 60 months. However, such protocols were associated with considerable long-term neurological complications especially in elderly patients (> 65 years)[17]. In an attempt to reduce the toxicity preserving the efficacy of treatment, a polychemotherapy regimen alone based on high-dose infusions and intraventricular administration of MTX was proposed[18]. It was shown that response rates and duration after polychemotherapy were comparable to polychemotherapy combined with cranial irradiation (CI). Magnetic resonance imaging (MRI) revealed white matter changes in one third of the treated patient population but clinical

symptoms were not present. Altogether, there was less neurotoxicity in the polychemotherapy group since there was no substantial decline of cognitive function in patients who did not receive cranial irradiation.

Initially, the administration of high-dose MTX in the therapy of malignant diseases was associated with considerable toxicity as well as drug-related deaths attributed mainly to severe myelosuppression and renal failure[19]. However, today high-dose MTX is considered to be generally well-tolerated when accompanied by an adequate hydratation, alkalisation, avoidance of drug interactions, drainage of the third space fluids (when present), pharmacodynamic monitoring and administration of an appropriate dosage of folinate rescue therapy[20].

Rescue mechanisms after high-dose methotrexate administration

The role of rescue therapy which follows high-dose MTX administration is to minimise or even prevent the systemic toxicity of the drug while preserving its cytotoxic efficacy in tumour cells. Since MTX is an antifolate, folates were considered to be appropriate as antidotes. Several agents have been suggested for this role but the most widely used is the calcium salt of folinic acid (N^5-formyltetrahydrofolic acid, Leucovorin®) due to its chemical stability (see Fig. 1.3).

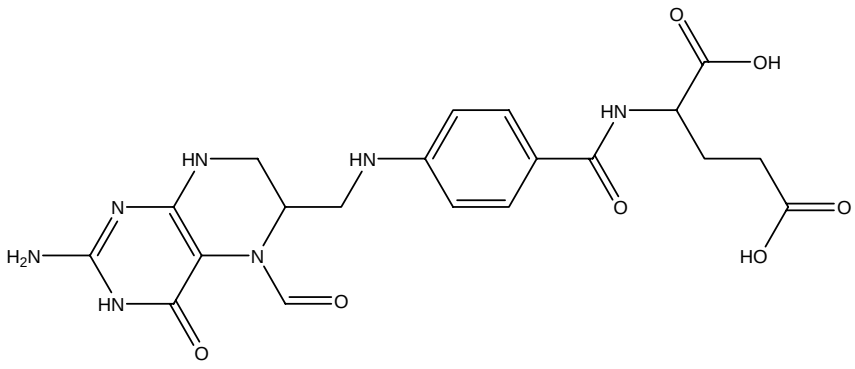

Fig. 1.3: Chemical structure of N^5-formyltetrahydrofolic acid.

The exact mechanism by which calcium folinate rescues normal cells from toxicity is not well understood. However, several hypotheses have been suggested: (a) Calcium folinate is easiliy converted into other reduced folates thus restoring the intracellular pool of tetrahydrofolates and thereby circumventing the MTX-induced inhibition of folate metabolism (see Fig. 1.2). (b) As a reduced folate calcium folinate is transported into the cell via the reduced folate carrier and can thereby competitively inhibit the entrance of MTX. (c) By competing at the level of folylpolyglutamate synthetase it could decrease the polyglutamation of MTX. (d) A direct competition and displacement of MTX at the level of DHFR has also been suggested[21]. However, the inhibition of DHFR is sustained in the presence of MTX polyglutamates[22].

The efficacy of calcium folinate rescue is influenced by its administered dose, the extracellular concentration of MTX and the duration of exposure to cytotoxic concentrations of the drug[21]. Since elevated plasma concentrations of MTX require an intensified rescue it is common clinical practice to monitor plasma levels of MTX after high-dose infusion and adjust the dosage of calcium folinate if necessary. Another important aspect is the time of rescue initiation. A delayed rescue has been associated with irreversible toxicity[23] whereas an early administration may rescue tumour cells as well. It has been suggested that starting rescue 36-42 h after the beginning of the high-dose MTX infusion would provide a sufficient exposure to MTX with minimal toxicity[23, 24].

1.1.4 Intrathecal administration of methotrexate

Although high-dose MTX therapy followed by rescue is well tolerated there is an intensive systemic exposure to the drug. Therefore, an attempt to circumvent the blood-brain barrier in order to avoid systemic drug exposure was made by intrathecal administration of MTX. Since this is a form of local chemotherapy, high concentrations of the drug in the CSF can be obtained with relatively low doses.

Initially, the dose of intrathecally administered MTX was based on body surface area similarly to high-dose MTX. However, Bleyer et al.[25] proposed an age-related dosage regimen based on the results obtained in pharmacokinetic studies. They showed that a fixed dose provided significantly less variability in the CSF concentration of MTX. This could be explained by a more rapid enlargement of the CSF volume in children (where intrathecal MTX is distributed) compared to the body surface area. Since children at the age of 3 years reach the CSF volume of an adult, the body surface area did not correlate with the volume in which MTX was distributed.
After intrathecal administration of low doses (6-12 mg) the concentration of MTX in the CSF is 100 times higher compared to plasma. Although low doses are injected intrathecally the drug is released slowly from the CSF and therefore a prolonged systemic exposure to the drug was observed when compared to an equivalent dose administered intravenously[26]. Nevertheless, compared to a high-dose infusion, intrathecal administration provided a reduced systemic exposure to the drug and considerably less systemic toxicity[27].
The introduction of the intrathecal administration of MTX was a major advance in the treatment of ALL patients with meningeal relapse. This led to a prophylactic or adjuvant use of intrathecally administered MTX in all ALL patients. In combination with cranial irradiation the use of intrathecal MTX reduced the incidence of meningeal relapse to less than 10%[28, 29].

Lumbar puncture

The common mode of intrathecal administration of cytotoxic drugs is lumbar puncture. There are several disadvantages of this administration method. First, a leakage out of the subarachnoid space (where CSF flows) into the surrounding subdural or epidural space is possible[30]. Second, because of the slow unidirectional flow of CSF the distribution within the CNS is limited. It was shown that only 10% of the concentration of MTX in the lumbar CSF was simultaneously present in the ventricle[26]. Third, due to the vascular uptake of the drug, the penetration into the brain parenchyma is poor[27, 31]. A potentially lower risk of leakage as well as an improvement of the distribution of the

drug to the ventricles can be obtained if the patient is kept in a prone or supine position for at least 30 min after the lumbar puncture, a procedure which is nowadays common practice[9].

The lumbar puncture is a painful procedure which is another disadvantage especially in the treatment of children or if frequent intrathecal administration is necessary.

Ommaya reservoir

In order to overcome the problems associated with the lumbar injection a direct intraventricular administration of the drug was introduced with the use of an Ommaya reservoir. The access to the CSF is provided through a surgical placement of a catheter into the lateral ventricle. The other end of the catheter is attached to a subcutaneously implanted reservoir[32] (see Fig. 1.4).

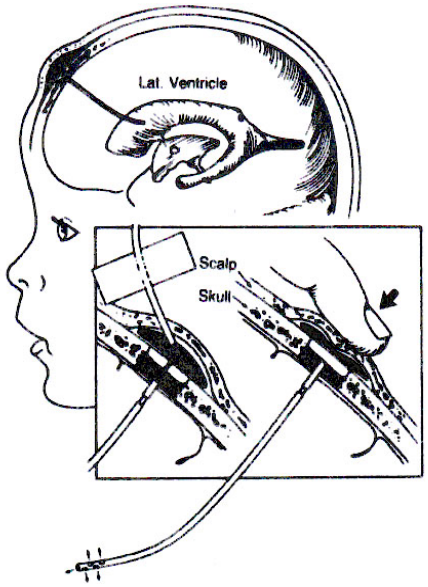

Fig. 1.4: Diagram of an Ommaya reservoir attached to a catheter[9].

The advantage of an intraventricular administration consists in a better delivery into the CSF as well as a better distribution of the drug throughout the CSF compartment[26]. It was also shown that children with recurrent CNS ALL who received intraventricular MTX had a reduced CNS relapse rate[33] and an prolonged duration of CNS remissions compared to intralumbar administration[34]. Moreover, higher CSF MTX concentrations were reported

and a higher inter-patient consistency was achieved after intraventricular administration compared to an equivalent intralumbar dose[11,26]. Nevertheless, Strother et al.[35] reported a considerable variability in the CSF MTX concentration both interindividually and intraindividually (in patients receiving several courses) after intraventricular drug administration. A possible explanation for this observation is the presence of CNS disease which may affect the CSF flow[36] and induce sustained CSF MTX concentrations[37-39].

Since the administration of a drug via Ommaya is less painful for the patient this approach is suitable for frequent CNS-directed therapy. Bleyer et al.[40] showed that more frequent administration of lower doses (6x1 mg every 12 h) induced a longer exposure to cytotoxic concentrations of MTX in the CSF. The total drug dose could be reduced. This so called C x T (concentration x time) approach was shown to be as effective in the treatment of meningeal ALL but less neurotoxic than a single-dose administration (12 mg/m^2).

The disadvantage of an Ommaya reservoir implantation is the need for a surgical treatment. Also high infection rates and a displacement of the catheter were observed.

1.2 Neurotoxicity of methotrexate

With the recognition that high-dose and intrathecal MTX play essential roles in the treatment of ALL, protocols have been developed in which the dose and frequency of the administration of MTX were more and more intensified. This further led to an increase of incidence of neurological complications in childhood ALL survivors. The severity of neurotoxicity varied from a transient and reversible form up to lasting damage leading to coma or even death. Similar observations were made in other patient populations who received intensified MTX regimens for the treatment of osteosarcoma or PCNSL.

The common classification of MTX-related neurotoxicity is related to the time of manifestation after therapy. Three forms are distinguished: acute, subacute and chronic neurotoxicity[41-45].

1.2.1 Acute neurotoxicity

High-dose MTX is associated with acute neurotoxicity which may occur during or within hours after the infusion. It is characterised by acute somnolence, confusion, fatigue, disorientation or seizures[41]. Elevated intracranial pressure has also been observed[41]. Acute episodes of cerebral oedema have been reported in patients with CNS disease. However, it is possible that this toxicity is at least partly mediated by the lysis of tumour cells in the CNS since it does not occur in patients without overt disease[41].

Another form of neurological morbidity, the so called acute arachnoiditis (chemical arachnoiditis, acute toxic syndrome), was described after the intralumbar or intraventricular administration of MTX[41]. This syndrome occurs in 5-40% of patients[41] usually within 2-4 h of the injection and lasts for 12-72 h[46]. The most common symptoms are headache, nausea, vomiting, fever, back pain, meningismus (pseudomeningitis) and dizziness[41]. Pathologically, pleocytosis (increased number of cells) and increased protein levels in the CSF, arachnoiditis and high intracranial pressure were observed[41, 47, 48]. Rarely this syndrome can present in a more dramatic form with massive polymorphonuclear pleocytosis in the CSF and high fever resembling bacterial meningitis[47-49]. The acute toxic syndrome is thought to be related to the dose and frequency of MTX administered as well as to the peak MTX levels in the CSF[25, 37, 50].

Although the acute form of MTX-related toxicity is usually reversible a fatal acute encephalomyelitis (inflammation of the brain and spinal cord) after a single intrathecal administration of MTX was reported[51].

1.2.2 Subacute neurotoxicity

After the administration of high-dose MTX a stroke-like syndrome was described which occured about one to two weeks after the infusion[45, 52-54]. Allen and Rosen[53] observed this toxicity in a total of eight patients after the first, second or third course of high-dose MTX. All patients developed hemiparesis (partial paralysis of one body-half). Seizures, speech disorders, cranial nerve and gaze palsies and decreased conciousness were also reported. In two patients hemiparesis migrated from one side to the other. All patients recovered spontaneously after 48-72 h and after subsequent MTX courses this syndrome did not reoccur. Similar neurological complications occurred in 4% of patients who received 8-9 g/m^2 of MTX for a variety of malignancies[45] and in 15% of patients who received 12.5 g/m^2 of MTX in the treatment of osteosarcoma[43].

Transient hemiparesis and speech disorder were also reported as a complication after intrathecal administration of MTX in one patient. Furthermore, this patient had focal cortical injuries which resembled an ischemic or stroke-like insult[54].

Intrathecal administration of MTX was also associated with a severe myelopathy followed by symptoms like pain in the legs, sensory changes, paraplegia (paralysis of both legs) and bladder dysfunction. The onset of symptoms occurred 30 min to 1-2 weeks after drug administration. Although partial or complete recovery was observed the disorder may have sequelae[42, 47, 55, 56]. Highly intensive short treatment sequences[41, 56], a prolonged CSF exposure of MTX[43] or long-term cumulative treatment[56] were considered to be risk factors for this form of neurotoxicity.

1.2.3 Chronic neurotoxicity

Chronic neurotoxicity may develop months to years following MTX therapy.

A transient leukoencephalopathy (pathologic alteration of the cerebral white matter) characterised by multifocal cerebral disturbances without signs of

dementia followed by a regained normal neurological status was reported after high-dose and intrathecal MTX[57, 58].

A severe form of leukoencephalopathy has also been associated with intensified high-dose MTX regimens[57, 59]. Allen et al.[57] observed this syndrome in seven patients several months after the initiation of therapy with 8-15 g/m^2 MTX. Patients presented first with subtle personality changes followed by progressive dementia, focal seizures, spastic quadriparesis and stupor. Only a partial recovery was seen in all patients after cessation of MTX therapy. Leukoencephalopathy was also seen in the computed tomographic scan (CT) of these patients as a hypodensity of the white matter. Demyelination (damage or destruction of the myelin sheath of neurons) and loss of cerebral parenchyma was manifested but no intracerebral calcifications were observed which are common after the combination of MTX therapy and cranial irradiation[47, 60-63]. High levels of MTX in the CSF were associated with this syndrome with an incidence of about 2%[57].

Severe leukoencephalopathy has been observed after intrathecal administration of MTX as well[64-66]. Symptoms varying from disorientation, lethargy, weakness to more severe such as personality changes, speech and movement disorders, hemiparesis, seizures and coma were described. In some cases the disease was progredient and fatal whereas other patients recovered partially or fully[64, 66, 67]. This severe form of leukoencephalopathy occurs rarely when only intrathecal MTX is used in the CNS disease prophylaxis[41]. However, when cranial irradiation is added to a high-dose and intrathecal MTX therapy the incidence is estimated up to 45%[41, 47, 65]. An overview of the symptoms of MTX-induced neurotoxicity is presented in Table 1.1.

Besides leukoencephalopathy, chronic toxicity after CNS prophylaxis may be associated with significant neuropsychological dysfunctions such as learning disability, cognitive disturbances and decrease in intelligence. Although such sequelae were observed especially in the combined MTX and cranial irradiation treatment[68-71], various authors showed that patients treated with

Table 1.1: Forms and symptoms of MTX-related neurotoxicity.

Neurotoxicity	Symptoms
Acute	High-dose MTX: Somnolence, confusion, fatigue, disorientation, seizures Intrathecal MTX: Chemical arachnoiditis: headache, nausea, vomiting, fever, back pain, dizziness
Subacute	High-dose and/or intrathecal MTX: Encephalopathy characterised by hemiparesis, speech and movement disorders, seizures, confusion, affective disturbances Intrathecal MTX: Myelopathy characterised by pain in the legs, sensory changes, paraplegia, bladder dysfunction
Chronic	High-dose and/or intrathecal MTX: Leukoencephalopathy characterised by confusion, somnolence or irritability, seizures, dementia, quadriparesis, visual disturbances, speech and movement disorders, coma, death Learning disability, cognitive disturbances, decrease in intelligence

MTX without radiation therapy may also develop learning disability and a decrease in intelligence quotient[72-74].

1.2.4 Possible mechanisms of neurotoxicity

Several hypotheses for the mechanism of induction of MTX-related neurotoxicity have been postulated. Some authors suggest a direct toxic effect of MTX on the CNS since experiments in animals or cell cultures showed that the drug may cause a direct axonal injury and affect the astrocytes[75-78].

Other investigators proposed that MTX-induced biochemical alterations may be responsible for the development of neurological complications[44]. Since MTX as a folate analogue impairs the folate cycle which is further related to

several other metabolic pathways there is a potential for possible indirect mediation of neurotoxicity. A detailed overview of the biochemical alterations induced by MTX is presented in this chapter.

Adenosine metabolism

Polyglutamated MTX and DHF inhibit the enzymes of the purine synthesis leading to an accumulation of glycineamide ribonucleotide (GAR) and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR). Selected steps in the purine synthesis and degradation are depicted in Fig. 1.5.

AICAR can inhibit 5'-adenylate deaminase and adenosine deaminase, enzymes mediating the catabolism of adenine ribotide and adenine riboside leading to elevated levels of adenosine monophosphate (AMP) and adenosine[79].

Elevated CSF concentrations of adenosine have been associated with clinical manifestation of acute neurotoxicity after high-dose MTX with or without intrathecal MTX[80, 81]. However, patients who received systemic MTX without developing signs of neurotoxicity had increased adenosine levels as well[81].

In two reports there was a partial or complete resolution of clinical symptoms after the administration of the adenosine receptor antagonist theophylline[80, 81]. These reports indicate that increased levels of adenosine may cause neurotoxicity, which is reversed by adenosine receptor antagonists.

Biopterin metabolism

MTX affects the biopterin pathway by inhibiting the regeneration of tetrahydrobiopterin (THB) from 7,8-dihydrobiopterin (DHB). The reduced biopterin is required for the hydroxylation of tyrosine, phenylalanine and tryptophan. The biosynthesis of bioamines and the metabolism of biopterins is shown in Fig. 1.6.

Tetrahydrobiopterin can be either synthetised de novo from guanosine triphosphate (GTP), a pathway not affected by MTX, or it can be recycled from dihydrobiopterin in three ways all of which are affected directly or indirectly by MTX. The drug directly inhibits the enzyme dihydropteridine reductase

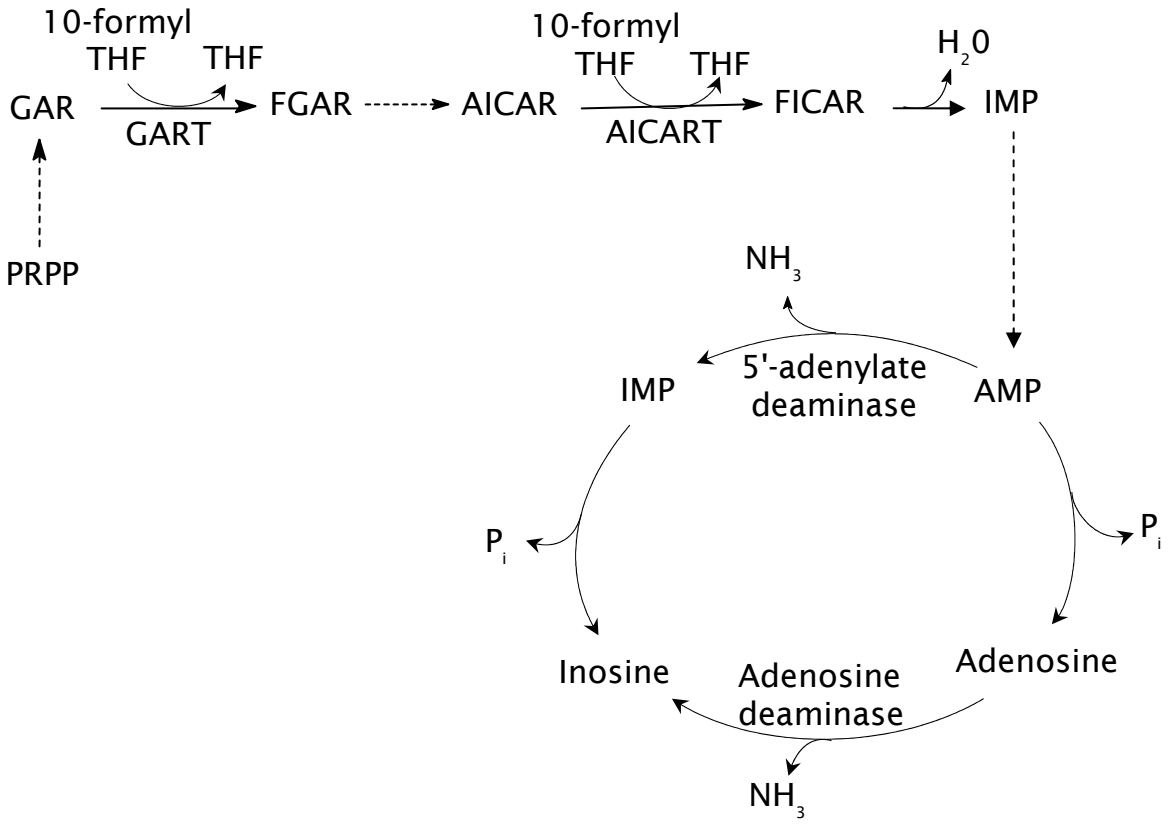

Fig. 1.5: Selected steps in purine synthesis and degradation.
Abbreviations: AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide; AICART: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; AMP: adenosine monophosphate; FGAR: formyl-glycineamide ribonucleotide; FICAR: 5-formamidoimidazole-4-carboxamide ribonucleotide; GAR: glycineamide ribonucleotide; GART: glycineamide ribonucleotide transformylase; IMP: inosine monophosphate; P_i: inorganic phosphate; PRPP: 5-phosphoribosyl-1-pyrophosphate; THF: tetrahydrofolate.
Interrupted arrows indicate indirect conversion.

(DHPR)[82] which is necessary for the reduction of quinoide dihydrobiopterin (qDHB) into the reduced form of biopterins, representing probably the main source of salvage. Moreover, quinoide dihydrobiopterin can be nonenzymatically converted into dihydrobiopterin which is reduced to

tetrahydrobiopterin by DHFR. Furthermore, tetrahydrobiopterin may be regenerated from quinoide dihydrobiopterin in the presence of the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR), whereby 5-methyl-THF is converted in 5,10-methylene-THF. This salvage process can be affected by MTX due to depletion of 5-methyl-THF.

Since tetrahydrobiopterin is necessary for the synthesis of neurotransmitters such as serotonin and dopamine, a MTX-induced alteration of the biopterin pathway could result in a diminished synthesis of the necessary neurotransmitters[83]. An impairment of the catecholamine and biopterin metabolism has been associated with clinical signs of subacute neurotoxicity reported in several cases of children who received high-dose MTX alone or in combination with intrathecal MTX[84,85]. Decreased concentrations of homovanillic acid and 5-hydroxyindoleacetic acid, the major metabolites of dopamine and serotonin, respectively were determined in the CSF of one patient directly after the infusion of MTX. Both metabolites returned into the normal range after the spontaneous resolution of symptoms[84]. Another patient who was comatose had a decreased level of tetrahydrobiopterin in the CSF. The child did not respond to calcium folinate and methylprednisolone therapy but an improvement of the clinical condition was achieved after the administration of L-dopa, carbidopa and 5-hydroxytryptophan (substitutive therapy for biopterin deficiency)[86].

In contrast to these case reports, some studies[85, 87-89] revealed different results suggesting that after the administration of MTX the biosynthesis of serotonin and dopamine remains unaffected. Furthermore, experiments in rats and neuroblastoma cell cultures, performed to induce a deficiency of tetrahydrobiopterin with MTX, revealed no significant decrease in the reduced biopterin[87,88] suggesting that MTX is unlikely to impair the hydroxylation of tyrosine and tryptophan[88].

Due to the contradictory literature data it still remains unclear whether or to which extent MTX is capable of altering the neurotransmitter biosynthesis by affecting the biopterin metabolic pathway.

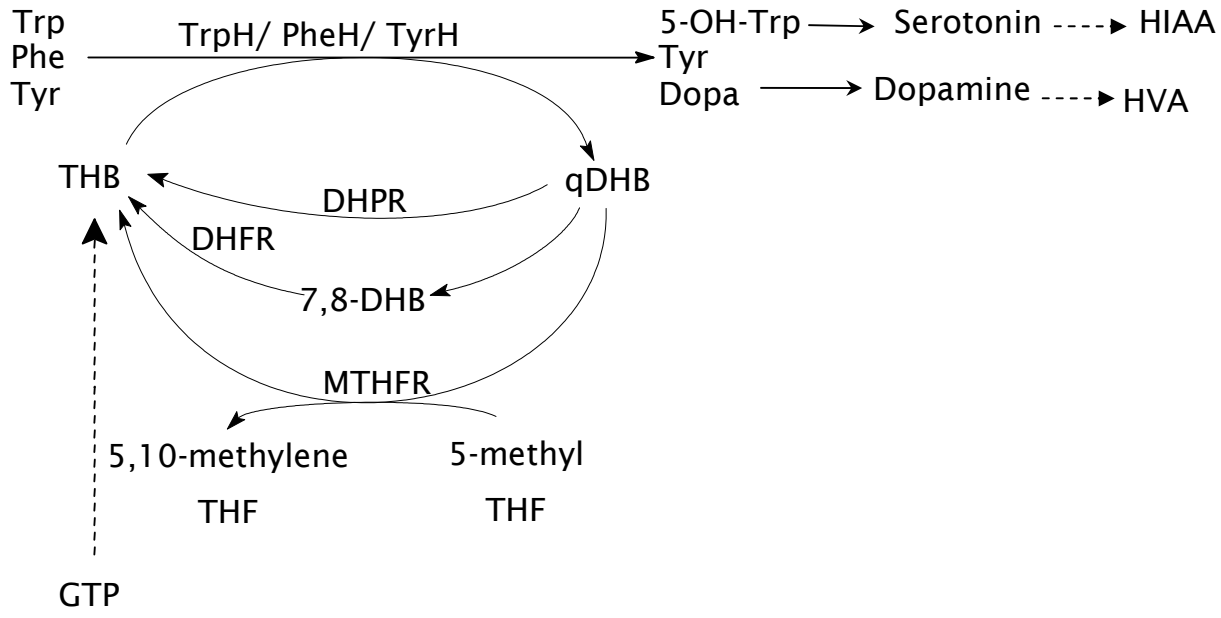

Fig. 1.6: Biosynthesis of bioamines and metabolism of biopterins. Abbreviations: DHFR: dihydrofolate reductase; 7,8-DHB: 7,8-dihydrobiopterin; DHPR: dihydropteridine reductase; GTP: guanosine triphosphate; HIAA: 5-hydroxyindoleacetic acid; HVA: homovanillic acid; MTHFR: 5,10-methylenetetrahydrofolate reductase; Phe: phenylalanine; PheH: phenylalanine hydroxylase; qDHB: quinoide dihydrobiopterin; THB: tetrahydrobiopterin; Trp: tryptophan; 5-OH-Trp: 5-hydroxytryptophan; TrpH: tryptophan hydroxylase; Tyr: tyrosine; TyrH: tyrosine hydroxylase.
Intermediate conversion is depicted with interrupted arrows.

Homocysteine metabolism

The homocysteine pathway is linked to the folate cycle via the coenzyme 5-methyl-THF which serves as the methyl group donor for the methylation of homocysteine to methionine. This reaction is catalysed by the enzyme methionine synthase and requires vitamin B_{12} as a cofactor. In the kidneys and the liver homocysteine can be metabolised to methionine via betaine-homocysteine methyltransferase (BHMT), but this enzyme has not been found in the brain of animals or man[90]. The reaction of methionine and adenosine triphosphate in the presence of the enzyme methionine adenosyltransferase yields S-adenosylmethionine (SAM), the most important

methyl donor in the metabolism of the cell. SAM can be further metabolised to S-adenosylhomocysteine (SAH). The hydrolysis of SAH results in adenosine and homocysteine (see Fig. 1.7).

The main route of catabolism of homocysteine is by entering the transsulphuration pathway starting with the conversion to cystathionine, catalysed by cystathionine-ß-synthase (CBS). Cystathionine is converted to cysteine which can subsequently be oxidised to cysteine sulphinic acid (CSA) and cysteic acid (CA). In analogy to the cysteine oxidation pathway homocysteine can be oxidised to homocysteine sulphinic acid (HCSA) and homocysteic acid (HCA). Enzymes catalysing this metabolic route of homocysteine have not been described, therefore a non-enzymatic conversion has been suggested[91]. Due to the high structural resemblance of CSA, CA, HCSA and HCA to aspartate and glutamate, known as excitants in the mammalian brain, these sulphur-containing amino acids exhibit strong excitatory effects as well[92].

By reducing the level of 5-methyl-THF and thereby disabling the remethylation of homocysteine MTX strongly interferes with the metabolism of this amino acid. By affecting the homocysteine pathway indirectly MTX could induce an elevation of the concentration of the amino acid as well as a decrease of SAM and increase of SAH in the CSF. All three compounds could be involved in the pathogenesis of neurotoxicity. The potential consequences of the alteration of homocysteine and its metabolites are presented below while those of SAM and SAH will be shown in chapter 1.4.

During the oxidation of homocysteine, reactive oxygen species are produced which could further oxidise neuronal unsaturated fatty acids resulting in nerve damage[93-95]. Furthermore, sulphur-containing excitatory amino acids have a high affinity to the N-methyl-D-aspartate (NMDA) receptor[96-99] a subtype of the glutamate receptor present in the CNS. The stimulation of the NMDA receptor induces an intracellular release of calcium ions leading further to an activation of cytotoxic enzymes and resulting in cell death[100-102]. NMDA receptor antagonists are effective anticonvulsive agents and it was

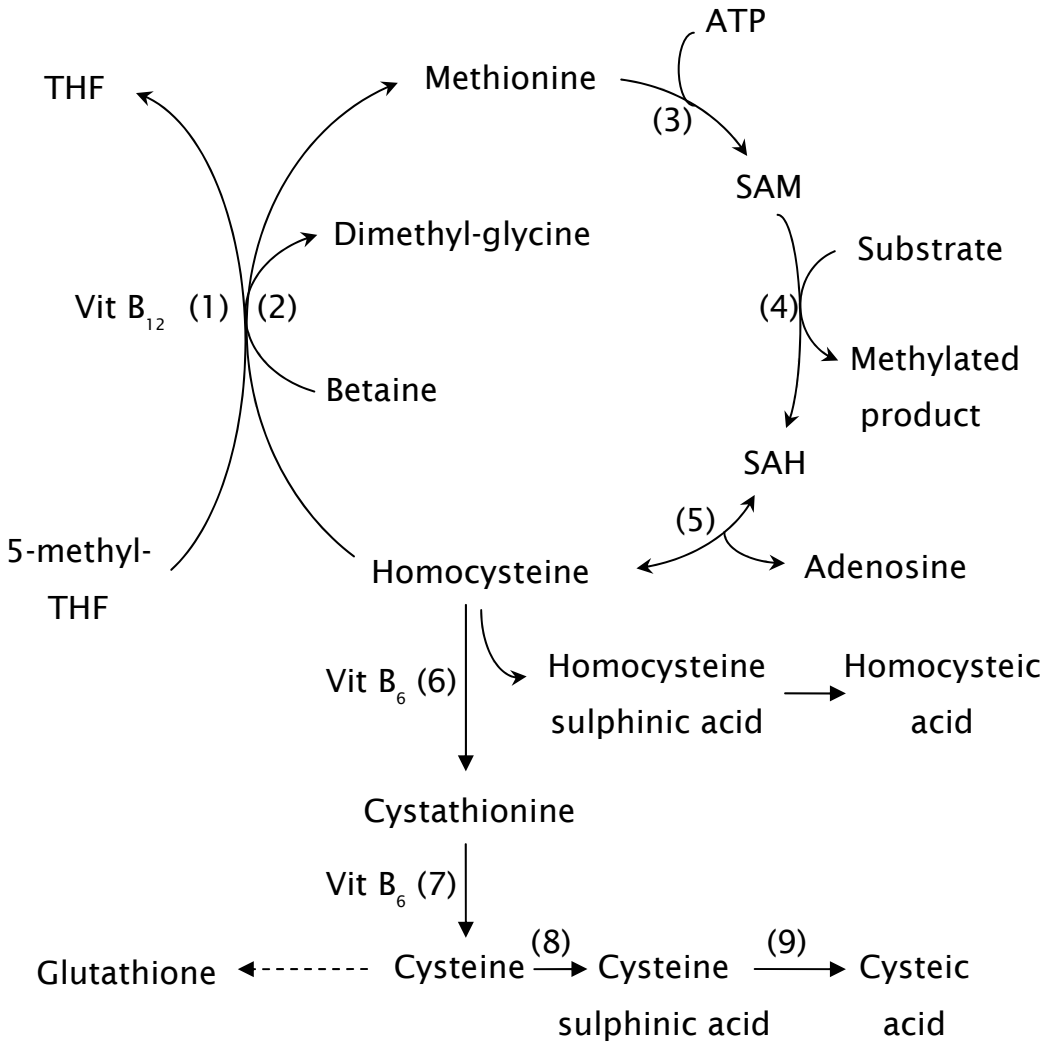

Fig. 1.7: Homocysteine metabolism.

Abbreviations: ATP: adenosine triphosphate; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; THF: tetrahydrofolate. Enzymes: (1): methionine synthase; (2): betaine-homocysteine methyltransferase; (3): methionine adenosyltransferase; (4): methyltransferase; (5): S-adenosylhomocysteine hydrolase; (6): cystathionine-ß-synthase; (7): cystathionase; (8): cysteine dioxygenase; (9): cysteine sulphinic acid dehydrogenase.

The conversion of cysteine to gluthatione requires steps which are not presented (interrupted arrow).

suggested that sulphur-containing excitatory amino acids are likely to play a role in the pathogenesis of seizures. Elevated concentrations of homocysteine in the CSF were reported in patients who received high-dose MTX[103-105]. Quinn et al.[106] observed elevated concentrations of homocysteine in a patient after intraventricular administration of MTX. Drachtman et al.[103] reported elevated homocysteine levels in patients with signs of subacute neurotoxicity compared to asymptomatic patients. These authors also observed a resolution of symptoms in patients after administration of the NMDA receptor antagonist dextromethorphan. This fact suggests that the observed neurotoxicity was at least partly mediated by the sulphur-containing excitatory amino acids.

Very high concentrations of HCSA and CA but especially CSA and HCA were found in the CSF of patients who received high-dose MTX[104, 105]. Patients with clinical signs of toxicity also had the most elevated concentrations of the metabolites. On the contrary, in a control group of healthy adults the sulphur-containing excitatory amino acids were not detected[104].

1.3 Folates

The folate metabolism is the main target of MTX and its polyglutamates and therefore special attention should be paid to the assessment of the potential role of the folate pathway impairment in the pathogenesis of the drug-related neurotoxicity. Since 5-methyl-THF (N^5-methyltetrahydrofolic acid) is the main transport form of reduced folates its concentration in serum and CSF has been used to assess the cellular folate status. The evaluation of the concentration of 5,10-methylene-THF (N^5,N^{10}-methylenetetrahydrofolic acid) could be important in determining the proliferative potential since it is a substrate for the thymidilate synthesis. The chemical structure of the two reduced folates is presented in Fig. 1.8.

Depletion of reduced folates has been observed in some metabolic disorders such as DHFR deficiency, hereditary folate malabsorption and nutritional folate deficiency[107]. Moreover, serum folate deficiency has also been

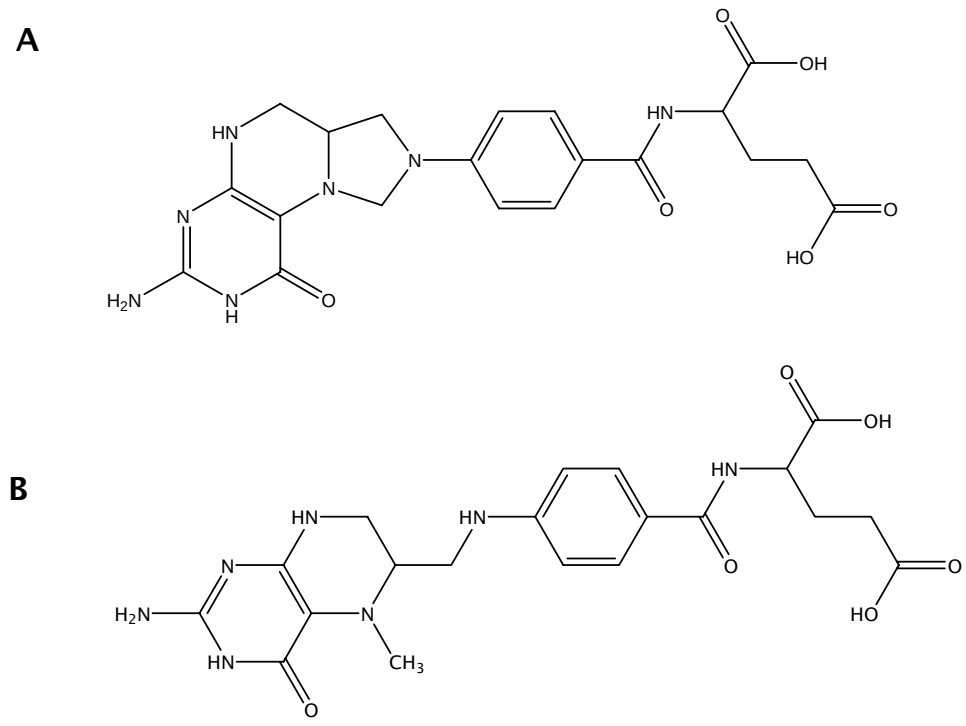

Fig. 1.8: The chemical structure of A: N^5,N^{10}-methylenetetrahydrofolic acid and B: N^5-methyltetrahydrofolic acid.

associated with psychiatric disorders such as depression and schizoid psychosis and symptoms such as insomnia, forgetfulness, irritability and mental retardation have been reported[108-114].

Clinical signs such as deceleration of head growth, behavioural changes, psychomotor retardation, cerebellar ataxia, dyskinesia, pyramidal signs and occasional seizures have been associated with decreased concentration of 5-methyl-THF in the CSF[115]. Depletion of the methyl donor was also observed in patients treated with MTX[104, 105]. Quinn et al.[106] reported a decrease of the CSF 5-methyl-THF concentration in one patient who received MTX intraventricularly without calcium folinate rescue. The authors also showed that the lower concentration persisted in the CSF for at least a week after the last intra-Ommaya MTX administration.

However, it is unclear if there is a direct relationship between the decreased concentration of reduced folates and MTX-induced neurotoxicity. On the other hand, it has been shown that the folate metabolism is closely related to other metabolic pathways. Moreover, patients with MTHFR deficiency were shown to have significantly decreased levels of SAM[116, 117] and significantly elevated levels of homocysteine[118]. This could be an indication that the reduced folates participate indirectly in the development of the MTX-related neurotoxicity by altering other metabolic pathways. Nevertheless, it is important to elucidate the relationship between the concentration of the reduced folates which are primarily affected by MTX and metabolite levels which are thought to be involved in the pathogenesis of MTX-associated neurotoxicity.

1.4 S-adenosylmethionine/S-adenosylhomocysteine

It has been postulated that the MTX-induced depletion of reduced folates possibly induces an impairment of the homocysteine pathway which could further lead to a decrease of the SAM and an increase of the SAH concentration. Because of the importance of the cellular role of SAM and SAH (also referred to as the methyl-transfer pathway), their CSF determination was an aim of this work and the consequences of their alteration are therefore presented in detail in this chapter.

Since SAM arises through the reaction between adenosine and methionine (see Fig. 1.7) it contains a methylated sulphur which is positively charged. Because of such chemical structure SAM is an instable compound which easily donates the methyl group converting to SAH (see Fig. 1.9).

SAM is thought to be the sole methyl donor in a variety of transmethylation reactions[119] involving at least 130 methyltransferase enzymes[120]. The biological activity of many compounds such as proteins, nucleic acids, fatty acids, phospholipids and polysaccharides is altered by methylation[121, 122]. Moreover, SAM is necessary for the inactivation of catecholamines and other

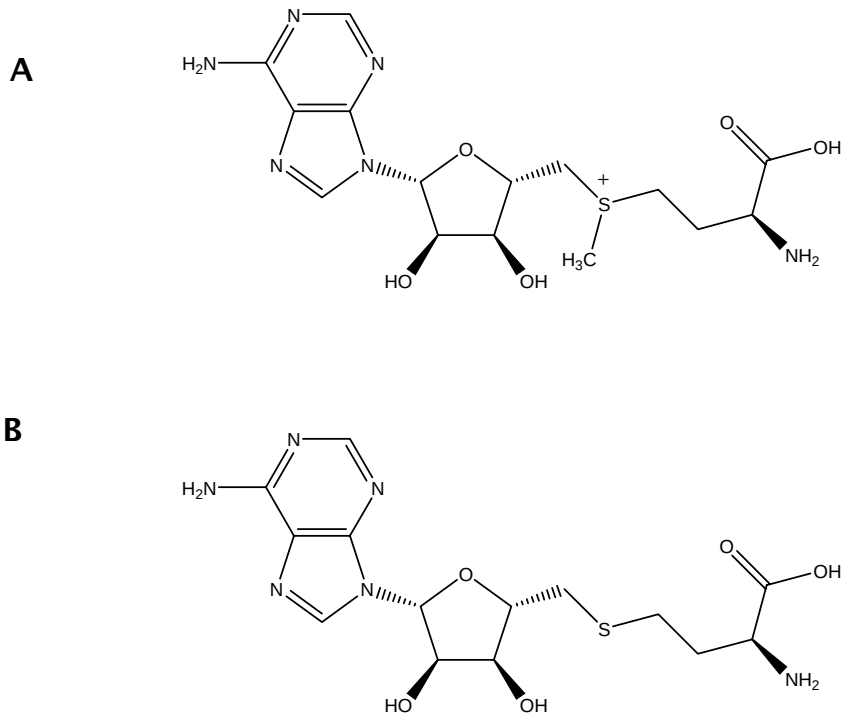

Fig. 1.9: Chemical stucture of A: S-adenosylmethionine and B: S-adenosylhomocysteine.

biogenic amines and it is indirectly involved in the polyamine, purine and pyrimidine synthesis[123-126]. SAH on the other hand is a powerful competitive inhibitor of methyltransferase enzymes[127, 128]. Therefore, a decrease of the SAM concentration and/or an increase of the SAH concentration could induce hypomethylation leading further to a variety of disorders in the cell.

It is known that SAM takes part as a methyl donor in the synthesis of choline which is a part of the structure of neuronal myelin sheath. The role of the myelin sheath is to protect axons and enable a rapid and efficient transmission of impulses along the nerve cell (see Fig. 1.10). It was postulated that hypomethylation could induce demyelination leading to neuronal damage. In effect, low CSF SAM concentrations were associated with demyelination of the cerebral white matter[117, 129].

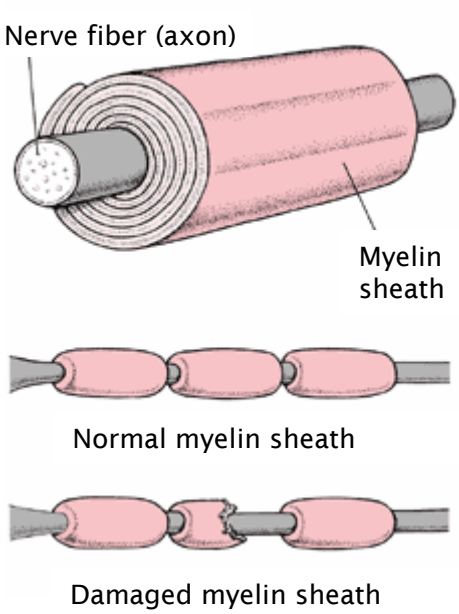

Fig. 1.10: Diagram of an axon with normal and damaged myelin sheath.

Demyelination has been observed in various diseases such as depression, Alzheimer's dementia, HIV infection, subacute combined degeneration of the spinal cord and multiple sclerosis[117, 129-135] all of which are assocciated with neurological disorders. Moreover, demyelination is a common feature observed in MTX-associated subclinical and clinical leukoencephalopathy[136-142] and it has therefore been postulated that the alteration of the CSF concentration of SAM and SAH may play a role in its pathogenesis.

Surtees et al.[136] reported a significant decrease of CSF SAM concentration in children with ALL who were intrathecally treated with MTX. They associated these findings with a subclinical demyelination which was observed in these patients. Similar findings regarding the CSF SAM concentration were reported in one patient who received MTX intraventricularly[106].

Kishi et al.[137] investigated both SAM and SAH in two ALL patients with clinically manifest leukoencephalopathy, a group of ALL patients without clinical symptoms of leukoencephalopathy and a control group. ALL patients had a significantly lower concentration of SAM compared to the reference group whereas the concentration of SAH was similar in both groups. The two

patients with severe neurotoxicity had a slightly lower CSF SAM concentration and a markedly higher SAH concentration compared to the ALL patients without manifest leukoencephalopathy, suggesting that a SAM/SAH ratio may be a marker for leukoencephalopathy rather than the concentration of SAM solely. However, the two neurotoxic patients received a combination of high-dose and intrathecal MTX as well as cranial irradiation whereas other ALL patients received no cranial irradiation. Since the influence of cranial irradiation on the development of leukoencephalopathy in these patients could not be assessed it remains unclear whether such severe neurotoxicity can be induced by high-dose and intrathecal MTX through an alteration of the SAM/SAH ratio.

If neurotoxicity associated with MTX is partly mediated by an alteration of the concentration of SAM and SAH it may be possible to prevent or at least reduce it by normalising the levels of these metabolites without thereby compromising the efficacy of MTX. It was shown in severely depressed patients that after oral or intravenous SAM administration its CSF concentration increased indicating the capacity of the metabolite to pass the blood-brain barrier[130]. Also betaine has shown to be effective in elevating the concentration of SAM in the CSF[129].

Surtees et al.[117] showed that the normalisation of SAM levels in the CSF was associated with remyelination. Furthermore, after substitution with SAM an antidepressant effect in patients with Parkinson's disease[138] and an improvement in cognitive dysfunction as well as mood and speed of mental processing in patients with Alzheimer's dementia was reported[139]. All these data indicate that a supplementation with SAM may be beneficial also for patients treated with MTX who develop a transient or lasting alteration of the methyl-transfer pathway.

In summary, it has been shown that the folate metabolism, which is impaired after MTX therapy, interferes with several other metabolic pathways. Therefore, it is probable that the pathogenesis of MTX-associated neurotoxicity is complex and multifactorial. Nevertheless, elucidating the

mechanisms of this type of toxicity could provide possibilites for its circumvention thus increasing the safety of the MTX therapy.

2 AIM AND OBJECTIVES

MTX administered as a high-dose infusion and/or intrathecally is gaining more and more importance in modern therapy regimens for ALL, PCNSL, osteosarcoma and medulloblastoma since it is associated with higher and prolonged remission rates. Patients seem to benefit from an intensified therapy regimen which comprises both a higher dose of the antifolate as well as a more frequent administration. Since such therapy intensification is also associated with a higher incidence of neurotoxicity it is essential to elucidate the pathogenetic mechanism of this undesired side effect in order to prevent it. It has been suggested that MTX-associated neurotoxicity is a consequence of the alteration of biochemical pathways related to the folate cycle.

Since clinical signs of neurotoxicity are thought to be a consequence of the MTX-induced damage to the brain tissue it is necessary to reveal MTX-related biochemical changes in the neuronal cells. However, this would require sampling of neuronal tissue which is not possible in humans. CSF (of all body fluids) reflects most closely the biochemical status in the brain tissue and was therefore regarded as the matrix in which such biochemical analysis could be performed.

The aim of this study was to assess the effect of systemic high-dose and/or intrathecal administration of MTX on the folate and homocysteine pathway in the CSF. The CSF analysis involved the determination of the concentration of

- Methotrexate
- 5-methyl-THF
- 5,10-methylene-THF
- Calcium folinate
- S-adenosylmethionine and S-adenosylhomocysteine
- Homocysteine
- Homocysteic acid and homocysteine sulphinic acid
- Cysteine

- Cysteic acid and cysteine sulphinic acid
- Glutathione

The analysis of homocysteine and sulphur-containing excitatory amino acids in the CSF was performed by Achim Becker[140].
CSF samples were obtained from patients who received high-dose and/or intrathecal MTX therapy for PCNSL or childhood ALL. PCNSL patients had an implanted Ommaya reservoir and an intensive protocol regarding administration of MTX which enabled frequent CSF sampling. From ALL patients samples were obtained by lumbar puncture throughout the therapy regimen.

In order to achieve the aim of the study the following objectives were defined:

- Development and validation of an high-performance liquid chromatography (HPLC) method for the analysis of 5,10-methylene-THF, 5-methyl-THF, calcium folinate and MTX in the CSF
- Development and validation of an HPLC method for the analysis of SAM and SAH in the CSF
- Development and validation of an HPLC method for the analysis of homocysteine and cysteine in the CSF (A. Becker)
- Development and validation of an analytical method using capillary electrophoresis for the analysis of sulphur-containing excitatory amino acids (A. Becker)
- Sampling and evaluation of the possible neurotoxic symptoms using common toxicity criteria (CTC) in cooperation with medical doctors
- Determination of the concentration of the above listed substances in the CSF
- The quantitative assessment of the effect of systemically and/or intrathecally administered MTX on the folate pathway and SAM/SAH.

3 MATERIALS AND METHODS

3.1 Materials

A brandname may be trademarked even when it is not noticed.

3.1.1 Reference substances

- Folinic acid, calcium salt (Sigma Aldrich, Steinheim, Germany)
- Methotrexate (Sigma Aldrich, Steinheim, Germany)
- N^5,N^{10}-methylenetetrahydrofolate (Dr. Schircks Laboratories, Jona, Switzerland)
- N^5-methyltetrahydrofolate (Sigma Aldrich, Steinheim, Germany)
- S-adenosyl-L-homocysteine (Sigma Aldrich, Steinheim, Germany)
- S-adenosyl-L-methionine, iodide salt (Sigma Aldrich, Steinheim, Germany)

3.1.2 Chemicals for the analysis of MTX and reduced folates

- Ascorbic acid (Merck, Darmstadt, Germany)
- Methanol LiChrosolv® HPLC grade (Merck, Darmstadt, Germany)
- Phosphoric acid 85% (V/V) (Fluka Chemie GmbH, Neu-Ulm, Germany)
- Potassium dihydrogen phosphate (Merck, Darmstadt, Germany)
- Sodium hydroxide (Merck, Darmstadt, Germany)
- Tris(hydroxymethyl)-aminomethane (Merck, Darmstadt, Germany)
- Water Ampuwa® (Fresenius Kabi AG, Bad Homburg, Germany)

3.1.3 Chemicals for the analysis of SAM and SAH

- Acetonitrile (Fisher Scientific, Longborough, UK)
- Chloroacetaldehyde 45% (Merck, Darmstadt, Germany)
- 1-Heptanesulphonic acid, sodium salt 98% (ACROS Organics, New Jersey, USA)
- Perchloric acid 70% (Sigma-Aldrich, Steinheim, Germany)
- Phosphoric acid 85% (V/V) (Fluka Chemie GmbH, Neu-Ulm, Germany)
- Potassium dihydrogen phosphate (Fluka Chemie GmbH, Neu-Ulm, Germany)
- Sodium acetate (Fluka Chemie GmbH, Neu-Ulm, Germany)
- Sodium chloride (Fluka Chemie GmbH, Neu-Ulm, Germany)
- Water Purelab™ Plus (USF, Ransbach-Baumbach, Germany)

3.1.4 Consumables and devices

- Analytical balance Kern 770 (Gottlieb Kern & Sohn, Albstadt, Germany)
- Centrifuge Microfuge® Lite (Beckman-Coulter, Fullerton, USA)
- Magnetic stirrer RMH71 (Gerhardt GmbH & Co. KG, Königswinter, Germany)
- One-channel pipets 1-1000 µL (Fischer Scientific, Schwerte, Germany)
- Pipet tips (Brand GmbH & Co, Wertheim, Germany)
- pH meter inoLab pH level 2 (WTW, Weilheim, Germany)
- Reaction tubes 1 mL (Eppendorf AG, Hamburg, Germany)
- Sample mixer (Gesellschaft für Laborbedarf, Würzburg, Germany)
- Ultrasonic bath Sonorex Super RK 103 H (Bandelin, Berlin, Germany)
- Vials 2 mL (Sarstedt, Nümbrecht, Germany)
- Waterbath MGW Lauda RM 6 (Werk Lauda, Lauda-Königshofen, Germany)
- Waterdestiller Purelab™ Plus (USF, Ransbach-Baumbach, Germany)

3.2 Instruments

3.2.1 HPLC system for the analysis of MTX and reduced folates

- Vacuum membrane degaser: SCM 1000
  (Thermo Separation Products, Inc.,
  Egelsbach, Germany)
- System contoller: SN4000
  (Thermo Separation Products, Inc,
  Egelsbach, Germany)
- Pumps: SpectraSYSTEM® P4000
  (Thermo Separation Products, Inc,
  Egelsbach, Germany)
- Autosampler: SpectraSYSTEM® AS3000
  (Thermo Separation Products, Inc,
  Egelsbach, Germany)
- Precolumn: Supelguard™ LC-18-DB;
  2 x 0.46 cm, 5 μm
  (Supelco, Bellefonte, USA)
- Column: Supelcosil™ LC-18-DB;
  15 x 0.46 cm, 3 μm
  (Supelco, Bellefonte, USA)
- Detectors: UV Detector: UV6000LP
  Fluorescence Detector: FL3000
  (Thermo Separation Products, Inc,
  Egelsbach, Germany)
- Software: ChromQuest®, Version 2.51
  (ThermoQuest Corporation, Egelsbach
  Germany)

3.2.2 HPLC system for the analysis of SAM and SAH

- Interface: D-6000 (Merck-Hitachi, Darmstadt, Germany)
- Pumps: L-6200 Intelligent Pump, L-6000 Pump (Merck-Hitachi, Darmstadt, Germany)
- Autosampler: AS-2000A (Merck-Hitachi, Darmstadt, Germany)
- Precolumn: Nucleosil™ LC-18-DB; 2 x 0.46 cm, 5 μm (Supelco, Bellefonte, USA)
- Column: Eclipse AAA LC-18-DB; 15 x 0.46 cm, 5 μm (Agilent Technologies Inc., Palo Alto, USA)
- Detector: Fluorescence Detector: L-7480 (Merck-Hitachi, Darmstadt, Germany)
- Software: Model D-7000 Chromatography Station Software (Merck-Hitachi, Darmstadt, Germany)

3.3 HPLC analysis for the determination of reduced folates and MTX in the CSF

3.3.1 Chromatographic conditions

In order to determine the CSF concentrations of 5,10-methylene-THF, 5-methyl-THF, calcium folinate and MTX an assay from Belz et al[141] was

adapted based on reversed-phase chromatography with fluorescence and ultraviolet (UV) detection*.

Since the three reduced folates have natural fluorescence at excitation and emission wavelengths of λ=294 nm and λ=356 nm respectively, the use of fluorescence detection without prior sample derivatisation was enabled. The signal intensity of calcium folinate was similar with both fluorescence and UV detector and the latter was used for its quantification at λ=310 nm. MTX does not show fluorescence at the above-specified conditions and was therefore quantified by using UV detection together with calcium folinate.

Sample preparation consisted in thawing the frozen sample at room temperature and pippeting 100 μL into a vial which was then put into the autosampler. 50 μL of the sample was injected into the system. The flow rate was kept constant at 1 mL/min. The analytes were eluted using a mobile phase which consisted of methanol (eluent A) and 10 mM phosphate buffer, pH 2.1 (eluent B). The buffer was prepared by dissolving 1.36 g of potassium dihydrogen phosphate in 1000 mL water and phosphoric acid was used to adjust the pH. Elution was performed by the use of the gradient presented in Table 3.1. The retention times of each of the analysed substances are presented in Table 3.2.

3.3.2 Sample collection

In order to avoid oxidation of the instabile tetrahydrofolates 5,10-methylene-THF and 5-methyl-THF, CSF samples were collected into vials containing ascorbic acid. A solution of ascorbic acid (25 mg/mL) was prepared by dissolving 250 mg in 10 mL water. This solution was aliquoted into vials each containing 100 μL and frozen at -20 ºC. Immediately before sampling, vials were thawed at room temperature. 500 μL CSF was added and

* In tables 5,10-methylene-THF will be abbreviated as 5,10-MeTHF, 5-methyl-THF as 5-MTHF and calcium folinate as Ca-folinate in order to obtain a better survey of data in the available space.

Table 3.1: Gradient for HPLC elution of 5,10-methylene-THF, 5-methyl-THF, calcium folinate and MTX

Time (min)	Methanol (%)	Phosphate buffer (10 mM, pH 2.1) (%)
0	87	13
12	87	13
20	60	40
22	87	13
23	87	13

Table 3.2: Retention times of 5,10-methylene-THF, 5-methyl-THF, calcium folinate and MTX during HPLC analysis.

Analyte	Retention time (min)
5,10-MeTHF	6.5
5-MTHF	8.8
Ca-folinate	18.0
MTX	21.0

samples were centrifuged at 15 500 x g for 10 min. The supernatant was frozen directly afterwards at -20 °C.

3.3.3 Validation

The method was validated according to the Guideline of the Food and Drug Administration (FDA Guidance for Industry, Bioanalytical Method Validation)[142]. Selectivity, stability of the compounds, recovery, limit of quantification, limit of detection, precision, accuracy and linearity were

assessed. The results were evaluated with the Software "Method Validation in Analytics" (MVA®), Version 2.0 (Novia GmbH Saarbrücken, Germany).

3.3.3.1 Selectivity

Selectivity of the method assures that the desired analytes are identified and quantified without interference of related compounds (degradants, metabolites, impurities etc.) or components of the matrix (endogenous substances etc.).
The selectivity of the method for each compound was assessed by comparison of chromatogrammes of buffer or CSF samples to which standard solutions of each compound were added and samples which contained buffer or CSF without the addition of analytes.

3.3.3.2 Stability

Some information refering to the stability of the analysed substances was found in the literature, such as the stability of stock solutions when stored at -20 ºC[141]. The stability of the analytes in CSF at room temperature and the stability when stored at 4 ºC was investigated.
To determine the stability at room temperature a frozen sample which served as reference was thawed and analyzed. Immediately after that a freshly prepared solution containing equal concentrations of the analytes as the reference sample was placed in the autosampler and analysed repeatedly for 10 h. This procedure was performed on the following two days as well.
To determine the stability at 4 ºC three samples were prepared analyzed and stored in the refrigerator. These samples were analysed on three subsequent days and compared to reference samples (containing equal analyte concentrations) which were stored at –20 ºC and thawed immediately before analysis. Thus, it was possible to distinguish between the instability of the analytes and the variation in the chromatographic system due to changes of temperature and air pressure.

Moreover, stability of the analytes in CSF was determined after two freeze and thaw cycles. Thereby it was possible to determine whether patient CSF samples could be analysed twice or three times if necessary without analyte loss. CSF samples were spiked with all four analytes in three different concentrations. An aliquot of each of the three CSF samples was analysed, the rest was frozen. The frozen aliquots were thawed after few days, analysed and the remaining content was frozen again. This was referred to as the first freeze and thaw cycle. The frozen samples were thawed once more and analysed. This was the second freeze and thaw cycle.
The stability was calculated in different time intervals with the following equation (Eq. 3.1):

$$\text{Stability } (\%) = \frac{CPA_x}{CPA_{REF}} \cdot 100 \qquad \text{Eq. 3.1}$$

CPA_x = corrected peak area of the CSF sample at temperature x

CPA_{REF} = corrected peak area of the reference CSF sample

3.3.3.3 Recovery

Recovery experiments were performed in order to determine if there was any difference in response between a buffer solution and the CSF matrix when equivalent concentrations of analytes were measured. No difference or a constant ratio enable calibration of the analytes in the buffer solution.
The CSF used in the experiment was obtained from six patients and pooled. The response in the pooled CSF was determined for each analyte at three different concentrations which corresponded to the lower, middle and upper range of the calibration curve. The determination of each concentration was repeated three times. Since 5,10-methylene-THF and 5-methyl-THF are endogenous in the CSF it was necessary to determine the response of these analytes in the unspiked CSF as well. The unspiked CSF was also analysed three times and the mean value of the response (peak area) was then substracted from the response of the spiked CSF samples. These results were

compared to the results of buffer solutions containing equal concentrations of analytes as the CSF samples and recovery was calculated according to the following equation (Eq. 3.2):

$$R\ (\%) = \frac{\overline{x}_{CSF}}{\overline{x}_{Buffer}} \cdot 100 \qquad \text{Eq. 3.2}$$

R = Recovery

$\overline{x}_{CSF}$ = Mean value of the peak area of the CSF sample

$\overline{x}_{Buffer}$ = Mean value of the peak area of the buffer sample

3.3.3.4 Limit of detection

The limit of the detection (LOD) is the minimal amount of a compound which can be detected with certainty. For the described HPLC method the limit of detection was estimated as signal-to-noise ratio 3:1.

3.3.3.5 Lower limit of quantification

The lower limit of quantification (LLOQ) is the lowest concentration of the analyte which can be precisely and accurately quantified. For the described HPLC method the limit of quantification was defined as the lowest concentration of the analyte which had a between-day coefficient of variation and relative error lower than 20%.

3.3.3.6 Precision

Precision experiments study the effect of random variations in the performance of a method. Precision is assessed through multiple analysis of a homogenous sample and standard deviation is the statistical parameter used for its description. The standard deviation (synonym coefficient of variation, CV) is calculated with the following formula (Eq. 3.3):

$$CV\ (\%) = \frac{\sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}}{\bar{x}} \cdot 100 \qquad \text{Eq. 3.3}$$

CV = coefficient of variation

$\bar{x}$ = mean value from n measurements

x_i = measured value

n = number of measurements

The precision of the method within a day (within-day precision, repeatability) and between days (between-day precision, reproducibility) was assessed. Within-day precision was obtained by preparing and analysing six CSF samples with equivalent concentrations of analytes. The unspiked CSF was analysed as well and the correction of the results was performed as described in chapter 3.3.3.3. Three different concentrations were selected corresponding to the lower, middle and upper span of the calibration curve. Between-day precision was obtained by the analysis of quality controls (see chapter 3.3.5.) on six subsequent days.

3.3.3.7 Accuracy

Accuracy shows how close the experimental value is to the value accepted as true or reference value. To determine the accuracy of the HPLC method CSF samples containing known nominal concentrations were analysed and their experimental value was calculated using the calibration curve. The statistical parameter which describes accuracy is the relative error (RE) which is calculated as following (Eq. 3.4):

$$RE\ (\%) = \frac{(C - C_{nom}) \cdot 100}{C_{nom}} \qquad \text{Eq. 3.4}$$

RE = relative error

C = value obtained from the analysis

C_{nom} = nominal value (true or reference value)

Accuracy is then calculated with the following equation (Eq. 3.5):

$$\text{Accuracy}(\%) = \text{RE} + 100 \qquad \text{Eq. 3.5}$$

3.3.3.8 Linearity

In order to validate a method it is important to establish the mathematical relationship between the measured response and the concentration of the analyte. If the measured response has a linear relationship with the concentration it can be described with the following mathematical equation (Eq. 3.6):

$$y_i = bx_i + a \qquad \text{Eq. 3.6}$$

y_i = measured response

b = slope coefficient

x_i = concentration of the analyte

a = intercept coefficient

Linearity of the method was tested by analysing seven CSF samples containing different concentrations of the analytes. Such calibration samples were freshly prepared and analysed on six subsequent days. To show the linearity of the method the coefficient of correlation (r) and the residuals were calculated.

The coefficient of correlation is a measure of the degree of correlation and is in the range of $-1 \leq r \leq 1$. A value of 0 indicates no correlation between the measured response and the analyte concentrations and values -1 or 1 indicate a perfect correlation with negative and positive slopes, respectively. The calculation of r is presented in Eq. 3.7:

$$r = \frac{\sum_{i=1}^{n}\{(x_i - \bar{x})(y_i - \bar{y})\}}{\sqrt{\sum_{i=1}^{n}\{(x_i - \bar{x})^2 (y_i - \bar{y})^2\}}} \qquad \text{Eq. 3.7}$$

r = coefficient of correlation

x_i = concentration of the analyte

$\bar{x}$ = mean of the analysed concentrations

y_i = measured response

$\bar{y}$ = mean of the measured responses

n = number of measurements

Furthermore, it was investigated whether a better fit of the calibration curve can be obtained by a weighted linear regression ($1/x$ or $1/x^2$).
Residuals are the difference between the measured and nominal concentrations. When the residuals are plotted versus the respective concentrations they should be randomly distributed around the value of zero. If this is not the case a linear relationship may not exist between response and concentration.

3.3.4 Calibration

3.3.4.1 Preparation of stock solutions

Since 5,10-methylene-THF and 5-methyl-THF are oxidable compounds stock solutions where prepared by dissolving the reference substances in a tris buffer (pH 7.6) containing ascorbic acid as antioxidant. The buffer was prepared by dissolving 15.8 g tris(hydroxymethyl)-aminomethane and 10 g ascorbic acid into almost 1000 mL water. The pH was adjusted with sodium hydroxide and the volume of the solution completed to 1000 mL. Stock solutions were prepared for each analyte separately in the following manner: Each reference substance was weighed and dissolved in 10 mL of the tris

buffer. This procedure was repeated three times so that at the end there were three vials for each reference substance which should contain the same concentration of the analyte. The concentration in each of the three vials was analysed by HPLC and if the variation coefficient was not greater than 3% they were accepted as stock solutions, aliquoted and kept at -20 ºC.

3.3.4.2 Preparation of calibrator solutions

The range for the calibration of each analyte was chosen so that the lowest concentration for each analyte was the lower limit of quantification and the highest concentration (upper limit of quantification, ULOQ) was above the expected concentrations in the CSF after metabolic changes. To obtain such a calibration curve 7 calibrator solutions (one for each calibration point) containing all four reference substances were prepared from stock solutions by dilution with tris buffer. 40 µL of a calibration solution was then added to 960 µL of pooled CSF. Another CSF sample was spiked with 40 µL of tris buffer and served as reference for substracting the endogenous CSF concentration of 5,10-methylene-THF and 5-methyl-THF. The final concentrations of the calibrator solutions are presented in Table 3.3.

3.3.4.3 Calculation of concentrations

The calibration curves were generated by analysing the 7 calibration points and the reference CSF in a random order. Peak area was determined with the ChromQuest® software. A substraction of the reference value was performed for each calibration point for 5,10-methylene-THF and 5-methyl-THF. Peak area was plotted against the concentration of the analytes and a linear regression analysis was performed using the MVA® software. The unknown concentrations were calculated from the regression equation.

Table 3.3: Calibrator concentrations for HPLC analysis of reduced folates and MTX in CSF.

Calibration point	Final concentration (nM)			
	5,10-MeTHF	5-MTHF	Ca-folinate	MTX
1	10.0	10.0	100.0	20.0
2	20.0	20.0	200.0	50.0
3	30.0	30.0	400.0	200.0
4	50.0	50.0	1000.0	1000.0
5	100.0	100.0	2000.0	2000.0
6	200.0	200.0	4000.0	6000.0
7	300.0	300.0	6000.0	10000.0

3.3.5 Quality control

A calibration curve was generated always prior to the analysis of patient samples. The calibration curve was accepted when the following criteria were fulfilled:

- Accuracy of LLOQ ≤ 20%
- Correlation coefficient r ≥ 0,99 (linear regression, weighted 1/x); deviation of residuals ≤ 15%
- At least 5 calibration points had to meet the requirement for the residuals including LLOQ and ULOQ

The CSF samples were thawed and left for no longer than 3 h at room temperature before analysis. The concentrations of the analytes were calculated from the calibration curve (weighted $1/x^2$).

To assure the quality of measurement quality control (QC) samples in two different concentrations were prepared before sample analysis was initiated. At least 5% of all analysed samples were QC samples The concentrations of the QC samples were in the lower and upper range of the calibration curve for each analyte. QC samples were prepared using the same procedure as for

the calibration points except that QC samples were prepared in a larger volume, aliquoted and frozen at -20 ºC. The QC samples were analysed between the analysis of the CSF samples. It was demanded that at least 67% of the calculated concentrations of QC samples are within ± 15% of the nominal value.
Some samples contained amounts of MTX which were above the ULOQ. Such samples were diluted with pooled CSF (1:5). Prior to the dilution the pooled CSF was analysed by HPLC and did not contain any traces of MTX.

3.4 HPLC-analysis for the determination of SAM and SAH in CSF

3.4.1 Chromatographic conditions

A method based on reversed phase chromatography was developed and validated for the analysis of SAM and SAH in CSF samples.
The fluorescence detection of both substances was enabled by their derivatisation with chloroacetaldehyde (the derivatisation process is described in chapter 3.4.3). The formed 1,N^6-etheno derivatives are highly fluorescent and were eluted on a reversed-phase column especially designed for the separation of amino acids. The analytes were eluted using a mobile phase which consisted of acetonitrile (eluent A) and an aqueous solution (pH 4.5) containing 40 mM of potassium dihydrogen phosphate and 8 mM of the ion-pair reagent 1-heptanesulphonic acid (eluent B). Eluent B was prepared by dissolving 5.44 g potassium dihydrogen phosphate and 1.6 g 1-heptanesulphonic acid in 1000 mL water. The pH of the solution was measured and adjusted to pH 4.5 when necessary using phosphoric acid. A gradient (presented in Table 3.4) was used for the elution of the analytes.

Table 3.4: Gradient for the elution of SAM and SAH by HPLC.

Time (min)	Acetonitrile (%)	Phosphate buffer (40 mM, pH 4.5) with 1-heptanesulphonic acid (8 mM) (%)
0	2	98
10	2	98
31	17	83
32	17	83
34	2	98

The injection volume was 100 µL. The flow rate was kept constant at 1 mL/min and SAM and SAH were detected using fluorescence detection at an excitation wavelength of 270 nm and an emission wavelength of 410 nm. The retention times were 23.5 min and 19.7 min for SAM and SAH, respectively.

3.4.2 Sample collection

Perchloric acid was used for the precipitation of proteins in the CSF. A 1.2 M solution was prepared by diluting 720 µL of perchloric acid (70%) with water up to 10 mL. This solution was aliquoted into vials, each containing 100 µL. The vials were marked and frozen at –20 ºC. Immediately before collecting a patient sample a vial was thawed. 500 µL of CSF were added and the content was centrifuged for 10 min at 15 500 x g. The supernatant was frozen at -20 ºC immediately after centrifugation.

3.4.3 Derivatisation of SAM and SAH

The derivatisation of SAM and SAH with chloroacetaldehyde and the formation of their fluorescent 1,N^6-etheno derivatives was well studied,

optimised and reported in several publications[143-146]. It has been shown that the incubation time and temperature as well as the pH of the reaction and the concentration of chloroacetaldehyde have a considerable impact on the derivatisation.

In accordance with the work of Wagner et al.[143] SAM and SAH were derivatised with 5.5 M chloroacetaldehyde at pH 3.5-4.0, 39 ºC for 16 h.

Chloroacetaldehyde (5.5 M) was prepared each day by diluting 1 mL of a 50% solution with 430 µL water. A 3 M sodium acetate solution was used for the adjustment of pH. This solution was prepared by disolving 24.6 g of sodium acetate in 100 mL water and was stored at room temperature. The derivatisation of each sample was performed as following: 25 µL of the 5.5 M solution of chloroacetaldehyde and 14 µL of the 3 M sodium acetate solution were added to 250 µL of each CSF sample.

All samples were stored for 16 h overnight in the waterbath at 39 ºC. The derivatisation reaction was stopped by freezing. The samples were thawed and analysed within 24 h after derivatisation. The derivatisation reaction is presented on the example of SAM in Fig. 3.1.

3.4.4 Development and optimisation of the method

In the developmental phase of the assay various columns were tested in order to achieve a good separation and selectivity.

Because of the polar properties of SAH and especially SAM which is ionised an attempt of separation of the two compounds was made on a amine column (Luna NH_2®, 150 x 4.6 mm, 5µm, Phenomenex, Torrance, USA) and two reversed-phase columns (Kromasil® C18, 125 x 4.0 mm, 3.5 µm, Agilent technologies, Palo Alto, USA and Eclipse AAA® LC-18-DB, 150 x 4.6 mm, 5 µm, Agilent technologies, Palo Alto, USA). Phosphate buffer (40 mM, pH 4.5) and isopropanol or acetonitrile were used as eluents and optimisation of separation was attempted with the use of gradients. In addition, 1-heptanesulphonic acid (8 mM) was added to the phosphate buffer

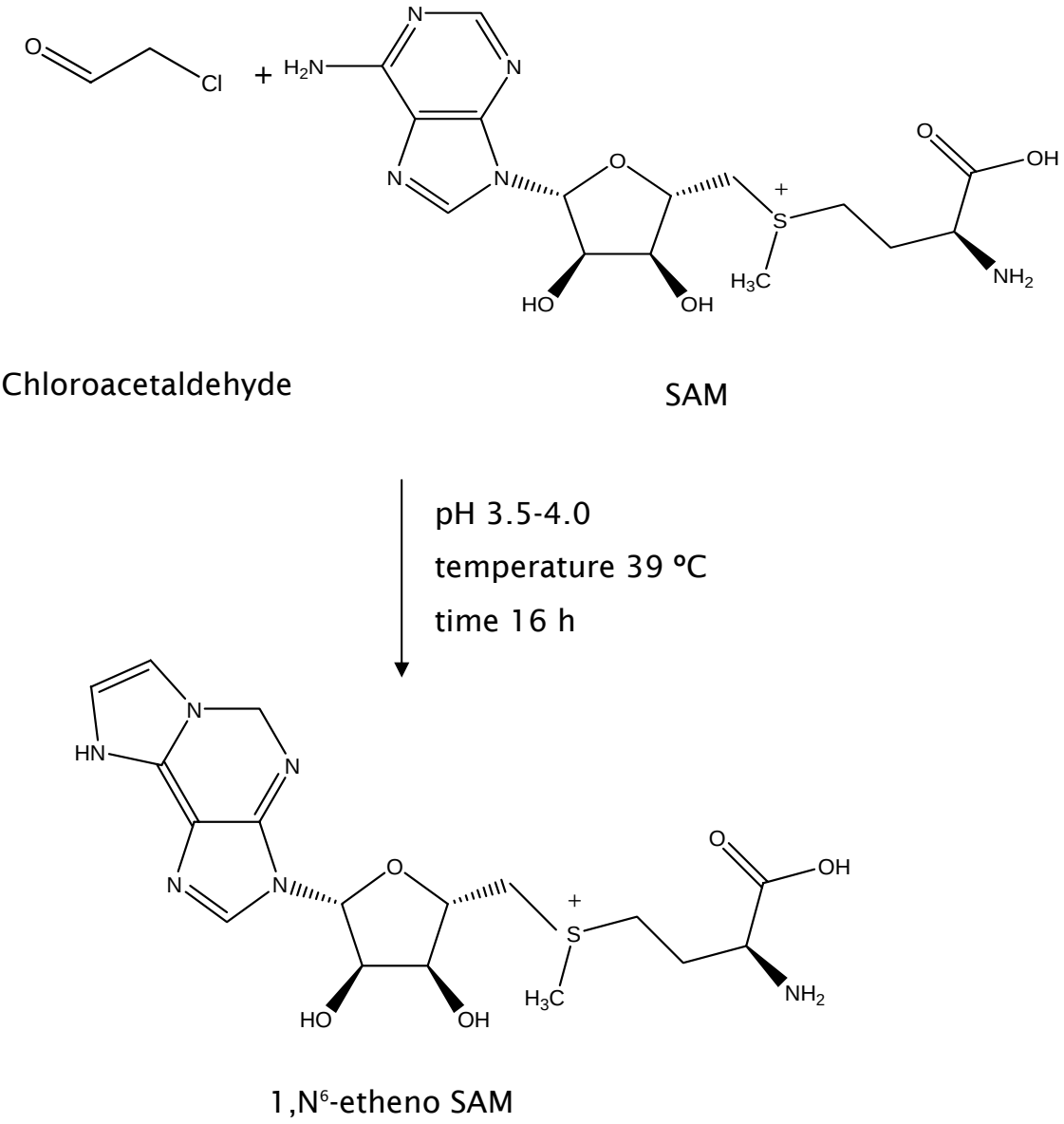

Fig. 3.1: Derivatisation of SAM with chloroacetaldehyde.

and separation with the respective eluent was attempted on the reversed phase columns.

3.4.5 Validation of the assay

The validation of the assay was performed according to the guideline of the Food and Drug Administration (Guidance for Industry, Bioanalytical Method Validation)[142].

3.4.5.1 Selectivity

For the assessment of selectivity chromatogrammes of pooled CSF were compared to chromatogrammes of the same pooled CSF spiked with standard solutions of SAM and SAH.

3.4.5.2 Stability

pH-dependent stability

Prior to preparing stock solutions of SAM and SAH their pH-dependent stability was investigated. The analytes were incubated for 48 h at 37 ºC in aqueous solutions with different pH (1-10). The incubation was followed by the derivatisation and measurement of the analytes.

Stability in acidic CSF samples

Moreover, the stability of the analytes in CSF samples to which perchloric acid (pH 1) was added and which were frozen at -20 ºC, was compared to the stability of *the same* CSF samples frozen without prior acidification (pH 7.4). This experiment was performed since some CSF samples for SAM and SAH analysis obtained from cooperation partners were frozen without prior deproteinisation with perchloric acid.

Room temperture stability in CSF samples

In addition the 1 h room temperature stability of SAM and SAH in the CSF with and without the addition of perchloric acid was investigated in order to determine if there is any loss of the analytes when samples are not frozen or

deproteinised immediately after collection, a situation which may occur in routine hospital practice.

Stability of 1,N^6-etheno derivatives of SAM and SAH in CSF

Furthermore, stability studies were performed in order to assess if the analytes were stable for 24 h and could be left in the autosampler after derivatisation and analysed during the night. CSF samples were spiked with three different concentrations of SAM and SAH. The concentrations corresponded to the lower, middle and upper range of the calibration curve. After derivatisation an aliquot of each sample was analysed and the rest was left on room temperature and the analysis was repeated after 24 h. Such procedure was repeated at two subsequent days.

Freeze and thaw stability

Moreover, the stability of SAM and SAH after two freeze and thaw cycles were analysed. CSF samples were spiked with SAM and SAH in three different concentrations. An aliquot of each of the three CSF samples was derivatised and analysed, the rest was frozen. The frozen aliquots were thawed after two days, analytes were derivatised and the rest was frozen one more time. This was referred to as the first freeze and thaw cycle. The frozen samples were thawed once more, derivatised and analysed. This was the second freeze and thaw cycle.

3.4.5.3 Recovery

The recovery experiment was performed in order to establish if the calibration curves could be obtained from calibrator solutions diluted with isotonic sodium chloride solution. The experiment was performed as described in chapter 3.3.3.3.

3.4.5.4 Limit of detection and lower limit of quantification

LOD and LLOQ were determined as described in chapter 3.3.3.4 and 3.3.3.5.

3.4.5.5 Precision and accuracy

Within-day and between-day precision and accuracy of the method were assessed as described in chapter 3.3.3.6 and 3.3.3.7. Since SAM and SAH are present in the CSF the peak areas had to be corrected by substraction of the value which was measured in the unspiked CSF.

3.4.6 Calibration

3.4.6.1 Preparation of stock solutions

Stock solutions were prepared by dissolving the analytes in water to which perchloric acid was added. Each analyte was weighed three times and the same procedure was used to exclude any imprecision in this step as described in chapter 3.3.4.1. After aliquotation stock solutions were frozen at –20 ºC.

3.4.6.2 Preparation of calibrator solutions

Seven calibration points were used to obtain regression curves for SAM and SAH, respectively. Calibration was performed using pooled CSF as matrix. The calibration range included the LLOQ as the lowest calibration point. The ULOQ was higher than the expected values after possible metabolic changes following MTX administration. The calibrator solutions were obtained by diluting stock solutions of the analytes and each calibrator solution contained both SAM and SAH. Into each of the 7 vials containing 240 µL of pooled CSF 10 µL of the respective calibrator solution was added. 240 µL of the CSF spiked with 10 µL of water and this sample served as reference to determine the endogenous concentrations of SAM and SAH. The final concentrations of the calibration curve for SAM and SAH, respectively, are presented in Table 3.5.

Table 3.5: Final concentration of SAM and SAH in CSF samples used for calibration

	Final concentration (nM)	
Calibration point	**SAM**	**SAH**
1	25.0	5.0
2	50.0	7.5
3	75.0	10.0
4	125.0	20.0
5	250.0	50.0
6	500.0	75.0
7	1000.0	100.0

3.4.6.3 Calculation of concentration

The calibration points and the reference CSF were analysed in a random order and the response in the reference sample was substracted from the calibration points response. Such corrected peak areas were plotted against the concentration of the analyte and the coefficient of regression and residuals were determined (see also chapter 3.3.4.3).

3.4.7 Quality control

Samples were derivatised as described in chapter 3.4.3.

The concentrations of SAM and SAH in the CSF samples of patients were obtained from calibration curves which were obtained every day prior to the analysis of patient samples. Calibration curves were weighted ($1/x^2$ for SAM and $1/x$ for SAH) and they were accepted if the criteria described in chapter 3.3.5 were met. QC samples were used to assure the quality of sample measurement. Two types of quality control samples were used. The first type consisted of QC samples which served to test the HPLC system. Solutions of three different concentrations corresponding to the lower, middle and upper

part of the calibration curve were prepared by diluting stock solutions of SAM and SAH with pooled CSF (as described in chapter 3.4.6.2). The samples were derivatised, aliquoted and frozen at -20 ºC. One aliquot of each concentration was analysed every day to assure the quality of the system.
The second type of QC samples served to assure the quality of the derivatisation. Three solutions of SAM and SAH were prepared in the same manner as for the system check. The solutions were then aliquoted and frozen at -20 ºC. One aliquot of each concentration was derivatised together with the CSF samples of patients and determined on the subsequent day. All together six QC samples each day were analysed inbetween the CSF samples of patients. The quality of the measurement was assured if at least 4 of 6 QC samples were within ± 15% of the nominal value.

3.5 Clinical studies

3.5.1 Adult patients with primary central nervous system lymphoma (PCNSL)

In cooperation with Prof. Dr. Schlegel (Department of Neurology, University Hospital, Rheinische Friedrich-Wilhelms-Universität, Bonn) CSF samples were obtained from five patients suffering from PCNSL. All of them received chemotherapy according to the Bonn protocol for PCNSL.

Patients characteristics

The characteristics of the five patients are shown in Table 3.6.
Patient 5 showed clinical signs of MTX-related chronic neurotoxicity in form of leukoencephalopathy during therapy confirmed by a radiology finding (MRI, T2-weighted).

Table 3.6: Characteristics of patients with PCNSL

Patient	Age (years)	Gender	BSA (m^2)	Morbidity
1	62	F	n.k.	Relapse of PCNSL of the B-cell line
2	40	F	1.62	PCNSL of the B-cell line
3	66	F	1.63	PCNSL of the B-cell line
4	73	F	1.94	Relapse of PCNSL of the B-cell line
5	64	F	1.79	PCNSL of the B-cell line

BSA: body surface area; n.k: not known

Bonn protocol for PCNSL

The Bonn protocol for PCNSL consisted of two cycles (1 and 2). Each cycle consisted of three chemotherapy blocks (A, B and C). The protocol is presented in Table 3.7.

Block A and B were identical regarding MTX therapy. MTX was administered in both blocks as high-dose infusion (5 g/m^2 over 24 h) followed by intraventricular courses (3 mg/day) via an Ommaya reservoir on three subsequent days. If the creatinine clearance which was determined before each block was low (decreased renal function) the dose of MTX was reduced. Patients older than 65 years received 3 g/m^2.

After high-dose MTX patients were rescued with calcium folinate (30 mg/m^2, i.v.). The rescue was given 34, 42, 48 and 54 h after the start of infusion. The MTX concentrations were monitored in the serum at 24, 42, 48 and 54 h after the initiation of infusion. If they were elevated (> 150 µM at 24 h; >1 µM at 42 h; >0.4 µM at 48 h or >0.2 µM at 54 h) rescue was intensified. In that case patients received calcium folinate (30 mg/m^2, i.v) every four hours until the concentration of MTX in serum was below 0.2 µM.

In contrast to block A and B, patients did not receive high-dose MTX in block C. They received four intraventricular courses of MTX through the Ommaya reservoir (day 3-6). Calcium folinate was not administered during this block.

Table 3.7: Bonn protocol for PCNSL

Drug	Dose	Day						
		1	2	3	4	5	6	7
Block A								
MTX*	5 g/m² i.v.	+						
Vincristine	2 mg/m² i.v.	+						
Ifosfamide	800 mg/m² i.v.		+	+	+	+		
Dexamethasone	10 mg/m² p.o.		+	+	+	+		
MTX	3 mg i.cv.		+	+	+			
Prednisolone	2.5 mg i.cv.		+	+	+			
Cytarabine	30 mg i.cv.					+		
Block B								
MTX*	5 g/m² i.v.	+						
Vincristine	2 mg/m² i.v.	+						
Cyclophosphamide	200 mg/m² i.v.		+	+	+	+		
Dexamethasone	10 mg/m² p.o.		+	+	+	+		
MTX	3 mg i.cv.		+	+	+			
Prednisolone	2.5 mg i.cv.		+	+	+			
Block C								
Cytarabine	3 g/m² i.v.	+	+					
Vindesine	5 mg/m² i.v.	+						
Dexamethasone	10 mg/m² p.o.			+	+	+	+	+
MTX	3 mg i.cv.			+	+	+	+	
Prednisolone	2,5 mg i.cv.			+	+	+	+	
Cytarabine	30 mg i.cv.							+

*or 3 g/m² for patients older than 65 years

Between each two blocks patients had two weeks time to recover. Patients with a relapse (patient 1 and 4) were treated according to a modified version of the Bonn protocol which included two cycles (1 and 2) consisting of blocks A and C each. For patient 1 the Bonn protocol for PCNSL was additionally adjusted. The adjustment consisted in the reduction of the dose of MTX (3 g/m^2) and the earlier start of rescue 60 mg, i.v. which occured one hour after the end of high-dose MTX infusion and was repeated every six hours. A total of five calcium folinate infusions was administered to this patient.

CSF sampling

Samples from patient 1 who received an intensified calcium folinate rescue after the high-dose infusion were obtained only at the end of a high-dose MTX infusion (0 h) and 1, 3.5, 7 and 24 h later. This patient received *no* intrathecal MTX. The five obtained samples were collected with ascorbic acid and used for the analysis of 5,10-methylene-THF, 5-methyl-THF, calcium folinate and MTX. After collecting samples were stored at -20 ºC. An overview of the sample collection times of patient 1 is shown in Fig. 3.2.

Samples from patients 2-5 were collected always shortly before the administration of intraventricular MTX therapy. An overview of the sampling time for block A and B is given in Table 3.8. In block C MTX was administered only intraventricularly and samples were obtained from day 3 to 7. Problems such as late inclusion in the study, complications during therapy and therapy break-up occured and it was not possible to obtain samples from all therapy blocks from each patient. An overview of the obtained samples is presented in Table 3.9.

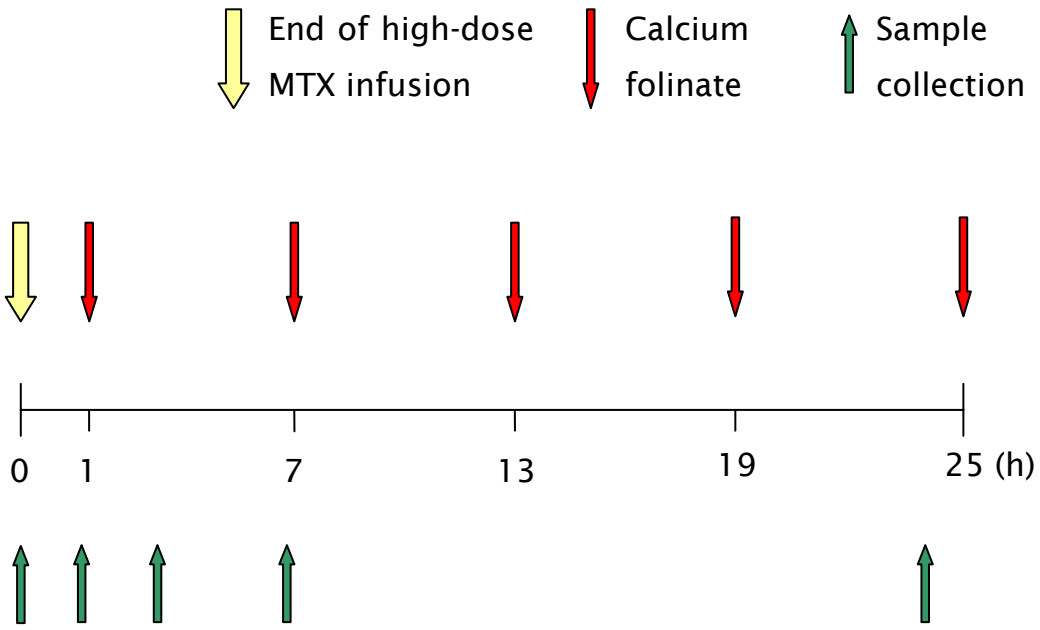

Fig. 3.2: Diagram of the sampling time and calcium folinate rescue of patient 1.

Table 3.8: Sample collection during the Bonn protocol for PCNSL

Block A/B	High-dose MTX	i.cv. MTX	Rescue	Sampling time
Day 1	+			No samples obtained
Day 2		+	+	1h after the end of the infusion and before i.cv. administration
Day 3		+	+ + +	Just before i.cv. administration Patient has received two infusions of calcium folinate prior to sampling
Day 4		+		Just before i.cv. administration Calcium folinate rescue completed prior to sampling
Day 5		+		Just before i.cv. administration

Table 3.9: Overview of collected CSF samples from patient 2-5.

Block	Day	Sample collection			
		Patient 2	Patient 3	Patient 4	Patient 5
A1	2	-	-	+	-
	3	+	-	+	-
	4	+	-	+	-
	5	+	-	+	-
B1	2	+	-	Patient did not receive block B1	+
	3	+	+		+
	4	+	+		+
	5	+	+		+
C1	3	+	+	+	+
	4	+	+	+	+
	5	+	+	+	+
	6	+	+	+	+
	7	+	+	+	+
A2	2	-	-	+	-
	3	-	+	+	+
	4	-	+	+	+
	5	-	+	+	+
B2	2	+	+	Patient did not receive block B2	Patient did not receive block B2 and C2
	3	+	+		
	4	+	+		
	5	+	-		
C2	3	+	+	-	
	4	+	+	-	
	5	+	+	-	
	6	-	+	-	
	7	+	+	-	

+: sample obtained; -: sample not obtained

3.5.2 Pediatric patients with acute lymphoblastic leukaemia (ALL) treated according to ALL BFM 2000

In cooperation with Prof. U. Bode (University Children's Hospital, Bonn, Germany) CSF samples were obtained from a population of pediatric patients with ALL who received high-dose MTX according to the therapy protocol ALL BFM 2000. In the collected CSF samples 5,10-methylene-THF, 5-methyl-THF, calcium folinate, MTX, SAM and SAH were determined.

Patients characteristics

A total of 27 patients suffering from ALL classified into standard-risk or middle-risk were included in this study. Since the therapy protocol is very similar for the two groups they were assessed together. Median age of the patients was 4 years ranging from 2-17 years. 74 CSF samples from 22 patients were obtained for the determination of reduced folates and MTX. SAM and SAH were analysed in 84 CSF samples obtained from 22 patients (see also Appendix B).

Protocol ALL BFM 2000

This protocol consisted of several components which differed between the risk groups but all patients started the therapy with protocol I which consisted of two phases (1 and 2).

Protocol I

During the first seven days of protocol I/phase 1 (also called prednisone prephase) all patients received prednisone which was started on day 1 with 25% of the calculated dose. The dose was then increased rapidly during the next few days to the final-dose of 60 mg/m^2/d p.o. or i.v. which was applied three times in equal daily doses (EDD). The end-dose was achieved at latest on day 5.

After the prednisone prephase, patients were randomised into two groups, one continued the therapy with prednisone (60 mg/m^2/d p.o in 3 EDD) while the other received dexamethasone (10 mg/m^2/d p.o. or i.v. in 3 EDD) instead. All patients received intrathecal MTX on day 1, 12 and 33 of phase 1, the dose was age-dependent (as presented in Table 3.10).

Table 3.10: Age-related dosage of intrathecal MTX in protocol ALL BFM 2000.

Age (years)	MTX (mg)
≥ 1	8
≥ 2	10
≥ 3	12

If the patient had an initial CNS disease or there was a suspicion of it or if lymphoblasts or hyperleucocytosis (≥ 50 000/μL) were identified in an initial lumbar puncture then intrathecal MTX was applied also on day 18 and 27. After the intrathecal administration of MTX patients had to rest with their head down for at least four hours (supine position).

No rescue therapy was applied during this part of treatment. Patients younger than 1 year were treated according to another protocol and therefore not included in the analysis.

During phase 1 patients were treated with vincristine (1.5 mg/m^2), daunorubicine (30 mg/m^2) and asparaginase (5000 IU/m^2). After completing phase 1 patients went into phase 2 in which they received cyclophosphamide (1 g/m^2), mercaptopurine (60 mg/m^2), cytarabine (75 mg/m^2) and intrathecal MTX. The complete therapy during protocol I is presented in Table 3.11.

After completing protocol I patients were categorised into risk groups and received further therapy according to the risk group they belonged. Three groups were defined: standard-risk (SR), middle-risk (MR) and high-risk (HR) according to patients' immunophenotype, immunogenotype, clinical picture and the response in the early phase of therapy. The characteristics of the patients are presented in Table 3.12.

SR and MR patients received similar therapy which consisted of protocol M and protocol II or III while HR patients received HR blocks which is not further described since CSF samples of HR patients were not included in the analysis.

Protocol M

About two weeks after the end of protocol I therapy was continued in SR and MR patients with protocol M. During this treatment phase patients received high-dose MTX (5 g/m^2 p.i. over 24 h), intrathecal MTX (dosage age-dependent see Table 3.10) and 6-mercaptopurine (25 mg/m^2 p.o.). High-dose MTX infusions were administered in two-week intervals. 10% of the high-dose MTX was administered in the first 30 min while the rest of the dose was infused over 23.5 h. The concentration of MTX in serum was monitored at: 24, 36, 42 and 48 h after the beginning of the MTX infusion.

Table 3.11: Protocol I of ALL BFM 2000.

Drug	Dosage	Day	Total no of admin.
	Phase 1		
Prednisone/ Prednisolone*	Beginning with 25%, dose escalation completed until day 5	1-7	7
	60 mg/m²/d p.o. in 3 EDD	8-28	63
	half of the dosage in 3 EDD	29 -31	9
Vincristine	1.5 mg/m² (max. dose 2 mg)	8, 15, 22, 29	4
Daunorubicine	30 mg/m² p.i. 1 h	8, 15, 22, 29	4
Asparaginase	5000 IU/m² p.i. 1 h	12, 15, 18, 21 24, 27, 30, 33	8
MTX	i.th. (see Table 3.10)	1, 12, 33 (18, 27)#	3 (5)
	Phase 2		
Cyclophosphamide	1 g/m² p.i. over 1h	36, 64	2
Mercaptopurine	60 mg/m² p.o.	36 –63	28
Cytarabine	75 mg/m² i.v.	38-41; 45-48 52-55; 59-62	16
MTX	i.th. (see Table 3.10)	45, 59	2

*or Dexamethasone (10 mg/m²/d p.o. or i.v. in 3 EDD) from day 8-28. From day 29-31 the dosage of dexamethasone was reduced to 50%. # only patients with CNS disease

Calcium folinate rescue (15 mg/m² i.v.) was started 42 h after the initiation of the MTX infusion if the serum MTX concentrations were as expected (see Table 3.13). If serum MTX level was higher than expected rescue was intensified and started at 36 h.

Table 3.12: Categorisation of ALL patients according to their characteristics.

Category	Characteristics of patients
Standard-risk (SR)	• Adequate response to a 7-day prednisone prephase and i.th. MTX on day 1: < 1000 WBC/µL blood on day 8 • Cytomorphological complete remission on day 33 • No presence of translocation t(9; 22) or t(4; 22) or fusion gene Bcr/Abl or MLL/AF4 • MRD-negative on day 33 and before protocol M, measured with at least two markers with a sensitivity of $\leq 10^{-4}$ Patients are categorised as SR only when all four criteria are fulfilled
Middle-risk (MR)	• Adequate response to a 7-day prednisone prephase and i.th. MTX on day 1: < 1000 WBC/ µL blood on day 8 • Cytomorphological complete remission on day 33 • No presence of translocation t(9; 22) or t(4; 22) or fusion gene Bcr/Abl or MLL/AF4 • Patient does not fulfill neither the MRD criterion for SR nor for HR Patients are categorised as MR only when all the above mentioned criteria are met
High-risk (HR)	• Inadequate response to a 7-day prednisone prephase and i.th. MTX on day 1: > 1000 WBC/µL blood on day 8 • Cytomorphologically no complete remission on day 33 which means non-response on that day • Presence of translocation t(9; 22) or fusion gene Bcr/Abl • Presence of translocation t(4; 11) or fusion gene MLL/AF4 • MRD $\geq 10^{-3}$ (high level of residual disease) prior to protocol M Patients are categorised as HR if only one of the criteria is fulfilled

MRD: minimal residual disease; WBC: white blood cell count

Table 3.13: Expected concentration in serum and rescue regimen after the high-dose MTX infusion.

Time after beginning of the MTX-infusion (h)	Expected concentration of MTX in serum (µmol/L)	Dose of calcium folinate (mg/m² i.v.)
24	≤ 150.0	-
36	≤ 3.0	-
42	≤ 1.0	15
48	≤ 0.4	15
54	≤ 0.4	15

Intrathecal MTX was administered 1 h after beginning of the high-dose MTX infusion. An overview of protocol M is given in Table 3.14. After completing protocol M, SR and MR patients were randomised into SR-1, SR-2, MR-1 and MR-2. SR-2 and MR-2 received protocol II while SR-1 and MR-1 received protocol III.

Table 3.14: Protocol M of ALL BFM 2000

Drug	Dosage	Day	Total no of admin.
6-mercaptopurine	25 mg/m² p.o.	1-56	56
MTX	5 g/m² i.v.	8, 22, 36, 50	4
MTX	i.th. (see Table 3.10)	8, 22, 36, 50	4

Protocol II

This protocol consisted of two phases (1 and 2) and was initiated about two weeks after the end of protocol M.

In phase 1 patients received dexamethasone (10 mg/m²/d p.o. or i.v. in 3 EDD), vincristine (1.5 mg/m² i.v., max. dose 2 mg), doxorubicine

(30 mg/m² p.i. over 1 h), asparaginase (10000 IU/m² p.i. 1 h) and if they had CNS disease intrathecal MTX (age-dependent dosage see Table 3.10). Phase 1 was followed by phase 2 in which patients received cyclophosphamide (1 g/m² p.i. over 1 h), thioguanine (60 mg/m² p.o.), cytarabine (75 mg/m² i.v.) and intrathecal MTX. An overview of protocol II is presented in Table 3.15. Calcium folinate rescue after intrathecal MTX was not administered in protocol II.

Table 3.15: Protocol II of ALL BFM 2000.

Drug	Dosage	Day	Total no of admin.
	Phase 1		
Dexamethasone	10 mg/m² p.o./i.v. 3 EDD	1-21	63
	Dosage reduced to 50%	22-24	9
Vincristine	1.5 mg/m² i.v. (max. dose 2 mg)	8, 15, 22, 29	4
Doxorubicine	30 mg/m² p.i. 1h	8, 15, 22, 29	4
Asparaginase	10000 IU/m² p.i. 1 h	8, 11, 15, 18	4
MTX*	i.th. (see Table 3.10)	1, 18	2
	Phase 2		
Cyclophosphamide	1 g/m² p.i. 1 h	36	1
Thioguanine	60 mg/m² p.o.	36-49	14
Cytarabine	75 mg/m² i.v.	38-41, 45-48	8
MTX	i.th. (see Table 3.10)	38, 45	2

*MTX was administered in phase 1 only to patients with overt disease; EDD: equal daily dose

Protocol III

This protocol was applied to SR-1 patients once and MR-1 patients twice. Protocol III was started approximately two weeks after the end of protocol M.

This protocol was also divided into two phases. In both of them patients received the same medication in the same dosage (except cyclophosphamide in phase 2 0.5 g/m² p.i. over 1 h instead of 1 g/m²) as in the respective phases of protocol II but the number of administrations and time intervals between them were different. An overview of protocol III is presented in Table 3.16.

Table 3.16: Protocol III of ALL BFM 2000.

Drug	Dosage	Day	Total no of admin.
	Phase 1		
Dexamethasone	10 mg/m² p.o/i.v. 3 EDD	1-15	45
	Dosage reduced to 50%	16-18	9
Vincristine	1.5 mg/m² i.v. (max dose 2 mg)	1, 8	2
Doxorubicine	30 mg/m² p.i. 1h	1, 8	2
Asparaginase	10000 IU/m² p.i. 1 h	1, 4, 8, 11	4
MTX*	i. th (see Table 3.10)	1	1
	Phase 2		
Cyclophosphamide	0.5 g/m² p.i. 1 h	15	1
Thioguanine	60 mg/m² p.o.	15-28	14
Cytarabine	75 mg/m² i.v.	17-20, 24-27	8
MTX	i.th. (see Table 3.10)	17, 24	2

*MTX was administered in phase 1 only to patients with overt disease

One week after completing protocol III patients who were classified into MR-1 received a so called 'interval therapy' over 10 weeks. This therapy consisted of 6-mercaptopurine (50 mg/m² p.o. daily) and MTX (20 mg/m² p.o. once a week). About a week after the end of the interval therapy patients received protocol III for the second time.

Two weeks after completing the last protocol patients started receiving a maintenance therapy which was the same as the above mentioned interval therapy and was administered until 24 months after diagnosis.

CSF sampling

CSF samples were obtained by lumbar puncture during the protocols I, M, II and III as presented in Table 3.17.

The samples were obtained as described in chapter 3.3.2 and 3.4.2. 5,10-methylene-THF, 5-methyl-THF, calcium folinate, MTX, SAM and SAH were measured.

Table 3.17: Sample collection during ALL BFM 2000.

ALL BFM 2000	Day
Protocol I	1, 12, 33, 45, 59
Protocol M	8, 22, 36, 50
Protocol II	38, 45
Protocol III	17, 24

Patients with manifest signs of neurotoxicity

In addition, samples were obtained from three patients with clinical signs of MTX-associated neurotoxicity. Their characteristics and sample schedule are presented in this chapter.

Case 1

A 15-year old boy, was diagnosed for the first time with ALL and classified as MR-1. Chemotherapy was initiated according to ALL BFM 2000. During protocol I the patient received a cumulative dose of 60 mg MTX intrathecally which was not accompanied by calcium folinate rescue. After completing protocol I without complications therapy was continued with protocol M in which the patient received high-dose MTX (5 g/m^2) combined with intrathecal MTX (12 mg) and calcium folinate rescue. Ten days after receiving the third

high dose of MTX the patient presented with signs of neurotoxicity. He complained of headache and dizziness, was agitated and irritable. The right arm and leg were paralysed (hemiparesis) as well as the left facial side. Seizures did not occur. Symptoms resolved completely within a few days and the therapy was continued according to the protocol. Ten days after the next (fourth) high-dose MTX infusion neurotoxicity reoccured. This time hemiparesis presented in the left body half and the right facial side. At this time-point an evaluation of neurological symptoms was performed by pediatric oncologists using common toxicity criteria (CTC).

MRI of the brain was performed revealing ischemic lesions in the white matter characterised as supraventricular infarctions. Signs of neurotoxicity persisted for about 24 h and resolved again abruptly and completely. Three days after the second toxicity episode CSF was collected by lumbar puncture. Furthermore, one patient sample was obtained during protocol I (day 45) and another one was obtained one month after neurotoxicity (protocol III day 17).

Case 2

A 13-year old boy was diagnosed for the first time with ALL. After being classified and randomised into the MR-1 arm he started receiving chemotherapy according to ALL BFM 2000. Similar to case one, this patient received a cumulative dose of 60 mg MTX intrathecally during protocol I without signs of neurological complications. One week after receiving his first high-dose (5 g/m^2) and intrathecal (12 mg) MTX administration followed by calcium folinate rescue the patient presented with paresis in the right hand which lasted about 36 h. After the second therapy course the same symptoms occured in the left hand and leg. Patient also developed a transient speech disorder. Within 72 h the clinical symptoms resolved but intention tremor and an altered reflex activity were observed.

Neurotoxicity evaluation was performed according to the CTC after the second toxicity episode by pediatric oncologists. MRT revealed changes in the white matter (demyelination) but neither ischemic lesions nor overt disease were present.

Four CSF samples were obtained one of them prior to neurotoxicity (Protocol I, day 33). One sample was obtained one day after resolution of symptoms following the second toxicity episode. One week later, MTX therapy was continued and a sample was obtained during the second high-dose infusion and just before intrathecal administration. Ten days later patient presented with nausea and emesis and a sample was obtained in conjunction with diagnostic lumbar puncture.

Case 3

The third patient with signs of neurotoxicity was a 35-year old female who received chemotherapy after her second ALL relapse. The patient had received MTX after the diagnosis of ALL as well as after the first relapse. The disease was firstly diagnosed at the age of 17 and the patient received MTX therapy accompanied by cranial irradiation. She was not treated in Bonn and it was not possible to obtain documentation from that period in order to assess possible neurological complications. Ten years later she developed her first relapse and was treated with high-dose (1 g/m^2) and intrathecal (12 mg) MTX without cranial irradiation. A total of nine high-dose infusions and 60 mg intrathecal MTX were administered. MTX-related neurological complications were not observed at that time. Six years later she started receiving therapy for the second relapse which did *not* include MTX. However, the patient had evident signs of MTX-related chronic neurotoxicity. Cognitive decline, memory loss, confusion, depressed level of conciousness, personality change, speech impairment and tremor were documented. The evaluation of neurotoxicity was performed according to CTC as in the former two cases. MRI revealed severe leukoencephalopathy in both brain hemispheres. Also circular lesions were present which were associated with an overt infection.

CSF was obtained at time of second relapse diagnosis. The second sample was obtained three months later. Ten days after collecting the second sample the patient presented with signs of toxicity probably due to a CNS infection. She had severe headache and pain followed by high fever. A lumbar puncture

was performed for diagnostic purposes. The CSF had an intensive yellow colour indicating hyperbilirubinemia which was later confirmed.

3.5.3 Pediatric patients with acute lymphoblastic leukaemia (ALL) treated according to TOTAL XV

CSF samples from a group of pediatric patients with ALL who were treated with high-dose MTX according to the protocol TOTAL XV were obtained in cooperation with Prof. M. Relling (St. Jude Children's Research Hospital, Memphis, USA). These children were part of a prospective clinical study in which the aim was primarily to analyse the homocysteine metabolism after high-dose MTX.

Patients characteristics

CSF samples for the determination of 5,10-methylene-THF, 5-methyl-THF, calcium folinate and MTX were obtained from 27 patients. The median age was 4 years, ranging from 1-14 years. A total of 53 samples was analysed. Because of the small number of samples all patients were assessed together regardless of their risk classification.

Protocol TOTAL XV

This protocol consisted basically of three therapy blocks: Remission/Induction, consolidation and maintenance therapy.

Remission/Induction (6-7 weeks)

Remission/Induction therapy was initiated by intrathecal administration of cytarabine (20-40 mg, age-dependent) and high-dose MTX-infusion (1 g/m^2 over 4 or 24 h). The latter is also referred to as Upfront-high-dose MTX. Calcium folinate rescue was started 42 h after beginning of the MTX infusion. The initial dose was 50 mg/m^2 i.v. and 15 mg/m^2 i.v. were administered every six hours later-on. All together 7 administrations of calcium folinate were foreseen and the concentration of MTX in serum was monitored throughout the rescue. If the level of MTX was higher than 1 µM at 42 h after the beginning of the infusion calcium folinate rescue was intensified.

Treatment was continued with prednisone (40 mg/m²), vincristine (1.5 mg/m²), daunorubicine (25 mg/m²) and asparaginase (10000 IU/m²) four days after high-dose MTX infusion. These drugs were administered for the subsequent 24 days. The second part of the Remission/Induction therapy consisted of high-dose cyclophosphamide (1 g/m²), cytarabine (75 mg/m²) and 6-mercaptopurine (60 mg/m²). An overview of the exact dosage regimen of the Remission/Induction therapy is presented in Table 3.18.

Table 3.18: Remission/Induction therapy

Drug	Dosage	Day	Total No of admin.
Cytarabine	20-40 mg	1	1
MTX	1 g/m² i.v.	1	1
Prednisone	40 mg/m² i.v.	5-32, 3 EDD	84
Vincristine	1.5 mg/m² i.v.	5, 12, 19, 26	4
Daunorubicine	25 mg/m² i.v.	5, 12	2
L-Asparaginase	10.000 IU/m² i.m.	6, 8, 10, 12, 14, 16 (19, 21, 23)*	6 (9)
Cyclophosphamide	1 g/m² i.v.	26	1
Cytarabine	75 mg/m² i.v.	27-30, 34-37	8
6-Mercaptopurine	60 mg/m² p.o.	26-39	14
TIT	i.th. see Table 3.19	19 (8, 26)#	1 (3)

*Administration of drug only if there were ≥ 5% blasts on day 19 of Remission/Induction

Only patients with CNS status 2 or 3 or Philadelphia chromosome. EDD: equal daily dose

Triple intrathecal therapy (TIT) consisting of MTX, hydrocortisone and cytarabine was given on day 19 of the Remission/Induction block (patients with CNS status 2 or 3 as well as patients with Philadelphia chromosome received this therapy additionally on day 8 and 26). Drug dosage was age-dependent (see Table 3.19). Rescue with calcium folinate (5 mg/m² p.o.) was administered 24 h and 30 h after the TIT.

Table 3.19: Dosage of the triple intrathecal therapy (TIT) according to age.

Age (months)	MTX (mg)	Hydrocortisone (mg)	Cytarabine (mg)
13-23	8	16	24
24-35	10	20	30
≥ 36	12	24	36

Similar to ALL BFM 2000 patients were categorised into three groups: Low-risk (LR), standard-risk (SR) and high-risk (HR). Risk was defined by patients' immunophenotype, immunogenotype, clinical picture and response in the early phase of therapy. The characteristics of the patients are presented in Table 3.20.

Consolidation (8 weeks)

During consolidation therapy patients received high-dose MTX infusion which was followed by the TIT on day 1, 15, 29 and 42. High-dose MTX was given as 24 h-infusion, where 10% of the whole dose was administered within the first hour (loading dose). The dosage of MTX depended on the risk classification of the patient and the MTX clearance which was determined after the first high-dose infusion of MTX (Upfront-high-dose MTX, see Remission/Induction). Furthermore, mercaptopurine (50 mg/m^2) was given every day. The chemotherapy regimen during the consolidation therapy is presented in Table 3.21.

Calcium folinate rescue was started 42 h after the beginning of high-dose MTX. A total of 5 doses of calcium folinate was administered (standard or high-risk, 15 mg/m^2 i.v. or p.o.; low-risk 10 mg/m^2 i.v. or p.o. every six hours). The rescue was intensified if the concentration of MTX in serum was > 1 µM 42 h after the beginning of the infusion.

Table 3.20: Categorisation of patients treated with TOTAL XV according to their characteristics.

Category	Characteristics of patients
Low-risk (LR)	• Age: 1-10 years • pre-B ALL • Leukocyte count < 50 x 10^9/L
Standard-risk (SR)	• Age: 1-16 years • pre-B ALL (with translocation t(1; 19) of the E2A-PBXI-fusion gene or MLL-rearrangement), T-ALL, testicular leukaemia, • hypoploidic chromosomes (< 45 chromosomes) • CNS 3 status (≥ 5 Leucocytes/µL CSF, blasts in CSF) • ≥ 5% blasts in the bone marrow on day 19 or 26 of R/I • minimal residual disease (≥ 0.01%) on day 46 of R/I
High-risk (HR)	• Age: 1-16 years • Patients with Philadelphia chromosome (Bcr-Abl) • ≥ 1% blasts in the bone marrow on day 46 of the R/I • ≥ 0.1% blasts in the bone marrow 16 weeks after R/I

R/I: Remission/Induction; MLL: mixed lineage leukaemia

Table 3.21: Consolidation therapy

Drug	Dose	Day	Total no of admin.
MTX	ca. 2.5 g/m^2 i.v (LR)* ca. 5.0 g/m^2 i.v. (SR, HR)#	1, 15, 29, 43	4
6-Mercaptopurine	50 mg/m^2 p.o.	1-56	56

*Desired steady-state concentration in plasma for low-risk patients: 33 µM
#Desired steady-state concentration in plasma for standard/high-risk patients: 65 µM

Maintenance therapy (120–146 weeks)

Maintenance therapy was started 7 days after the last high-dose MTX infusion of the consolidation block. The chemotherapy in this block depended strongly upon the risk categorisation of the patient. Patients received a treatment schedule during week 1-6 which was repeated during week 11-16 (seeTable 3.22). During week 7-9 and 17-19 Reinduction I and II therapy was given (presented in Table 3.23).

Table 3.22: Maintenance therapy during week 1-6 and 11-16

Drug	LR			SR, HR		
	Dosage	Day of week	Total no of admin.	Dosage	Day of week	Total no of admin.
MTX	40 mg/m² i.v.	1#	4	---	---	---
6-Mercaptopurine	75 mg/m² p.o.	1-7	42	50 mg/m² p.o.	1-7	42
Doxorubicine	---	---	---	30 mg/m² i.v.	1*	2
Vincristine	2 mg/m² i.v.	1*	2	2 mg/m² i.v.	1*	2
L-Asparaginase	---	---	---	2500 IU/m² i.m.	1	6
Dexamethasone	8 mg/m² p.o.	1-5* 3EDD	30	12 mg/m² p.o.	1-5* 3EDD	30

*only in the 1st and 4th week; # only in the 2nd, 3rd, 5th and 6th week

From week 21-28 patients received a regimen presented in Table 3.24 which was repeated five times. Subsequently (from week 68-100) patients received 6-mercaptopurine (daily 75 mg/m², p.o.), MTX (once a week 40 mg/m², i.v.) and dexamethasone (every four weeks, 8 or 12 mg/m², p.o.) in combination

Table 3.23: Reinduction therapy I (week 7-9) and II (week 17-19)

Drug	LR			SR, HR		
	Dosage	Day of week	Total no of admin.	Dosage	Day of week	Total no of admin.
Reinduction I						
Doxorubicine	30 mg/m² i.v.	1	1	30 mg/m² i.v.	1, 8	2
Vincristine	1.5 mg/m² i.v.	1, 8, 15	3	1.5 mg/m² i.v.	1, 8, 15	3
L-Asparaginase	10000 IU/m² i.m.	3 days per week	9	25000 IU/m² i.m.	1, 8, 15	3
Dexamethasone	8 mg/m² p.o.	1-8, 15-21	48	8 mg/m² p.o.	1-8, 15-21	48
TIT	see Table 3.19	1	1	see Table 3.19	1	1
Reinduction II						
Doxorubicine	30 mg/m² i.v.	1	1	---	---	---
Vincristine	1.5 mg/m² i.v.	1, 8, 15	3	1.5 mg/m² i.v.	1, 8, 15	3
L-Asparaginase	10000 IU/m² i.m.	3 days per week	9	25000 IU/m² i.m.	1, 8, 17	3
Dexamethasone	8 mg/m² p.o.	1-8; 15-21	48	8 mg/m² p.o.	1-8, 15-21	48
Cytarabine	---	---	---	2 g/m² i.v. 2 EDD	15, 16	4
TIT	See Table 3.19	1	1	See Table 3.19	1	1

TIT: triple intrathecal therapy; EDD: equal daily doses

Table 3.24: Maintenance therapy during week 21-28.

Drug	LR			SR, HR		
	Dosage	Day of week	Total no of admin.	Dosage	Day of week	Total no of admin.
MTX	40 mg/m² i.v.	1[a]	6	40 mg/m² i.v.	1[b]	4
Vincristine	2 mg/m² i.v.	1*	2	2 mg/m² i.v.	1*	2
Cyclophosphamide	---	---	---	300 mg/m² i.v.	1[#]	2
Dexamethasone	8 mg/m² p.o.	1-5*	10	12 mg/m² p.o.	1-5*	10
Cytarabine	---	---	---	300 mg/m² i.v.	1[#]	2
6-Mercaptopurine	75 mg/m² p.o.	1-7	56	75 mg/m² p.o.	1-7	56

* only in the 24th and 28th week; # only in the 23rd and 27th week; [a] only in the 21st -23rd week and 25th -27th week; [b] only in the 21st, 22nd, 25th and 26th week

with vincristine (2 mg/m², i.v). Girls completed the therapy after 100 weeks, boys received 6-mercaptopurine and MTX for another 46 weeks. Triple intrathecal therapy was applied at least seven times but not more than sixteen times which depended on the risk profile of the patient.

CSF sampling

Not more than three CSF samples were obtained from each patient by lumbar puncture. Samples were collected before the Remission/Induction therapy and on day 1 and day 15 of the consolidation block. CSF was obtained immediately before the intrathecal administration of MTX.

For the analysis of 5,10-methylene-THF, 5-methyl-THF, calcium folinate and MTX, samples were collected into vials containing ascorbic acid as described

in chapter 3.3.2. SAM and SAH were not determined since CSF samples stabilised with perchloric acid were not obtained (see also chapter 3.4.2).

3.6 Statistical data analysis

Statistical analysis of data was performed by the use of Excel® 2002 (Microsoft, Redmond, USA) and SPSS 12.0 for Windows® (SPSS Inc., Chicago, USA).

3.6.1 Descriptive statistics

In order to describe the obtained data measures of central tendency were used. Central tendency considers two important aspects of a continuous variable which are: the center of the distribution and how the observations are dispersed within the distribution.
To describe the center of distribution sample median and sample mean (see Eq. 3.8) were considered

Sample median ($\tilde{x}$): The sample median is the middle of a distribution: half of the scores are above the median and half are below. If there is an even number of observations the median is the average of the two central scores

Sample mean ($\overline{x}$):

$$\overline{x} = \frac{\sum_{i=1}^{n} x_i}{n} \qquad \text{Eq. 3.8}$$

x_i = individual values

n = total number of sample observations

The following measures were used for the description of how the observations were dispersed within a distribution:

Standard deviation (SD):

$$SD = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}} \qquad \text{Eq. 3.9}$$

$\bar{x}$ = mean value

x_i = individual value

n = total number of sample observations

Relative standard deviation (RSD): see Eq. 3.3

Range: The range represents the difference between the largest (maximum) and the smallest (minimum) value.

The data were graphically presented with the use of box-and-whisker plots. The box plot is a rectangular box in which the upper line represents the third quartile (75% of observations are below) and the lower line the first quartile (25% of observations are below). The horizontal line in the box represents the second quartile (median). The vertical lines (whiskers) extend from the top or bottom of the box to the maximum and minimum value, respectively. Values that are above or below one and a half box length (calculated from top or bottom of the box) are defined as outliers and marked with a circle. Values that are above or below the range of three box lengths are defined as extreme values and marked with a starsign (see Fig. 3.3).

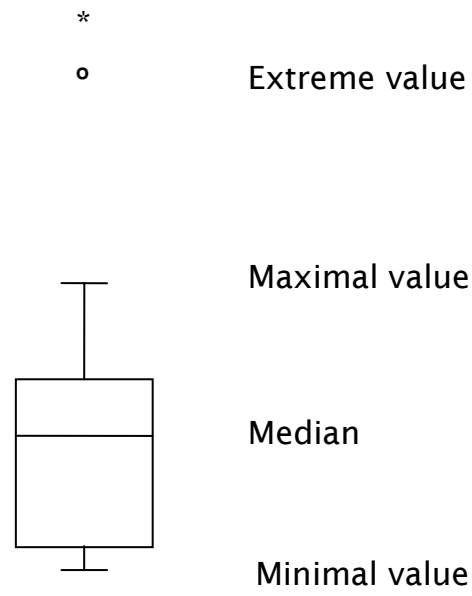

Fig. 3.3: Box-and-whisker plot

3.6.2 Inferential statistics

Inferential statistics was used to infer (make predictions) about a population based on the data obtained from a sample of the population. By convention the null hypothesis is stated as no real difference between two or more populations. The alternative hypothesis (research hypothesis) is stated as the opposite to the null hypothesis. The null hypothesis is tested using inferential statistics. In this work the null hypothesis was rejected at an error probability (p) of ≤ 0.05 which means that there was a probability of no more than 5% of rejecting a true null hypothesis.

In order to assess whether data were normally distributed the Shapiro-Wilk-Test was used for samples with no more than 50 observations and the Kolmogorov-Smirnov-Test for samples with more than 50 observations. The null hypothesis in both tests was rejected at a significance level $p < 0.05$ meaning that the distribution was significantly different from normal distribution. Small samples (less than 10 values) were treated as if they were not normally distributed.

Various statistical tests were used to investigate if there was any difference in the measured parameter between two or more samples. The selection of an appropriate test depended upon the type of variables (continuous or discrete), their distribution and number. Moreover, it was considered whether the variables were dependent or independent from each other. An overview of the tests used is presented in Fig. 3.4.

3.6.3 Correlation analysis

Correlation was used to determine the degree to which two variables showed a possible interrelationship. The correlation coefficient (r) was used to measure the strength of the relationship between two variables as well as the direction of the relationship. This is a well-defined mathematical index which can take values from -1.00 to +1.00. If r = 1.00 there is a perfect positive correlation, r = -1.00 indicates a perfect negative (inverse) correlation and r=0 means that there is no linear correlation between the two variables. If the variables were normally distributed the correlation coefficient was calculated according to Pearson. For variables measured on an ordinal scale or not normally distributed Kendall rank correlation was used to quantify the possible relationship between two variables.
In Table 3.25 the interpretation of correlation coefficients is summarised. The statistical significance of a given correlation was also tested and the level of significance was set to $p < 0.05$.

3.6.4 Regression analysis

Regression analysis was used to evaluate the relationship between an independent (predictor) and a dependent (response) variable. A least-squares regression line was computed to describe a linear relationship (see Eq. 3.6) between the predictor variable (x) and the response variable (y).

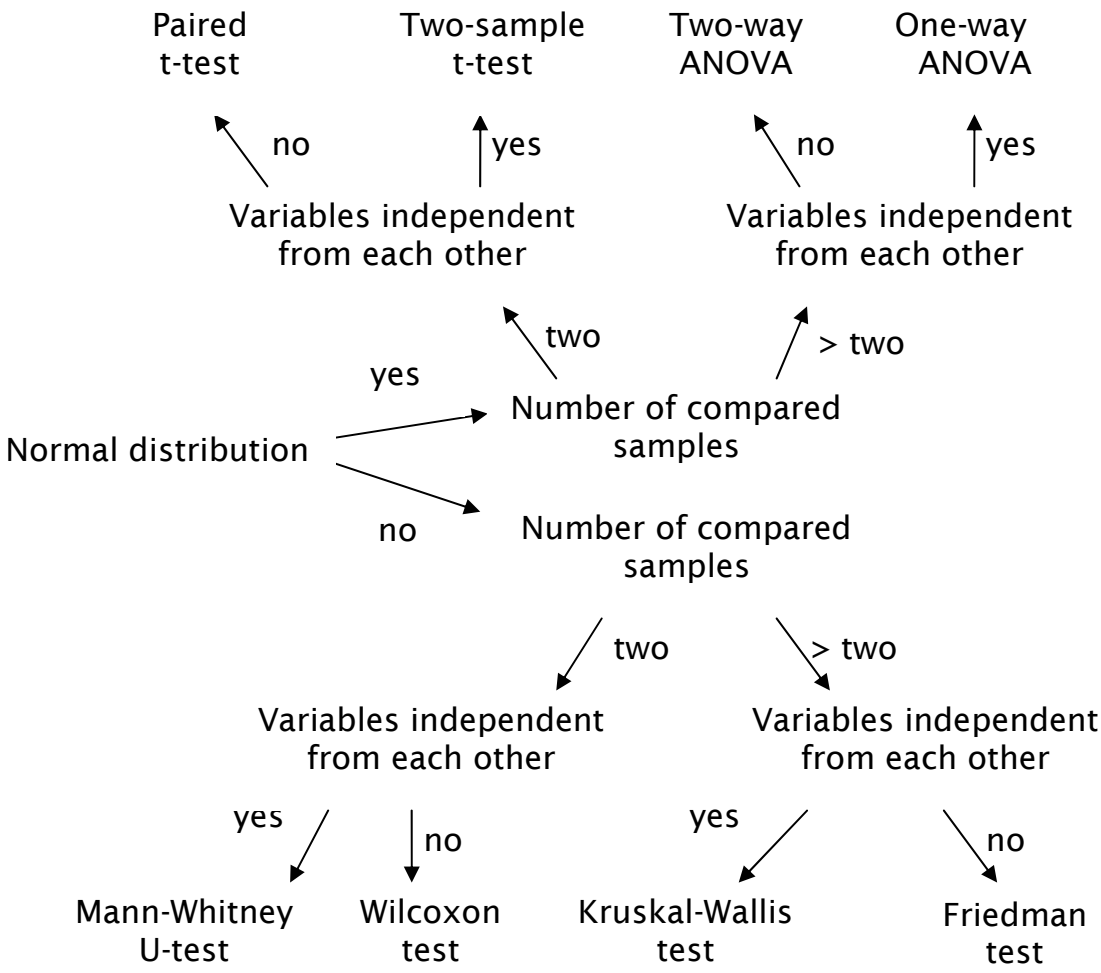

Fig. 3.4: Decision algorithm for the selection of the appropriate statistical test.

Table 3.25: Interpretation of correlation coefficients

Coefficient of correlation (r)	Degree of correlation
< 0.2	Slight, negligible
0.2 – 0.4	Weak
0.4 – 0.7	Moderate
0.7 – 0.9	High, strong
> 0.9	Very high, very strong

4 RESULTS

4.1 Analysis and validation

HPLC analysis was used for the determination of reduced folates and MTX as well as SAM and SAH. The results of method development and validation are presented in this chapter.

4.1.1 HPLC analysis for the determination of reduced folates and MTX in CSF

Belz et al.[141] developed a method for the determination of 5-methyl-THF, calcium folinate and MTX using HPLC with UV and fluorescence detection. This method was used as a basis and was further modified to enable the quantification of 5,10-methylene-THF.
Fluorescence detection was used for the determination of 5-methyl-THF and 5,10-methylene-THF, whereas calcium folinate and MTX were quantified with the use of UV detection.

4.1.1.1 Method development

The mobile phase from Belz et al.[141] was modified. Methanol was used for elution instead of acetonitrile since it has a higher polarity index (6.6) and a lower eluotrope equivalent (5.5) in comparison to acetonitrile (6.2 and 7.3, respectively)[147]. Therefore, retention times of the analytes were prolonged when an equivalent concentration of methanol was used compared to acetonitrile. This further enabled a better separation of peaks in the chromatogramme. Such change in mobile phase required an optimisation of the gradient. The resulting gradient is presented in Table 3.1.
In order to define the optimal excitation and emission wavelength for the endogenous reduced folates a scan was performed with the fluorescence detector. The results revealed an optimal excitation (λ = 294 nm) and emission wavelength (λ = 356 nm) which was in accordance with literature

data[141]. The UV detector was used in parallel with the fluorescence detector. In that way MTX which has no natural fluorescence could be analysed simultaneously with the reduced folates. A scan was performed to find the optimum wavelength (λ = 310 nm). Calcium folinate was also detected by UV absorption whereas the peaks of 5,10-methylene-THF and 5-methyl-THF were seen only at higher concentrations indicating that UV detection is not sensitive enough for determining lower concentrations of these compounds.

4.1.1.2 Selectivity

Chromatogrammes presented in Fig. 4.1 show CSF samples before and after spiking with 5-methyl-THF, 5,10-methylene-THF, calcium folinate and MTX. 5-methyl-THF and 5,10-methylene-THF are endogenous substances in the CSF and were identified by the enlargement of the respective peaks after spiking.

4.1.1.3 Stability

In order to investigate the stability of the analytes in CSF in presence of ascorbic acid, samples were kept at room temperature or 4 ºC and analysed. Two freeze and thaw cycles were performed as well. The stability investigation is described in chapter 3.3.3.2.

Stability of the analytes at room temperature

Table 4.1 shows the stability of the analytes at room temperature. The range is obtained from three different concentrations of each analyte which corresponded to the lower, middle and upper range of the calibration curve. Stability was calculated with the use of Eq. 3.1. If there was no tendency during the stability observation period and if the results were within the range of instrument precision then the analytes were considered to be stable.

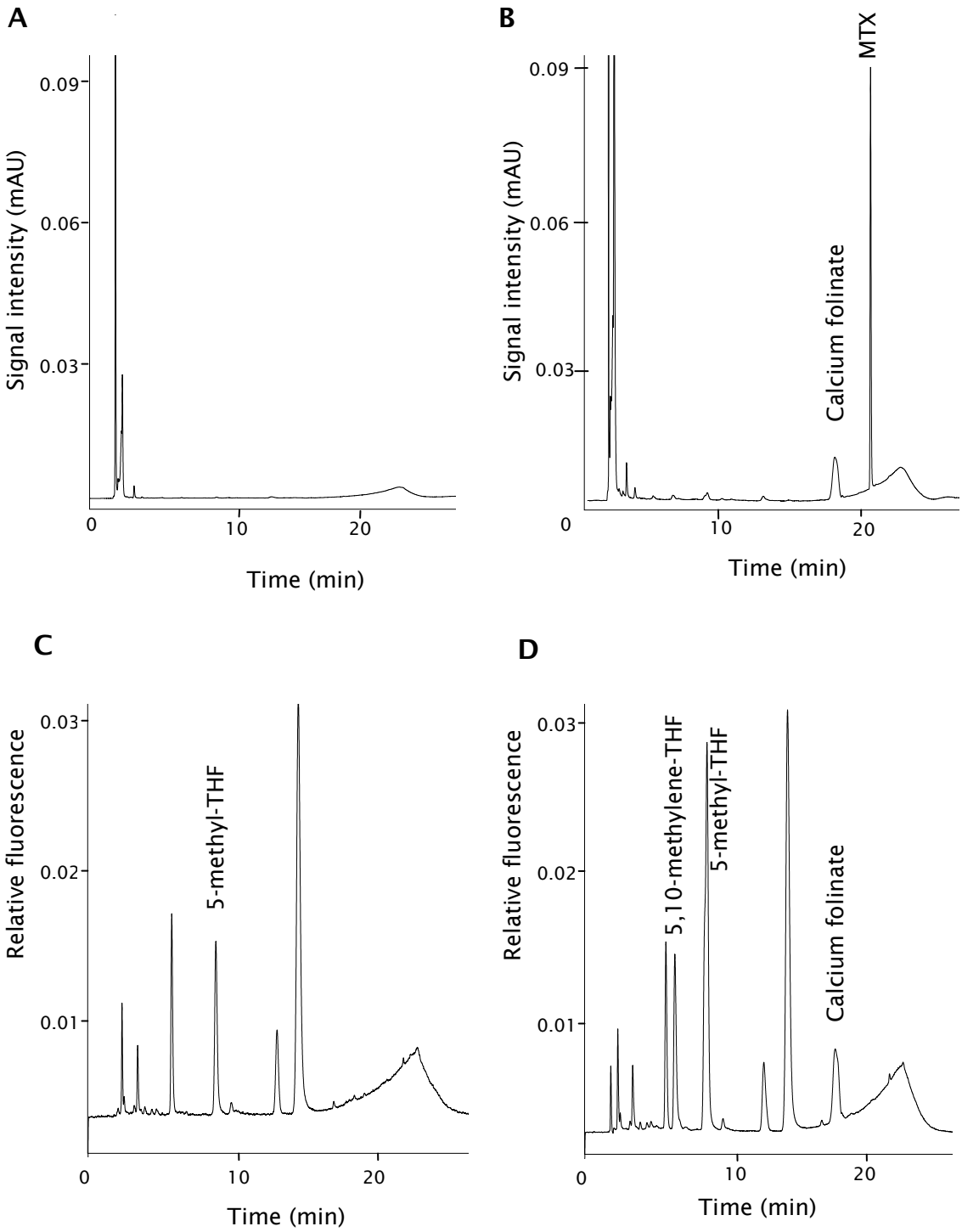

Fig. 4.1: Characteristic chromatogrammes of pooled CSF (A: UV detection, C: fluorescence detection) and pooled CSF spiked with 25 nM 5,10-methylene-THF, 25 nM 5-methyl-THF, 500 nM calcium folinate and 500 nM MTX (B: UV detection, D: fluorescence detection).

Table 4.1: Room temperature stability of 5,10-methylene-THF, 5-methyl-THF, calcium folinate and MTX in CSF (n=3).

Time (h)	Stability at room temperature (%)			
	5,10-MeTHF	5-MTHF	Ca-folinate	MTX
1	98.5-101.2	97.7-100.9	98.8-102.4	99.9-100.3
2	95.7-98.2	97.1-99.7	99.0-101.5	99.3-99.8
3	95.6-96.7	95.6-99.8	99.8-100.4	99.7-102.6
4	91.8-93.8	95.5-98.4	97.1-99.9	95.8-100.1
5	89.6-91.1	95.4-97.5	98.2-99.5	99.6-100.2
6	88.5-90.3	95.9-96.8	98.0-99.1	97.6-100.1
7	86.7-88.4	95.6-96.3	98.4-100.3	100.0-103.4
8	83.6-85.7	95.9-96.5	97.7-100.0	99.4-100.1
9	83.4-84.8	94.6-96.1	99.6-101.3	100.1-102.5
10	80.5-82.9	95.1-95.9	95.6-100.8	97.5-99.7

As expected calcium folinate and MTX were stable throughout the 10 h period at room temperature (97.1-103.4%). 5-methyl-THF had an acceptable stability throughout the observation period (94.6-100.9%) but a slight loss tendency was observed. 5,10-methylene-THF was instable over 10 h but a loss of less than 5% was observed within 3 h. Therefore, all patient CSF samples were kept for no more than 3 h at room temperature prior to analysis. Since one sample was analysed for 30 min a maximum of six samples was placed in the autosampler.

Stability of analytes at 4 ºC

The results of the stability investigation at 4 ºC are presented in Table 4.2.
Since a loss tendency was observed for 5,10-methylene-THF at 4 ºC as well, patient samples were not kept for longer than 24 h in the refrigerator. All CSF samples in which reduced folates and MTX were analysed were kept for not longer than 3 h at room temperature or 24 h at 4 ºC.

Table 4.2: Stability of 5,10-methylene-THF, 5-methyl-THF, calcium folinate and MTX at 4 ºC in CSF (n=3).

Time (h)	Stability at 4 ºC (%)			
	5,10-MeTHF	5-MTHF	Ca-folinate	MTX
24	95.0-97.4	99.1-104.1	99.6-102.3	98.5-101.0
48	92.8-95.7	98.2-99.9	98.6-101.4	99.5-101.6
72	91.1-93.2	95.6-97.9	99.8-105.2	94.1-103.5

Freeze-and-thaw stability

The freeze-and-thaw stability of the analytes is presented in Table 4.3. The results indicate acceptable stability (95.8-105.4%) of all four analytes in the CSF after two cycles of thawing and freezing. Therefore, it was possible to analyse patient samples up to three times if necessary. Because of the instability at room temperature it was important that the samples were refrozen as soon as possible after thawing.

Table 4.3: Stability of 5,10-methylene-THF, 5-methyl-THF, calcium folinate and MTX in the CSF after freezing and thawing of CSF samples (n=3).

Cycle	Freeze and thaw stability (%)			
	5,10-MeTHF	5-MTHF	Ca-folinate	MTX
1	97.1-99.1	98.6-101.7	99.4-103.5	99.6-100.9
2	95.8-97.4	96.4-98.3	100.1-105.4	100.2-101.5

4.1.1.4 Recovery

Recovery was determined as described in chapter 3.3.3.3. The results are presented in Table 4.4.

The results of the recovery experiments reveale that the response (specified as peak area) of calcium folinate and MTX in CSF is constant in the range of 95.0-112.5% compared to the response of the equivalent concentration of the analytes in tris-buffer. On the other hand, the recovery of 5,10-methylene-THF (60.0-78.8%) and 5-methyl-THF (85.4-94.8%) decreased with the lower analyte

Table 4.4: Recovery of 5,10-methylene-THF, 5-methyl-THF, calcium folinate and MTX in CSF (n=3).

5,10-MeTHF		5-MTHF		Ca-folinate		MTX	
Conc. (nM)	Recovery (%)	Conc. (nM)	Recovery (%)	Conc. (nM)	Recovery (%)	Conc. (nM)	Recovery (%)
20.0	60.0	20.0	85.4	200.0	112.5	50.0	95.0
100.0	71.6	100.0	89.2	1000.0	98.7	2500.0	102.7
250.0	78.8	250.0	94.8	5000.0	100.1	7500.0	100.1

concentrations. Due to the results of the recovery experiments it was neccessary to calibrate 5,10-methylene-THF and 5-methyl-THF in CSF in order to determine their concentration in patient samples. Since all four compounds were determined simultaneously the calibration curves were prepared using pooled CSF as matrix.

4.1.1.5 LOD and LLOQ

The criteria for establishing LOD and LLOQ are presented in chapter 3.3.3.4 and 3.3.3.5, respectively.The limits of detection and the lower limits of quantification for the reduced folates and MTX are presented in Table 4.5. The precision of the determination of LLOQ defined as coefficient of variation was in the range 12.7-19.8% for all four compounds whereas accuracy was 85.0-90.1%. The method met the requirements of the FDA guideline (CV ≤ ± 20% and accuracy ≤ ± 15%).

4.1.1.6 Precision

Precision was investigated as described in chapter 3.3.3.6. Coefficients of variation were calculated with the use of Eq. 3.3.

Table 4.5: LOD and LLOQ (with precision and accuracy data) of the reduced folates and MTX in CSF (n=6).

	5,10-MeTHF	5-MTHF	Ca-folinate	MTX
LOD (nM)	2.0	2.0	20.0	5.0
LLOQ (nM)	10.0	10.0	100.0	20.0
Precision, CV (%)	19.8	17.5	17.7	12.7
Accuracy (%)	85.0	86.6	87.8	90.1

Within-day precision

The results of the within-day precision investigation of reduced folates and MTX are presented in Table 4.6. The coefficients of variation for the injection precision were < 5% for the middle and upper concentrations of all four analytes. The lowest concentration of all substances had a variation coefficient < 10%.

Table 4.6: Within-day precision of reduced folates and MTX in CSF (n=6).

5,10-MeTHF		5-MTHF		Ca-folinate		MTX	
Conc. (nM)	**CV (%)**	**Conc. (nM)**	**CV (%)**	**Conc. (nM)**	**CV (%)**	**Conc. (nM)**	**CV (%)**
20.0	9.7	20.0	6.6	200.0	4.4	50.0	4.6
100.0	4.9	100.0	3.3	1000.0	1.8	2500.0	1.8
250.0	3.1	250.0	2.8	5000.0	0.9	7500.0	0.4

Between-day precision

The results of the between-day precision are presented in Table 4.7. The between-day precision was analysed in order to assess the reproducibility of the method. Since all calculated coefficients of variation were in the range 1.3-14.7% the method met the requirements of the FDA guideline according to which the CV should not be higher than 15% [142].

Table 4.7: Between-day precision of reduced folates and MTX in CSF (n=6).

5,10-MeTHF		5-MTHF		Ca-folinate		MTX	
Conc. (nM)	CV (%)	Conc. (nM)	CV (%)	Conc. (nM)	CV (%)	Conc. (nM)	CV (%)
20.0	14.7	20.0	10.5	200.0	8.8	50.0	6.7
100.0	9.8	100.0	6.3	1000.0	3.5	2500.0	2.8
250.0	6.9	250.0	3.9	5000.0	1.3	7500.0	1.3

4.1.1.7 Accuracy

Accuracy was obtained by analysing three different CSF concentrations of the analytes on six subsequent days (see also chapter 3.3.3.7). The mean values of obtained coefficients of variation are presented in Table 4.8. Accuracy was in the range 88.6-103.4% which is within the range of the FDA guideline (85.0-115.0%).

Table 4.8: Accuracy of the HPLC method for the determination of reduced folates and MTX in CSF (n=6).

5,10-MeTHF		5-MTHF		Ca-folinate		MTX	
Conc. (nM)	Accur. (%)	Conc. (nM)	Accur. (%)	Conc. (nM)	Accur. (%)	Conc. (nM)	Accur. (%)
25.0	88.6	25.0	102.5	250.0	89.5	60.0	102.6
75.0	103.4	75.0	89.6	2000.0	93.7	3000.0	97.4
200.0	99.4	200.0	100.5	4000.0	100.8	8000.0	99.5

4.1.1.8 Linearity

The linear relationship between concentrations and the response of the analytes was investigated in the CSF on six subsequent days. Calibration curves were obtained as described in chapter 3.3.4. The results are presented in Table 4.9.

Since the calibrator concentrations of analytes were in a wide range the curves were fitted using weighting[148]. In that way low concentrations had a higher impact on the calibration curve compared to unweighted regression. The best fitting was found with $1/x^2$ since the residuals were in the range -14.9–12.1 (< 15%) and the means (n=42) between 2.1-4.9%.
A characteristic calibration curve for 5-methyl-THF obtained with linear regression (weighting $1/x^2$) is shown in Fig. 4.2.

Table 4.9: Coefficients of correlation and residuals of calibration curves for different regression models (n=6).

Analyte	Concentration range (nM)	Coefficient of correlation (r)	Residuals	
			Range (%)	Mean (%)
linear, not weighted				
5,10-MeTHF	10-300	0.9989-0.9994	-35.4-28.8	15.7
5-MTHF	10-300	0.9991-0.9999	-22.3-9.8	13.7
Ca-folinate	100-6000	0.9994-0.9999	-15.6-21.7	10.1
MTX	20-10000	0.9994-0.9999	-9.1-17.1	12.7
linear, weighted 1/x				
5,10-MeTHF	10-300	0.9981-0.9990	-27.6-26.8	8.7
5-MTHF	10-300	0.9989-0.9992	-19.6-4.2	7.5
Ca-folinate	100-6000	0.9991-0.9995	-10.3-16.1	5.1
MTX	20-10000	0.9992-0.9999	-7.9-10.2	3.7
linear, weighted $1/x^2$				
5,10-MeTHF	10-300	0.9965-0.9986	-14.9-12.1	4.9
5-MTHF	10-300	0.9971-0.9989	-14.7-7.4	4.2
Ca-folinate	100-6000	0.9980-0.9992	-8.6- 9.3	3.8
MTX	20-10000	0.9983-0.9989	-5.2-7.4	2.1

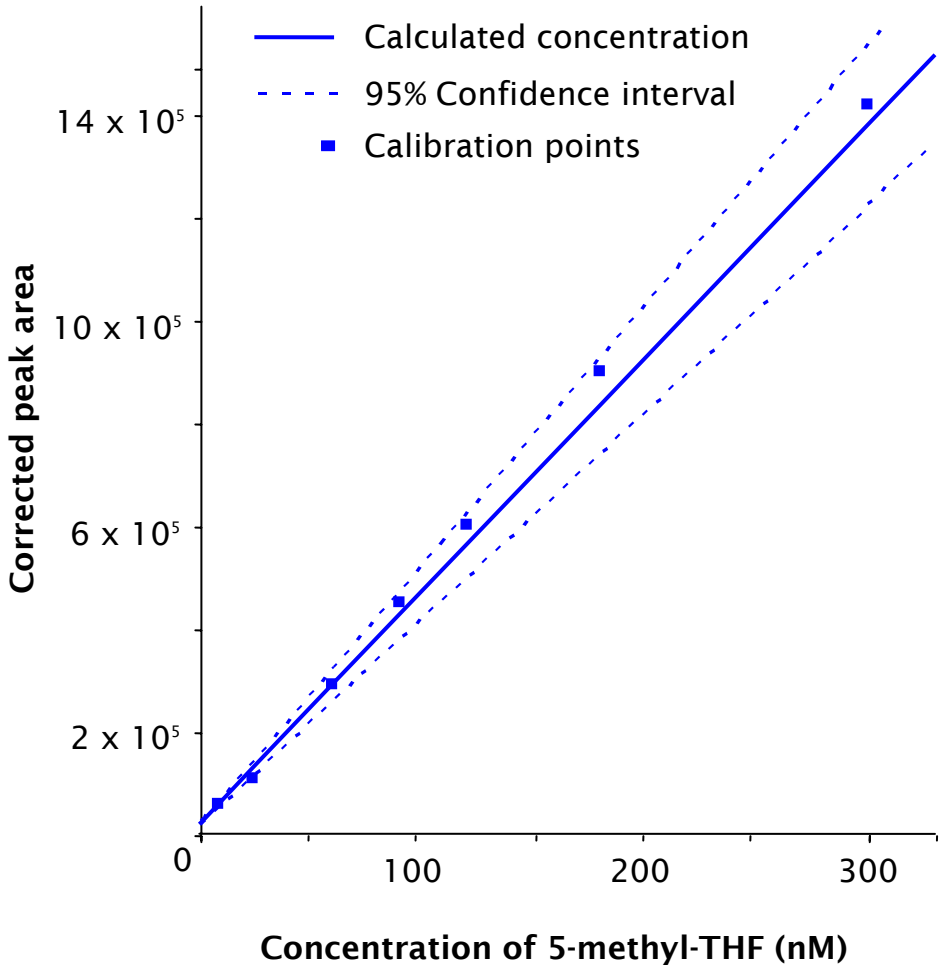

Fig. 4.2: Characteristic calibration curve for 5-methyl-THF (linear regression modell, weighted $1/x^2$).

4.1.2 HPLC analysis for the determination of SAM and SAH in CSF

4.1.2.1 Method development

The determination of SAM and SAH as their 1,N^6-etheno derivatives has been described in several literature reports[132, 137, 143, 149]. However, in most of them the concentrations of the analytes were assessed after separate runs. The goal in the developmental phase of this work was to achieve a simultaneous determination of both analytes. Therefore, columns were tested in order to

obtain an optimal separation of the 1,N^6-etheno derivatives of SAM and SAH in the CSF (see also 3.4.4).

Due to the polarity of the compounds an attempt was made to separate the two analytes using the Luna NH_2® column. Phosphate buffer (40 mM, pH 4.5) and isopropanol or acetonitrile served as mobile phase. The composition of the mobile phase was varied and several gradient systems were run. However, no satisfactory separation of the substances was obtained.

Further, a reversed-phase column was tested using the same mobile phase as for the amine column. A separation of the peaks was possible but the peaks were neither symmetric nor sharp and it was not possible to obtain the needed sensitivity for the quantification of SAH.

A much better peak symmetry and higher sensitivity for both analytes was observed with the use of a reversed-phase column especially designed for the separation of amino acids (Eclipse AAA®). The mobile phase consisted initially of a phosphate buffer (40 mM, pH 4.5) and acetonitrile but despite the use of different gradients SAM eluted always within 5 minutes and it was impossible to obtain an adequate selectivity for this analyte. Since the positive charge of SAM was thought to be responsible for the quick elution of this analyte the ion-pairing agent 1-heptanesulphonic acid (8 mM) was added to the phosphate buffer. The elution time of SAM was prolonged for more than 30 minutes using heptanesulphonic acid. Moreover, SAM eluted later than SAH. The mobile phase gradient was optimised and the final gradient presented in Table 3.4 resulted in a selective, reproducible separation of SAM and SAH with an acceptable run time (1,N^6-etheno-SAH had a retention time of 19.7 min while 1,N^6-etheno-SAM was detected after 23.5 min).

Fig. 4.3 shows a characteristic chromatograme of SAM and SAH in the CSF in form of their 1,N^6-etheno derivatives on the Eclipse AAA® column.

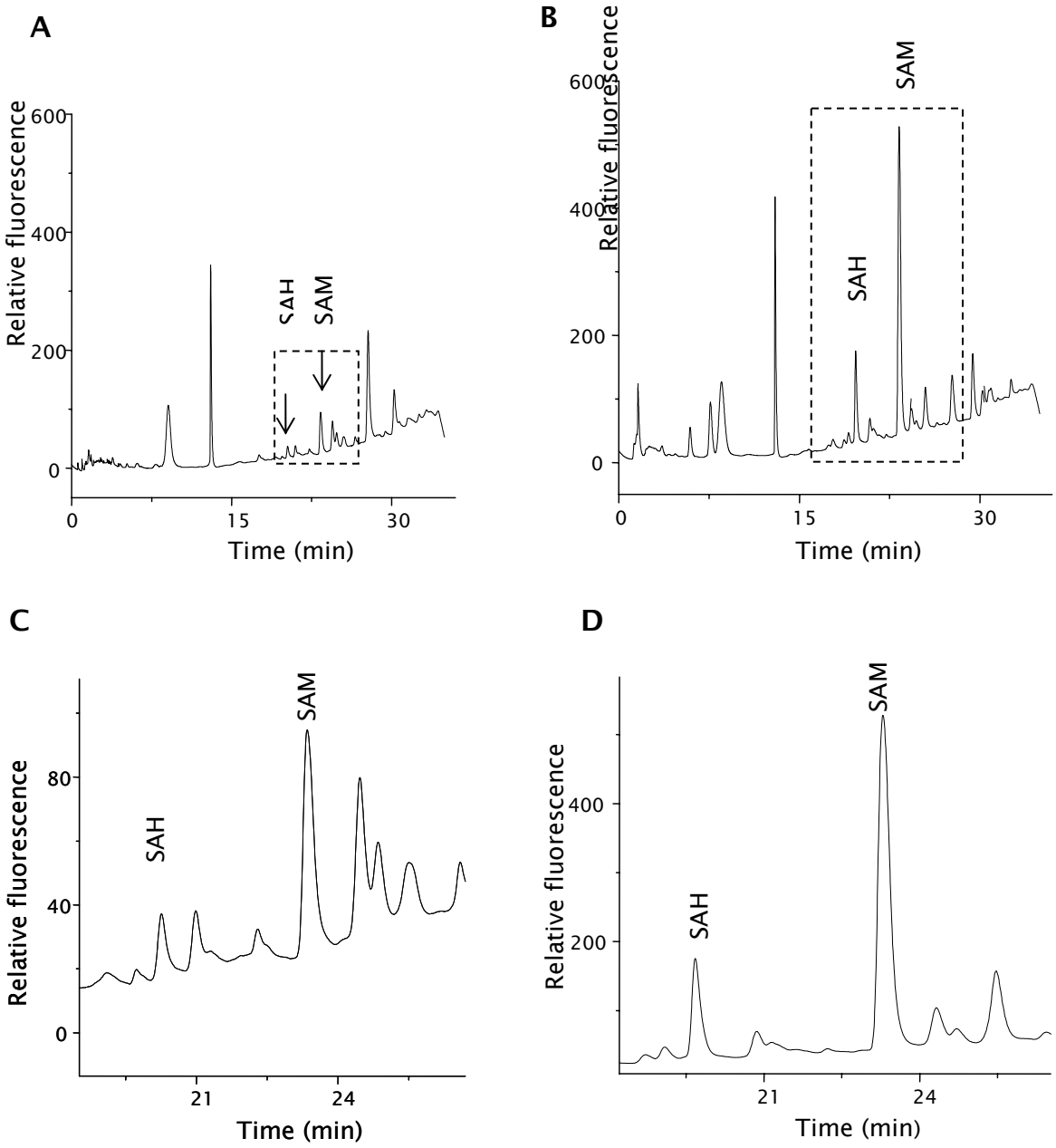

Fig. 4.3: Characteristic chromatogrammes of SAM and SAH in: pooled CSF (A), pooled CSF spiked with 15 nM SAH and 125 nM SAM (B). C and D depict the marked areas of A and B, respectively, in which the peaks of interest are shown more precisely.

4.1.2.2 Selectivity

Selectivity was shown by comparison of the CSF samples of five patients with *the same* CSF samples which were spiked with SAM and SAH (see Fig. 4.3).
In chromatogramme B it is shown that the peaks identified as SAM and SAH have a higher peak area in comparison to the peaks in chromatogramme A. No interfering compounds were detected which could interfere with the identification or quantification of SAM and SAH.

4.1.2.3 Stability

pH-dependent stability

In order to prepare stable stock solutions the influence of pH on the stability of SAM and SAH was investigated. Five aqueous solutions containing 15 nM SAH and 125 nM SAM were prepared. The pH of the solutions was adjusted to 1, 3, 5, 7 and 10, respectively, with the use of perchloric acid or sodium hydroxide. They were kept at 37 ºC for 48 h to accelerate any possible degradation of the analytes. The experiment was repeated three times.
There was a strong influence of pH on the stability of SAM. In the solution with pH 1 the highest response defined as peak area was observed (stability 100%) which decreased rapidly with increasing pH. In the alkaline solution no SAM was detected (stability 0%). In contrary to SAM, the stability of SAH was in the range of 91.2-132.1%. A somewhat higher response was obtained at pH 3 but because of the instability of SAM pH 1 was chosen for both analytes. The results are presented in Table 4.10.
As a consequence of these results perchloric acid was added to each CSF sample drawn for SAM and SAH analysis prior to freezing. In that way samples were deproteinised and pH 1 was obtained in which SAM is stable. Since some samples obtained from cooperation partners were not collected with perchloric acid, it was investigated whether the pH-dependent instability noticed at 37 ºC also occured at -20 ºC. For that reason, CSF samples collected from one patient with PCNSL at different time-points were analysed. Each sample was divided into several vials after collection. In one vial Table

4.10: pH-dependent stability of SAM and SAH after 48 h at 37 ºC in aqueous solutions (n=3).

pH	pH-dependent stability (%)	
	SAM	SAH
1.0	100.0	100.0
3.0	48.5	132.1
5.0	30.8	107.5
7.0	2.5	95.4
10.0	0.0	91.2

perchloric acid was added while another one was frozen without acidification. Samples were kept frozen approximately one year. After thawing, perchloric acid was added into the previously non-acidified aliquot as well in order to deproteinise the CSF sample and equalise the pH in both CSF samples prior to derivatisation. The matched-pair samples were analysed: in the samples kept at pH 1 the SAM concentration was 153.1-966.6 nM (median 843.6 nM) while a SAM concentration of 105.5-618.2 nM (median 407.5 nM) was determined in samples kept at physiological pH. The SAH concentration in the samples kept at pH 1 was in one sample below 5 nM (LLOQ) and 6.2-11.0 nM (median 8.0 nM) in all others while the samples kept at physiological pH exhibited a SAH concentration of 11.2-43.7 nM (median 24.7 nM). The results are presented in Table 4.11. Furthermore, CSF samples of six other patients suffering from ALL which were stored over a year at -20 ºC were analysed. Similarly, the decrease of the SAM and increase of the SAH concentration was observed in the CSF samples which were not acidified prior to freezing. The SAM concentration in samples kept at pH 1 was 51.5-222.7 nM (median 145.4 nM) and 31.7-85.2 nM (median 66.0 nM) in samples kept at pH 7.4. The concentration of SAH was below LLOQ in three and 5.0-7.2 nM in the other three samples kept at pH 1 whereas samples kept at pH 7.4 had the SAH concentration 5.2-16.2 nM (median 8.5 nM). The results are presented in Table 4.12.

Table 4.11: CSF SAM and SAH concentrations in samples stored at -20 ºC at least one year (all samples obtained from one patient).

Sample	Concentration (nM)			
	SAM		SAH	
	pH 1	pH 7.4	pH 1	pH 7.4
1	153.1	105.6	Below LLOQ	12.1
2	290.7	198.7	7.0	11.2
3	381.0	237.4	6.2	24.1
4	641.2	417.1	6.6	16.6
5	856.7	539.1	8.0	32.7
6	856.8	356.5	10.2	18.7
7	903.0	618.2	7.8	27.9
8	966.6	482.8	8.0	17.5
9	899.1	372.1	8.3	43.7
10	854.3	418.3	8.2	26.4
11	866.8	321.4	9.1	17.2
12	831.7	515.7	9.5	25.3
13	645.4	512.5	6.3	26.2
14	672.7	398.7	11.0	38.2

An investigation of the stability of SAM and SAH in the CSF at room temperature for 1 h was performed in order to exclude any possible change of the concentration of analytes due to enzymatic conversion. CSF samples were collected from six patients. In one vial with CSF, perchloric acid was added immediately and the sample was frozen at -20 ºC while another vial with the same CSF sample was kept at room temperature for 1 h and then frozen. The samples were thawed at latest six weeks after freezing and perchloric acid was added into CSF samples kept at physiological pH. The analysis of the samples revealed no significant difference between the groups (Wilcoxon test, p=0.917 and p=0.345 for SAM and SAH respectively).

Table 4.12: CSF SAM and SAH concentrations in samples from six patients stored at -20 ºC, at least one year.

Patient	SAM (nM)		SAH (nM)	
	pH 1	pH 7.4	pH 1	pH 7.4
1	192.5	75.5	6.0	16.2
2	222.7	85.2	7.2	11.9
3	110.7	71.9	Below LLOQ	5.2
4	180.0	53.5	5.0	9.0
5	99.4	60.1	Below LLOQ	7.2
6	51.5	31.7	Below LLOQ	7.9

The concentration of SAM was 81.4-253.6 nM (median 133.9 nM) in the deproteinised and 88.0-221.5 nM (median 141.3 nM) in the samples kept 1 h at room temperature. The concentration of SAH was 5.0-8.2 nM (median 6.1 nM) in the acidified samples and 5.0-8.4 nM (median 6.3 nM) in the samples kept for 1 h at room temperature. The results are presented in Table 4.13.

Stability of 1,N^6-etheno derivatives of SAM and SAH at room temperature

The stability of derivatised SAM and SAH in the CSF was investigated at room temperature for a period of 24 h in order to establish if the derivatised samples were stable enough to be kept in the autosampler and analysed over night. Stability was investigated at three different concentrations which corresponded to the lower, middle and upper range of the calibration curve. Each experiment was repeated three times and the results (shown as mean values, n=3) are presented in Table 4.14. The 24 h stability of SAM and SAH in form of their 1,N^6-etheno derivatives at room temperature ranged from 98.6 to 104.1% and from 97.2 to 100.3%, respectively. Since no loss tendency was observed it was concluded that the analytes could be kept in the autosampler and analysed within the investigated time period.

Table 4.13: CSF SAM and SAH concentration in six samples immediately frozen at pH 1 or kept for 1 h at room temperature and frozen at pH 7.4 for a period of maximal six weeks.

Patient	Concentration (nM)			
	SAM		SAH	
	Frozen immediately pH 1	1 h at room temper. pH 7.4	Frozen immediately pH 1	1 h at room temper. pH 7.4
1	81.4	88.0	6.0	5.0
2	205.8	202.6	6.1	6.9
3	84.8	77.7	8.2	8.4
4	253.6	221.5	5.0	5.7
5	95.6	104.4	6.4	6.8
6	172.2	178.1	5.0	5.1

Table 4.14: Stability of the 1,N^6-etheno derivatives of SAM and SAH in CSF at room temperature (n=3).

Time (h)	Stability of 1,N^6-etheno derivatives (%)	
	SAM	SAH
3	95.0-102.9	96.0-101.2
8	95.5-97.0	93.7-103.4
24	97.2-100.3	98.6-104.1

Freeze-and-thaw stability of SAM and SAH

The stability of SAM and SAH in CSF after two freeze and thaw cycles was investigated as well (see also chapter 3.4.5.2). The results are presented in Table 4.15.

Table 4.15: Stability of SAM and SAH after freeze and thaw (-20 ºC) cycles (n=3).

Cycle	Freeze-and-thaw stability (%)	
	SAM	SAH
1	97.2-101.1	95.1-108.5
2	99.0-105.2	97.3-104.0

SAM and SAH remained stable (99.0-105.2% and 97.3-104.0%, respectively) in the CSF after the second freeze-and-thaw cycle, thus enabling a repeated analysis of SAM and SAH in patient samples when necessary.

4.1.2.4 Recovery

The response (peak area) of SAM and SAH in CSF was compared to the response when 0.9% NaCl was used as matrix in order to determine if the latter could be used as calibration matrix (see also chapter 3.4.5.3). The results of the investigation are presented in Table 4.16. The recovery of SAM and SAH in the CSF increased with higher concentrations of the analytes (60.0-78.2% and 67.2-80%, respectively). Since the recovery was not constant pooled CSF was used as matrix for all further investigations.

Table 4.16: Recovery of SAM and SAH in the CSF compared to 0.9% NaCl (n=3).

SAM		SAH	
Concentration (nM)	Recovery (%)	Concentration (nM)	Recovery (%)
50.0	60.0	11.0	67.2
120.0	61.3	55.0	71.6
500.0	78.2	110.0	80.0

4.1.2.5 Limit of detection and lower limit of quantification

LOD and LLOQ which were determined as described in chapter 3.3.3.4 and 3.3.3.5 are presented in Table 4.17.

Table 4.17: LOD and LLOQ (with precision and accuracy data) of the HPLC method for analysis of SAM and SAH (n=6).

	SAM	SAH
LOD (nM)	5.0	1.0
LLOQ (nM)	25.0	5.0
Precision CV (%)	19.0	10.5
Accuracy (%)	82.0	88.0

4.1.2.6 Precision

Within-day precision

The within-day precision in the CSF was investigated by using of quality controls as described in chapter 3.4.5.5. The results are presented in Table 4.18.

Table 4.18: Within-day precision of SAM and SAH in CSF (n=6).

SAM		SAH	
Concentration (nM)	**CV (%)**	**Concentration (nM)**	**CV (%)**
60.0	14.0	15.0	9.1
200.0	9.4	35.0	8.7
650.0	3.8	85.0	6.1

The within-day precision was 3.8-14.0% for SAM and 6.1-9.1% for SAH which was acceptable according to the criteria of the FDA guideline (≤ 15%).

Between-day precision

The results of the between-day precision are shown in Table 4.19.

The coefficients of variation presented in Table 4.19 indicated that SAH could be determined precisely using this method whereas the determination of SAM in lower concentrations was less precise compared to SAH but still acceptable according to the FDA guideline (≤ 20% at LLOQ).

Table 4.19: Between-day precision of SAH and SAM in CSF (n=6).

SAM		SAH	
Concentration (nM)	**CV (%)**	**Concentration (nM)**	**CV (%)**
25.0	19.0	5.0	10.5
50.0	11.3	8.0	6.6
125.0	13.5	20.0	10.0
500.0	7.9	70.0	5.6
750.0	7.9	100.0	3.3

4.1.2.7 Accuracy

The accuracy of the method was determined using quality controls. The results are presented in Table 4.20. The calculated concentrations of SAM were 98.3-111.0% of the nominal concentration, wheras 88.6-92.5% were found for SAH. Since the calculated concentrations were always within the range of ±15% of the nominal concentrations, the method was qualified as accurate for the determination of both analytes.

4.1.2.8 Linearity

Six calibration curves were analysed in order to determine the appropriate regression model for the calculation of concentrations of SAM and SAH (see also chapter 3.4.6.3). The results are presented in Table 4.21.

Table 4.20: Accuracy of the HPLC method for the determination of SAM and SAH (n=6).

SAM		SAH	
Concentration (nM)	**Accuracy (%)**	**Concentration (nM)**	**Accuracy (%)**
60.0	111.0	15.0	90.0
200.0	108.5	35.0	88.6
250.0	98.3	85.0	92.5

Table 4.21: Coefficients of correlation and residuals of the calibration curves of SAM and SAH.

Analyte	Concentration range (nM)	Coefficient of correlation (r)	Residuals	
			Range (%)	Mean (%)
		linear, not weighted		
SAH	5-100	0.9990-0.9999	-14.5-20.4	8.3
SAM	25-750	0.9989-0.9999	-42.0-12.7	19.5
		linear, weighted 1/x		
SAH	5-100	0.9983-0.9995	-14.0-7.4	7.2
SAM	25-750	0.9981-0.9993	-32.6-19.6	13.8
		linear, weighted $1/x^2$		
SAH	5-100	0.9981-0.9993	-14.7-7.3	6.8
SAM	25-750	0.9978-0.9990	-14.5-7.0	6.5

The results showed better coefficients of correlation using the non-weighted model but the range and mean of residuals were lower in both weighted models. The range of residuals for SAH was < ±15% when fitted with the 1/x or $1/x^2$ weighted model and the means of residuals were comparable for both models (6.8 and 7.2%, respectively). Thus, all concentrations of CSF SAH

were calculated using 1/x weighting. For SAM the best fit of data (range of residuals < ±15%; mean 6.5%) was achieved using the $1/x^2$ weighted model.
A characteristic calibration curve obtained for SAH by linear regression (weighted 1/x) and SAM ($1/x^2$) is shown in Fig. 4.4 and 4.5.

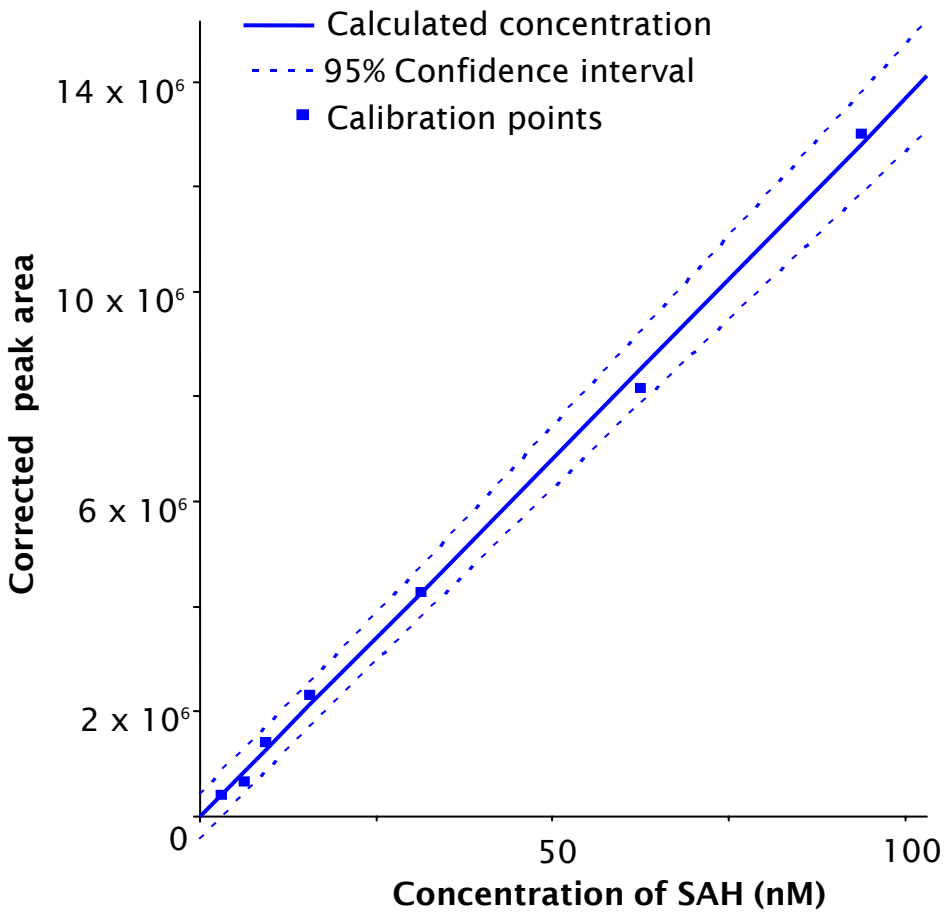

Fig. 4.4: Characteristic calibration curve of SAH (linear regression model, weighted 1/x).

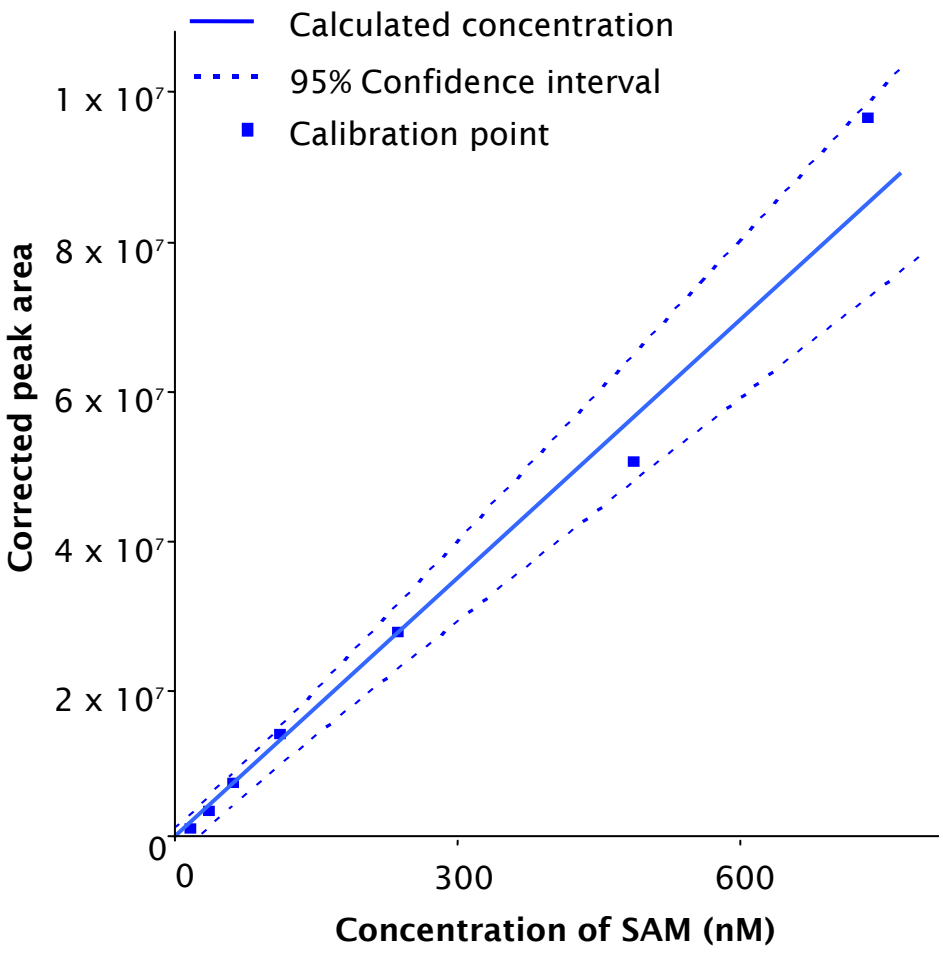

Fig. 4.5: Characteristic calibration curve of SAM (linear regression model, weighted $1/x^2$).

4.2 Adult patients with PCNSL

In order to obtain an insight into the biochemical alterations of the folate and methyl-transfer pathway after administration of high-dose and/or intraventricular MTX CSF samples were obtained from patients with PCNSL. Since these patients received frequent intraventricular therapy prior to which CSF could be obtained, it was possible to become several samples *during* MTX therapy blocks. This enabled monitoring of the analytes closely after

therapy. Because of the low incidence of PCNSL only five patients were recruited in this study in the period from June 2001 to October 2003. All patients had an implanted Ommaya reservoir from which CSF samples were obtained. One patient (no 1) did not receive intraventricular therapy and samples were obtained only within 24 h after the end of one high-dose MTX infusion. Another patient (no 5) had manifest neurotoxicity in form of leukoencephalopathy and will therefore be assessed separately.

4.2.1 CSF concentrations of reduced folates and MTX

Block A/B

Blocks A and B of the Bonn protocol for PCNSL were assessed together since they did not differ regarding MTX therapy. Treatment commenced with the 24 h infusion of high-dose MTX (3 or 5 g/m^2) on day 1. A CSF sample was obtained on day 2 at the end of the infusion and just before the intraventricular administration of MTX (3 mg). Calcium folinate rescue was administered between sampling on day 2 and 4. On day 3-5 samples were obtained prior to intraventricular MTX.

In patient 1 an exception to the above described schedule was made. This patient received the high-dose MTX (3 g/m^2) infusion which was followed by an intensified calcium folinate rescue initiated one hour after the end of the high-dose infusion (see also chapter 3.5.1). Since five samples were obtained from this patient within 24 h after the end of the high-dose MTX infusion it was possible to monitor closely the effect of high-dose MTX followed by calcium folinate on the CSF concentration of 5,10-methylene-THF and 5-methyl-THF. Within 24 h after the end of the high-dose MTX infusion the concentration of MTX decreased from 2.9 µM (end of infusion) to 0.2 µM (24 h) as shown in Fig. 4.6 A. The concentrations of all three reduced folates increased in the same period of time (Fig. 4.6 B). The concentrations of 5,10-methylene-THF and 5-methyl-THF were below LLOQ at the end of the MTX infusion and increased slowly after rescue administration. Six hours after receiving the first infusion of calcium folinate a quantification of the

concentrations of 5,10-methylene-THF and 5-methyl-THF was possible and they were 22 nM and 28 nM, respectively, whereas no calcium folinate was detected in the CSF. The levels of 5,10-methylene-THF, 5-methyl-THF and calcium folinate increased to 28 nM, 44 nM and 118 nM, respectively, 23 h after the beginning of the rescue.

The CSF analysis of MTX in three other PCNSL patients revealed drug levels in the range 0.7-2.0 µM (median 1.5 µM) at the end of the high-dose infusion (see Fig. 4.7 A). The aim of the subsequent frequent low dose intraventricular MTX administration was to provide a constant CSF cytotoxic drug concentration (1 µM) during the treatment block. Instead the MTX concentration ranged from 0.3-20.3 µM (median 2.3 µM) indicating a large intra- and interindividual variability both within and between the blocks. Due to the sampling schedule it can be assumed that the measured MTX concentrations were the lowest in the CSF on day 3, 4 and 5. Nevertheless, in some samples extremely high MTX concentrations were determined (15.0, 18.7 and 20.3 µM). On the other hand, one patient had low MTX levels in all samples (0.3-0.8 µM). A cumulation of MTX in the CSF was not observed but the variability increased from day to day.

The analysis of reduced folates in the three PCNSL patients revealed that 5,10-methylene-THF was below LLOQ (10 nM) and in some cases below LOD (2 nM) whereas calcium folinate was below LOD (20 nM) in all samples. Decreased levels of 5-methyl-THF (between LOD (2 nM) and LLOQ (10 nM)) were determined in all samples collected directly after the end of the high-dose MTX infusion (see Fig. 4.7 B). On day 3, after the patients had received systemic calcium folinate rescue, the concentration of 5-methyl-THF increased to a median of 54.8 nM (32.2-121.8 nM) which is in the physiological range according to literature data[141, 150, 151]. On the following two days the CSF 5-methyl-THF concentration remained in a similar range (day 4: median 65.3 nM (22.6-90.5 nM) and day 5: median 67.6 nM (26.7-81.9 nM)).

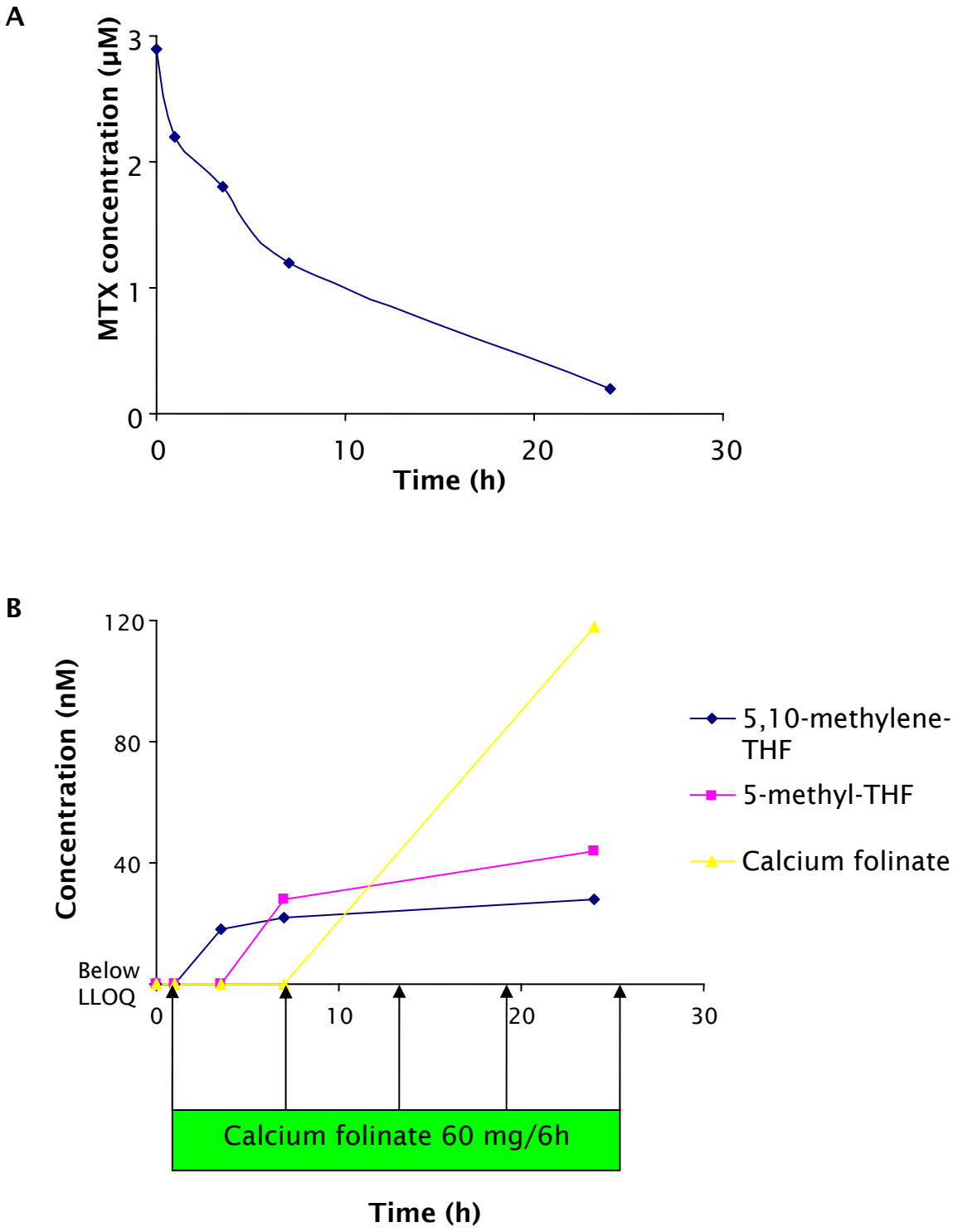

Fig. 4.6: CSF concentration of MTX (A) and reduced folates (B) within 24 h after the end of high-dose MTX infusion followed by intensified rescue in patient 1.

Intraindividually, the highest concentration of 5-methyl-THF was observed on day 3 or 4 and was followed by a decline tendency observed at latest on day 5. An exception to this pattern occured in two blocks where the concentration of 5-methyl-THF increased throughout the block. Individual data are presented in Appendix A.

Since the concentration of 5-methyl-THF was below LLOQ on day 2 statistical evaluation was performed for day 3-5 by using the Friedman test. There was no significant difference between the CSF 5-methyl-THF concentration on these days ($p=0.565$, $n=7$). MTX data were not statistically evaluated since only four matched-pair samples were available throughout the blocks.

Block C

During block C patients received intraventricular MTX (3 mg/day) from day 3-7 which was not accompanied by rescue. Samples were obtained on each day just before intraventricular drug administration.

In analogy to block A and B there was a large intra- and interindividual variability of the CSF MTX concentration during block C. An overview of the drug concentrations throughout block C is presented in Fig. 4.8 A Individual data are shown in Appendix A.

Patients 3 had MTX levels in the range 1.3-9.1 µM. Patient 2 had very elevated CSF MTX concentrations (8.3-39.4 µM) during block C of the first cycle. Since no signs of toxicity were observed in this patient, the extreme levels of MTX were possibly a consequence of a catheter misplacement of the Ommaya reservoir. This disorder was the cause of absence of intrathecal therapy during the next block after which the Ommaya was reimplanted and the patient received further therapy according to the Bonn protocol. Patient 4 had low CSF MTX concentration (0.2 to 0.7 µM) in all samples similar as in block A/B.

The CSF analysis of reduced folates revealed that 5,10-methylene-THF and calcium folinate could not be determined in any sample. The concentrations of 5-methyl-THF in the CSF of the three patients on day 3 of block C were in the range 59.2-91.5 nM (median 73.8 nM) which was within the normal range according to literature data[141,150,151]. During block C 5-methyl-THF

concentration decreased to 15.3-48.5 nM (median 26.0 nM) on day 7 which was two to threefold less than at the beginning of the same block (see also Fig. 4.8 B and Appendix A).
The statistical analysis of the concentrations of MTX and 5-methyl-THF during block C was not performed due to the small number of matched-pair samples (n=3).

4.2.2 CSF concentrations of SAM and SAH

The CSF samples of four PCNSL patients obtained during therapy which were stabilised with perchloric acid (1.2 M) were used for the analysis of the concentration of SAM and SAH. Samples from patient 1 were not acidified prior to freezing and SAM and SAH were therefore not determined. The sample analysis was performed with the use of HPLC with fluorescence detection as described in chapter 3.4.

Blocks A/B

In contrast to the alteration of the 5-methyl-THF concentration in block A/B a trend in the concentration of SAM was not observed at the same time. The CSF concentrations of SAM and SAH during block A/B are depicted in Fig. 4.9. Individual data are presented in Appendix A.
A large intraindividual variability of the CSF SAM concentration *among* blocks was observed in patient 3. During block B of the first cycle the CSF SAM concentration was 516.3-697.2 nM. In block A of the second cycle lower levels of SAM were measured (148.1-358.7 nM). In the following therapy block B the SAM concentration was again higher (641.2-856.8 nM). In patient 4 a different pattern was observed. In block A of the first cycle the CSF SAM concentrations were 279.4-304.4 nM. In block A of the second cycle the variability of the CSF SAM concentration was higher (144.3-441.9 nM). Patient 2 had relatively constant CSF SAM concentrations in blocks A/B of the first cycle and block B of the second (183.1-236.2 nM, 170.8-230.4 nM and 199.7-261.9 nM respectively). Samples were not obtained during block A of the second cycle due to the Ommaya reservoir misplacement.

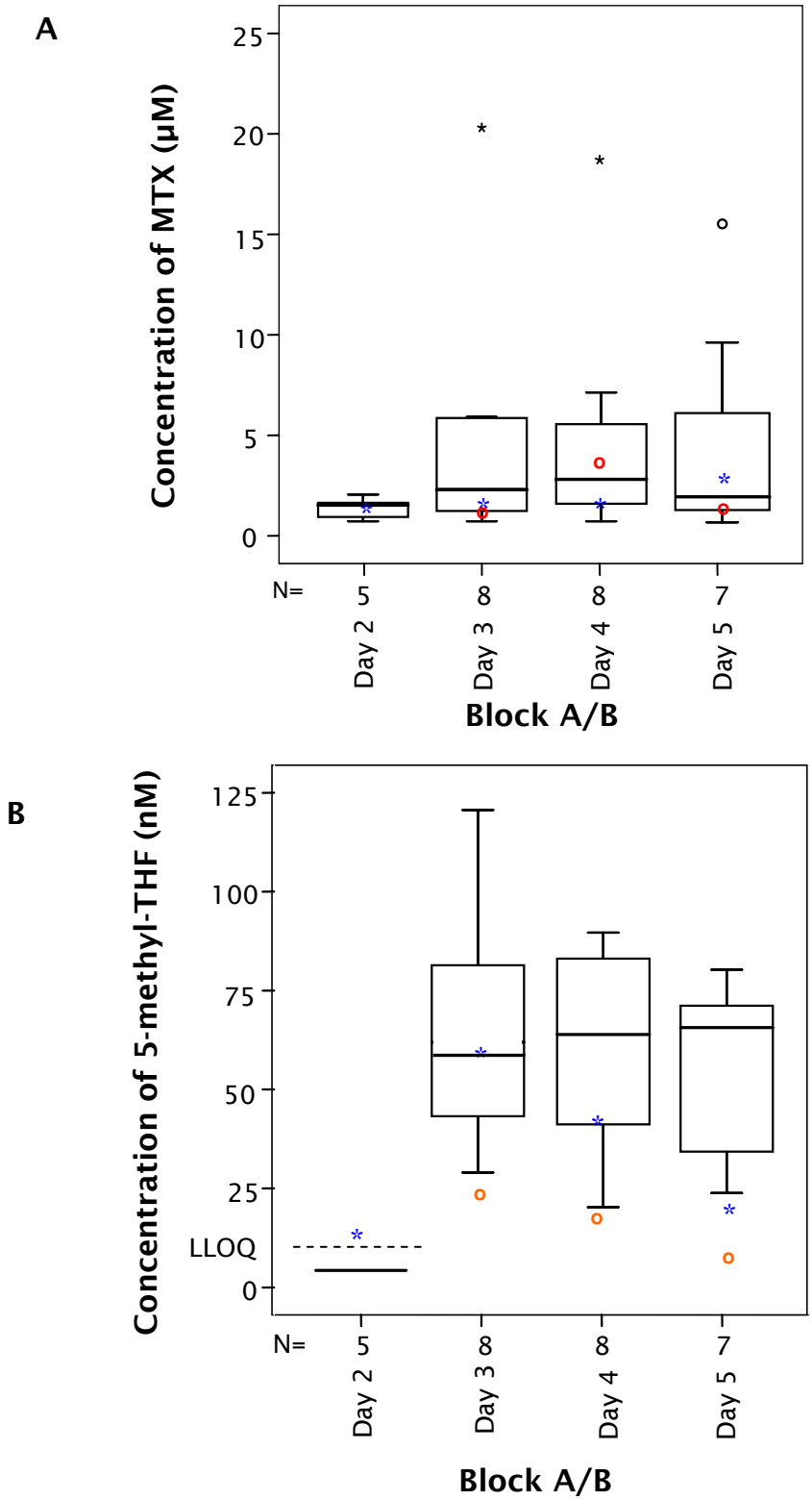

Fig. 4.7: Concentration of MTX (A) and 5-methyl-THF (B) in three PCNSL patients during blocks A/B. Blue stars and red circles represent the concentration of the analytes in the neurotoxic patient (no 5) in block B1 and A2, respectively (see also chapter 4.2.3).

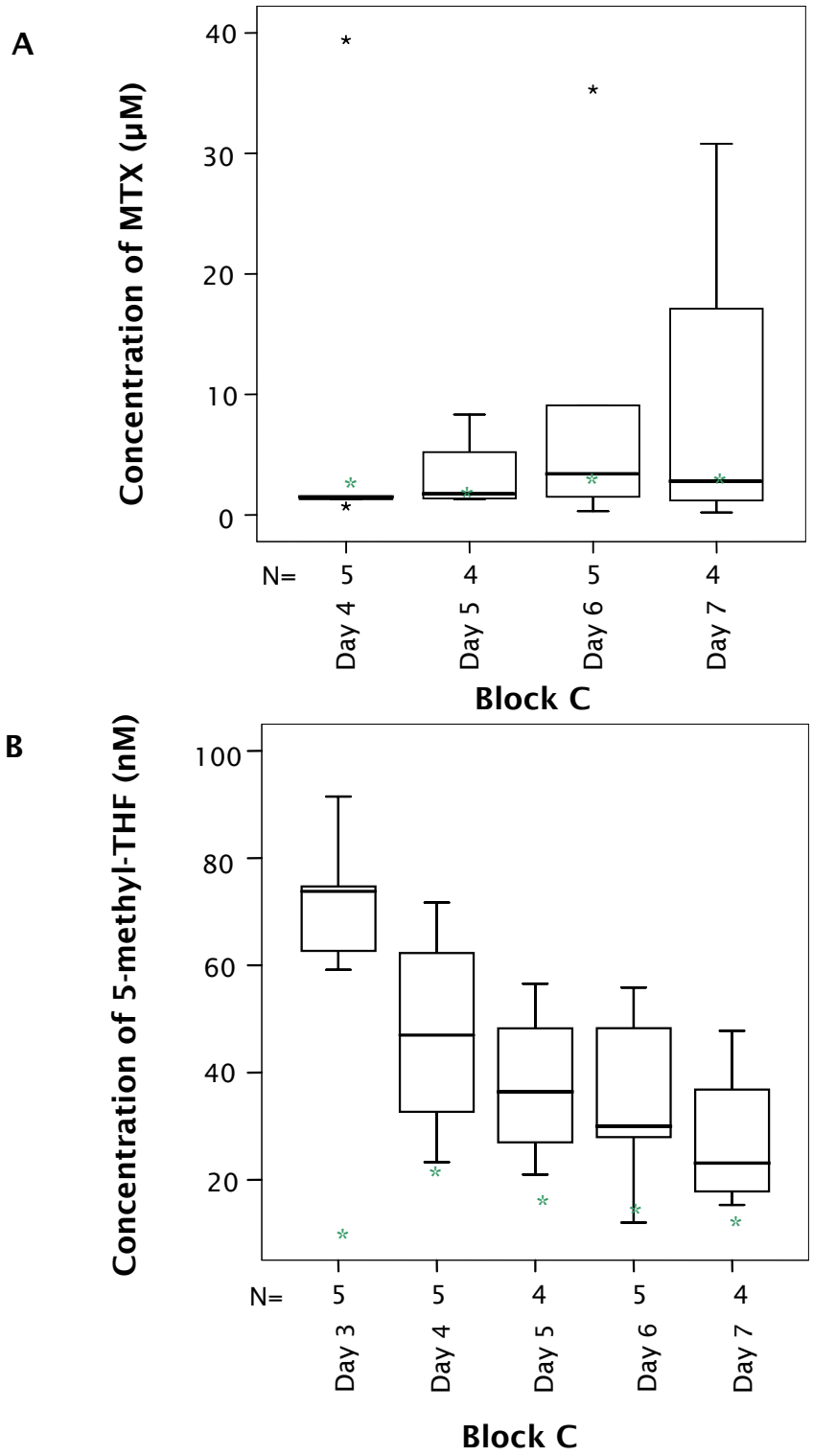

Fig. 4.8: CSF concentration MTX (A) and 5-methyl-THF (B) in three PCNSL patients during block C. Green stars represent the concentration of analytes in the patient with neurotoxicity (patient 5, see chapter 4.2.3).

There was no significant change of the concentration of SAM in blocks A/B from day 2-5 (Friedman test; p=0.564, n=5).

The concentration of SAH was below LLOQ (5 nM) in all samples obtained from patient 2 as well as in the sample obtained on day 5 of Block A1 and during the complete block A2 from patient 4. In all other samples from block A/B SAH concentrations ranged between 5.0 and 12.5 nM (median 7.0 nM). Similar to the concentration of SAM no time dependence in the SAH concentrations was observed within the block. No statistical evaluation of the SAH concentration during block A/B was performed due to the small number of samples for a matched-pair comparison (n=3 for Friedman test).

Block C

Similar as in block A/B there was no time-dependence in the CSF SAM concentration in block C. The concentrations of SAM determined during block C in the three patients are presented in Fig. 4.10. The concentrations of SAH are not depicted due to the small number of samples. Individual data are presented in Appendix A.

The concentrations of SAM during blocks C were in the range of 182.4 to 966.6 nM (median 369.4 nM) in patients 2-4. The concentration of SAH was 5.3-11.0 nM (median 7.1 nM) in patients 3 and 4 and no trend was observed within or between blocks. Patient 2 had CSF SAH levels below LLOQ.

A statistical analysis of the SAM and SAH concentrations during block C was not performed due to the small number of matched-pair samples.

4.2.3 CSF analysis of reduced folates, MTX, SAM and SAH in a PCNSL patient with neurotoxicity

Patient 5 developed chronic neurotoxicity in form of leukoencephalopathy during therapy. The evaluation of neurotoxicity according to CTC was not available, instead radiology findings were obtained. The MRI (T2-weighted) performed after block C1 showed a confluent increase of the signal which was interpreted as massive demyelination. The manifestation of clinical leukoencephalopathy was followed by a discontinuation of chemotherapy which occured after block A2.

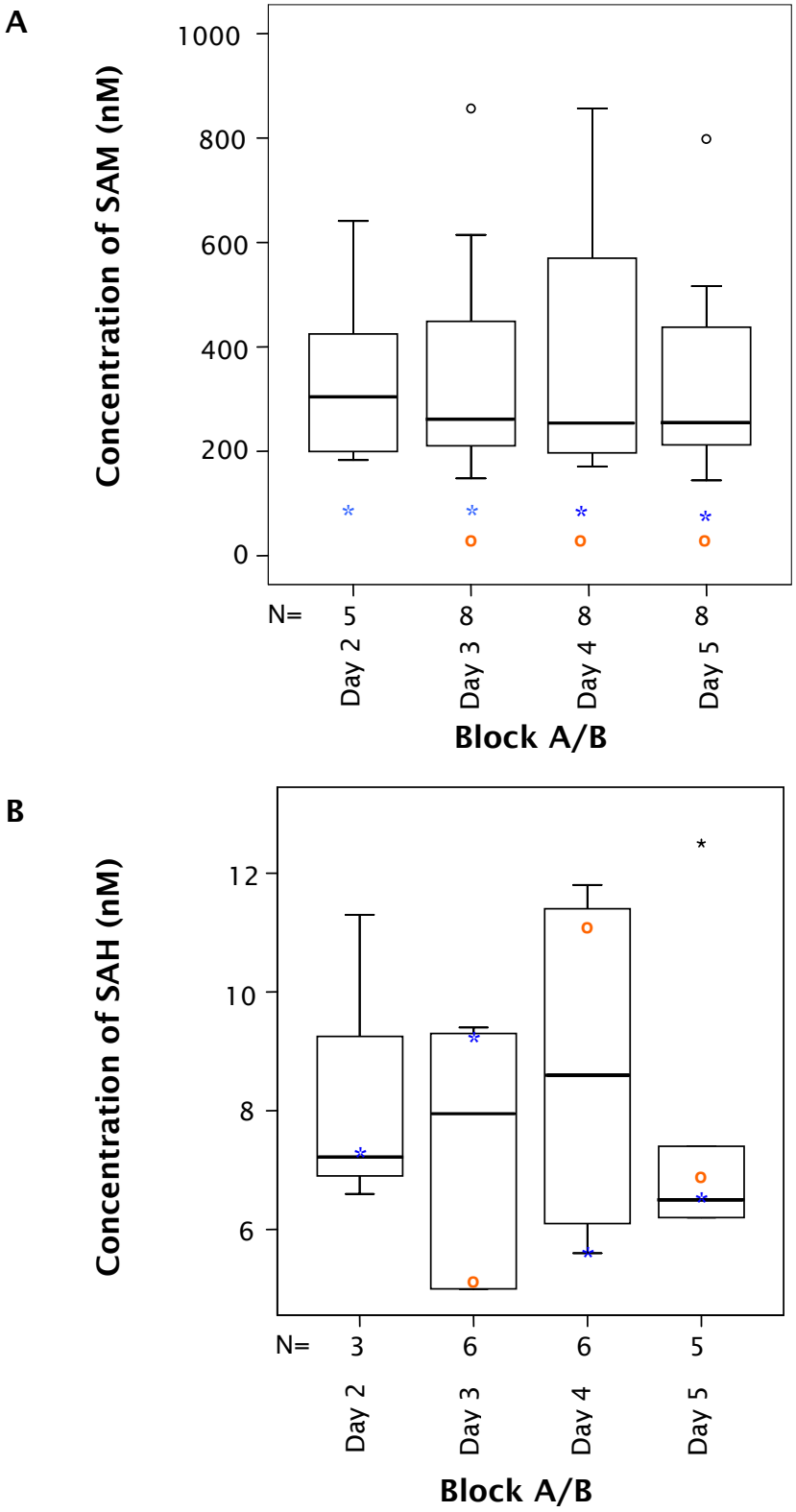

Fig. 4.9: Concentrations of SAM (A) and SAH (B) in patients 2, 3 and 4 in block A/B. The blue stars and red circles represent the concentrations in the patient with neurotoxicity during block B1 and A2, respectively (see chapter 4.2.3).

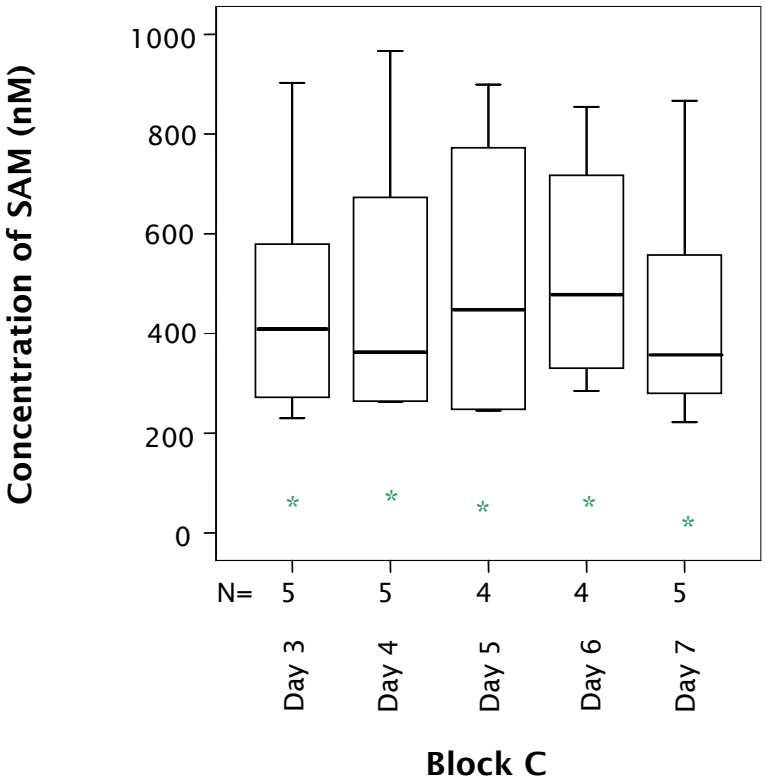

Fig. 4.10: SAM concentrations in patients 2, 3 and 4 during block C. Green stars represent the SAM concentration in the patient with neurotoxity (block C1, see chapter 4.2.3).

CSF samples were obtained during block B1, C1 and A2.
During the three therapy blocks the CSF concentration of MTX in patient 5 was 1.1-3.8 µM which was similar to the concentrations determined in the other three PCNSL patients (see Fig. 4.7 A and Fig. 4.8 A). Low CSF 5-methyl-THF concentrations were observed throughout therapy. Compared to the other PCNSL patients (median 67.6 nM on day 5, block A/B) patient 5 had a threefold lower 5-methyl-THF concentration at the end of block B1 (see Fig. 4.7 B). Approximately two weeks later on day 3 of block C1 the concentration of the reduced folate was again low (13.5 nM vs median 73.8 nM) and persisted in a low range throughout the block (22.7, 17.7, 16.2 and 14.0 nM on day 4, 5, 6 and 7 respectively, see Fig. 4.8 B). Extremely low 5-methyl-THF concentrations were observed in block A2 as well.

No trend in CSF SAM concentration was observed within any treatment block. However, the SAM level decreased from block to block (90.8-127.4 nM in block B1, 44.7-54.7 nM in block C1 and 34.1-47.6 nM in block A2, see Fig. 4.9 A and Fig. 4.10). Compared to the three other PCNSL patients who had a median CSF SAM concentration of 261.9 nM in block A/B and 376.2 nM in block C the patient with manifest leukoencephalopathy had considerably lower SAM levels.

The concentrations of SAH were 5.0-11.4 nM during all three blocks and neither a difference to the other patients nor a time-dependence was observed.

4.3 Pediatric ALL patients treated according to ALL BFM 2000

In cooperation with Prof. Bode (University Children's Hospital, Bonn) CSF samples were obtained from patients with childhood ALL treated according to ALL BFM 2000. In contrast to the PCNSL collective where a monitoring of MTX, reduced folates, SAM and SAH within days was possible, CSF samples from ALL patients could be obtained only 1 to 3 weeks after MTX administration. This group of ALL patients received intrathecal MTX also without calcium folinate rescue whereas combined systemic high-dose and intrathecal MTX treatment was followed by calcium folinate. Therefore, it was possible to compare the effect of the two modalities on the concentration of reduced folates, SAM and SAH throughout therapy.

27 patients categorised as low or medium risk ALL were included in the study. All CSF samples were obtained by lumbar puncture. Usually several samples were obtained from each patient. However, it was not always possible to obtain enough CSF for the analysis of all analytes. Therefore, 74 samples from 22 patients were obtained for the analysis of reduced folates and in 84 samples from 22 patients SAM and SAH concentrations were measured (Data shown in Appendix B). In addition, samples were obtained

from three patients with manifest neurotoxicity which were evaluated separately.

Patients included in this study were randomised into two arms. Both groups chemotherapy started with protocol I which was followed by protocol M. After that one study arm received protocol II while the other received protocol III. Since protocols I, II and III are similar regarding MTX treatment (see chapter 3.5.2) they were evaluated together. In protocol M high-dose and intrathecal MTX was followed by calcium folinate rescue.

4.3.1 CSF concentrations of reduced folates and MTX

Protocol I and II/III

During protocol I and II/III patients received at least seven intrathecal MTX administrations prior to which samples were obtained. The first sample (day 1/protocol I) was obtained prior to any MTX therapy. Therefore, the concentration of the analytes in these samples served as a reference to which all other results were compared.

Similar as in the PCNSL population, the concentrations of 5,10-methylene-THF were below LLOQ or even LOD in all analysed samples. MTX was not detected in any sample which was not surprising due to the fact that the drug was administered at least one week prior to sampling. The concentrations of 5-methyl-THF were quantified in all samples. A trend in the level of the reduced folate was observed during protocol I. The data are graphically depicted in Fig. 4.11. Just prior to the first intrathecal MTX administration the concentration of 5-methyl-THF was 35.2–91.9 nM (median 56.4 nM; n=8). 12 days later the measured levels of the reduced folate were approximately twofold lower (12.0–40.7 nM; median 24.2 nM; n=7). Since the obtained samples were not obtained from the same patients the Mann-Whitney U-test was used for statistical evaluation and revealed a significantly decreased 5-methyl-THF concentration 12 days after intrathecal MTX administration (p=0.002). Interestingly, an increase in the concentration of 5-methyl-THF was observed in protocol I from day 33 onwards resulting in

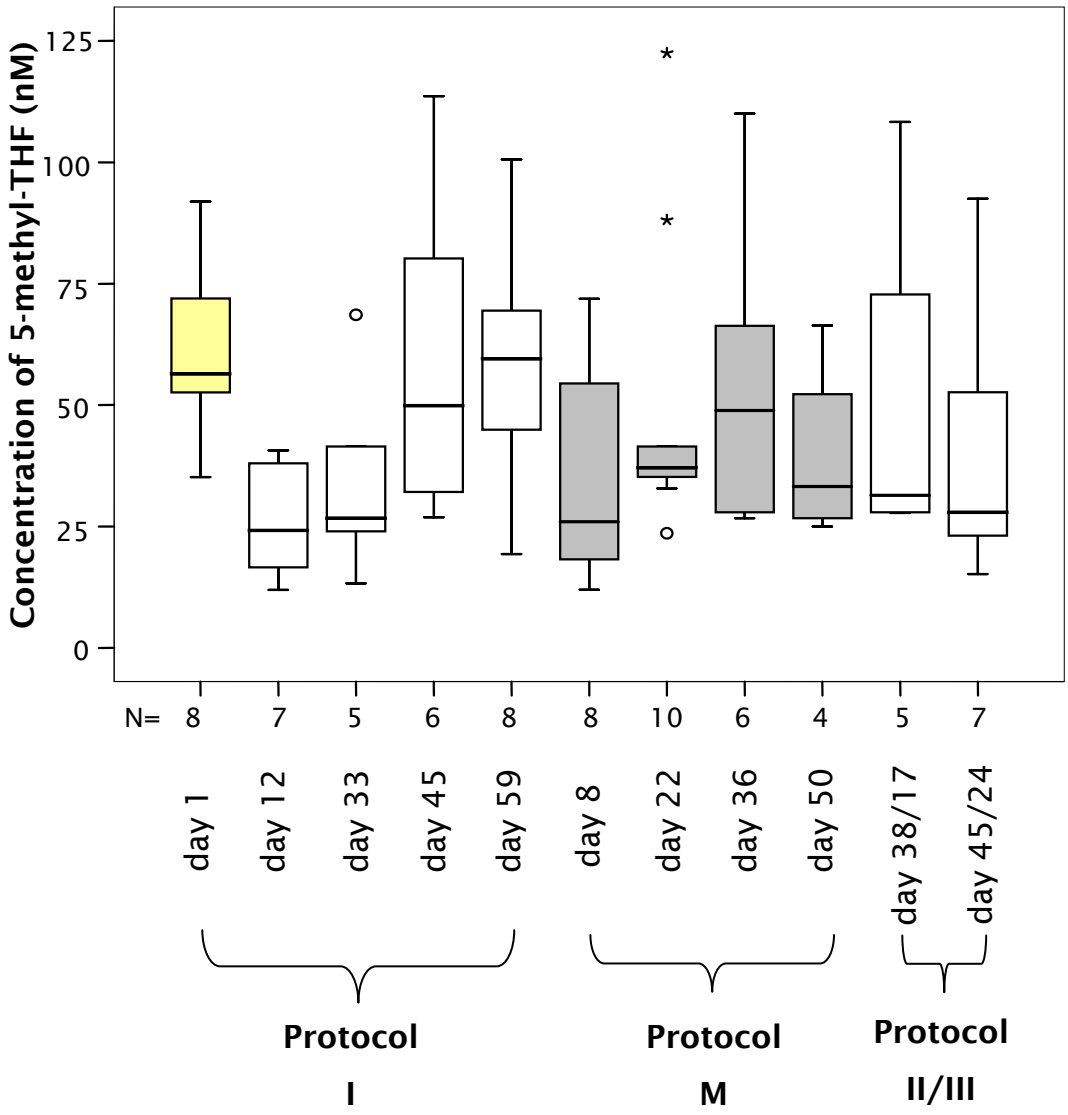

Fig. 4.11: CSF 5-methyl-THF concentrations during ALL BFM 2000. The yellow box-and-whisker plot depicts the concentration prior to any MTX therapy, white are the boxes in protocols with intrathecal MTX administration while the grey boxes represent the protocol with high-dose and intrathecal drug administration followed by rescue.

a nonsignificant difference between concentrations during protocol I (day 33, 45 and 59) and those prior to therapy.

Unfortunately, only from three patients samples were obtained both on day 1 and 12 (matched-pair). In two cases the concentration of 5-methyl-THF was

decreased almost twofold on day 12 compared to day 1. In the third case it was low on both days In four cases an intraindividual comparison of the concentrations prior to MTX therapy and at the end of protocol I (day 59) was possible and it revealed tendencially higher reduced folate levels at the end of therapy. The matched-pair concentrations of 5-methyl-THF on day 1, 12 and 59 are shown in Table 4.22.

Table 4.22: Concentration of 5-methyl-THF in the CSF of four ALL patients on day 1, 12 and 59 of protocol I.

Patient	Conc. 5-methyl-THF (nM)		
	Day 1	Day 12	Day 59
1	35.2	40.8	44.2
2	65.3	36.8	66.1
3	59.6	39.2	-
4	52.2	-	68.9
5	53.3	-	70.0

In protocol II/III a large interindividual variability was observed and the CSF 5-methyl-THF concentration was not significantly different compared to day 1 of protocol I.

Protocol M

Patients treated according to ALL BFM 2000 received in protocol M high-dose MTX treatment followed by intrathecal MTX administration and calcium folinate rescue. Samples were obtained from these patients on day 8, 22, 36 and 50, approximately 1 h after the beginning of the high-dose infusion and before intrathecal MTX administration. Similar as in the CSF samples obtained during protocol I and II/III, 5,10-methylene-THF could not be quantified during protocol M and was above LOD only in a few samples. Calcium folinate was not detected in any sample which was not surprising since the last rescue infusion dated at least two weeks prior to sample

collection. The concentration of MTX varied from 0.09-1.6 µM (median 0.5 µM). At the same time the 5-methyl-THF level was 12.0-122.5 nM (median 37.3 nM) indicating large interindividual variability which was probably due to the fact that MTX had already reached the CNS (see Fig. 4.11). Statistical analysis revealed no significant difference between the CSF 5-methyl-THF concentration on day 8, 22 and 36 of protocol M (Kruskal-Wallis test, p=0.285). Furthermore, the concentration of 5-methyl-THF was not significantly different at begin of therapy and during protocol M (Kruskal-Wallis test, p=0.132). Day 50 was excluded from analysis due to the small number of samples (n=4). An intraindividual monitoring of the concentrations of 5-methyl-THF was not possible since there was no patient from whom samples were obtained at all five time-points.

4.3.2 CSF concentrations of SAM and SAH

Protocol I and II/III

CSF samples collected with perchloric acid were used for the determination of SAM and SAH. The CSF SAH concentration could be quantified only in 19 samples (5.0-11.4 nM) whereas it was between LOD and LLOQ in the other 65 samples. The concentration of SAM was quantified in all samples and in analogy to the evaluation of 5-methyl-THF, the concentration measured on day 1 of protocol I was used as reference since patients had received no MTX therapy priorily. An overview of the concentrations of SAM during all protocols of ALL BFM 2000 is shown in Fig. 4.12.

The median SAM concentration in the reference samples was 188.6 nM (n=10). In samples obtained on day 12 the SAM concentrations (median 114.4 nM, n=8) were significantly lower compared to the reference value (Mann-Whitney U-test, p=0.012). On day 33 and 45 the difference was significant as well (p=0.031 and p=0.033, respectively). In addition, a matched-pair comparison was possible in seven patients samples from whom samples were obtained both on day 1 and 12 of protocol I (shown in Table 4.23). The significant difference between the SAM concentrations before and

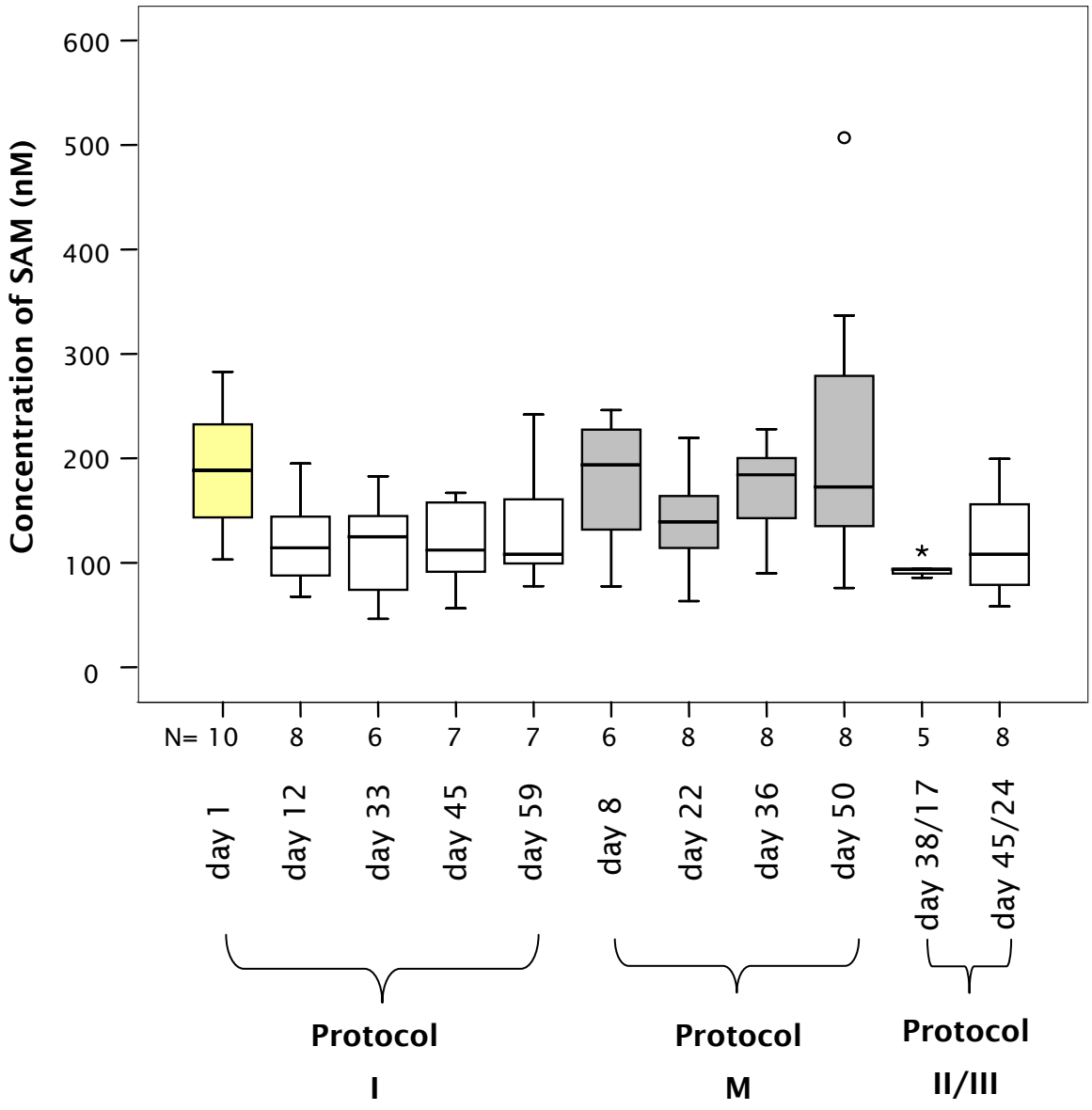

Fig. 4.12: CSF concentrations of SAM during protocol ALL BFM 2000. The yellow box-and-whisker plot stands for the concentrations prior to MTX therapy, grey box plots represent the concentrations in protocol M and white boxes are the concentrations of SAM during protocols with intrathecal MTX therapy only.

Table 4.23: CSF SAM concentrations in seven ALL patients on day 1 and 12 of protocol I.

Patient	Concentration of SAM (nM)	
	Day 1	Day 12
1	194.9	143.4
2	278.4	136.1
3	201.9	152.2
4	224.4	120.6
5	156.9	86.1
6	282.8	131.5
7	103.1	89.5

12 days after intrathecal MTX was confirmed in this group as well (Wilcoxon test, p=0.018).

In protocol II/III the median concentration of SAM was 93.7 nM (n=5) on day 17/38 and 108.1 nM (n=8) on day 45/24. On both days the CSF SAM concentrations were significantly lower compared to the beginning of therapy ($p<0.001$ and $p=0.012$, respectively). This result is interesting in view of the fact that the patients had not received intrathecal MTX in protocol II/III prior to day 38/17.

There was no significant difference between the CSF SAM concentrations from day 12-59 of protocol I and the concentration in protocol II/III (Kruskal-Wallis test, p=0.898).

Protocol M

The analysis of CSF samples obtained during protocol M enabled the assessment of the SAM concentrations two weeks after high-dose and intrathecal MTX therapy followed by calcium folinate rescue.

The concentrations of SAH were again below LLOQ in the majority of samples of the children population. SAM was determined in all analysed samples and was in the range 63.3-506.9 nM (median 177.6 nM) in protocol M (see

Fig. 4.12). Statistical evaluation was performed to determine whether there are significant differences among the CSF SAM concentrations during protocol M. The SAM concentrations in block M were also compared to the beginning of therapy (day 1, protocol I). While there were only two patients from whom samples were obtained on day 1 of protocol I as well as during protocol M it was not possible to perform an intraindividual comparation of SAM concentrations after MTX treatment followed by rescue. Therefore, the analysis was performed by using the Kruskal-Wallis test which revealed no significant difference among the concentrations of SAM on day 8, 22, 36, and 50 of protocol M ($p=0.380$). Furthermore, the SAM concentrations during protocol M did not differ significantly from those at the beginning of therapy ($p=0.501$).

4.3.3 CSF concentrations of reduced folates, MTX, SAM and SAH in ALL patients with neurotoxicity

Case 1

A 15-year old boy developed signs of subacute neurotoxicity during protocol M of ALL BFM 2000 (see also chapter 3.5.2). An evaluation of the neurotoxic symptoms was perfomed by the pediatric oncologists according to the CTC. The results (patient's symptoms and their severity) are presented in Table 4.24.

The CSF analysis of 5-methyl-THF, SAM and SAH was performed in three samples obtained from the patient. In the first sample obtained on day 45 of protocol I (approximately ten weeks prior to toxicity) the concentration of 5-methyl-THF (68.5 nM) was comparable to the median (56.4 nM) obtained from the ALL population prior to MTX therapy (day 1, protocol I) and somewhat higher than the median (49.9 nM) determined in the ALL population on the same therapy day. In the second sample obtained three days after the second episode of subacute neurotoxicity the concentration of

5-methyl-THF was less than one third compared to the first sample (19.1 nM) and about twofold lower than the median concentration in protocol M

Table 4.24: Evaluation of neurotoxicity according to CTC in case 1.

Symptom	CTC Grading	Description of severity according to CTC
Headache	2	Moderate pain
Ataxia (incoordination)	2	Mild symptoms interfering with function, but not interfering with activities of daily living
Dizziness	3	Interfering with activities of daily living
Irritability	1	Mild, easily consolable
Mood alteration (anxiety, agitation)	1	Mild mood alteration not interfering with function
Cranial neuropathy	3	Present, interfering with activities of daily living
Motor neuropathy	4	Paralysis
Pyramidal tract function	4	Bedridden or disabling; paralysis
CNS cerebrovascular ischemia	2	Not described

(37.3 nM) according to which the patient was treated at the time-point of toxicity. One month after the resolution of symptoms the concentration of 5-methyl-THF (49.6 nM) returned into the physiological range. Samples were not obtained during or shortly after the first toxicity episode.

An overview of the MTX treatment and CSF SAM and 5-methyl-THF concentration in the three samples from case 1 are presented in Fig. 4.13. The concentration of SAM followed a different pattern compared to 5-methyl-THF. The first sample contained 35.8 nM SAM which was more than threefold lower than the median 112.2 nM determined on day 45 of protocol I. Shortly after the toxicity the concentration of SAM was higher (79.3 nM) compared to the first sample but it was still about twofold lower than the median in block M (177.6 nM). No difference of the SAM level was observed one month after toxicity on day 17 of protocol II (82.8 nM). However, on the

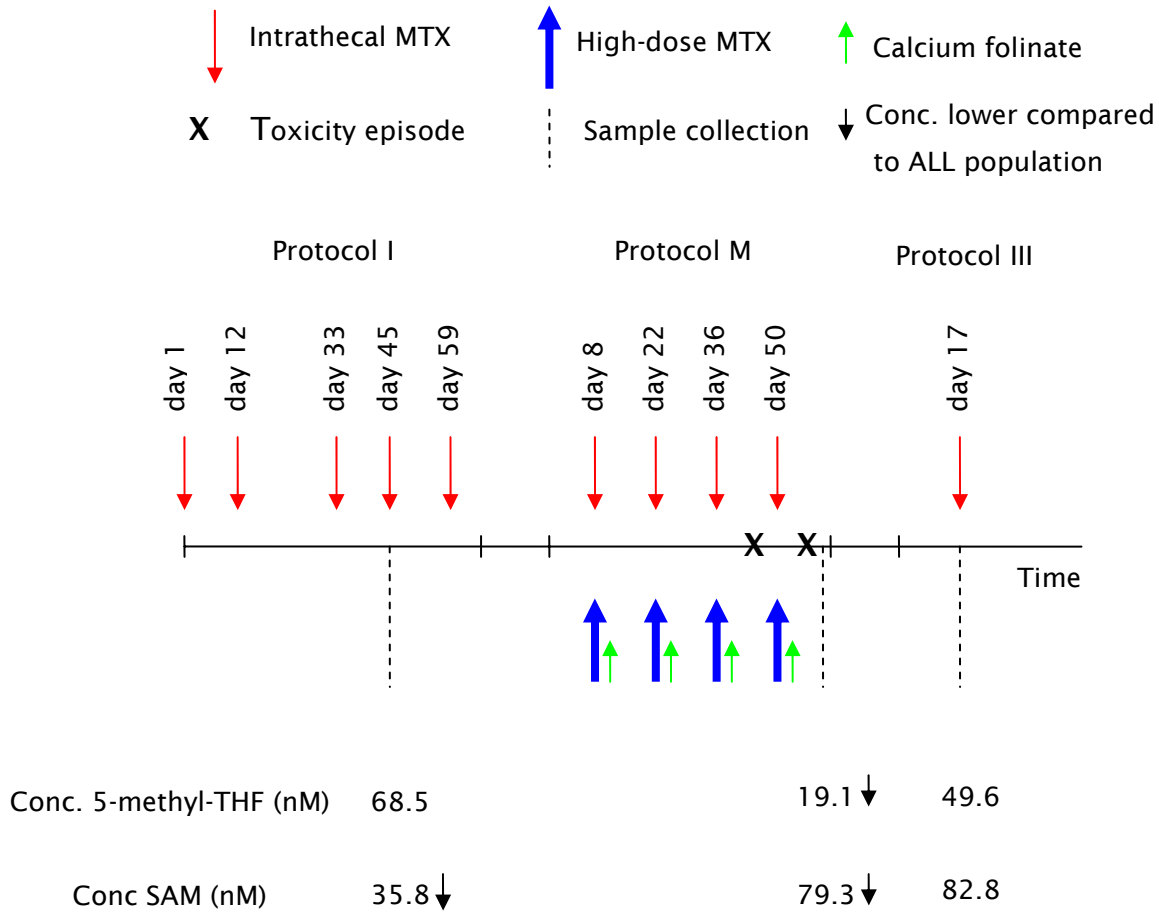

Fig. 4.13: MTX teatment, sample collection and CSF 5-methyl-THF and SAM concentrations prior to and after the second neurotoxicity episode in case 1.

same day the median SAM level in the ALL population was 93.7 nM. The concentration of SAH was above LLOQ in all three samples (6.7, 8.6 and 6.4 nM chronologically). A comparison of the SAH concentrations betweencase 1 and the pediatric ALL population without toxicity was not possible due to the inability of determining the SAH concentrations in most samples of the latter group.

Case 2

In analogy to case 1, this patient developed subacute neurotoxicity during protocol M (ALL BFM 2000). The results of the evaluation of neurotoxicity according to CTC are presented in Table 4.25.

Table 4.25: CTC neurotoxicity evaluation in case 2.

Symptom	CTC Grading	Description of severity according to CTC
Motor neuropathy	3	Objective weakness, interfering with activities of daily living
Speech impairment	3	Receptive or expressive dysphasia, impairing ability to communicate

The first CSF sample was obtained on day 33 of protocol I. The concentration of 5-methyl-THF (13.1 nM) was barely above LLOQ, and about twofold lower than the median (26.7 nM) on day 33 of protocol I. The concentration of the reduced folate (27.5 nM) just after resolution of toxicity was increased compared to the first sample but still lower than the median in protocol M (37.3 nM) according to which the patient was treated at that time. One week later, a further increase of the 5-methyl-THF concentration was observed (34.4 nM) but another 10 days later the patient experienced nausea and emesis and the CSF concentration of the reduced folate declined to 17.2 nM.
In contrast to case 1, the concentration of SAM in the first sample (146.9 nM) was comparable to the median obtained on day 33 (124.9 nM). In two samples obtained shortly after the toxic episode the SAM concentration (74.8 nM and 79.4 nM) was more than twofold lower than the median during block M (177.6 nM). In the fourth sample an increase of the SAM level was observed (106.9 nM) but it was still lower compared to the median during the protocol. The CSF SAH concentration was above LLOQ in all four obtained samples (12.6 nM, 10.4 nM, 5.8 nM and 12.4 nM chronologically).
An overview of the MTX treatment as well as the alterations observed in the 5-methyl-THF and SAM concentrations are depicted in Fig. 4.14.

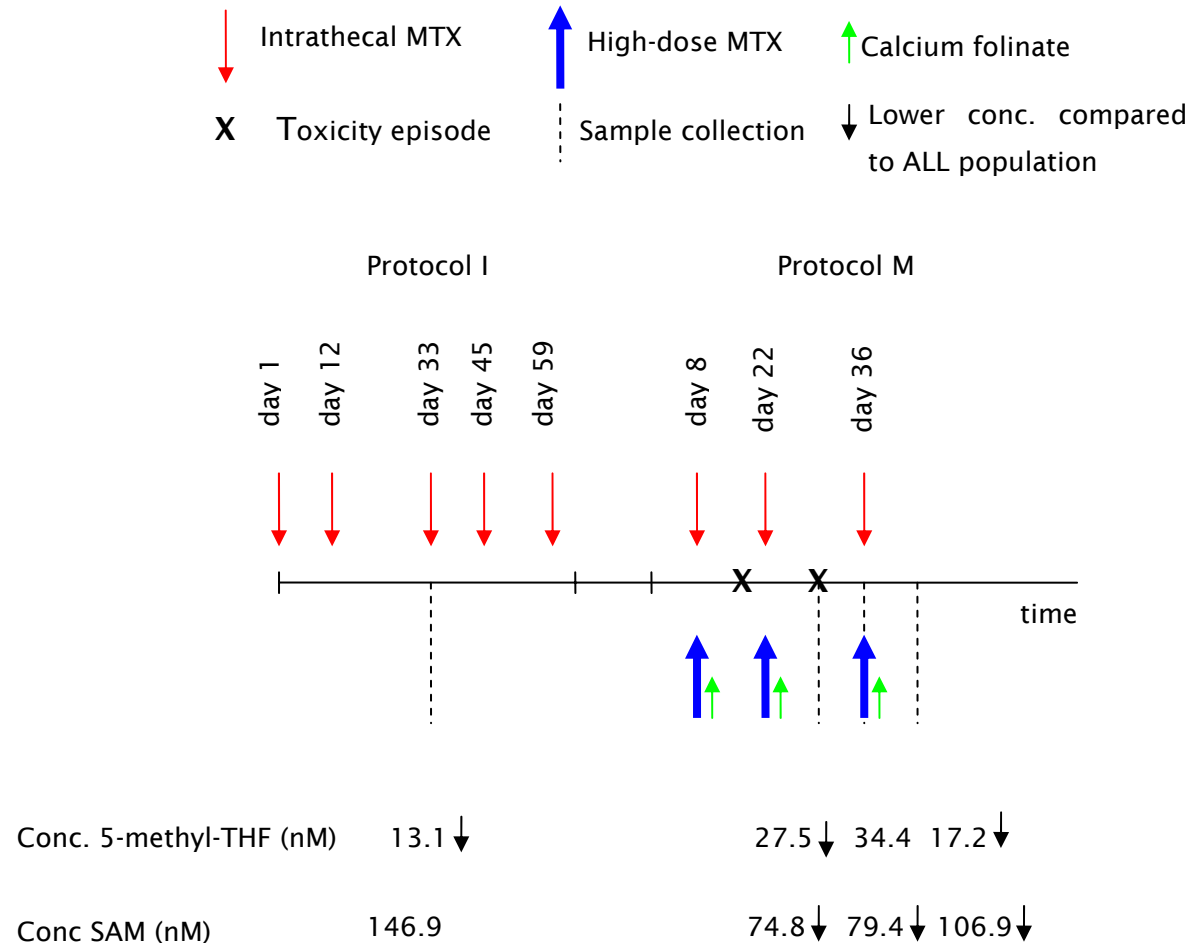

Fig. 4.14: MTX treatment, sample collection and CSF 5-methyl-THF and SAM concentrations prior to and after the second neurotoxicity episode in case 2.

Case 3

This patient was an adult female who presented with the second ALL recidive and was treated according to a protocol which did not contain MTX. She received MTX during therapy for the first ALL diagnosis as well as in the treatment of the first recidive. At the time of diagnosis of the second recidive, the patient had signs of MTX-related chronic neurotoxicity (manifest leukoencephalopathy). The evaluation of chronic neurotoxicity according to CTC is presented in Table 4.26.

Three CSF samples were obtained from the patient within a time period of four months. CSF analysis of 5-methyl-THF revealed a constant concentration (29.3-31.8 nM) in all three samples. Compared to the median concentration

Table 4.26: CTC evaluation of chronic neurotoxicity in case 3.

Symptom	CTC Grade	Description of severity according to CTC
Ataxia (incoordination)	3	Moderate symptoms interfering with activities of daily living
Cognitive disturbance	3	Cognitive disability resulting in significant impairment of work performance; cognitive decline >2 SD*
Confusion	3	Confusion or delirium interfering with activities of daily living
Depressed level of consciousness	3	Obtundation or stupor; difficult to arouse; interfering with activities of daily living
Irritability	1	Mild; easily consolable
Leukoencephalopathy-associated radiological findings	2	Moderate ventriculomegaly; and/or focal T2 hyperintensities extending into centrum ovale; or involving 1/3 to 2/3 of susceptible areas of cerebrum
Memory loss	2	Interfering with function but not with activities of daily living
Mood alteration (anxiety, agitation)	1	Mild mood alteration not interfering with function
Depression	2	Interfering with function but not with activities or daily living
Neuropathic pain	3	Severe pain
Personality change	2	Change but not disruptive to patient or family
Speech impairment	2	Awareness of receptive or expressive dysphasia, not impairing the ability to communicate
Tremor	2	Moderate tremor, interfering with daily function but not interfering with activities of daily living

SD: standard deviation

(56.4 nM) of the reduced folate in ALL patients prior to therapy (chosen as reference despite of the age of 35 years but due to the fact that CSF samples were obtained by lumbar puncture), the patient had approximately twofold lower concentrations of 5-methyl-THF. The SAM concentration was considerably lower in the first two samples (below LLOQ and 34.2 nM, respectively) compared to the median (188.6 nM) obtained from ALL patients

prior to any MTX therapy. In the third sample the SAM concentration was higher (184.5 nM). At that time-point the patient had an overt infection and signs of hyperbilirubinemia.
The concentrations of SAH were above LLOQ in all three samples (7.5-14.3 nM). The results of the CSF analysis are summarised in Table 4.27.

Table 4.27: CSF analysis of 5-methyl-THF, SAM and SAH in case 3.

Time	Concentration (nM)		
	5-methyl-THF	SAM	SAH
At time of the second recidive diagnosis	31.8	Below LLOQ	10.1
Three months later	31.7	34.2	7.5
Ten days later during acute toxicity not MTX-related	29.3	184.5	14.3

4.4 Pediatric ALL patients treated according to TOTAL XV

In cooperation with Prof. Dr. M. Relling (St. Jude Children's hospital, Memphis, USA) CSF samples were obtained from childhood ALL patients treated according to protocol TOTAL XV. SAM and SAH were not determined in this population since samples collected with perchloric acid were not obtained.
ALL patients received high-dose MTX infusion followed by calcium folinate rescue at the beginning of therapy (Remission/Induction phase, see also chapter 3.5.3). 19 days later they received intrathecal MTX as a part of the triple intrathecal therapy followed by calcium folinate rescue. The first CSF sample from these patients was obtained prior to any MTX therapy (day 1 of Remission/Induction) while the second was obtained about 8-9 weeks later at

the beginning of the consolidation phase (day 1). On day 1 of the consolidation block patients received another high-dose infusion of MTX in combination with triple intrathecal therapy and calcium folinate rescue. The third sample was obtained on day 15 of the consolidation block.

Alltogether 42 CSF samples were analysed from 18 patients. Complete sampling (3 samples per patient) was obtained only from six patients. From the remaining twelve patients samples were collected at two time-points. In summary, 10 CSF samples were obtained at day 1 of Remission/Induction (R/I), and 16 each were analysed from day 1 (C1) and day 15 (C15) of consolidation, respectively.

As in the other two collectives, 5,10-methylene-THF was below LLOQ in all analysed CSF samples. Calcium folinate which was administered at least two weeks prior to sampling was not detected in any sample. The concentration of 5-methyl-THF at the three time-points was highly variable 17.8-103.9 nM (mean 59.8 nM). The explorative data analysis revealed a normal distribution of the 5-methyl-THF concentration in the ALL population. Therefore, statistical analysis was performed by using of the two-way ANOVA. No significant difference of the analyte concentration among the three sampling time-points (p=0.932) was observed (see also Table 4.28:). A graphical presentation of the CSF concentrations of 5-methyl-THF in patients treated according to TOTAL XV is shown in Fig. 4.15.

4.5 Correlations among the CSF concentrations of MTX, 5-methyl-THF, SAM and SAH

In order to get a better insight into the biochemical alterations after MTX administration potential correlations between the concentrations of the drug, 5-methyl-THF, SAM and SAH in the CSF were investigated. The CSF samples

Table 4.28: Concentrations of 5-methyl-THF in the CSF of six patients during therapy according to TOTAL XV.

Patient	Concentration of 5-methyl-THF (nM)		
	R/I	C1	C15
1	67.5	67.1	67.2
2	62.7	62.0	66.4
3	86.2	84.6	86.8
4	59.8	61.8	56.2
5	68.5	68.1	79.3
6	43.0	36.1	43.5

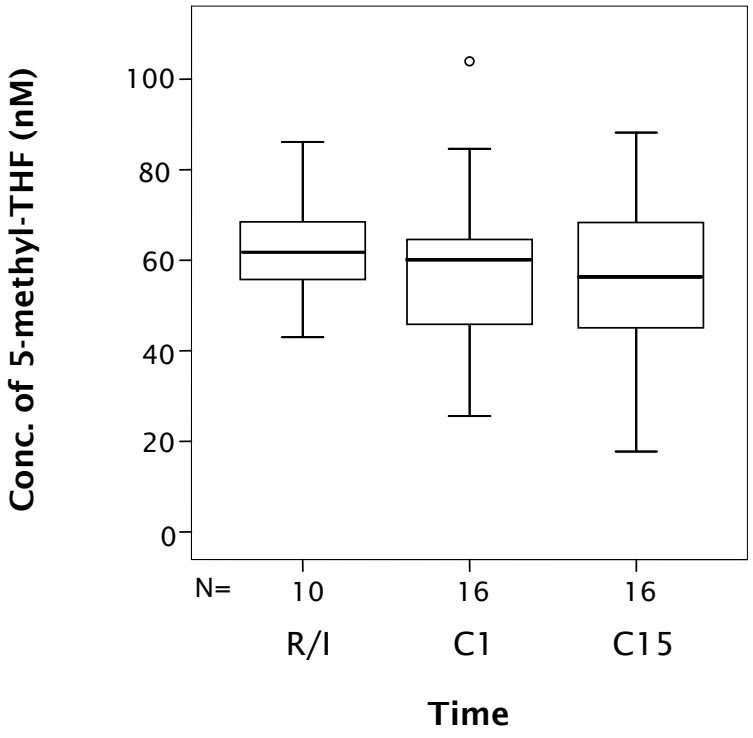

Fig. 4.15: Concentration of 5-methyl-THF in the CSF of patients treated according to TOTAL XV.

obtained from PCNSL patients were thought to be appropriate for such analysis since a monitoring of MTX, 5-methyl-THF, SAM and SAH concentrations was possible due to the sampling schedule. The correlation analysis was performed for patient 2, 3 and 4 together. Due to the two different MTX treatment modalities in block A/B and C the correlation investigation was performed first for all therapy blocks together and thereafter for block C alone. The rational for the latter analysis was the fact that calcium folinate was not administered in block C and would therefore not have interfered with the effects of the drug. All correlation analyses were performed by using of the rank correlation according to Kendall (see also chapter 3.6.3). All substance combinations were investigated (see Table 4.29). A significant correlation of concentrations was found only by SAM and SAH in block C. However, this correlation was not significant when all therapy blocks were assessed together.

Table 4.29: Correlation of concentrations of 5-methyl-THF, MTX, SAM and SAH during therapy in all blocks together and in block C.

Compounds	**Rank correlation according to Kendall**	
	All blocks	**Block C**
5-methyl-THF/MTX	r=-0.155, p=0.167, N=39	r=-0.292, p=0.106, N=17
5-methyl-THF/SAM	r=-0.037, p=0.717, N=45	r=-0.186, p=0.214, N=23
5-methyl-THF/SAH	r=-0.037, p=0.797, N=25	r=-0.375, p=0.053, N=15
MTX/SAM	r=-0.193, p=0.064, N=45	r=-0.239, p=0.181, N=18
MTX/SAH	r=-0.165, p=0.277, N=23	r=0.463, p=0.051, N=15
SAM/SAH	r=0.255, p=0.058, N=28	r=0.394, p=0.042, N=15

5 DISCUSSION

In the following chapter the results presented in chapter four will be discussed in the same order.

5.1 HPLC method for the CSF determination of reduced folates and MTX

A method for the determination of MTX, calcium folinate, 5-methyl-THF and 5,10-methylene-THF was developed and validated. The CSF concentration of 5-methyl-THF was assessed due to the fact that it is the main transport form of reduced folates and it is also linked to other metabolic pathways such as homocysteine. The CSF concentration of 5,10-methylene-THF necessary for thymidilate synthesis might be another marker for MTX-induced cytotoxicity since its reduction directly affects the DNA synthesis. It was therefore investigated simultaneously.

Several methods for the quantification of plasma and CSF 5-methyl-THF concentrations based on microbiologic assays and reversed phase HPLC were found in the literature[141, 152, 153]. Due to the poor reproducibility of the microbiological assays an HPLC method was considered. Since the concentrations of 5-methyl-THF in the CSF are in the nanomolar range sensitive reversed phase HPLC methods using electrochemical or fluorescence detection are necessary for its determination[141, 152, 154]. Although the use of electrochemical detection provides generally higher sensitivity compared to fluorescence it also has disadvantages. The mobile phase has to be degased constantly, the water used for the buffer has to be of highest purity and it is difficult to obtain a stable baseline when the detector is set at elevated potentials. Under such conditions it is difficult to develop a robust method. On the other hand, it is known that the CSF concentrations of 5-methyl-THF exceed the respective plasma concentrations by two to fourfold (in healthy adults 30-80 nM[141, 150, 155], in children 60-80 nM[136, 151]). Thus, the use of fluorescence detection was supposed to provide enough sensitivity for

its quantitation in CSF[141]. In addition, tetrahydrofolates exhibit natural fluorescence. Thus, no derivatisation is necessary for their determination. MTX is not a reduced folate and does not exhibit fluorescence at 294/356 nm. Therefore, it and had to be determined by using an UV detector.

The method for the simultaneous determination of 5,10-methylene-THF, 5-methyl-THF, calcium folinate and MTX used in this study was based on the work of Belz et al[141]. The modification consisted in adding one more analyte (5,10-methylene-THF) and substituting acetonitrile for methanol in the mobile phase. A gradient which was optimised during method development was used for the elution of the analytes. The method was validated and the results presented in chapter 4.1.1 show that it is acceptable according to the international criteria for method validation.

The main problem when developing a method for the determination of tetrahydrofolates is the instability of these compounds. It was shown that 5,10-methylene-THF was the most instable of the analytes at room temperature whereas calcium folinate was the most stable one. The stability of calcium folinate was not surprising since it is the main reason why this compound is administered as rescue. Because of the instability of two reduced folates at room temperature samples could not be kept in the autosampler overnight. As a consequece the number of samples which could be analysed per day was lower.

The sensitivity of the method was high enough for the quantfication of 5-methyl-THF and MTX in the CSF. However, the concentration of 5,10-methylene-THF was rarely quantifiable and in many cases even not detectable. Literature data regarding the concentration of this compound in the CSF were not found. The fact that 5,10-methylene-THF could be determined in one PCNSL patient who received frequent calcium folinate rescue within 24 h after the end of the high-dose MTX infusion indicates that it is possible to determine 5,10-methylene-THF in the CSF using the described method after highly intensive rescue regimens. Possible explanations for the low CSF concentration are that 5,10-methylene-THF

could be trapped in the cell having a poor affinity for the reduced folate carrier and rapid metabolism or consumption for thymidilate synthesis. Calcium folinate was also quantified in only one (and the same) patient as 5,10-methylene-THF. This indicates that also calcium folinate may be quantified in the CSF using the described method only after very intensive rescue regimens. Such result is plausible since it was estimated that only approximately 1% of systemic calcium folinate passes the blood-brain barrier[156]. Peripherally, it is rapidly converted into 5-methyl-THF which then passes the blood-brain barrier by means of active transport[156-158]. The method was sensitive enough for the quantification of nanomolar ranges of MTX and therefore appropriate for the monitoring of the drug concentration in the CSF of patients treated with systemic high doses or intrathecally.

For the quantification of 5,10-methylene-THF in the CSF a more sensitive method is necessary. This could be achieved by using a more sensitive fluorescence detector and increasing the volume of injection which was in this case kept at 50 µL to provide a longer duration of the precolumn and column. Moreover, exctraction procedures could be considered in order to obtain concentrated samples.

The advantage of the described method is its simplicity. Due to natural fluorescence samples are directly injected into the autosampler without any previous time-consuming derivatisation. Moreover, the sensitivity of the method is sufficient for the determination of 5-methyl-THF and MTX so that there is no need for a pre-concentration of the analytes in the sample. Furthermore, the method revealed to be very robust which is a considerable advantage in routine analysis.

In summary, the described reversed-phase HPLC method is adequate for determining the concentration of the main form of tetrahydrofolates (5-methyl-THF) and MTX in the CSF of patients after systemic high-dose or intrathecal administration of MTX followed by calcium folinate rescue. Due to its simplicity and low cost it is suitable for routine use in monitoring the above mentioned analytes.

5.2 HPLC method for the CSF determination of SAM and SAH

A decreased concentration of SAM or altered SAM/SAH ratio has been associated with disorders such as demyelination seen in patients with MTX-related leukoencephalopathy, Alzheimer's dementia, depression and Parkinson's disease[117, 129-131, 137-139, 159]. Therefore, the determination of SAM and SAH in tissue, plasma and CSF has gained more and more interest in the past few decades. Various methods for the analysis of SAM alone or together with SAH mostly based on reversed phase HPLC were found in the literature[143-146, 160-163]. In brain tissue where the concentrations of these compounds are relatively high simple methods using HPLC with UV detection are sufficient for their quantification[160-163]. In CSF and plasma, the concentration of SAM and SAH is about 1000-fold lower than in the tissue and therefore more sensitive methods are required. An overview of methods used for urine, plasma or CSF determination of SAM and SAH are presented in Table 5.1.

The most sensitive published method for the determination of SAM and SAH employed HPLC with electrochemical detection (see Table 5.1). Melnyk et al.[165] analysed SAM and SAH in plasma, lymphocytes and tissue using a method which did not require extraction or derivatisation of the compounds. Furthermore, both SAM and SAH were separated and quantified in the same run. However, the most important disadvantage of the method was the use of a relatively high potential (+920 mV) for the oxidation of the compounds. Such high potential is often associated with poor selectivity since most endogenous CSF compounds are oxidised as well. Moreover, it stresses the analytical cell of the electrochemical detector resulting in a short duration of live. Further, disadvantages consisted in using the highest quality of water, filtering and constant degasing of the mobile phase to achieve the required robustness of the method. However, because of the high sensitivity of the method its use was initially planned for the analysis of the CSF concentration

Table 5.1: Methods for SAM and SAH quantification in urine, plasma and CSF

Authors	Method	LOD	Matrix	Comment
Wagner et al. (1987) [143]	HPLC-FD	SAM 0.1 pM SAH not analysed	Urine	Derivatisation with CAA at 40 ºC for 12-16 h.
Weir et al. (1992)[164]	HPLC-FD	Not stated	CSF	Derivatisation with CAA at 39 ºC for 8 h SAM and SAH were determined in separate runs
Capdevila et al. (1998)[149]	HPLC-FD	SAM 5 pM SAH not stated	Plasma	tedious extraction of plasma samples prior to derivatis-ation with NDA for 10 min Determination in separate runs
Kishi et al. (2000)[137]	HPLC-FD	SAM 15 nM SAH 7 nM	CSF	Derivatisation whith CAA for 60 min at 60 ºC Separation of SAM and SAH in the same run on a vinyl copolymer column
Castro et al. (2002)[144]	HPLC-FD	SAM 5 nM SAH 2.5 nM	Plasma	Derivatisation with CAA 4h/40 ºC SAM resulted as impure peak and had to be purified and measured again.
Melnyk et al. (2000) [165]	HPLC-ECD	SAM 200 fM SAH 40 fM	Plasma	Redox potential set at 400 and 920 mV SAM and SAH separation in the same run
Struys et al. (2002) [166]	MS-MS	SAM 7.5 nM SAH 2.5 nM	CSF	Simultaneous SAM and SAH analysis

CAA: chloroacetaldehyde; ECD: electrochemical detection; FD-fluorescence detection; LOD: limit of detection; MS-MS: tandem mass spectrometry NDA: naphtalene dialdehyde;

of SAM and SAH in this study. During the method development several problems occured. First, the baseline stability was poor requiring a long time for equilibration. Second, when standard solutions or CSF samples were injected a broad peak appeared in the chromatogramme which disabled the detection of the desired analytes. This interfering peak derived from the perchloric acid added to the standard solutions and CSF samples. The confirmation was obtained by analysing a solution of perchloric acid alone and standard solutions containing no perchloric acid. In the study of Melnyk et al. samples were collected with the addition of trichloroacetic acid[165]. Since all CSF samples in this study were collected before and during method development using perchloric acid it would have been difficult to analyse them using this method.

Therefore, an attempt was made to develop and validate an HPLC method using fluorescence detection which would be sensitive enough for determining the CSF concentrations of SAM and SAH. Several methods for the quantification of adenosine, SAM and SAH based on reversed-phase HPLC with fluorescence detection have been described in the literature[143-146]. Since SAM and SAH do not exhibit natural fluorescence derivatisation of the analytes is required prior to analysis. The reaction of these compounds with chloroacetaldehyde resulting in their fluorescent 1,N^6-etheno derivatives has been well studied[137, 143, 144, 146, 164]. However, the sensitivity of these methods for CSF SAH analysis was questionable since the determined SAH concentration often coincided with the lower limit of detection of the method. Moreover, the separation of these compounds seemed to be difficult since they were often analysed in separate runs with different mobile phases. It was shown in the work of Wagner et al.[143] that the derivatisation reaction with chloroacetaldehyde depends on the concentration of the derivatising agent, the pH as well as time and temperature of derivatisation. The authors showed that optimal derivatisation conditions are obtained with a 5.5 M solution of chloroacetaldehyde, a pH between 3.5 and 4.0, and a 12-16 h incubation at 39 ºC. These conditions were reproduced for the derivatisation of SAM and SAH in this work. Although some authors used shorter incubation

times such as 4 or 8 h at 39 °C[144, 164] or even 1 h at 60 °C[137] this was not attempted in this method since according to Wagner et al. a loss of the analytes due to incomplete derivatisation occurs.

The disadvantage of chloroacetaldehyde as derivatising agent is its unspecificity since all CSF compounds containing adenosine in their structure can build the 1,N^6-etheno derivatives. The similar structure of the derivatives would further present a difficulty for the separation of the compounds. In fact, tedious separation procedures of the 1,N^6-etheno derivatives of SAM and SAH have been reported in several articles[143, 164, 167].

In order to obtain the separation of 1,N^6-etheno SAM and 1,N^6-etheno SAH in the same run, different columns were tested. An column with an endcapped amine phase, a reversed-phase column and a reversed-phase column especially designed for the separation of amino acids were tested (see also chapter 3.4.4 and 4.1.2). Since both 1,N^6-etheno derivatives are polar compounds their separation was attempted on a relatively polar column. Although different mobile phases and different gradients were examined, no successful separation of the compounds on the polar column was achieved. Also the reversed-phase column turned out to be inadequate. Although the 1,N^6-etheno derivatives of SAM and SAH were separated from each other poor selectivity was observed. Peaks were neither sharp nor symmetric and the required sensitivity could not be obtained. In contrast, good results were obtained with the use of the reversed-phase column especially designed for the separation of amino acids. The selectivity was adequate, the investigated peaks were sharp and symmetric and a much higher sensitivity was achieved. This is in accordance with other authors who investigated the separation of SAM and its metabolites (without prior derivatisation) on different reversed-phase columns observing a similar effect[168]. Furthermore, the influence of the column properties was also investigated for 1,N^6-etheno derivatives of adenosine and adenine. A good separation of the compounds was achieved with the use of a vinyl alcohol copolymer column[146].

The mobile phase which was selected for the separation of the compounds consisted of acetonitrile and a phosphate buffer containing the ion-pairing

reagent heptanesulphonic acid. Since 1,N^6-etheno SAM is a cation which elutes very quickly disabling a successful separation from other endogenous compounds, the role of the heptanesulphonic acid was to neutralise the analyte by forming an ion-pair. The ion-pair had a higher affinity towards the column compared to 1,N^6-etheno SAM resulting in a longer retention time. Before the introduction of heptanesulphonic acid into the mobile phase 1,N^6-etheno SAM eluted before 1,N^6-etheno SAH whereas in the presence of the ion-pairing reagent the peak of the SAM derivative was detected later than that of the SAH derivative.

The method was further optimised regarding the mobile phase gradient in order to obtain a good separation of compounds in the shortest possible time. Finally, the retention time for 1,N^6-etheno SAH was 19.7 min and for 1,N^6-etheno SAM 23.5 min. One run was completed in 35 min which can be considered as acceptable run time in routine. The validation of the analytical method followed its development.

It is known that temperature and pH influence the stability of SAM. It was shown that the highest stability of SAM was obtained in acidic solution (pH 1) whereas in alkaline solution (pH 10) it was completely degraded after 48 h at 37 ºC. Moreover, a reduced SAM concentration in CSF samples stored at -20 ºC for at least one year at physiological pH was measured compared to *the same* samples stored at -20 ºC and pH 1. Such result indicates that SAM is instable in the CSF when stored frozen at physiological pH. On the other hand, significantly higher CSF SAH concentrations were determined in samples kept frozen at pH 7.4 compared to the same samples kept frozen at pH 1. Nevertheless, the increase of the SAH concentration was by far not equimolar to the loss of SAM. This indicated that the decomposition of SAM in the CSF was only partly due to the conversion to SAH.

An explanation for the pH- and temperature-dependent decomposition of SAM was found in the work of Parks et al[169]. They showed that after heating (temperature not stated) at pH 4 or pH 7 for 30 min the hydrolysis to methylthioadenosine and homoserine takes place (Fig. 5.1, cleavage at site A). A brief heating (100 ºC) in alkaline solutions results in the formation of

methionine (Fig. 5.1, cleavage at site B) whereas alkaline hydrolysis at lower temperatures (25 °C) results in the formation of adenine and S-ribosylmethionine (Fig. 5.1, cleavage at site C). The formation of SAH (Fig. 5.1, cleavage at site D) and decarboxylated SAM (Fig. 5.1, cleavage at site E) were considered to be due to enzymatic reaction and were not detected in vitro[169] . Based on the results of Parks et al. it was hypothesized that the degradation observed at -20 °C could be due to enzymatic degradation *prior to* freezing.

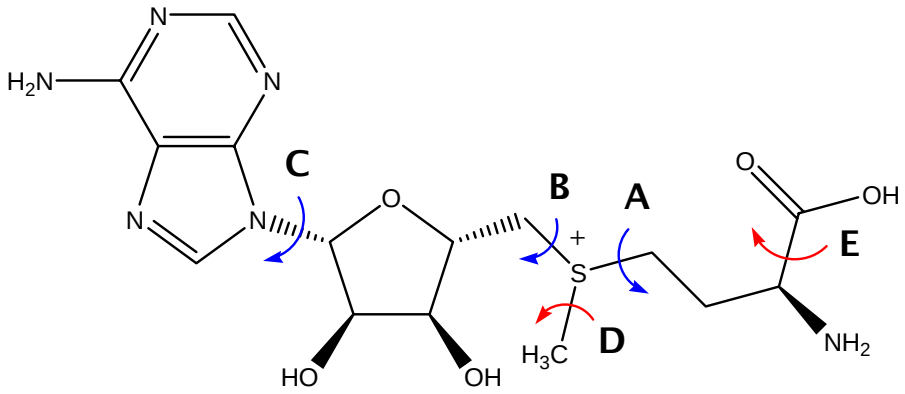

Fig. 5.1: Degradation of S-adenosylmethionine: hydrolysis sites A, B and C, enzymatic cleaving possible at sites D and E.

Such hypothesis was additionally based on the fact that there are over 100 enzymes belonging to the family of methyltransferases which could have been responsible for the demethylation of SAM[120]. Perchloric acid is used to precipitate proteins in biological samples and would therefore have inactivated all enzymes in a sample offering an explanation for the different results in acidic and neutral samples. If the enzymatic decomposition was responsible for the observed alteration of the CSF SAM and SAH concentrations a CSF sample which was kept at room temperature for an hour should have had altered SAM and SAH levels compared to the same sample which was immediately deproteinised with perchloric acid, centrifuged and frozen. However, neither decreased SAM nor increased SAH

concentrations were measured in the samples kept 1 h at room temperature compared to the aliquots which were acidified and frozen. This led to the conclusion that it is not the enzyme activity but possibly a chemical degradation which induces the decrease of the concentration of SAM and increase of the concentration of SAH in the samples which are kept for a longer time at -20 ºC without prior addition of perchloric acid.

However, the mechanism of SAM metabolism in the CSF seems to be complex and the reason why a loss of SAM in the CSF at physiological pH was observed only after long-time storage (at least one year) remains unclear and requires further investigation. Nevertheless, due to the instability of SAM special attention had to be paid to the storage of biological samples in which SAM and SAH were to be quantified. Using CSF samples without stabilisation with perchloric acid for the investigation of the methyl-transfer pathway after MTX therapy would lead to the false conclusion that the altered SAM and SAH concentrations are a consequence of MTX activity. Therefore, when CSF samples are stored at -20 ºC they should be acidified (pH 1) priorily or measured within a short time-period after collection.

Although the HPLC method for determination of CSF SAM and SAH in this work fulfills the requirements according to the international criteria for the biovalidation of analytical methods it was observed that the coefficient of variation of the between-day precision for 1,N^6-etheno SAM was relatively high (7.9-19%). The somewhat poorer reproducibility of the method for 1,N^6-etheno SAM was probably due to the use of the ion-pairing reagent. Similar findings have been described in the literature[144].

The method was shown to be sensitive for the CSF analysis of SAM in patients after systemic high-dose and/or intrathecal MTX treatment since SAM was quantified in all samples. In contrast, SAH was only detected in most samples but not quantified. From four adult patients three had quantifiable CSF SAH levels (all three patients were > 60 years) whereas in the children ALL population this was the case only in 19 of 84 samples. The limit of detection of SAH was 1 nM whereas the limit of quantification was 5 nM suggesting that in most of the analysed CSF samples in this population the

SAH concentration ranged between 1 and 5 nM. Compared to literature data[137, 164, 166], the CSF SAH concentration was significantly lower in this study (see also Table 5.2).

Table 5.2: CSF SAH concentrations determined in several studies in different patient populations.

Author	Method	Sample storage	Population	CSF SAH conc. (nM)
Keating et al. 1991[132]	HPLC-FD	pH 1, -20 ºC	29 adult patients with supposedly normal CSF SAH levels	Range 8.9-41.1 Mean 19.0
Kishi et al. 2000[137]	HPLC-FD	Physiological pH, -70 ºC	18 reference children 7 children with ALL receiving MTX therapy	Mean 17.3 Mean 18.9
Struys et al. 2000[166]	MS-MS	Physiological pH, -20 ºC	10 children with unknown neurological dysfunction	Range 8.9-14.1 Mean 11.3
This work	HPLC FD	pH 1, -20 ºC	22 children with ALL 4 adults with PCNSL both groups receiving MTX	Range <1.0-15.2* Range <1.0-12.5*

*median not calculated since most values were below LLOQ, ECD: electrochemical detection; FD: fluorescence detection; MS-MS: tandem mass spectrometry

Keating et al.[132] and Kishi et al.[137] measured higher CSF SAH concentrations in a reference population compared to the results in this study. The latter group also reported no significant difference in the CSF SAH level between the pediatric reference population and patients with ALL. On the other hand,

Struys et al.[166] measured somewhat lower CSF concentrations of SAH in a pediatric reference population. In a personal communication these authors reported that also lower SAH levels (5 nM) were observed in other patients[170]. Such observation is in accordance with the results in this study. Moreover, it cannot be excluded that the somewhat lower SAH concentrations in this study result from the difference in storage conditions.

In summary, a method for the determination of SAM and SAH as their 1,N^6-etheno-derivatives using reversed phase HPLC with fluorescence detection has been developed and validated. The advantage of this method was the separation of the analytes within one run of acceptable duration. It was shown that the successful separation and the achievement of appropriate sensitivity depended primarily on the selection of the column and that especially packed columns are necessary for obtaining satisfactory results. Such columns are cost-intensive but performing two separate runs for analysis of SAM and SAH or tedious clean-up procedures are time-consuming and at the end more expensive.

Since the 1,N^6-etheno derivatives are stable at room temperature for at least 24 h samples can be kept in the autosampler and analysed overnight enabling the determination of a larger number of samples (25-30) per day. The disadvantage of the method is certain the time-consuming derivatisation process. Also it is concluded that this method is not sensitive enough for the CSF analysis of SAH in the children population who had received MTX as high-dose infusion and/or intrathecally. Therefore, in order to determine the low SAH concentrations a more sensitive method is necessary. As the optimal derivatisation process has been applied, an adequate column regarding peak symmetry and sharpness has been used and a large sample volume (100 µL) has been injected the achievement of higher sensitivity could be attempted by using a more sensitive fluorescence detector.

5.3 CSF concentrations of MTX, reduced folates, SAM and SAH in PCNSL patients

The biochemical alteration of the folate and methyl-transfer pathway in the CNS after MTX therapy was studied in patients treated according to the Bonn protocol for PCNSL. Due to the frequent intraventricular drug administration and the presence of an Ommaya reservoir it was possible to obtain CSF samples within 4-5 subsequent days during therapy blocks. Therefore, the CSF concentrations of reduced folates and SAM and SAH closely after MTX therapy could be monitored. Moreover, patients received a combination of high-dose (3-5 g/m^2) and intraventricular MTX (3 mg) followed by rescue in some therapy blocks (A and B) whereas intraventricular MTX without rescue was administered in block C. Therefore, the influence of rescue could be assessed. Furthermore, in one patient the effect of an intensified rescue with calcium folinate (60 mg/6 h, 5 administrations) on the concentration of reduced folates was monitored within 24 h after the end of the high-dose (3 g/m^2) MTX infusion.

MTX concentration

At the end of the high-dose infusion the CSF concentrations of MTX were 0.7 to 2 µM (median 1.5 µM, n=6). Seidel et al.[171] reported similar concentrations in a pediatric ALL population who received 6-8 g/m^2 infusions. They determined a CSF concentration of 0.1 to 1.2 µM per 1 g/m^2 MTX two hours before the end of a 24 h infusion. Millot et al.[172] measured also a mean of 1.5 µM of MTX in the CSF at the end of a 24 h infusion of 5 g/m^2 in pediatric ALL patients. During block C the CSF drug concentrations were 0.2 to 9.1 µM 24 h after intra-Ommaya administration. Strother et al.[35] reported peak CSF MTX levels of 423.6 µM (median) after an intra-Ommaya administration of 2 to 10 mg (median dose 6 mg) which decreased to 0.4 to 138.2 µM (median 4.6 µM) 24 h later.

A large intra- and interindividual variability of the CSF MTX concentration was observed in all blocks. In several studies variability of MTX concentrations in

the serum and CSF after high-dose MTX infusions alone or in combination with intrathecal MTX has been reported[171, 173, 174]. However, it has been shown that intraventricular administration reduces the concentration variability[40]. This was not confirmed in the present study since in block C were only intraventricular MTX was administered a large intra- and interindividual variability was observed as well (0.2-9.1 µM). Furthermore, in some samples in block C extremely high MTX concentrations were determined (18.7-39.4 µM). Nevertheless, such results are in accordance with the findings of Strother et al.[35] who also observed a large intra- and interindividual variability after intra-Ommaya MTX administration (0.4-138.2 µM 24 h after administration of 2-10 mg).

Concentration of reduced folates

In six CSF samples obtained at the end of the high-dose MTX infusion the concentration of 5-methyl-THF was below LLOQ (10 nM). On day 3 of block C the median CSF 5-methyl-THF concentration in the same patients was 73.8 nM. Since this sample was obtained more than two weeks after the high-dose and intraventricular MTX treatment followed by rescue the CSF level of the reduced folate was considered physiological for these patients and was used as reference. A more than sevenfold decrease of the CSF concentration of the main reduced folate was present at the end of the high-dose (3-5 g/m^2) MTX infusion. Surtees et al.[136] also reported a significant decrease of 5-methyl-THF in the CSF of children suffering from ALL at the end of a high-dose MTX infusion (6-8 g/m^2). However, the extent to which the reduced folate concentration was depleted was not stated.

The administration of calcium folinate induced an increase in the concentrations of 5-methyl-THF in all patients. In the sample obtained approximately 15 h after initiation of rescue (30 mg/m^2, see also chapter 3.5.1) the concentration of 5-methyl-THF was in the physiological range (day 3 block A/B) but calcium folinate was not detected. It is known that calcium folinate is systemically converted to 5-methyl-THF which then passes the blood-brain barrier[156, 158, 175, 176]. The results of the CSF investigation are in

accordance with the findings of Mehta et al[156] who reported primarily an increase of 5-methyl-THF in the CSF after intravenously administered rescue. Also Allen et al.[177] observed that the CSF concentration of the reduced folate increased after oral calcium folinate rescue. An overview of the literature data referring to CSF 5-methyl-THF concentrations after calcium folinate rescue is presented in Table 5.3.

An increase in the concentration of 5,10-methylene-THF was observed only in PCNSL patient 1 (see 4.3.1) who received intensified rescue. In this patient the concentration of 5,10-methylene-THF was 28 nM 23 h after start of rescue indicating that the concentration of the metabolite was above the physiological range (literature data for CSF concentrations of 5,10-methylene-THF are not available). It is probable that calcium folinate was converted to both 5-methyl-THF and 5,10-methylene-THF resulting in their elevation. Such hypothesis was made due to the fact that in the folate cycle 5,10-methylene-THF is *irreversibly* converted into 5-methyl-THF[157, 178-180].

Calcium folinate which was always administered as a racemic form was detected only after intensified rescue (120 nM, 23 h after rescue start). Thyss et al[158]. could not detect calcium folinate in the CSF within 24 h after an infusion of 100 or 250 mg in healthy individuals who were not administered MTX. In contrast Mehta et al.[156] found low concentrations of calcium folinate in the CSF after the administration of 50-100 mg/m^2 following intraventricular MTX administration and reported that the CSF concentration of calcium folinate represented less than 1% of the corresponding serum concentration. This offers an explanation for the fact that in this work calcium folinate was not detected in the CSF of PCNSL patients who received standard rescue.

During block C in which the patients received intraventricular MTX only a constant decrease of the concentration of 5-methyl-THF was observed. At the end of block C a two to threefold lower CSF concentration of 5-methyl-THF was measured (median 23.2 nM on day 7 vs 73.8 nM on day 3). At that time-

Table 5.3: CSF 5-methyl-THF concentrations after high-dose or intraventricular MTX treatment followed by calcium folinate rescue.

Authors	MTX therapy	CSF 5-methyl-THF conc. after MTX therapy	Calcium folinate rescue	CSF 5-methyl-THF conc. after rescue
Mehta et al.[156]	6.25 or 7 mg/m² i.cv.	Not reported	50 or 100 mg/m² i.v.	200-600 nM few hours after rescue
Allen et al.[177]	8-12 g/m² i.v.	Not reported	10 mg/6 h p.o. Total 11 doses	≈ 30 nM 72 h after rescue start
Thyss et al.[158]	2.5 g/m² i.v.	Not reported	Dose not stated	50 nM two weeks after third high-dose MTX infusion
Surtees et al.[136]	6-8 g/m² i.v.+ 7.5-12.5 mg i.th.	Not reported	Dose not stated	Not significantly different compared to prior therapy
This work	3-5 g/m² i.v. + 3 mg i.cv. PCNSL patients	Below 10 nM at the end of the MTX infusion	30 mg/m² i.v. 3x prior to sampling	56 nM approx. 15 h after rescue start
	5 g/m² i.v. + 8-12 mg i.th. ALL patients (see also chapter 5.4)	Not measured	15 mg/m² i.v. 3x	Not significantly decreased two weeks after therapy

point (day 7) patients had received a cumulative dosis of 12 mg MTX intraventricularly. Also Quinn et al.[106] reported low CSF 5-methyl-THF concentration in one patient after intra-Ommaya MTX administration (2 mg/day, 3 administrations) without rescue. However, in contrast to the gradual decrease observed in PCNSL patients they observed a rapid decrease from approximately 75 to 20 nM in 24 h. Moreover, they observed low CSF concentrations of 5-methyl-THF after nine days as well.

A decrease of the CSF concentration of reduced folates was expected since intraventricular MTX without rescue was administered during block C. It was also demonstrated that this decrease was not as striking as after the 24 h high-dose MTX infusion. This was surprising since it has been shown that higher MTX levels are present in the CSF after intraventricular administration of low doses compared to systemic high-dose infusion[35, 171, 172]. On the other hand, it is known that folate enter the CSF from blood through active transport[181, 182]. It has also been shown that high-dose MTX causes folate depletion in serum[183]. Moreover, 1-5% of the high-dose MTX infusion reaches the CNS[171, 172]. Therefore, it is possible that the depletion of CSF reduced folates after systemic high-dose treatment is the consequence of both systemic (serum) and CNS-directed effects of the drug on the pool of reduced folates. In contrast, intraventricular MTX would cause only a depletion of folates in the CSF whereas the systemic exposure is much lower compared to a high-dose infusion.

SAM and SAH concentrations

A significant alteration in the CSF concentration of SAM was not observed during a treatment block in any of the patients (data graphically depicted in Appendix A). The opposite finding is of Quinn et al.[106] in a case report of one patient receiving intra-Ommaya MTX without rescue. They observed an approximately threefold decrease of the CSF SAM concentration one day after administration of 2 mg MTX. This was the only report found in the literature in which the CSF SAM concentration was determined closely after intraventricular MTX therapy.

In block A/B all patients received calcium folinate rescue which induced an increase in the CSF 5-methyl-THF concentration to the normal range on day 3 of the therapy block. Since the concentration of the reduced folate was low for a relatively short time period, it is imaginable that there was not enough time to induce an alteration of the SAM and SAH concentration in the CSF in block A/B. In block C the 5-methyl-THF concentration decreased slowly from day to day and low levels persisted probably for some time. Therefore it cannot be excluded that an alteration of the SAM and SAH concentration occured at a later time-point between blocks when samples were not obtained. An indication for such hypothesis was the lower CSF SAM concentration measured at the beginning of the block A of the second cycle in patient 3 (see appendix A). This was interesting in view of the fact that block A is the only block following block C in which intraventricular MTX therapy was administered without rescue. Assuming that the low CSF 5-methyl-THF concentration from block C persisted for some time and caused further a decrease of the SAM concentration this may explain the low CSF SAM concentration at the beginning of block A2. However, in patient 4 (see appendix A) a decreased SAM concentration in block A2 was not observed. Nevertheless, the measured CSF MTX concentrations of patient 4 were much lower (0.2-0.7 µM) in block C1 compared to patient 3 (1.3-9.4 µM). Also the CSF 5-methyl-THF concentration of patient 4 on day 7 of block C1 was 47.8 nM (normal range) whereas only 15.3 nM were measured in patient 3. It is possible that the CSF concentration of 5-methyl-THF concentration in patient 4 after block C1 did not decrease enough to cause an alteration of the SAM concentration. Unfortunately, patient 2 did not receive intraventricular therapy during block A2 due to an Ommaya misplacement and therefore samples could not be obtained during this block.

Similar observations were not made for the CSF SAH concentrations. Kishi et al.[137] measured CSF SAH concentrations in ALL patients after high-dose and intrathecal MTX therapy. These authors reported no significant difference between the CSF SAH levels in a pediatric reference population and cancer

patients treated with a combination of high-dose MTX infusion (2 g/m^2) and intrathecal MTX administration (12 mg/m^2) followed by rescue. Only this report was found in the literature in which CSF SAH concentrations were measured after MTX therapy.

PCNSL patient with neurotoxicity

Since one of the PCNSL patients manifested signs of chronic neurotoxicity during therapy it was interesting to compare her CSF concentrations of reduced folate, SAM and SAH to the other non-toxic patients. It was observed that she (PCNSL patient 5) had very low CSF levels of 5-methyl-THF throughout therapy. Morever, in block B1 (first block in which samples were obtained) a decrease of the CSF concentration of the reduced folate was observed on day 4 and 5 so that the patient finished the treatment block with almost threefold lower CSF 5-methyl-THF concentration compared to the others. Furthermore, she presented at therapy block C1 two weeks later with a more than fourfold lower 5-methyl-THF concentration (compared to other PCNSL patients) which persisted throughout the block. Since the CSF concentration of the reduced folate could not be monitored during the treatment pause it is unknown whether the concentration was low during the entire time between blocks. However, this is likely since the patient did not receive calcium folinate inbetween. Furthermore, a decrease of the CSF SAM concentration was observed from block to block. In block A2 the SAM concentration was the lowest (35.1-47.6 nM) which was significantly lower compared to the median of 261 nM determined in the non-toxic PCNSL patients during block A/B. At that time-point the patient had manifest signs of leukoencephalopathy. Kishi et al.[137] reported decreased CSF SAM levels in two ALL patients with MTX-associated leukoencephalopathy as well. However, these authors also observed about twofold higher CSF SAH concentrations in the toxic patients compared to the non-toxic ALL patients and the reference population. In contrast, the SAH concentration was in the same range during all blocks as in the non-toxic PCNSL patients in which it could be quantified. In contrast to the PCNSL patient, the neurotoxic ALL patients of Kishi et al.

received cranial irradiation. It was shown in animal experiments that total body irradiation induced a reduction of folate levels in the liver[184]. It is possible that a similar effect occurs with CNS folates after radiotherapy. Therefore, it is imaginable that after a combination of MTX and cranial irradiation the folate metabolism is altered to a higher extent inducing further metabolic changes such as accumulation of SAH which is not seen in the non-irradiated patients. Due to scarce literature data it remains unclear whether or under which circumstances MTX could alter the CSF SAH concentration, requesting further investigations to elucidate this issue.

5.4 CSF concentrations of reduced folates, SAM and SAH in ALL patients

In analogy to the Bonn protocol for PCNSL there were phases during ALL BFM 2000 in which patients received intrathecal MTX (8-12 mg, age-dependent) without calcium folinate rescue and others in which intravenous high-dose (5 g/m^2) and intrathecal MTX was followed by calcium folinate (15 mg/m^2, see chapter 3.5.2). Therefore, the two therapy modalities were assessed separately in ALL patients as well. In contrast to the PCNSL collective where samples were obtained on subsequent days during treatment blocks with MTX, samples from ALL patients were obtained one to three weeks after drug administration.

During protocol M in which patients received calcium folinate rescue samples were always obtained two weeks after MTX therapy and no significant alteration in the CSF 5-methyl-THF concentration was observed compared to the reference samples obtained prior to any MTX therapy. However, the variability in the CSF concentration of the reduced folate was pronounced during this protocol probably due to the fact that the CSF samples were drawn after the start of a new MTX infusion. Since the infusion started approximately only one hour prior to sample collection there was not enough time to cause a significant depletion of the CSF concentration of 5-methyl-THF.

Patients treated according to TOTAL XV received a similar MTX regimen during the consolidation block (see chapter 3.5.3). High-dose (2.5 or 5.0 g/m^2) and intrathecal MTX (8-12 mg) accompanied by folinate rescue (10 or 15 mg/m^2 5 times) were administered. Two weeks later a CSF sample was obtained. An intraindividual comparison (six patients) of the CSF 5-methyl-THF concentrations during therapy revealed no significant difference between the reduced folate concentrations during consolidation therapy compared to samples obtained prior to any MTX therapy.

It was shown in the PCNSL population who received a similar MTX regimen during block A and B that the depleted CSF concentrations of 5-methyl-THF significantly increased after administration of calcium folinate rescue. Moreover, Thyss et al.[158] observed an accumulation of 5-methyl-THF in the CSF after several high-dose MTX courses followed by rescue. Since the CSF samples of ALL patients in this study were drawn weeks after the administration of folinate rescue the result of normal CSF 5-methyl-THF concentration two weeks after therapy was not surprising.

In contrast to protocol M (concomitant systemic infusion) patients receiving intrathecal MTX had a significantly decreased CSF 5-methyl-THF during protocol I 12 days after the first MTX administration compared to pretreatment. After further intrathecal administration of MTX during protocol I and II/III, somewhat higher concentrations of 5-methyl-THF were determined and they were not significantly different from the inital values obtained prior to therapy. In addition, in three cases an intraindividual comparison prior to first intrathecal administration and twelve days later was possible. In two out of three cases the concentration of the reduced folate was decreased after twelve days, but it was already low prior to therapy. Moreover, intraindividual comparison between the start and end of protocol I revealed slightly higher CSF concentrations of the reduced folate at the end of the protocol. Interestingly, Surtees et al.[136] reported similar findings in an ALL children collective after intrathecal MTX therapy. They also observed a significant decrease of the CSF 5-methyl-THF concentration initially which was not present later-on although patients continued receiving intrathecal MTX

therapy. Quinn et al.[106] observed a decreased CSF concentration of 5-methyl-THF seven days after the last intra-Ommaya MTX administration (2 mg/day on three subsequent days). Six days later the concentration was almost as high as the pretreatment value. In another study these authors also observed a significant decrease of 5-methyl-THF in the CSF within 7 days after oral MTX prior to delayed calcium folinate rescue[104] (see also Table 5.4).

A decrease of the CSF reduced folate concentrations which persists for some time is evident after intrathecal MTX administration without rescue. However, it is unclear why concentrations of 5-methyl-THF tend to increase during protocol I. Certain, the small number of evaluated samples could lead to an erroneous conclusion since it is possible that a significant decrease of the CSF 5-methyl-THF concentration at later time-point during protocol I and II/III could not be shown due to the sample number. Nevertheless, the intraindividual data indicate a trend which cannot be explained with the present knowledge and therefore further investigation of CSF 5-methyl-THF if concentrations in shorter time-intervals after MTX therapy would be valuable.
In the pediatric ALL population treated according to ALL BFM 2000 receiving a high-dose infusion followed by intralumbar MTX administration and calcium folinate rescue in protocol M, no significant change in the CSF SAM concentration was found compared to the concentration of the methyl donor before drug treatment. In contrast, significantly decreased CSF concentrations of SAM were determined during protocol I and II or III in which intrathecal MTX was administered without calcium folinate rescue. Although the number of samples which were included in the statistical analysis was small an intraindividual comparison was possible in seven patients prior to the first intrathecal administration and twelve days later. In all of them a decrease of the SAM concentration was observed. Kishi et al.[137] reported decreased CSF SAM concentration in ALL patients who were treated with high-dose and intrathecal MTX followed by rescue. The findings of Surtees et al.[136] who observed a significant decline of the CSF SAM concentration in ALL patients who received intrathecal MTX without calcium folinate compared

Table 5.4: Overview of literature data of CSF 5-methyl-THF concentrations after MTX therapy *without* concomitant rescue.

Authors	MTX therapy	Result
Quinn et al.[106]	2 mg i.cv. on three subsequent days	Decreased concentrations one day and nine days after the first MTX administration (25 nM vs. 75 nM)
Quinn et al[104].	MTX p.o. (exact dose not stated)	Significantly decreased concentrations within a week of therapy (54.2 nM vs. 82.8 nM)
Surtees et al.[136]	7.5-12.5 mg i.th.	Decreased concentrations in initial protocol phases
This work	3 mg i.cv. on four subsequent days (PCNSL patients)	Significantly decreased concentrations on the fifth day (23.2 nM vs. 73.8 nM)
	8-12 mg i.th. (ALL patients)	Decreased concentrations 12 days after first therapy course (24.2 nM vs. 56.4 nM)

to patients who received high-dose MTX followed by rescue were similar to those in this work. An overview of literature data of CSF SAM concentrations after MTX therapy is presented in Table 5.5. It can be postulated that the CSF SAM concentrations decrease only after a persistent exposure to decreased CSF 5-methyl-THF concentrations which probably occurs after intrathecal MTX administration without concomitant rescue. The decreased SAM concentrations one to three weeks after intrathecal administration support such hypothesis. However, it was observed in this study that concentrations of SAM were decreased on day 17/38 of protocol II/III. Since these samples

Table 5.5: Overview of literature data of CSF SAM concentrations after MTX therapy.

Authors	MTX therapy	Result
Quinn et al.[106]	2 mg i.cv. No rescue.	~threefold lower concentrations one day after drug administration
Kishi et al.[137]	2 g/m² i.v. + 12 mg/m² i.th. + rescue 15 mg/m² x 8	~threefold lower concentrations 10 days after therapy course
Surtees et al.[136]	6-8 g/m² i.v. + 7.5-12.5 mg i.th. + rescue (dose not stated)	No significant difference to reference samples
	7.5-12.5 mg i.th., no rescue	Significantly lower concentrations
This work	5 g/m² i.v. + 8-12 mg i.th.+ rescue 15 mg/m² x 2 (Protocol M, ALL BFM 2000)	No significant difference 14 days after therapy course compared to reference
	8-12 mg i.th., no rescue (Protocol I, ALL BFM 2000)	Significantly lower concentrations 12-21 days after therapy

were collected prior to intrathecal MTX administration and more than a month after a rescued high-dose and intrathecal combination the result was surprising. The reason for the decreased SAM concentration remains unclear but it is probably not MTX-related.

Since the concentration of SAH was quantified in only 23% of the obtained CSF sample no conclusions could be drawn from these data. However the available results suggest no major change in SAH concentrations following MTX treatment.

ALL patients with neurotoxicity

Two patients treated according to ALL BFM 2000 developed signs of subacute neurotoxicity approximately ten days after receiving a high-dose and intrathecal MTX therapy course followed by calcium folinate rescue. In one patient (case 1) low SAM concentrations were determined in all collected samples whereas the level of 5-methyl-THF was low only shortly after the toxic episode. In contrast, in case 2 the reduced folate concentration was low in all samples whereas the level of SAM was decreased only just after toxicity. The common finding in both cases was that decreased concentrations of both substances together were observed only briefly after the toxicity episode which was almost two weeks after the MTX treatment course. Moreover, it was shown in this work that non-toxic patients had normal concentrations of 5-methyl-THF and SAM two weeks after the same MTX treatment. SAH could be quantified in all samples of both patients. Since most SAH concentrations in the non-toxic ALL population were below LLOQ a quantitative comparison was not possible.

Low CSF 5-methyl-THF levels were also reported by Quinn et al.[105] in one pediatric ALL patient who developed signs of subacute neurotoxicity four days after receiving the first course of triple intrathecal therapy which included 8 mg MTX. This patient presented with seizures and CSF was obtained by lumbar puncture shortly afterwards. The analysis revealed a concentration decrease of the reduced folate from 80 nM (prior to therapy) to 11 nM. In addition, an increase of concentrations of homocysteine and of the excitatory amino acids homocysteic and cysteine sulphinic acid was observed.

An overview of literature data regarding CSF 5-methyl-THF, SAM and SAH concentration in patients with manifest MTX-related neurotoxicity is presented in Table 5.6.

It is possible that both patients in this study developed toxicity due to MTX-related biochemical alterations. Interesting is the fact that both patients received intrathecal MTX (a total of 60 mg) during protocol I which was well

Table 5.6: CSF concentrations of 5-methyl-THF, SAM and SAH in patients with MTX-associated neurotoxic symptoms.

Author	Toxicity	CSF 5-methyl-THF conc. (nM)	CSF SAM conc. (nM)	CSF SAH conc. (nM)
Quinn et al.[105]	1 case of subacute toxicity	11.0	Not reported	Not reported
Kishi et al[137].	2 cases of chronic toxicity	Not reported	32.9 65.8	27.5 34.4
This work	2 cases of subacute toxicity	19.1 27.5	79.3 74.8	8.6 10.4
	2 cases of chronic toxicity	29.3-31.8 18.7-27.7	<25-184.5 34.1-47.6	7.5-14.3 5.0-11.4

tolerated. Toxicity occurred after receiving a combination of high-dose infusion and intrathecal MTX followed by rescue. In contrast, Strunk et al.[185] reported a patient treated according to the same protocol with a similar neurotoxicity episode which occured after intrathecal therapy in protocol I indicating that the development of toxicity is independent of the time of treatment.

It was shown in the PCNSL collective that the high-dose (5 g/m^2) MTX infusion induces a more extensive depletion of the 5-methyl-THF concentration than intraventricular MTX. After 3-4 infusions of calcium folinate (30 mg/m^2) rescue which followed the systemic high-dose MTX infusions the concentration of 5-methyl-THF was replenished. The ALL patients received usually two infusions of 15 mg/m^2 calcium folinate. Nevertheless, the non-toxic ALL population had normal CSF levels of reduced folates two weeks after the high-dose indicating a complete repletion of the pool of reduced folates. If it is postulated that in both patients rescue failed for some reason to restore the pool of reduced folates after the high-dose MTX infusion then it is possible that very low CSF 5-methyl-THF persisted for some time. This

could have induced the impairment of folate-related biochemical pathways leading further to manifest signs of toxicity.
The third patient with MTX-related chronic toxicity had an approximately twofold lower CSF 5-methyl-THF concentration in all three obtained samples compared to the non-toxic patients. The SAM level was more than fourfold decreased in two of the three samples but it has to be mentioned that the patient had an overt infection and hyperbilirubinemia at the time of the third sample collection and it cannot be excluded that it influenced the CSF concentration of the compound. The CSF SAH concentration was quantified in all three obtained samples similar as in the two patients with subacute neurotoxicity. The patient received high-dose and intrathecal MTX therapy six and sixteen years prior to toxicity and at the time of first diagnosis cranial irradiation was performed as well. However, signs of MTX-related neurotoxicity never occurred during the therapy with the drug. Due to the long time period between last MTX therapy and sample collection it is uncertain whether the observed biochemical alterations are a consequence of MTX treatment. Nevertheless, the observed changes were similar as in the other patients with signs of neurotoxicity.

5.5 Correlation between MTX, reduced folates, SAM and SAH concentrations in the CSF

The CSF concentrations of MTX, 5-methyl-THF, SAM and SAH of PCNSL patients were examined for potential correlations in order to get a better insight into the biochemical alterations of the folate and one-carbon metabolism after MTX therapy.
There was no significant correlation between CSF MTX, 5-methyl-THF, SAM and SAH concentrations in PCNSL patients after MTX therapy. It was shown in studies that there is no significant correlation between the concentrations of 5-methyl-THF and SAM in the CSF or blood in a reference population of healthy children and adults whereas a correlation existed between SAM and

SAH in plasma but not in erythrocytes[151, 186]. However, since it is probable that time is required for the decrease of the SAM concentration after reduced folate depletion it cannot be excluded that a correlation between the metabolite concentrations exists after MTX therapy but was not evident at the same time during this monitoring.
The lack of correlation between the CSF concentrations of reduced folates, SAM and SAH in healthy humans as well as in MTX-treated patients makes the assessment of biochemical alterations due to therapy more difficult. Moreover, it indicates that MTX-induced changes in the homocysteine pathway are probably multifactorial and cannot be predicted only by monitoring MTX and reduced folates in plasma and CSF.

5.6 Potential clinical consequences of biochemical alterations after MTX treatment

It was shown in PCNSL as well as in ALL patients that MTX induces biochemical alteration of the folate pathway. Moreover, the methyl-transfer pathway is impaired as well after administration of intrathecal MTX without concomitant rescue. However, the question arises what the clinical relevance of such biochemical alteration is in terms of MTX-related neurotoxicity.
It was shown in PCNSL patients that both high-dose and intrathecal MTX induces a depletion of 5-methyl-THF in the CSF. In all patients of this study with clinical signs of MTX-related neurotoxicity decreased CSF 5-methyl-THF concentrations were observed both during and shortly after toxicity. Low CSF reduced folate levels have been associated with neurological complications involving seizures, cerebellar ataxia and psychomotor retardation[115]. Moreover, axonal neuropathy, myelopathy and leukoencephalopathy characterised by demyelinating lesions of the white matter were associated with folate deficiency[187-190]. However, the pathogenetic mechanism by which a decrease of the reduced folate concentrations could induce neurotoxicity remains unclear. It was postulated that a depletion of the pool of reduced

folates mediates toxicity indirectly by inducing a biochemical alteration of the folate-linked metabolic pathways[44, 191, 192]. It was shown in PCNSL patients that the decrease of the CSF levels of 5-methyl-THF was associated with the increase of the homocysteine concentration after intrathecal MTX administration[140]. Decreased CSF 5-methyl-THF and SAM concentrations were observed in ALL patients after administration of intrathecal MTX in this study. Also an alteration of the adenosine and biopterin pathway was associated with reduced folate deficiency[80, 115, 193]. Moreover, it has been postulated that neurotoxicity occurs after a delayed CSF excretion of MTX[37, 194, 195]. A prolonged MTX exposure in the CSF may hence induce a more extensive depletion of the pool of reduced folates. Furthermore, calcium folinate which replenishes the CSF concentration of 5-methyl-THF has been shown to be beneficial in preventing and reversing MTX-induced toxicity in some cases[196, 197]. In view of the above presented facts a simultaneous monitoring of the CSF concentration of 5-methyl-THF and MTX could be useful in establishing the role of the folate depletion in drug-associated neurotoxicity.

It was postulated that a decrease of CSF SAM concentrations occurs after MTX therapy[44, 136, 137, 192]. In ALL patients of this study a decrease of the SAM concentrations was observed in protocols in which MTX had been administered without concomitant rescue. Decreased CSF SAM concentrations have been reported in patients with depression, HIV and subacute combined degeneration of the spinal cord[117, 130-132, 134, 198]. In all these patient collectives demyelination was observed which was associated with the methyl-transfer pathway impairment[117, 129, 133, 134, 159]. An impairment of the myelin sheath has been observed also in MTX-treated patients without clinical signs of neurological complications. It was shown in studies that 9-50% of the patients treated with high-dose and intrathecal MTX followed by rescue develop subclinical transient changes of the white matter characterised as demyelination[58, 73, 74, 199]. Such findings were associated with the administration of intrathecal MTX and remyelination occurred after discontinuation of therapy[58]. The clinical impact of the transient white matter changes remains unclear.

Approximately 60% of childhood ALL survivors had a decreased overall and verbal intelligence quotient and/or arithmetic achievement after MTX therapy without concomitant cranial irradiation[73, 200]. There was no clear correlation between these findings and white matter changes. Nevertheless, there are indices that patients with white matter changes may be more susceptible to certain neuropsychological deficits, primarily involving attention[74, 201]. The results of neuropsychological testing and diagnostic imaging indicate that although clinically not worrisome pathologic changes of the white matter occur in a large number of patients treated with MTX especially when it is administered intrathecally. Since this route of administration was associated with decreased CSF SAM concentrations in this study, a potential correlation between the decrease of the SAM concentrations and the subclinical transient changes observed with MRI remains an interesting topic for further investigation.

Two patients in this study presented with signs of transient subacute neurotoxicity characterised by hemiparesis and speech disorders after receiving high-dose and intrathecal MTX followed by calcium folinate rescue. Several cases with similar symptomatology were reported in the literature in which toxicity was associated with high-dose MTX[43, 45, 52, 202, 203]. Nevertheless, the same clinical picture was observed after intrathecal MTX administration as well[54, 185, 204, 205] indicating that both routes of drug administration may induce similar alteration. Transient subacute neurotoxicity was associated with changes of the white matter in MRI which indicated damage of the myelin sheath[45, 52, 56, 58, 73, 185, 203, 205, 206]. However, severe necrosis of the white matter resulting in tissue loss is not associated with such disorder[58]. In accordance to the literature, some changes in the white matter were observed in the two patients with subacute transient neurotoxicity in this study as well. The analysis of a CSF sample obtained shortly after toxicity revealed in both patients an approximately twofold lower SAM concentrations compared to the ALL population prior to treatment. One of the patients had decreased SAM levels more than two months prior to toxicity as well. Due to these facts it is possible that the alteration of the methyl-transfer pathway

played an important role in the genesis of neurological complications in both patients.

Two patients in the present work had signs of severe chronic neurotoxicity. Clinically, signs of cognitive dysfunction dominated. Cases of severe MTX-associated chronic neurotoxicity have been reported in the literature[57, 207-212]. Usually such toxicity was observed after combined MTX therapy and cranial irradiation[47, 196, 207, 210, 213] which was administered in one of the cases in this study as well. However, cases of toxic leukoencephalopathy were observed after MTX regimens which were not accompanied by radiotherapy as well[57, 208, 212]. This was the case in the PCNSL patient in this study. MRI scans in both patients revealed leukoencephalopathy characterised by severe demyelination. These findings are common in patients presenting with this form of neurological complications[63, 209, 214]. CSF analysis of both patients revealed the lowest SAM concentrations measured in this study. Both patients had a more than fourfold lower SAM concentration compared to non-toxic patients at the time-point when toxicity was clinically present. Compared to the patients with subacute neurotoxicity lower CSF SAM concentrations and a much more pronounced demyelination were encountered in the leukoencephalopathic patients, a further indication that the impairment of the methyl-transfer pathway is involved in the development of MTX-associated neurotoxicity. However, further studies are necessary to provide a better understanding of the relationship between the decrease of CSF SAM concentrations, the degree of demyelination and clinical symptoms. For that purpose a monitoring of the CSF SAM concentrations in patients receiving intensive MTX therapy seems to be useful. Furthermore, it was shown that the CSF SAM concentrations were increased following the administration of oral SAM[130, 198, 215]. The question arises whether patients who have low CSF SAM concentrations i.e. after administration of intrathecal MTX without calcium folinate rescue could profit from such substitution. In this way the methylation capacity may be preserved without compromising the efficacy of MTX.

6 Summary

The introduction of intrathecal (3-12 mg) and/or intravenous high-dose (≥ 1 g/m^2) MTX administration followed by calcium folinate rescue into therapy protocols has significantly improved cure rates of malignancies such as ALL, PCNSL, medulloblastoma and osteosarcoma. Nevertheless, this therapy is associated with an increased frequency of neurotoxicity which is classified as acute, subacute and chronic according to clinical symptoms and time of their appearance. Usually acute and subacute toxicity is reversible whereas the chronic form which involves leukoencephalopathy characterised by demyelination may be present in a severe form ending with coma or even death. Due to the potential severity of neurotoxicity the elucidation of the pathogenesis remains an important topic in order to develop prevention strategies and increase the safety of MTX treatment.

It was postulated that MTX by interfering with the folate metabolism induces an alteration of the folate-related metabolic pathways which may further lead to the genesis of neurological complications. Moreover, the supposed depletion of the only cellular methyl-donor SAM may result in the decreased methylation of choline which further leads to malformation of the neuronal myelin sheath (demyelination).

The aim of this work was to get a better insight into the biochemical alterations of the folate and methyl-transfer pathway in the CSF after administration of MTX and to investigate whether these alterations are related to neurological complications. For this purpose analytical methods were developed and CSF samples from adult PCNSL patients and pediatric ALL patients were collected and analysed.

The effect of MTX and calcium folinate rescue therapy on the folate metabolism was assessed by the quantification of 5,10-methylene-THF and 5-methyl-THF. The four compounds were determined simultaneously using HPLC with UV and fluorescence detection. The method was validated according to the FDA guideline for validation of bioanalytical methods. The main advantage of the method is the simple sample preparation due to the

natural fluorescence of the reduced folates which enables the use of the method in the routine monitoring of the compounds.

SAM and SAH were also determined by HPLC with the use of fluorescence detection. The method was validated according to the FDA guideline as well. The compounds were quantified as their 1,N^6-etheno derivatives after incubation with chloroacetaldehyde. An appropriate selection of the stationary phase was crucial in obtaining the desired selectivity and sensitivity of the method. The main advantage of the developed method is the simultaneous separation of both analytes in an acceptable run time (35 min).

Method development and validation was followed by the analysis of patient CSF samples. Five adult PCNSL patients whith an implanted Ommaya reservoir and treated according to the Bonn protocol were included in the study. One of them received an intensified calcium folinate rescue initiated one hour after the end of the high-dose MTX infusion (3 g/m^2) and it was possible to monitor the drug and reduced folates within 24 h after the end of the high-dose infusion. 5,10-methylene-THF and calcium folinate could be determined in the CSF of the patient. In the same time period MTX decreased rapidly whereas an increase of the 5-methyl-THF concentration was observed as a consequence of rescue. From the other four patients CSF samples were obtained on four or five subsequent days during a treatment block enabling close monitoring after therapy. In some blocks patients received high-dose MTX (3-5 g/m^2) infusions followed by calcium folinate rescue and intraventricular MTX administration (3 mg/day) while in others MTX was administered only intraventricularly and was not accompanied by rescue. This enabled a distinction between the two treatment modalities regarding biochemical alterations. It was observed that high-dose MTX caused a massive depletion of the CSF 5-methyl-THF concentration determined at the end of infusion which was repleted after rescue administration. The 5-methyl-THF concentrations decreased to a lower extent 24 h after intraventricular MTX administration. However, at the end of the block the reduced folate concentration was 2-3 fold lower compared to the beginning.

The low reduced folate concentrations persisted for some time since no calcium folinate was administered. In contrast to the depletion of folates an alteration of the SAM and SAH concentration was not observed during blocks. Nevertheless, in one patient lower CSF SAM concentrations were observed approximately two weeks after the treatment block with intraventricular MTX suggesting that the SAM concentrations decreased only after a persistent depletion of 5-methyl-THF. One patient had clinical signs of chronic toxicity in form of leukoencephalopathy. Low 5-methyl-THF concentrations were determined in this patient throughout the therapy. The SAM concentrations decreased during therapy and were extremely low at the time of appearance of neurological complications whereas the SAH concentration did not alter significantly during therapy.

CSF samples were also obtained from two pediatric ALL collectives. Sampling was performed one to three weeks after the last MTX therapy enabling an insight into the biochemical alterations of the folate and methyl-transfer pathway weeks after treatment. It was observed that the concentrations of 5-methyl-THF and SAM were in the normal range two weeks after the treatment with high-dose (2.5 or 5 g/m^2) and intrathecal (8-12 mg) MTX followed by calcium folinate rescue. In contrast, in protocols with intrathecal MTX (8-12 mg) not accompanied by folinate rescue the 5-methyl-THF concentrations were significantly decreased 12 days after the first intrathecal MTX administration but not later-on whereas SAM was significantly decreased throughout these protocols. Such results support the hypothesis that the depletion of SAM probably occurs only after persistently low 5-methyl-THF concentrations.

Three ALL patients manifested signs of neurotoxicity. Two exhibited subacute neurotoxicity characterised by hemiparesis and speech disorder which resolved spontaneously. Both patients had low levels of 5-methyl-THF and SAM shortly after the toxic episode. One patient exhibited a leukoencephalopathy probably as a consequence of earlier MTX therapy. Low 5-methyl-THF and SAM concentrations were found in this patient as well. Moreover, demyelination was present in all four neurotoxic patients

indicating that the low CSF SAM concentration may indeed contribute to this disorder.

In conclusion, the results suggest that the biochemical alterations of the folate and methyl-transfer pathway may play an important role in the genesis of neurotoxicity of MTX. Nevertheless, further prospective studies with more patients are required to clarify the relationship between decreased SAM concentrations, demyelination and its role in subacute and especially chronic MTX-related neurotoxicity. In addition, the possible role of low reduced folate and SAM concentrations in the prediction of neurologic complications deserves further investigation.

7 Literature

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Appendix

Appendix A

Results of the CSF analysis of reduced folates, MTX, SAM and SAH in PCNSL patients treated in the Department of Neurology, University Hospital, Rheinische Friedrich-Wilhelms-Universität, Bonn.

Graphical presentation of the data of patient 2-5 in Fig. 1-8

CSF concentrations of reduced folates, MTX, SAM and SAH in patients treated according to the Bonn protocol for PCNSL

Pat. 1	(h) after end of HD-MTX infusion	Concentration 5,10-MeTHF (nM)	5-MTHF (nM)	Ca-folinate (nM)	MTX (µM)	SAM (nM)	SAH (nM)
1	0	n.d.	n.d.	n.d.	2.9	-	-
1	1	n.d.	n.d.	n.d.	2.3	-	-
1	3.5	n.q.	n.q.	n.q.	1.7	-	-
1	7	22.0	28.0	n.q.	1.2	-	-
1	24	28.0	44.0	118.0	0.2	-	-

Pat. 2	Time Block/day	Concentration 5,10-MeTHF (nM)	5-MTHF (nM)	Ca-folinate (nM)	MTX (µM)	SAM (nM)	SAH (nM)
2	A1/3	n.d.	32.4	n.d.	2.6	236.2	n.q.
2	A1/4	n.d.	22.7	n.d.	6.7	183.1	n.q.
2	A1/5	n.d.	26.7	n.d.	1.6	220.2	n.q.
2	B1/2	n.d.	n.q.	n.d.	0.7	182.9	n.q.
2	B1/3	n.d.	65.6	n.d.	5.5	184.5	n.q.
2	B1/4	n.d.	55.1	n.d.	18.7	170.8	n.q.
2	B1/5	n.d.	44.2	n.d.	9.2	230.4	n.q.
2	C1/3	n.d.	63.0	n.d.	n.d.	229.8	n.q.
2	C1/4	n.d.	47.2	n.d.	39.4	263.3	n.q.
2	C1/5	n.d.	40.0	n.d.	8.3	245.2	n.q.
2	C1/6	n.d.	-	n.d.	35.3	284.8	n.q.
2	C1/7	n.d.	26.0	n.d.	30.8	280.1	n.q.
2	B2/2	n.d.	n.q.	n.d.	0.9	199.7	n.q.
2	B2/3	n.d.	44.4	n.d.	1.5	261.9	n.q.
2	B2/4	n.d.	50.7	n.d.	2.5	217.2	n.q.
2	B2/5	n.d.	82.2	n.d.	15.1	203.8	n.q.
2	C2/3	n.d.	75.0	n.d.	n.d.	271.9	n.q.
2	C2/4	n.d.	62.5	n.d.	1.5	264.3	n.q.
2	C2/5	n.d.	56.8	n.d.	2.1	250.3	n.q.
2	C2/6	-	-	-	-	-	-
2	C2/7	n.d.	48.5	n.d.	3.4	221.6	n.q.

Pat.	Time	Concentration					
3	Block/day	5,10-MeTHF (nM)	5-MTHF (nM)	Ca-folinate (nM)	MTX (µM)	SAM (nM)	SAH (nM)
3	B1/2	-	-	-	-	-	-
3	B1/3	n.q.	97.9	n.d.	5.6	614.4	7.0
3	B1/4	n.d.	35.9	n.d.	2.2	697.2	6.1
3	B1/5	n.d.	71.3	n.d.	1.6	516.3	6.2
3	C1/3	n.d.	59.5	n.d.	n.d.	579.3	5.3
3	C1/4	n.d.	32.8	n.d.	1.3	672.7	7.4
3	C1/5	n.d.	21.0	n.d.	1.4	645.4	9.5
3	C1/6	n.d.	12.0	n.d.	9.1	579.7	6.3
3	C1/7	n.d.	15.3	n.d.	3.4	557.3	11.0
3	A2/2	-	-	-	-	-	-
3	A2/3	n.d.	48.3	n.d.	20.3	148.1	5.0
3	A2/4	n.q.	90.9	n.d.	3.5	210.4	7.0
3	A2/5	n.d.	75.4	n.d.	2.3	358.7	6.2
3	B2/2	n.d.	n.q.	n.d.	2.0	641.2	6.6
3	B2/3	n.d.	63.8	n.d.	1.3	856.7	8.9
3	B2/4	n.d.	84.8	n.d.	2.0	856.8	10.2
3	B2/5	-	-	-	-	798.3	12.5
3	C2/3	n.q.	74.1	n.d.	n.d.	903.0	7.8
3	C2/4	n.d.	23.4	n.d.	1.5	966.6	8.0
3	C2/5	n.d.	33.2	n.d.	1.3	899.1	8.3
3	C2/6	n.d.	28.2	n.d.	1.5	854.3	8.2
3	C2/7	n.d.	20.6	n.d.	2.2	866.8	9.1

Pat.	Time	Concentration					
4	Block/day	5,10-MeTHF (nM)	5-MTHF (nM)	Ca-folinate (nM)	MTX (µM)	SAM (nM)	SAH (nM)
4	A1/3	n.q.	122.4	n.d.	0.8	283.5	9.3
4	A1/4	n.q.	75.9	n.d.	0.3	295.3	11.8
4	A1/5	n.d.	30.0	n.d.	0.4	279.4	n.q.
4	C1/3	n.q.	91.8	n.d.	n.d.	409.1	6.4
4	C1/4	n.d.	72.1	n.d.	0.7	362.6	7.1
4	C1/5	n.d.	35.9	n.d.	n.q.	182.4	5.3
4	C1/6	n.d.	56.2	n.d.	0.3	376.2	6.4
4	C1/7	n.d.	48.1	n.d.	0.2	357.1	5.7
4	A2/2	n.d.	n.d.	n.d.	1.5	424.8	n.q.
4	A2/3	n.d.	69.3	n.d.	0.5	261.3	n.q.
4	A2/4	n.d.	83.7	n.d.	0.3	441.9	n.q.
4	A2/5	n.d.	67.6	n.d.	0.4	144.3	n.q.

Pat.	Time	Concentration					
	Block/Day	5,10-MeTHF (nM)	5-MTHF (nM)	Ca-folinate (nM)	MTX (µM)	SAM (nM)	SAH (nM)
5	B1/2	n.d.	13.3	n.d.	1.1	127.4	7.2
5	B1/3	n.d.	57.5	n.d.	1.9	122.1	9.4
5	B1/4	n.d.	41.8	n.d.	1.3	119.0	5.6
5	B1/5	n.d.	23.0	n.d.	2.6	90.8	6.5
5	C1/3	n.d.	13.5	n.d.	n.d.	44.7	6.5
5	C1/4	n.d.	22.9	n.d.	3.2	54.2	7.9
5	C1/5	n.d.	17.8	n.d.	1.4	48.9	7.1
5	C1/6	n.d.	16.2	n.d.	2.8	51.3	7.5
5	C1/7	n.d.	14.0	n.d.	1.8	48.7	7.3
5	A2/2	-	-	-	-	-	-
5	A2/3	n.d.	18.8	n.d.	1.4	34.1	5.0
5	A2/4	n.d.	27.8	n.d.	3.8	38.8	11.4
5	A2/5	n.d.	14.0	n.d.	1.9	47.6	7.4

n.d.: not detected

n.q.: not quantified

-: sample not obtained

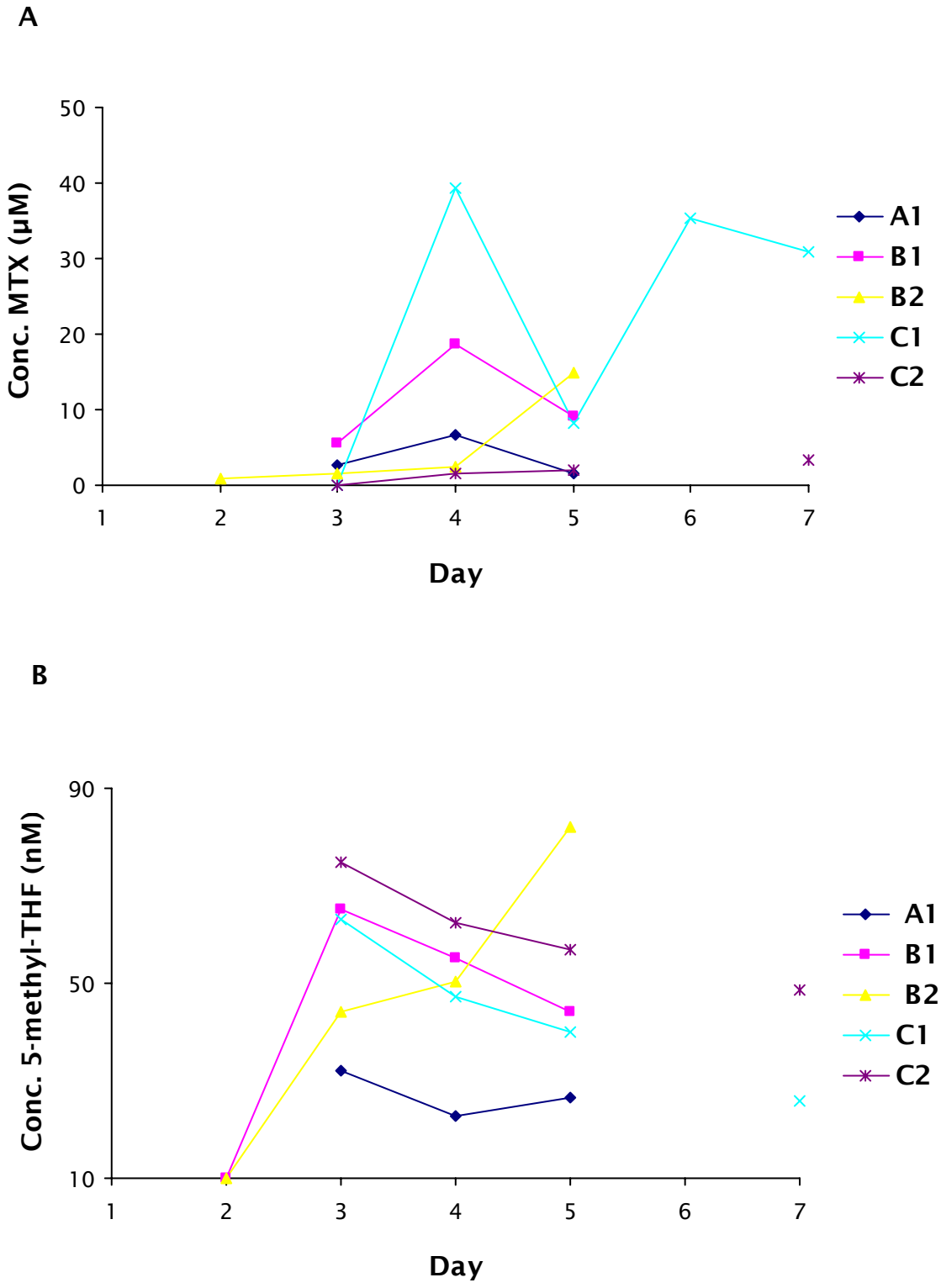


Fig.1: CSF concentration of MTX (A) and 5-methyl-THF (B) in patient 2.

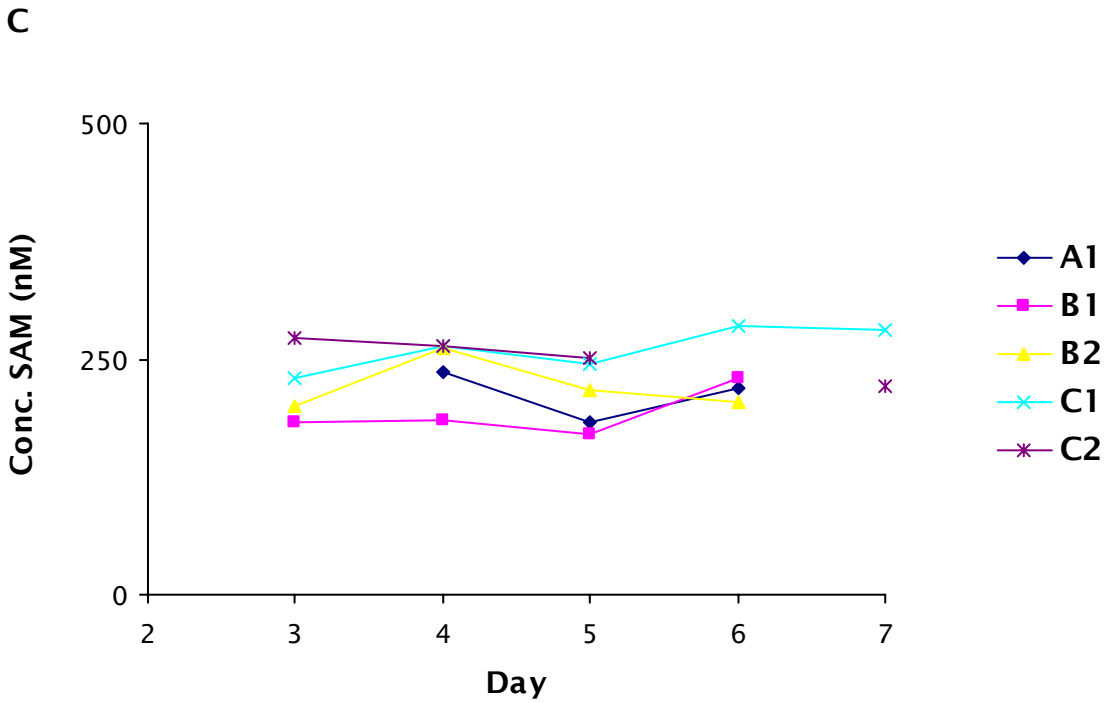


Fig.2: CSF concentration of SAM (C) in patient 2.

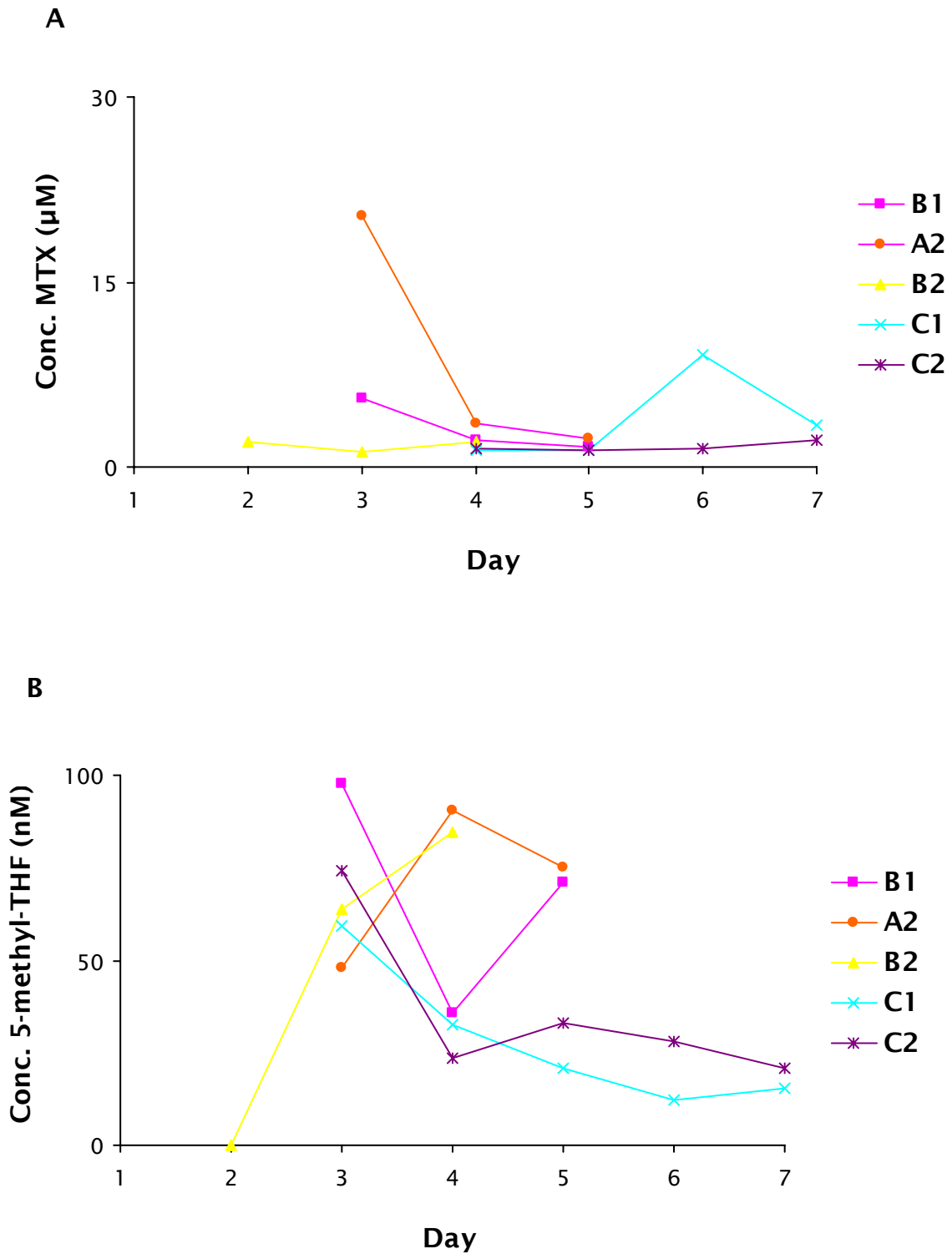


Fig. 3: CSF concentration of MTX (A) and 5-methyl-THF (B) in patient 3.

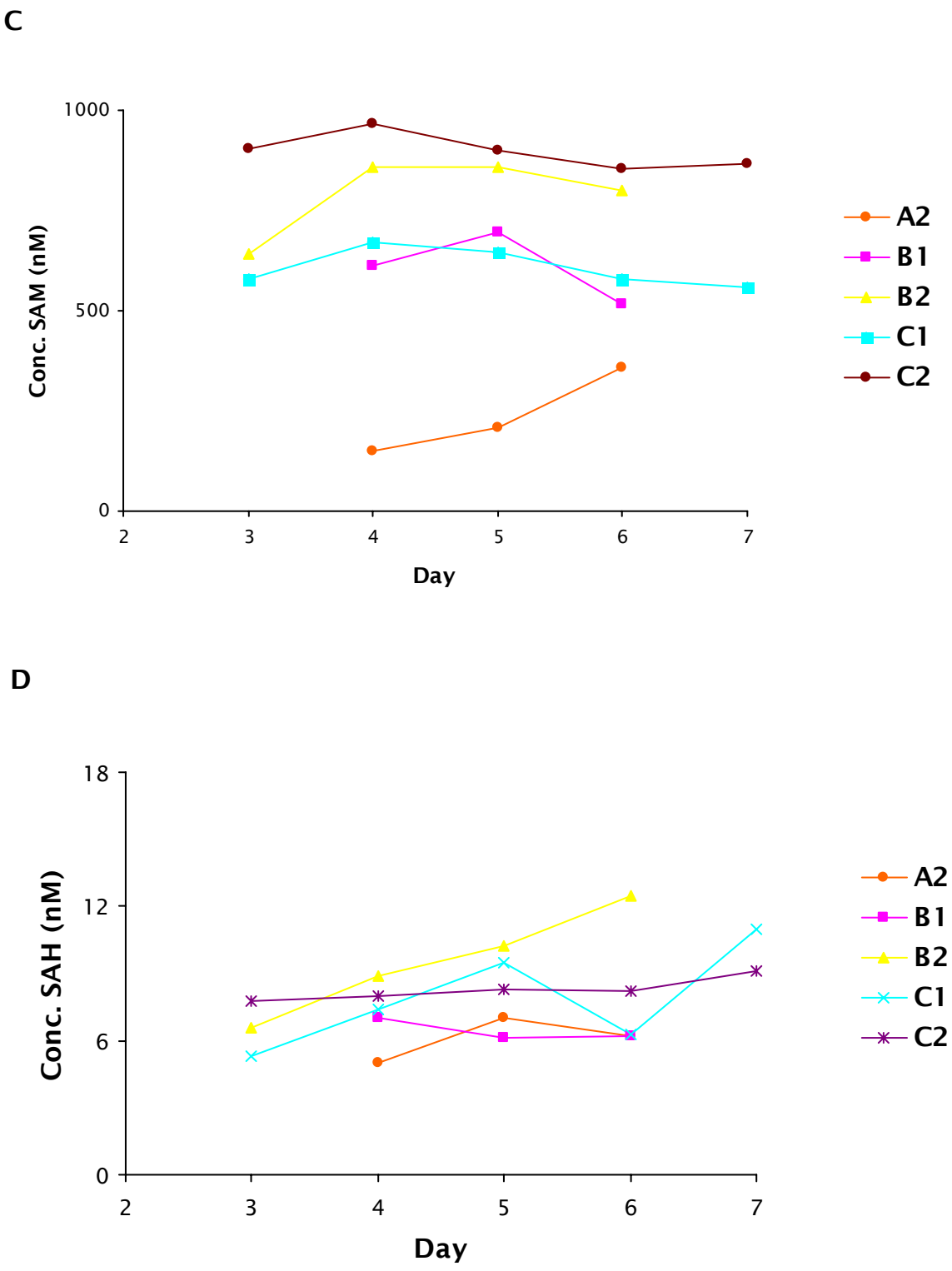


Fig.4: CSF concentration of SAM (C) and SAH (D) in patient 3.

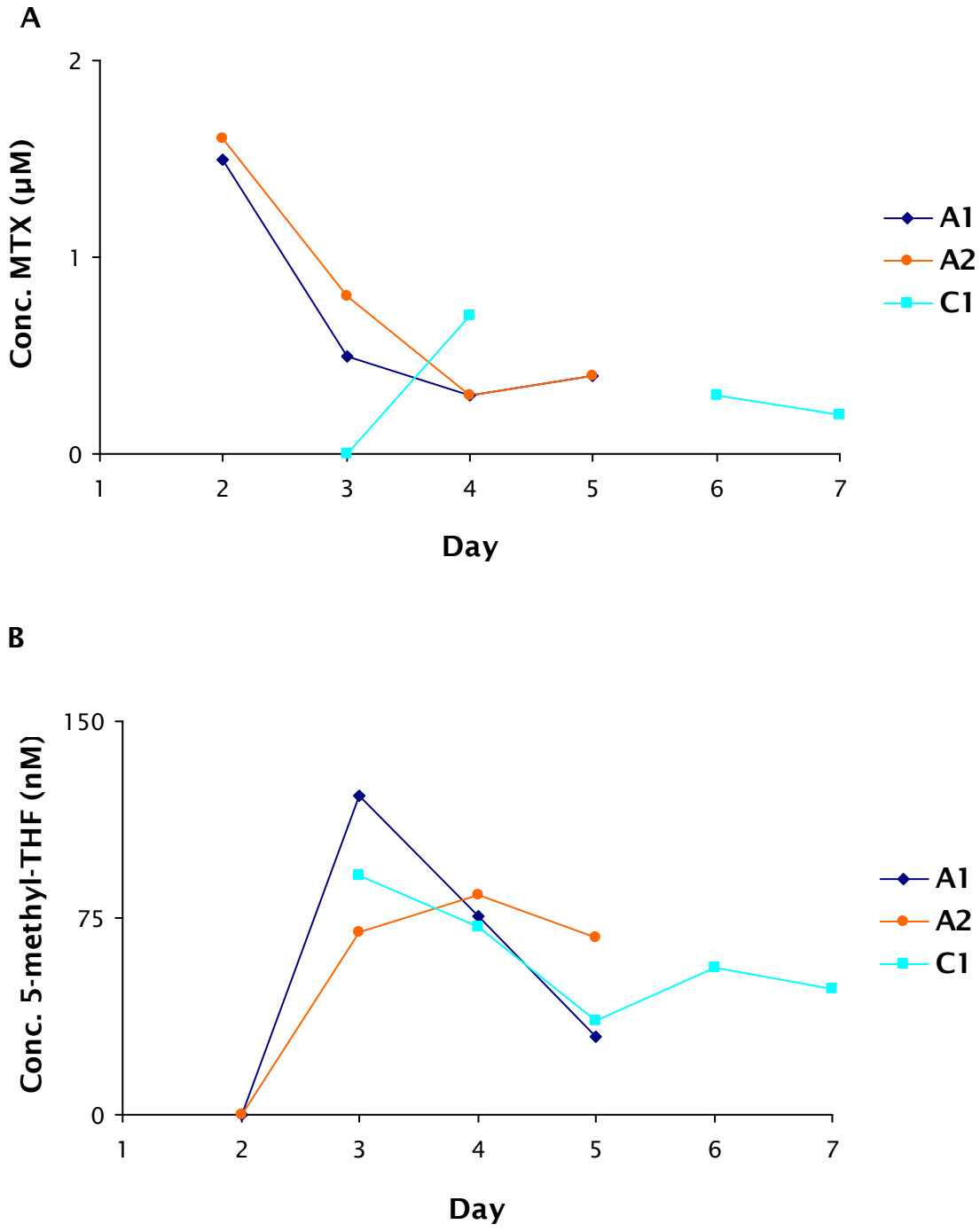
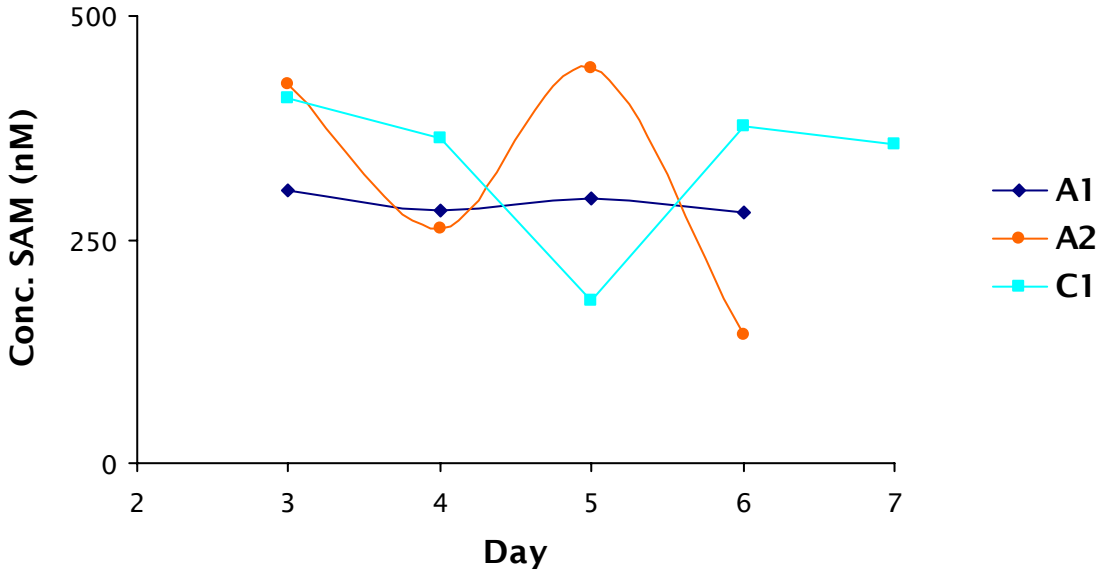


Fig. 5: CSF concentration of MTX (A) and 5-methyl-THF (B) in patient 4.

C



D

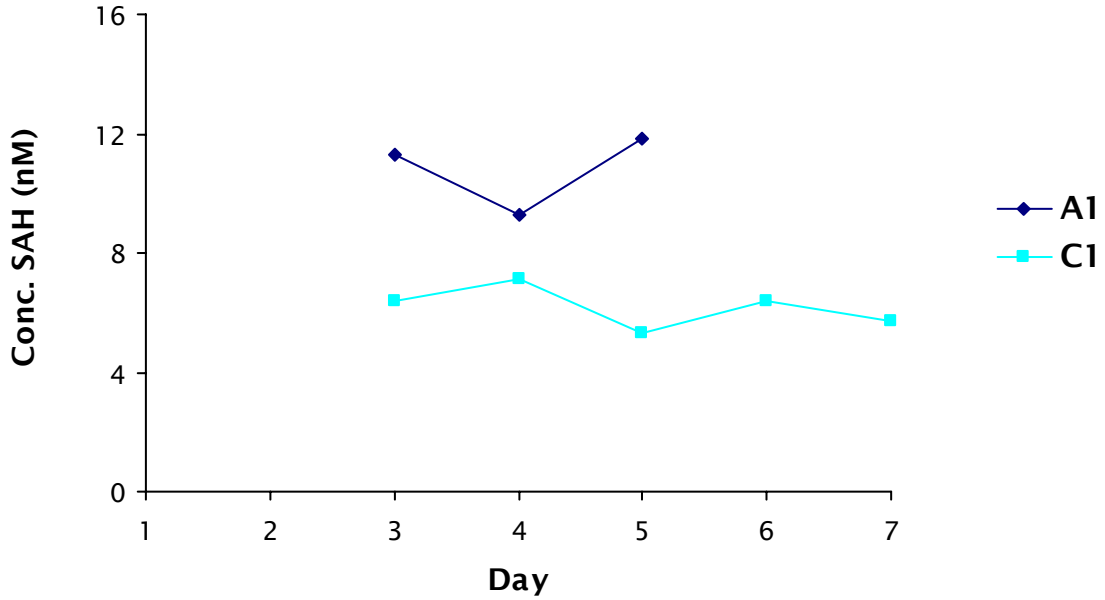


Fig. 6: CSF concentration of SAM (C) and SAH (D) in patient 4.

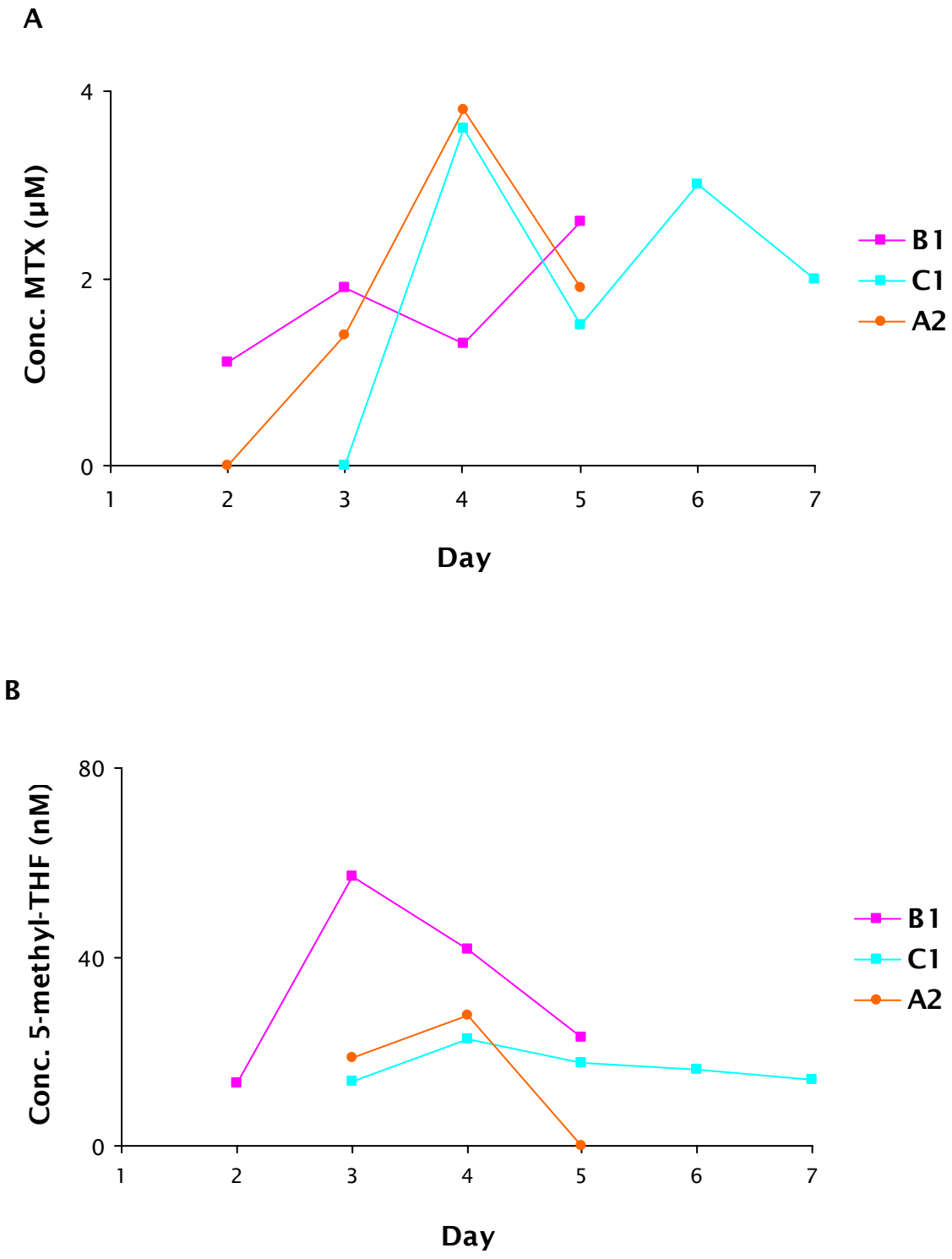
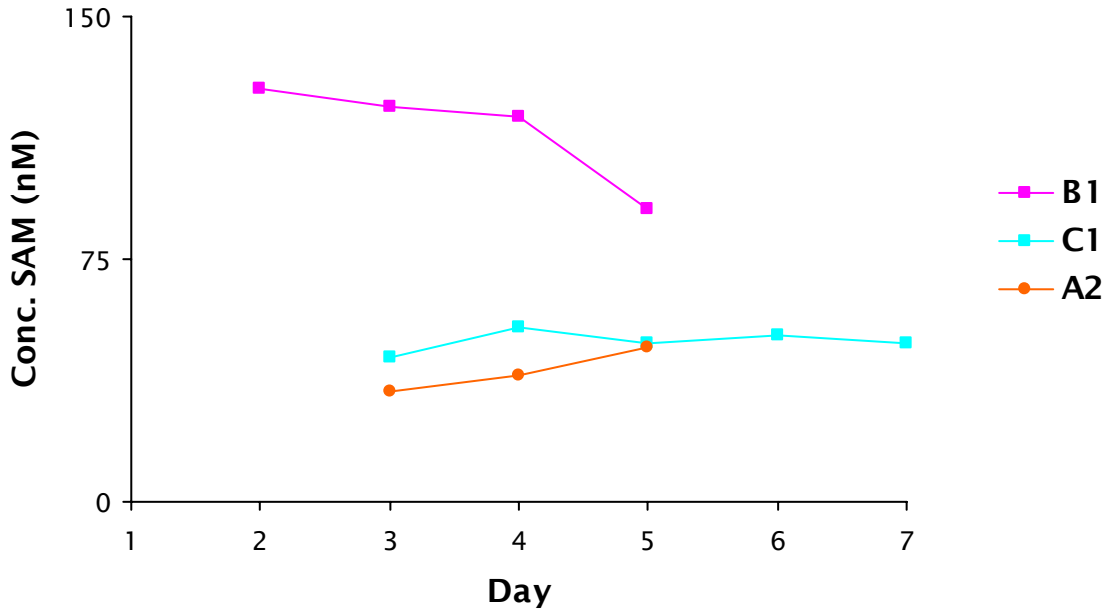


Fig. 7: CSF concentration of MTX (A) and 5-methyl-THF (B) in patient 5.

C



D

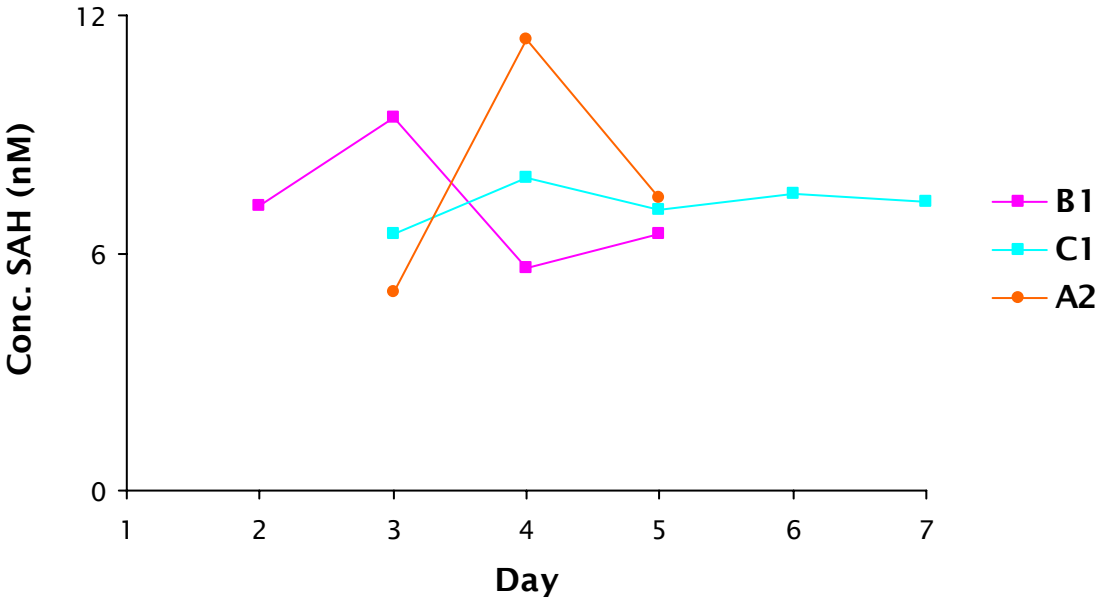


Fig. 8: CSF concentration of SAM (C) and SAH (D) in patient 5.

Appendix B

Results of the CSF analysis of 5-methyl-THF, MTX, SAM and SAH in patients treated according to ALL BFM 2000. 5,10-methylene-THF and calcium folinate were not quantified in any of these samples.

CSF concentrations of 5-methyl-THF, MTX, SAM and SAH in patients treated according to ALL BFM 2000.

Pat.	Time	Concentration			
		5-MTHF (nM)	MTX (µM)	SAM (nM)	SAH (nM)
1	Protocol I, day 1	65.3	n.d.	194.9	n.q.
1	Protocol I, day 12	36.8	n.d.	143.4	n.q.
1	Protocol I, day 45	42.0	n.d.	166.8	n.q.
1	Protocol I, day 59	66.1	n.d.	-	-
1	Protocol M, day 8	71.9	1.6	-	-
2	Protocol I, day 12	24.2	n.d.	-	-
2	Protocol I, day 33	41.5	n.d.	-	-
2	Protocol I, day 59	19.3	n.d.	104.6	n.q.
2	Protocol M, day 8	12.0	3.8	77.4	n.q.
2	Protocol M, day 22	41.5	n.q.	132.8	5.6
2	Protocol M, day 50	-	-	75.8	5.0
2	Protocol II, day 38	108.3	n.d.	-	-
2	Protocol II, day 45	76.9	n.d.	-	-
3	Protocol I, day 1	-	-	156.9	n.q.
3	Protocol I, day 12	11.9	n.d.	86.1	n.q.
3	Protocol I, day 33	13.3	n.d.	74.2	n.q.
3	Protocol M, day 8	20.8	0.8	-	-
3	Protocol M, day 22	36.2	0.2	-	-
3	Protocol M, day 50	38.1	0.4	-	-
4	Protocol I, day 12	-	-	108.2	6.0
4	Protocol M, day 8	n.q.	0.3	-	-
4	Protocol M, day 22	32.8	0.5	151.1	5.3
4	Protocol M, day 36	26.7	0.3	90.0	5.5
4	Protocol II, day 45	27.9	n.d.	-	-
5	Protocol I, day 1	35.2	n.d.	-	-
5	Protocol I, day 12	40.7	n.d.	-	-
5	Protocol I, day 45	57.8	n.d.	-	-
5	Protocol I, day 59	44.2	n.d.	-	-
5	Protocol M, day 8	28.5	0.3	131.8	8.3
5	Protocol M, day 22	37.8	0.3	145.7	7.2
5	Protocol M, day 36	54.2	0.3	200.7	6.1
5	Protocol M, day 50	-	-	131.8	8.5
6	Protocol I, day 12	15.7	n.d.	67.4	n.q.
6	Protocol I, day 45	80.2	n.d.	111.0	n.q.
6	Protocol I, day 59	100.6	n.d.	77.5	n.q.
6	Protocol M, day 8	55.7	0.6	246.2	n.q.
6	Protocol M, day 22	88.1	0.3	-	-
6	Protocol M, day 36	-	-	227.9	n.q.
7	Protocol I, day 1	91.9	n.d.	175.2	n.q.
7	Protocol I, day 33	68.6	n.d.	128.9	n.q.

Pat.	Time	Concentration			
		5-MTHF (nM)	MTX (µM)	SAM (nM)	SAH (nM)
8	Protocol I, day 1	-	-	224.4	n.q.
8	Protocol I, day 12	17.5	n.d.	120.6	n.q.
8	Protocol I, day 33	24.0	n.d.	120.9	n.q.
8	Protocol I, day 45	-	-	149.3	n.q.
8	Protocol I, day 59	53.0	n.d.	-	-
8	Protocol M, day 8	-	-	199.7	n.q.
8	Protocol M, day 22	23.6	0.7	219.4	n.q.
8	Protocol M, day 36	43.6	0.7	182.5	n.q.
8	Protocol M, day 50	-	-	221.1	n.q.
9	Protocol I, day 45	26.9	n.d.	-	-
9	Protocol M, day 8	15.7	0.3	-	-
9	Protocol M, day 22	39.4	0.3	-	-
9	Protocol M, day 38	31.4	n.q.	-	-
10	Protocol I, day 59	45.7	n.d.	-	-
10	Protocol M, day 8	53.3	n.q.	-	-
10	Protocol M, day 50	25.0	0.5	154.6	10.6
10	Protocol II, day 38	72.8	n.d.	27.7	n.q.
11	Protocol I, day 45	32.1	n.d.	-	-
11	Protocol I, day 59	-	-	205.5	n.q.
11	Protocol M, day 8	-	-	227.4	n.q.
11	Protocol M, day 22	36.4	0.5	176.6	n.q.
11	Protocol M, day 36	27.9	8.5	178.6	n.q.
11	Protocol M, day 50	28.4	3.2	190.5	n.q.
11	Protocol III, day 17	-	-	111.3	n.q.
11	Protocol III, day 24	-	-	171.6	n.q.
12	Protocol I, day 1	52.2	n.d.	201.9	n.q.
12	Protocol I, day 12	-	-	152.1	n.q.
12	Protocol I, day 33	-	-	182.8	n.q.
12	Protocol I, day 45	-	-	112.2	n.q.
12	Protocol I, day 59	68.9	n.d.	108.1	n.q.
12	Protocol III, day 24	92.5	n.d.	76.6	n.q.
13	Protocol I, day 33	26.7	n.d.	-	-
13	Protocol M, day 22	122.5	0.7	-	-
13	Protocol M, day 36	110.0	0.5	-	-
14	Protocol I, day 1	78.6	n.d.	-	-
15	Protocol I, day 45	113.6	n.d.	56.4	n.q.
16	Protocol M, day 36	66.3	0.6	-	-
17	Protocol I, day 1	59.6	n.d.	90.0	n.q.
17	Protocol I, day 12	39.2	n.d.	-	-
17	Protocol I, day 45	-	-	72.0	n.q.
17	Protocol III, day 17	-	-	94.3	9.0
17	Protocol III, day 24	-	-	140.3	n.q.

Pat.	Time	Concentration			
		5-MTHF (nM)	MTX (µM)	SAM (nM)	SAH (nM)
18	Protocol I, day 1	53.0	n.d.	232.4	5.0
18	Protocol M, day 36	-	-	199.7	n.q.
18	Protocol M, day 50	-	-	506.9	n.q.
18	Protocol III, day 17	-	-	93.7	n.q.
18	Protocol III, day 24	-	-	199.6	n.q.
19	Protocol I, day 1	53.3	n.d.	129.3	n.q.
19	Protocol I, day 59	70.0	n.d.	94.1	n.q.
19	Protocol M, day 22	35.2	1.9	106.6	n.q.
19	Protocol III, day 24	-	-	94.7	n.q.
20	Protocol M, day 22	-	-	63.3	n.q.
20	Protocol III, day 17	27.9	n.d.	85.7	5.2
20	Protocol III, day 24	28.4	n.d.	81.2	15.5
21	Protocol III, day 24	15.2	n.d.	-	-
22	Protocol M, day 8	23.5	2.4	-	-
22	Protocol M, day 36	-	-	186.0	n.q.
22	Protocol M, day 50	66.4	0.1	138.3	7.5
22	Protocol III, day 17	27.8	n.d.	89.7	n.q.
22	Protocol III, day 24	18.5	n.d.	121.5	n.q.
23	Protocol I, day 1	-	-	278.4	n.q.
23	Protocol I, day 12	-	-	136.1	n.q.
23	Protocol I, day 33	-	-	144.8	n.q.
23	Protocol I, day 45	-	-	166.1	n.q.
23	Protocol I, day 59	-	-	115.8	n.q.
24	Protocol III, day 45	-	-	58.3	6.0
25	Protocol M, day 8	-	-	187.9	n.q.
25	Protocol M, day 50	-	-	336.7	n.q.
26	Protocol I, day 1	-	-	282.8	11.5
26	Protocol I, day 12	-	-	131.5	7.4
26	Protocol I, day 59	-	-	242.0	12.3
26	Protocol M, day 22	-	-	121.7	n.q.
26	Protocol M, day 36	-	-	106.8	n.q.
27	Protocol I, day 1	-	-	103.1	n.q.
27	Protocol I, day 12	-	-	89.5	n.q.
27	Protocol I, day 33	-	-	46.3	n.q.

n.d.: not detected

n.q.: not quantified

-: sample not obtained

Appendix C

Results of the CSF analysis of 5-methyl-THF in pediatric ALL patients treated according to the 'TOTAL XV' Protocol. 5,10-methylene-THF, calcium folinate and MTX were not quantified in any of these samples.

CSF concentration of 5-methyl-THF in children treated according to TOTAL XV.

Patient	Time	Conc. of 5-methyl-THF (nM)
1	Remission/Induction, day 1	67.5
1	Consolidation, day 1	67.1
1	Consolidation, day 15	67.2
2	Remission/Induction, day 1	62.7
2	Consolidation, day 1	62.0
2	Consolidation, day 15	66.4
3	Remission/Induction, day 1	86.2
3	Consolidation, day 1	84.6
3	Consolidation, day 15	86.8
4	Remission/Induction, day 1	59.8
4	Consolidation, day 1	61.8
4	Consolidation, day 15	56.2
5	Remission/Induction, day 1	68.5
5	Consolidation, day 1	68.1
5	Consolidation, day 15	79.3
6	Remission/Induction, day 1	43.0
6	Consolidation, day 1	36.1
6	Consolidation, day 15	43.5
7	Consolidation, day 1	103.9
7	Consolidation, day 15	88.2
8	Consolidation, day 1	59.8
8	Consolidation, day 15	69.5
9	Consolidation, day 1	61.6
9	Consolidation, day 15	17.8
10	Consolidation, day 1	44.1
10	Consolidation, day 15	56.5
11	Consolidation, day 1	35.0
11	Consolidation, day 15	36.7
12	Consolidation, day 1	60.4
12	Consolidation, day 15	46.7
13	Consolidation, day 1	25.6
13	Consolidation, day 15	52.9
14	Consolidation, day 1	47.7
14	Consolidation, day 15	53.1
15	Remission/Induction, day 1	85.6
15	Consolidation, day 1	56.7
16	Remission/Induction, day 1	50.7
16	Consolidation, day 1	57.5
17	Remission/Induction, day 1	60.9
17	Consolidation, day 15	43.0
18	Remission/Induction, day 1	55.7
18	Consolidation, day 15	58.6