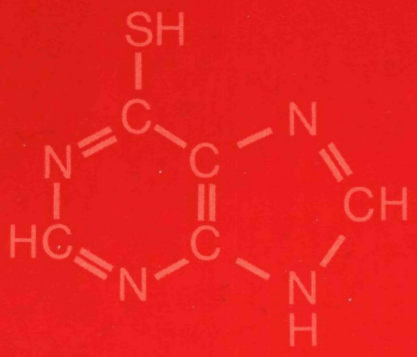

6-MERCAPTOPURINE AND 6-THIOGUANINE:

A FRESH LOOK ON VINTAGE ANTICANCER DRUGS

TEUNIS J. SCHOUTEN

6-MERCAPTOPURINE AND 6-THIOGUANINE:

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proefschrift
ter verkrijging van de graad van
doctor in de geneeskunde
aan de Katholieke Universiteit te Nijmegen
op gezag van de rector magnificus
Prof. Dr. J.H.G.I. Giesbers
volgens besluit van het college van dekanen
in het openbaar te verdedigen
op donderdag 23 mei 1985
des namiddags te 4.00 uur

door

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geboren te Meerkerk (Z.H.)

1985
druk: Stichting Studentenpers Nijmegen

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The studies were supported by a grant (SNUKC 81-2) from the Queen Wilhelmina Foundation, the Netherlands Organization against Cancer (KWF/NOK).
Printing-expenses were partly subsidized by the "Ank van Vlissingen Fonds".

Mijn vader promoveert.

Mijn vader heeft maar weinig tijd
en zorgt ook voor maar weinig gezelligheid.
Hij werkt en werkt maar door,
soms baal ik er wel van hoor.
Geen weekend meer weg,
Ai, wat een pech.
Het is telkens: „Geen geneur aan mijn kop,
ga weg en donder op".
Ja, mijn vader promoveert,
Na al die lasten hoop ik dat hij er ook nog
wat van leert.

Nu is hij klaar,
gelukkig maar.

Toke Schouten.

CONTENTS

ALL	: acute lymphoblastic leukaemia
AML	: acute myeloid leukaemia
AUC	: area under (the concentration-time) curve
CNS	: central nervous system
CSF	: cerebrospinal fluid
DCLSG	: Dutch Childhood Leukaemia Study Group
DTT	: dithiothreïtol
HGPRT	: hypoxanthine guanine phosphoribosyl transferase
HPLC	: high-performance liquid chromatography
i.v.	: intravenous
6MGR	: 6-mercaptoguanine riboside (=6-thioguanine riboside)
6MP	: 6-mercaptopurine
6MPR	: 6-mercaptopurine riboside
MTX	: methotrexate
PRPP	: phosphoribosylpyrophosphate
SNWLK	: Stichting Nederlandse Werkgroep Leukemie bij Kinderen (Dutch Childhood Leukaemia Study Group)
TCA	: trichloroacetic acid
6TG	: 6-thioguanine
6TGR	: 6-thioguanine riboside (=6-thioguanosine)
6TX	: 6-thioxanthine
6TU	: 6-thio-uric acid

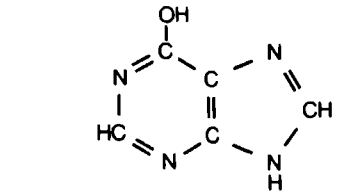
CHAPTER I

6-MERCAPTOPURINE AND 6-THIOGUANINE: BACKGROUND OF THE STUDIES

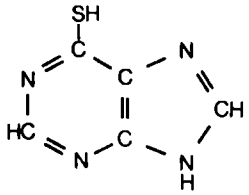
6-Mercaptopurine (6MP) and 6-thioguanine (6TG) were synthesized for use as analogs of natural purines (1, 2, 3). The hypothesis was that analogs might be metabolized initially in the same way as there natural counterparts, but yet could derange the metabolism of the malignant cells, ultimately leading to cell death. Such analogs are called antimetabolites. 6MP is used in the treatment of acute leukaemia and the first results in patients were reported in 1953 by Burchenal et al (4). In the sixties the role of 6MP changed from inducing haematological remission to maintaining such a remission (5, 6). Nowadays 6MP is administered in the maintenance treatment of acute lymphoblastic leukaemia (ALL) essentially in the same way and in the same dose as it was used 30 years ago. This means administration of low doses by the oral route (5, 6). 6MP is combined with methotrexate (MTX), the latter drug being given once or twice a week (5, 6). The combination of 6MP and MTX has been used on empirical grounds for nearly 20 years, but recently arguments for a synergistic biochemical interaction have been raised (7, 8). Such a biochemical synergistic interaction can be demonstrated in vitro only at much higher concentrations than those obtainable in vivo after administration of the drugs in customary doses and schedules. Further investigations in vitro, and translation of the results into in vivo relevant dosages and schedules, are certainly warranted (7, 8).
The other thiopurine we studied, 6TG, is mainly used in the treatment of acute myeloid leukaemia (AML). The customary use of 6TG, instead of 6MP, in the treatment of AML is based more on convention than on a deliberate choice (3). 6TG too is administered usually by the oral route.

METABOLISM

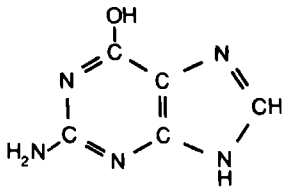
6MP and 6TG are analogs of hypoxanthine and guanine, respectively (fig. 1). Both drugs differ from their natural analogs by having a SH group instead of an OH group attached at the 6^{th} position of the purine ring. Both drugs can be metabolized initially in the same way as their natural counterparts, but ultimately cause a derangement of the metabolism



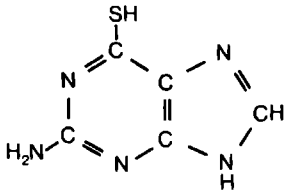
hypoxanthine



6-mercaptopurine



guanine



6-thioguanine

Fig. 1. Structural formulas of 6-mercaptopurine and 6-thioguanine, with their natural counterparts hypoxanthine and guanine, respectively.

of the cell, eventually leading to the death of this cell. Before discussing the various mechanisms of cell kill we will discuss briefly the normal purine metabolism.

The natural purines can be synthesized from simple precursors, starting from PRPP, by a ten step route of metabolism called the de novo synthesis (fig.2) (9). The first purine resulting from this pathway is inosine monophosphate (IMP) which can be converted into adenine and guanine nucleotides by the purine interconversion enzymes (fig.2).

The first step in the purine de novo synthesis is catalyzed by the enzyme phosphoribosylpyrophosphate glutamyl amidotransferase and this rate limiting enzyme in the purine de novo synthesis is susceptible to feedback inhibition by IMP and IMP analogs. This is relevant for the biochemical effects, which can be produced by thiopurine metabolites.

The de novo synthesis is a multi-step energy requiring pathway, and the economy of the cells is served by the existence of the so-called salvage pathway. In this pathway the natural purine bases are "salvaged" from degradation to xanthine and uric acid by conversion to their respective

ABBREVIATIONS USED IN FIG. 2 STARTING FROM TOP OF THE SCHEME

R-5-P	ribose-5-phosphate
PRPP	α-D-5-phosphoribosyl pyrophosphate
PRA	5-phospho-β-D-ribosylamine
GAR	5'-phosphoribosylglycinamide
FGAR	5'-phosphoribosyl-N-formylglycineamine
FGAM	5'-phosphoribosyl-N-formylglycineamidine
AIR	5'-phosphoribosyl-5-aminoimidazole
CAIR	5'-phosphoribosyl-4-carboxy-5-aminoimidazole
SAICAR	5'-phosphoribosyl-4-(N-succinocarboxamide-)5-aminoimidazole
AICAR	5'-phosphoribosyl-5-amino-4-imidazole carboxamide
FAICAR	5'-phosphoribosyl-5-formamido-4-imidazole carboxamide
IMP	inosine-5'-monophosphate
sAMP	adenylosuccinate
XMP	xanthosine-5'-monophosphate
(d)AMP	(deoxy)adenosine-5'-monophosphate
(d)ADP	(deoxy)adenosine-5'-diphosphate
(d)ATP	(deoxy)adenosine-5'-triphosphate
(d)GMP	(deoxy)guanosine-5'-monophosphate
(d)GDP	(deoxy)guanosine-5'-diphosphate
(d)GTP	(deoxy)guanosine-5'-triphosphate

NUMBER	ENZYME
1	phosphoribosylpyrophosphate aminotransferase
2	IMP-dehydrogenase
3	adenylosuccinate synthetase
4	adenylosuccinase
5	hypoxanthine guanine phosphoribosyl transferase (HGPRT)
6	AMP-deaminase
7	5'-nucleotidase
8	adenosine kinase
9	adenosine deaminase
10	adenine phosphoribosyl transferase
11	purine nucleoside phosphorylase
12	xanthine oxidase
13	guanase

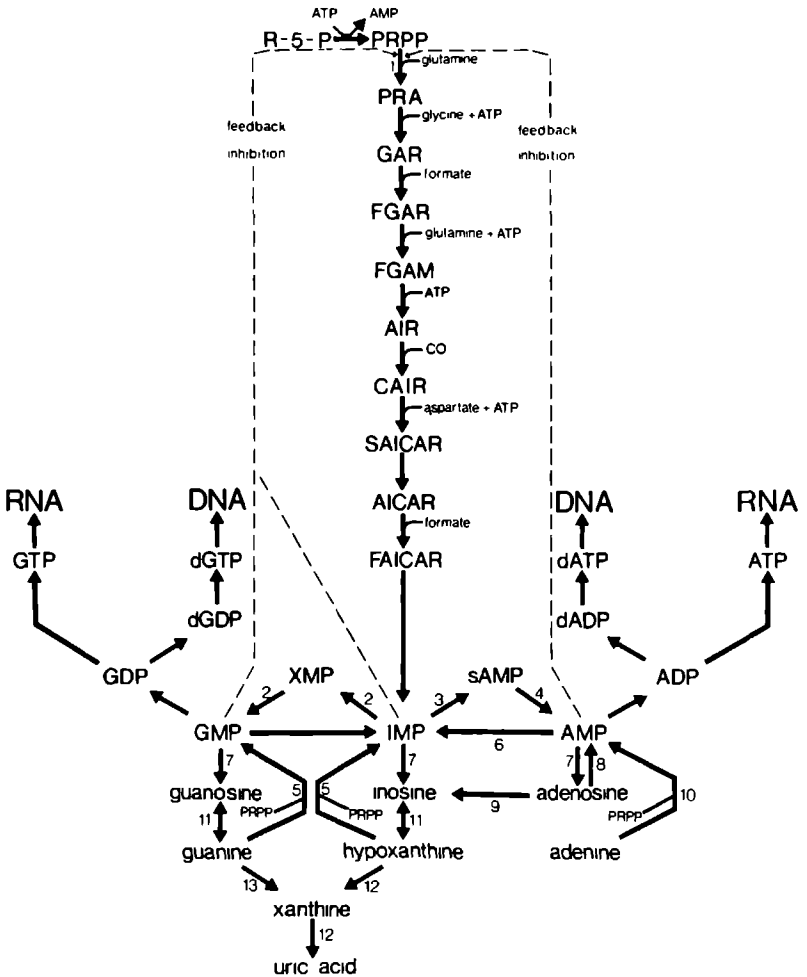


Fig. 2. The vertical chain of reactions starting with PRPP and terminating in IMP represents the purine de novo synthesis. The reactions indicated horizontally from IMP to GMP and AMP, are the interconversions. The reactions from guanine, hypoxanthine and adenine back to the corresponding ribonucleotide monophosphates are called the salvage pathway. For abbreviations and meaning of the numbers see opposite page.

monophosphates (fig.2). Hypoxanthine guanine phosphoribosyl transferase (HGPRT) catalyzes the ribophosphorylation of guanine and hypoxanthine into GMP and IMP, respectively. Adenine phosphoribosyltransferase is involved in the ribophosphorylation of adenine to AMP. Phosphoribosylpyrophosphate (PRPP) functions as a co-substrate for these "salvage" reactions.

Both 6MP and 6TG can substitute in the purine salvage pathway for their natural analogs hypoxanthine and guanine (fig.3), for review see Paterson and Tidd (1975) and LePage (1977) (1, 2). The salvage enzyme hypoxanthine guanine phosphoribosyl transferase and the cosubstrate PRPP are of course of critical importance for the activation of the thiopurines to there monophosphates. 6MP is converted to 6-thio-IMP and 6TG to 6-thio-GMP. 6-Thio-IMP can be methylated to 6-methyl-thio-IMP or inter-

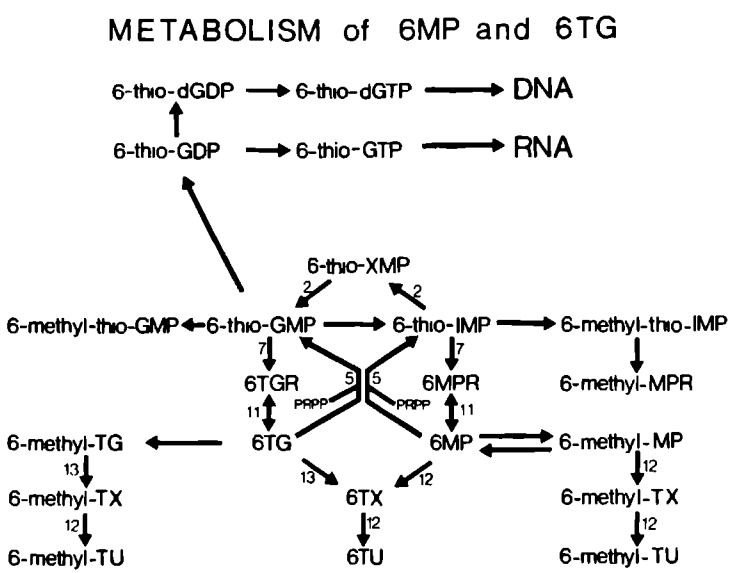


Fig. 3. The metabolic scheme is analogous to figure 2, with 6TG and 6MP instead of the natural counterparts, guanine and hypoxanthine, respectively. (Abbreviations: 6(-methyl-)TU:6-(methyl-)thio-uric acid, 6(-methyl-)TX:6-(methyl-)thioxanthine, 6(-methyl-)MP(R):6-(methyl-)mercaptopurine (riboside), 6(-methyl-)TG(R): 6-(methyl-)thioguanine (riboside).

converted to 6-thio-GMP. 6-thio-GMP can be methylated to 6-methyl-thio-GMP or further metabolized to 6-thio-GDP and 6-thio-GTP and eventually be incorporated into RNA. At the level of 6-thio-GDP the action of a reductase can lead to formation of 6-thio-dGDP and a kinase then produces 6-thio-dGTP, which can be incorporated into DNA.

As mentioned before, IMP is capable of feed-back inhibition of the purine de novo synthesis, and one may expect the same inhibition from 6-thio-IMP and 6-thio-GMP. This is indeed the case, especially 6-methyl-thio-IMP exerts a strong feed-back inhibition of the purine de novo synthesis. For 6-methyl-thio-GMP this inhibition is not observed (1, 10). As a result of the inhibition of the first step in the de novo synthesis, PRPP can accumulate and this leads to increased synthesis of 6-thio-IMP by the salvage pathway: the so-called "self enhancement". The strong biochemical perturbations in the purine metabolism caused by 6-methyl-thio-IMP and similar metabolites result in growth inhibition, but not in irreversible damage leading to cell death (1, 2). So the ultimate site of cytotoxic effect of 6MP and 6TG is most probably their incorporation into DNA (as 6-thio-dGTP) and, to a lesser extent, into RNA (as 6-thio-GTP) (1, 2, 11, 12, 13, 14, 15, 16).

As the incorporation into DNA is the main site of cytotoxicity for 6MP and 6TG, it follows that 6MP and 6TG can only be effective when cells pass through the S-phase of the cell cycle. It is especially in this phase that precursors of DNA and DNA itself are assembled. The specificity of 6MP and 6TG for those phases of the cell cycle in which nucleotides and DNA are synthesized for DNA duplication, has been demonstrated repeatedly (12, 13, 14).

The antimetabolites 6MP and 6TG, if used in the appropriate concentration in vitro or dose and schedule in vivo, will only exert a cytostatic effect on tumour cells which are actively proliferating. Moreover, as the individual cells from a cell population are randomly distributed in the various phases of the cell cycle, 6MP and 6TG must be present at least for as long as the duration of the cell cycle for those particular cells.

The catabolism of 6MP and 6TG is very similar to that of hypoxanthine and guanine (fig.2 and 3) (1, 2, 9). So 6MP is susceptible to xanthine oxidase. This enzyme is present in large amounts in intestinal epithelial cells and in liver cells. Xanthine oxidase oxidizes 6MP in two

steps to 6-thio-uric acid (6TU). 6TU and the preceding intermediate metabolite 6-thioxanthine (6TX) are excreted in urine (1).
6TG is first deaminated by guanase, an enzyme which is not that abundant in the human body as is xanthine oxidase. Guanase catalyzes the deamination of 6TG to 6-thioxanthine, and 6-thioxanthine is further degraded to 6-thio-uric acid by xanthine oxidase (1).

METHODS TO MEASURE CYTOTOXIC EFFECTS OF DRUGS IN VITRO

In considering the methods by which a cytostatic effect is measured, one should realize very well that cure of malignant disease by chemotherapy can only be attained if all malignant cells, including the last one, are destroyed (17, 18). The concept of log kill has been especially helpful in our understanding of the effects of cytotoxic drugs (17). So, methods which actually can measure cell kill in several logs are likely the best ones to attain a correlation with the cytotoxicity relevant in vivo. Clonogenic assays of malignant cells seem best suited to transpose in vitro data to in vivo studies (19, 20, 21, 22, 23). However, these clonogenic assays are not without problems, especially for solid tumour samples obtained from humans (24, 25).
In leukaemia it is somewhat easier to obtain samples for clonogenic assays (26, 27). Some authors have successfully used those assays in chemosensitivity testing (28, 29). They draw attention to the factor of duration of drug exposure, especially for S-phase specific drugs. Both groups found better predictive values by continuous exposure to cytosine arabinoside than by 1 hour pulse exposure (28, 29). Park and coworkers conclude from their data that on the one hand it may be important to simulate the in vivo situation for getting better predictive results with in vitro tests, but their data indicate on the other hand the possibility that this in vitro leukaemia clonogenic assay might be used as a guide to determine the most efficacious schedule of clinical drug administration (28).

STARTING POINT AND EVOLUTION OF THE STUDIES ON 6-MERCAPTOPURINE AND 6-THIOGUANINE

6MP is one of the two drugs which are extensively used in the maintenance treatment of childhood ALL (5, 6). We planned our project on 6MP, because only a few studies on the pharmacokinetics and metabolism of 6MP in this clinical situation were available (1, 2 ,3). The know-how in the field of purine metabolism existing in our institution formed a basis to start our investigations on the purine analogs, 6MP and 6TG. We performed our study on the use of 6MP in ALL patients receiving maintenance treatment according to the operative protocol of the "Stichting Nederlandse Werkgroep Leukemie bij Kinderen" (Dutch Childhood Leukaemia Study Group).

As the first objective we were obliged to develop a reliable method for the determination of 6MP, as there was no standard method to quantitate 6MP in biological fluids. Such a method has to be specific and sensitive, especially because the low dose of 6MP applied in the maintenance treatment of ALL results in very low plasma concentrations. We solved this problem by using high-performance liquid chromatography (HPLC) (Chapter II). The presence of a protective agent for 6MP, from the moment the sample of biological fluid was taken and throughout the total analytical procedure, was a major step. By using a relatively large injection volume the sensitivity of the method became adequate. The method described in Chapter II was applied to study plasma and CSF concentrations of 6MP in patients and in animals (Chapters IV, V, VI).Later the method of analysis was refined to include the quantitation of 6MP riboside (Chapter III). Moreover the method was changed in such a way that 6TG and 6TG riboside could be quantitated by the same analytical procedure. This has practical advantages when performing investigations both on 6MP and 6TG as we did in the second part of the study.

The orientating study on patients receiving 6MP as part of their maintenance treatment raised several questions, which should be elucidated in further studies. In our study a very important finding was the good penetration of 6MP into CSF in patients, with a ratio of CSF to simultaneous plasma concentrations of about 0.40 (Chapter IV). However the concentrations of 6MP measured were much lower than the concentrations

which exert a cytotoxic effect in in vitro studies on mouse leukaemia and lymphoma cells (11, 30, 31).

The very low plasma concentrations observed in patients led to further exploration in an experimental animal model (Chapter V). With regard to dose and schedule of 6MP administration we conformed us to the operative protocol of maintenance therapy. This means we used a dosage of about 2 mg/kg/day in dogs and administered this dose by the oral route as a rule. To study the plasma concentration-time curves of 6MP adequately, we resorted to i.v. bolus injections, still at the customary dose. Although the concentrations which we measured after i.v. bolus injections were much higher than after oral administration, these concentrations of 6MP were still relatively low in comparison with the concentrations used in in vitro cytotoxicity studies. Moreover, the concentrations of 6MP after i.v. bolus injections declined to very low levels after just a few hours. In vitro studies had shown that not only concentrations of 6MP in the order of 100-200 μM were necessary for a maximal effect, but also that the cells should be exposed to these concentrations for a long time. In biological terms the period of exposure should be in the order of 1-2 median cell cycle lengths (11).

The results from our studies in patients and dogs are pointing to changing the usual dosages, routes and schedules of administration of 6MP. We also included 6TG in our further search for a reliable application of purine antagonists. Both substances are closely related structurally and the mechanism of cytotoxicity is very similar. 6TG might have some advantage over 6MP as the biochemical pathway leading to incorporation into DNA is more straightforward for 6TG than for 6MP. In vitro studies using mouse lymphoma cells had demonstrated that 6TG exerts its maximal cell kill at lower concentrations and that the maximal cell killing effect is somewhat greater for 6TG (11).

During the last years the customary dose and way of administration were already in discussion in the literature (32, 33). Studies were reported on the low bioavailability of 6MP after oral administration. For this reason parenteral administration of 6MP and 6TG during prolonged periods of time seemed worthwhile. A report by Frei and Cancellos on dose as a critical factor in cancer chemotherapy (18) and the high concentrations used in in vitro studies (11) made us to consider the effect of high doses of 6MP and 6TG. In the report by Frei and Canellos the concept of

"maintenance" chemotherapy is rejected in curatively intended chemotherapy. The original goal of maintenance treatment was not directed at cure, but rather at prolonging the time to relapse. The authors state that there is no clear evidence from any study that maintenance treatment will increase the cure rate. The philosophy of maintenance treatment is not based on good therapeutic principles derived from fundamental biologic studies and is not in accord with the results of more recent and sophisticated clinical trials (18).

In Chapters VI, VII and VIII we report on studies in monkeys (6MP) and goats (6TG and 6MP). The drugs, 6MP and 6TG, were administered by infusion. In our study in monkeys, using radiolabeled 6MP, an infusion period of one and four hours was used (Chapter VI). A relatively high dose rate of 6MP was given, i.e. 5 mg/kg/hour. In Chapter VII the results of two hours infusions of 6TG into goats, at a dose rate of 2 mg/kg/hour, are reported. In Chapter VIII really prolonged, i.e. 24 hours, infusions of 6MP are applied at different dose rates. The confirmation of the finding of good penetration of 6MP and also 6TG into CSF in these studies in goats is worth to be mentioned beforehand.

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CHAPTER II

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PLASMA 6-MERCAPTOPURINE IN CLINICALLY RELEVANT CONCENTRATIONS

Abreu RA De, Baal JM van, Schouten TJ, Schretlen EDAM, Bruyn CHMM de.
Published in: J Chromatogr 1982;227:526-533

Journal of Chromatography, 227 (1982) 526—533
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1090

Note

High-performance liquid chromatographic determination of plasma 6-mercaptopurine in clinically relevant concentrations

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(First received June 25th, 1981; revised manuscript received July 31st, 1981)

Since 1953 the drug 6-mercaptopurine (6MP) has been used extensively in the treatment of leukemia [1]. This purine analogue was first applied to induce and maintain remission of the leukemic process. During the last decade 6MP has been used for maintaining remissions induced with other agents [2].

The first pharmacokinetic studies in man were reported by Hamilton and Elion [3]. They used radioactive 6MP (^{35}S) to study plasma and urine levels of the drug in two patients with leukemia. Column and paper chromatography were used to separate urinary metabolites. The intravenous route of administration has mostly been used in experimental studies. In clinical practice, however, oral therapy is the rule [2, 4].

Later pharmacokinetic studies are scant. The main reason for this might be the lack of specific and sensitive methods for determination of 6MP. A colorimetric determination of 6MP in plasma has been described [5]. Problems of specificity, especially interference of natural purine metabolites, were circumvented by using the patients' own pre-treatment plasma for the reference determinations. The lower limit of detectability was in the range of 0.2—0.5 μg/ml. For duplicate determinations about 20 ml blood were necessary and about 50 ml for producing reference plasma samples. Finkel [6] described a fluorimetric method with approximately the same sensitivity

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(± 1.0 μg/ml), which required less blood per determination.

In recent years, several high-performance liquid chromatographic (HPLC) methods for the determination of 6MP in blood have been described [7—9]. Although these HPLC methods are relatively easy to perform, the sensitivity is still not optimal (0.2 μg/ml) [7]. Two systems, mainly designed for the determination of azathioprine, require a time-consuming extraction method with 12% and 50% recovery, respectively [8, 9]. The lower limit of detectability is in the range of 5 ng/ml. Only small amounts of serum or plasma are necessary per determination (0.5—1 ml).

In the present paper we describe an HPLC method designed for the identification and quantitation of 6MP in plasma. The lower detection limit is approximately 3 ng/ml. An application of the method is illustrated by means of pharmacokinetic studies, using a common clinical dose level [2].

EXPERIMENTAL

Chemicals

6MP, 6-mercaptopurine riboside (6MPR), and 6-mercaptoguanine ribosibe (6MGR) were purchased from Sigma (St. Louis, MO, U.S.A.); dithiothreitol (DTT) was from Boehringer (Mannheim, G.F.R.); all other chemicals were from E. Merck (Darmstadt, G.F.R.), all of the highest analytical grade.

Sample preparation

Venous blood samples of 2 ml were collected in tubes containing heparin and also 120 μg of DTT to prevent oxidation of 6MP. After mixing, the blood sample was centrifuged (5 min, 2000 *g*) and the plasma was pipetted into micro test-tubes (type 3810, Eppendorf, Hamburg, G.F.R.), and kept on ice for 5 min. Ice-cold 50% (w/v) trichloroacetic acid (TCA) was added in an amount of one-tenth of the plasma volume. After mixing vigorously, the suspension was kept on ice for another 10 min. Precipitated protein was removed by centrifugation (10 min, 2000 *g*) and the supernatant (TCA extract) was adjusted to a pH of between 6 and 7 with 4.0 *M* potassium hydroxide.

Stock solutions

Stock solutions of 6MP were prepared from which standard solutions were obtained by dilution. Standard solutions were used for calibration and standardisation of the HPLC method. 6MP (10 mg) was dissolved in a solution of 0.025 *M* potassium diphosphate containing 60 mg/l DTT, and adjusted to a pH of about 10.0 with 4.0 *M* potassium hydroxide.

After 6MP had completely been dissolved, the pH of the solution was adjusted to pH 6.6 with phosphoric acid and diluted to a concentration of 1 mg/ml. The stock solution was kept at a temperature of 4°C and was stable for at least two weeks.

6MP solutions for intravenous injection

Solutions for intravenous injection were freshly prepared; 60 mg of 6MP were dissolved in 0.25 ml of a 5.0% solution of sodium hydroxide. When

diluted with sterile physiological saline to a 6MP concentration of 10 mg/ml, the pH of the solution was about 10.0. The solution was used shortly after preparation. For the animal experiments 5 ml of the solution were injected, while passing through a sterile Millipore type Millex-GS membrane filter (pore size 0.22 μm).

Instrumentation

The experiments were performed on a Spectra Physics SP-8000 high-performance liquid chromatograph equipped with an auto-injector with Valco valve, an automated data system with integrator and a two-channel printer-plotter. The volume of the sample loop was 460 μl. Column effluents were monitored with two fixed SP 8210 UV detectors (Spectra Physics) set at wavelengths of 312 nm and 280 nm, respectively. Both detectors had an 8-μl flow cell.

HPLC procedure

Chromatography was carried out at a constant flow-rate of 1.5 ml/min on two Spherisorb 10-ODS (particle size 10 μm) reversed-phase columns (250 mm × 4.6 mm I.D.) in series. A 460-μl sample of neutralized TCA extract was injected on the columns and was eluted with 0.050 *M* potassium phosphate buffer (pH 6.35). Before use the phosphate buffer was filtered through a Millipore type HA membrane filter (pore size 0.45 μm) and during elution the buffer was degassed by continuous helium purging. The maximum run-time was 30 min. Integrated peak areas at 312 nm were used for calculating the concentrations.

RESULTS AND DISCUSSION

Stability

At low pH 6MP is poorly soluble. The solubility is much better at basic pH but then 6MP is easily oxidized. Therefore, we found it necessary to stabilize stock solutions and blood samples with DTT. The addition of DTT remarkably increased the stability, and the average recovery from standards added to plasma increased from 20% (without DTT) to 94% (with 60 μg of DTT per ml of plasma).

Chromatographic conditions

The scan patterns of human plasma spiked with 6-thiouric acid, 6-thioxanthine, 6MP, 6MPR and 6MGR (Fig. 1a) show a good separation. The plasma samples are free from compounds interfering with the detection of these components under the chromatographic conditions used (Fig. 1b).

Calibration and quantification

Calibration curves were made using standard solutions of increasing concentration. The peak areas were calculated by automatic integration at 312 nm. The linearity is good; little, if any, difference exists between determinations carried out in plasma and those carried out in aqueous solution. The

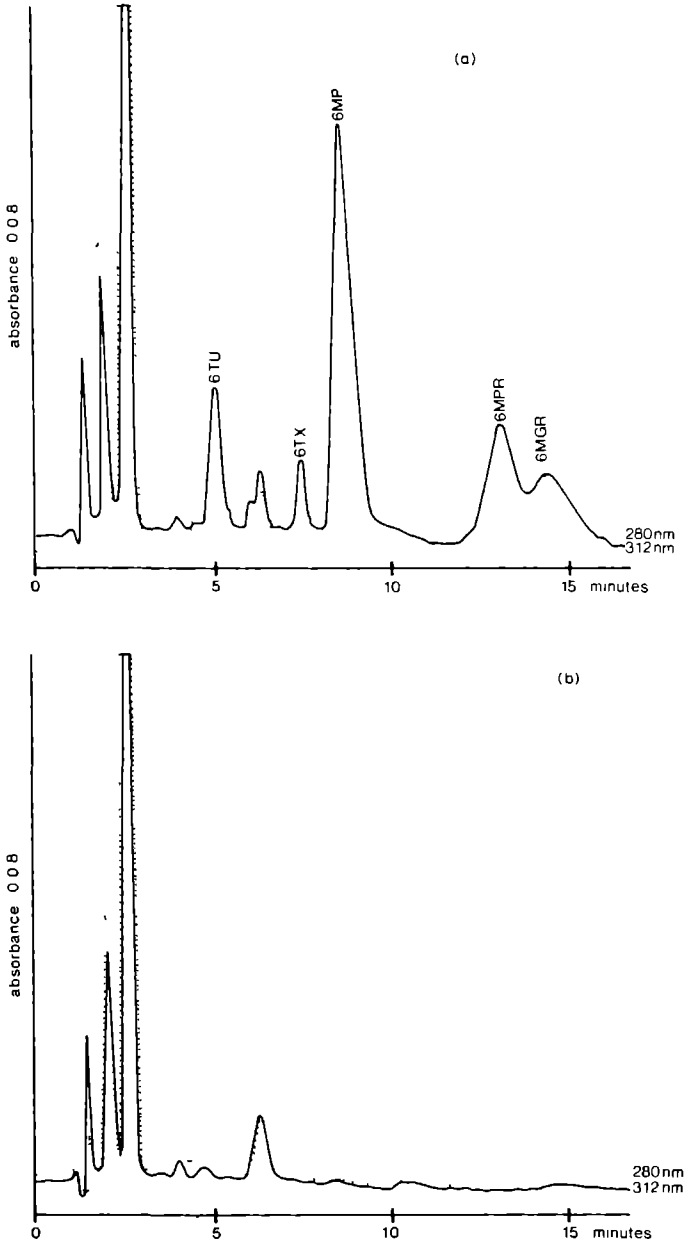

Fig. 1. Elution profiles of the separation of 6MP, 6MPR and 6MGR on Spherisorb-ODS columns (for separation conditions, see Experimental). (a) Chromatogram of human plasma sample with added 6-thiouric acid (6TU), 6-thioxanthine (6TX), 6MP, 6MPR and 6MGR. (b) Chromatogram of human plasma sample without addition of mercaptopurines.

absorbance 0 01
0
5
10
15 minutes
DTT
280nm
312nm
(a)

absorbance 0 04
0
5
10
15 minutes
6MP
280nm
312nm
(b)

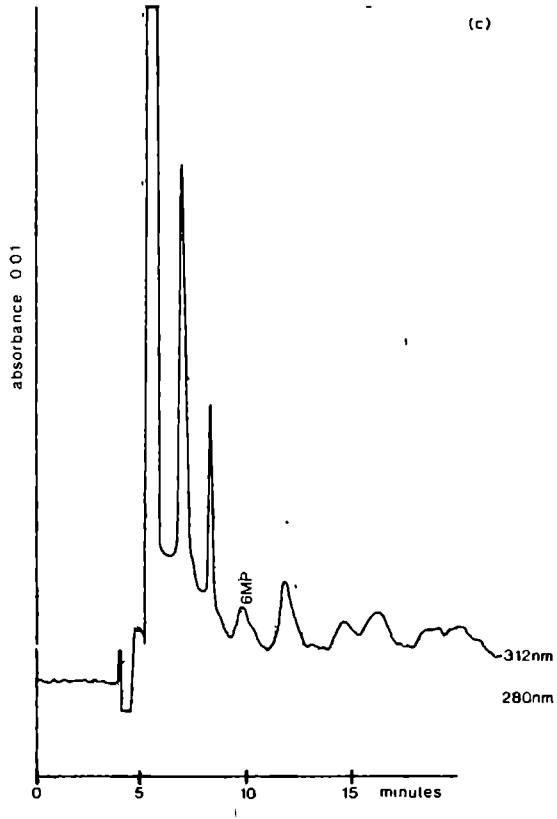

Fig. 2. HPLC of 6MP in plasma of a 28-kg Labrador dog. (a) Plasma sample collected before 6MP administration (b) Plasma sample collected 10 min after intravenous injection of 50 mg of 6MP. (c) Plasma sample collected 5 h after intravenous injection of 50 mg of 6MP.

recovery of 6MP added to plasma is 94 ± 5%. The relationship between 6MP plasma concentration in the range 3—1800 ng/ml and peak area is: concentration = ($4.59 \cdot 10^{-4}$ int. area + 3.30) ng/ml. The correlation coefficient is 0.9994. In a low concentration range (3—100 ng/ml) the correlation coefficient between concentration and peak area is 0.9970. In this concentration range the relationship between 6MP concentrations and peak area is: concentration = ($4.63 \cdot 10^{-4}$ int. area + 2.6) ng/ml. The lower detection limit is approximately 3 ng/ml using a 460-μl loop.

Analysis of plasma levels of 6MP after an intravenous bolus injection in a Labrador dog

Typical scans of TCA extracts before injection (a), and 10 min (b) and 5 h (c) after injection are shown in Fig. 2. The profiles presented in the figure are representative of time-dependent decline of the 6MP levels. By means of computer analysis, the concentration—time curve can be fitted in a model with two half-lives for 6MP. The semi-logarithmic plot in Fig. 3 expresses the relationship between the 6MP levels in plasma and the time after intravenous injection. The calculated half-life times are 21 min and 130 min, with correlation coefficients of 0.985 and 0.968, respectively.

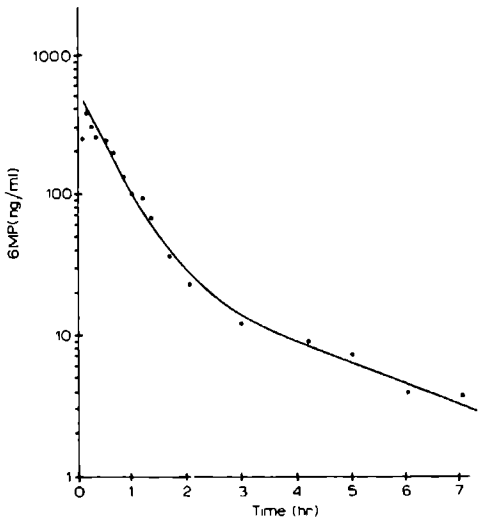

Fig. 3. Plasma levels of 6MP as a function of time after intravenous injection of a 28-kg Labrador dog with 50 mg of 6MP.

CONCLUSIONS

The sensitivity of the method presented here is more than sufficient to follow the pharmacokinetic behaviour of 6MP in plasma for several hours after a clinical dose. Although HPLC methods are commonly used [7—12], the extraction methods do not seem optimal since low recoveries are reported

in the ng/ml range [8, 9]. The extraction procedure is very critical due to the lability of the mercapto (SH) group of 6MP. The addition of DTT is important since, as a reducing agent, it stabilizes the SH group of 6MP, and it increases the recovery from 20 to 94%. Another important factor is to keep the extraction procedure as short and as simple as possible. It is advisable to perform the extraction in the cold in order to reduce the risk of oxidation at room temperature. Recently Ding and Benet [8] have advised the use of dithioerythritol. This compound has a stabilizing effect comparable to that of DTT, so it might have contributed to increasing the recovery. However, a recovery of only 12% was obtained [8]. With our method it should be possible to measure 6MP in plasma from leukemic patients and to perform pharmacokinetic studies of 6MP during maintenance treatment. Further studies to define the pharmacokinetic behaviour of 6MP in Labrador dogs and also in patients with acute leukemia using the method described in this paper are at present underway in our laboratory.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. M.J.M. Oosterbaan from the Division of Clinical Pharmacy, who performed the computer analysis of the plasma concentration—time curve, and Dr. J.A.J.M. Bakkeren for critically reading the manuscript.

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CHAPTER III

SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF 6-MERCAPTOPURINE, 6-THIOGUANINE, 6-MERCAPTOPURINE RIBOSIDE AND 6-THIOGUANOSINE IN BIOLOGICAL FLUIDS

Baal JM van, Leeuwen MB van, Schouten TJ, Abreu RA De. Published in: J Chromatogr 1984;336:422-428

Journal of Chromatography, 336 (1984) 422—428
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2319

Note

Sensitive high-performance liquid chromatographic determination of 6-mercaptopurine, 6-thioguanine, 6-mercaptopurine riboside and 6-thioguanosine in biological fluids

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(First received May 4th, 1984; revised manuscript received July 16th, 1984)

For parallel studies on 6-mercaptopurine (6MP) and 6-thioguanine (6TG), a system is desirable to measure both drugs, as well as their metabolites 6-mercaptopurine riboside (6 MPR) and 6-thioguanosine (6TGR). The method we described previously [1] is not suitable for measuring 6TG and 6TGR, because 6TG was not separated from 6MP, and because the peak of 6TGR was very broad. Other methods for the determination of 6-thiopurines have been reported, but are less suitable for our purposes either because the lower limit of detectability is not low enough [2—6] or because extraction of the samples is too time-consuming [7—10]. In the present paper we describe a high-performance liquid chromatographic (HPLC) method for the identification and quantitation of 6MP, 6TG, 6MPR and 6TGR in plasma, cerebrospinal fluid (CSF) and urine.

EXPERIMENTAL

Chemicals

6MP and 6TG were products of Fluka (Hicol, Rotterdam, The Netherlands); 6MPR and 6TGR were from Sigma (St. Louis, MO, U.S.A.); xanthine oxidase and dithiothreitol (DTT) were from Boehringer (Mannheim, F.R.G.); all other chemicals were from E. Merck (Darmstadt, F.R.G.); helium was from Hoekloos (Amsterdam, The Netherlands). Water used for all solutions was purified through a Milli-Q-System (Millipore, Bedford, MA, U.S.A.).

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HPLC procedure

Experiments were performed on a Spectra Physics HPLC SP 8000B (Spectra Physics, Santa Clara, CA, U.S.A.), connected to an automatic sampler MSI 660 (Kontron, Electrolab, London, U.K.). Column effluents were monitored with a fixed-wavelength ultraviolet (UV) detector SP 8210 (Spectra Physics) at 312 nm and a variable-wavelength UV/VIS detector Model 770 (Spectra Physics) set at 342 nm. Detector signals were plotted on a two-channel printer—plotter of the HPLC apparatus. Peak areas were correlated to concentrations, as will be discussed later.

The columns were packed in our laboratory with Nucleosil 10 C_{18}, particle size 10 μm (Chrompack, Middelburg, The Netherlands). During packing a reservoir was connected directly on top of the column tube, and a slurry of Nucleosil (3 g of Nucleosil in 10 ml of methanol) was pumped into the column (250 mm × 4.6 mm I.D.) by means of the HPLC pump. Methanol 50% (v/v) was used as eluent and the pressure was kept at about 21 MPa by controlling the flow-rate.

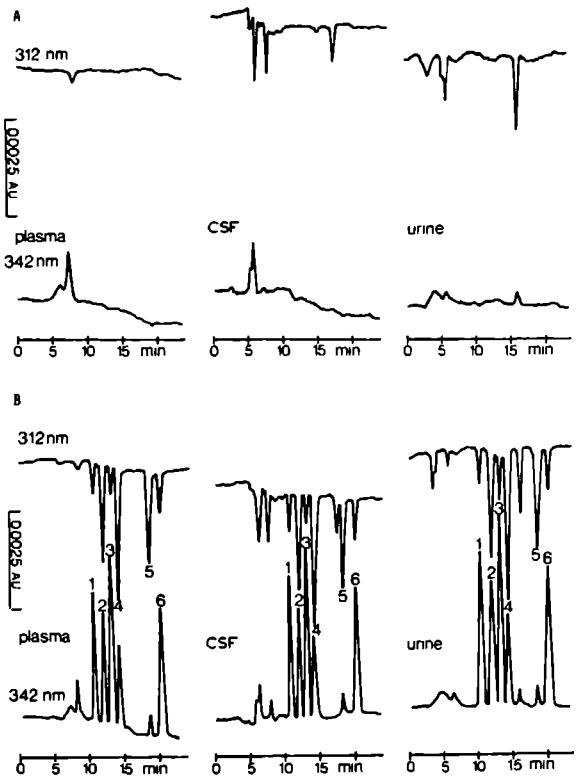

Fig. 1. Scan patterns of separations of 6-thiopurines, following the mobile phase sequence of Table I (injected volume 195 μl). (A) Blanks of plasma, CSF and urine samples; (B) plasma, CSF and urine samples, spiked with 6TU (1), 6TX (2), 0.7 μM 6TG (3), 0.4 μM 6MP (4), 0.4 μM 6MPR (5) and 0.7 μM 6TGR (6). 6TX and 6TU are products of xanthine oxidation reaction on 6MP. The absorption is expressed in absorbance units (AU). The scale is indicated in the figures.

TABLE I

MOBILE PHASE SEQUENCE USED FOR SEPARATION OF 6-THIOPURINES

A = 0.025 *M* phosphoric acid, pH 2.75; B = methanol 50% (v/v); and C = 0.10 *M* potassium dihydrogen phosphate, pH 6.6.

Time (min)	A (%, v/v)	B (%, v/v)	C (%, v/v)
0.0	100.0	0.0	0.0
5.0	98.0	2.0	0.0
10.0	30.0	3.5	66.5
20.0	30.0	3.5	66.5

Chromatography was carried out on two columns in series, at a constant flow-rate of 1.7 ml/min and at a temperature of 33°C (in a water bath).

To achieve a separation such as seen in Fig. 1, we eluted with 0.025 *M* phosphoric acid (pH 2.75), methanol 50% (v/v) and 0.10 *M* potassium dihydrogen phosphate (pH 6.6) following the mobile phase sequence of Table I. The eluents were degassed before and during HPLC runs by continuous helium purging. Before use, solutions were filtered through a Millipore filter (type HA, pore size 0.45 μm). Total run time (including equilibration time for the next run) was 40 min. It is advisable to wash columns with methanol 50% (v/v) after several runs. Depending on the injected volume, 195 μl or 500 μl, we wash the columns after every twenty or ten runs, respectively.

Stock solutions

The 6-thiopurines were dissolved in 0.025 *M* potassium dihydrogen phosphate and the solutions were adjusted to a pH of about 11 with 4.0 *M* potassium hydroxide. After the 6-thiopurines had dissolved completely, the solutions were re-adjusted to a pH between 6.5 and 7.0 with 4.0 *M* phosphoric acid and diluted to a thiopurine concentration of 0.1 mg/ml. Before addition of DTT (final concentration 60 mg/l), 20 μl of each solution were diluted to 500 μl with 0.05 *M* potassium dihydrogen phosphate pH 4.6, and the exact thiopurine concentrations were determined spectrophotometrically.

Parameters on which the exact concentrations were calculated are [11]: 6MP: λ_{max} = 322 nm, ϵ_{max} = 21.5 mM^{-1} cm^{-1}. 6TG: λ_{max} = 342 nm, ϵ_{max} = 25.6 mM^{-1} cm^{-1}. 6MPR: λ_{max} = 322 nm, ϵ_{max} = 27.6 mM^{-1} cm^{-1}. 6TGR: λ_{max} = 342 nm, ϵ_{max} = 26.7 mM^{-1} cm^{-1}.

Stock solutions kept at a temperature of 4°C were stable for at least two weeks.

Enzymatic preparation of 6-thioxanthine (6TX) and 6-thiouric acid (6TU)

6-Mercaptopurine was catabolized enzymatically by xanthine oxidase (EC 1.2.3.2) into 6TX and 6TU according to the procedure described by the manufacturer of the enzyme.

Sample preparation

Venous blood samples of 2 ml were collected in tubes containing heparin

plus 120 μg of DTT. After mixing, the blood samples were centrifuged (5 min, 2000 *g*) and plasma was pipetted into micro test-tubes (type 3810, Eppendorf, Hamburg, F.R.G.). CSF samples of 0.5 ml were collected in micro test-tubes containing 30 μg of DTT. Plasma and CSF samples were put on ice and deproteinized by adding one-tenth of the sample volume of freshly prepared ice-cold 50% (w/v) trichloroacetic acid (TCA).

From the urine samples, 2 ml were taken and pipetted into tubes containing 120 μg of DTT. The urine samples were filtered through a Millipore filter (pore size 0.22 μm) before measurement.

DTT was added to increase the stability and the recovery of the assay [1, 8]. If not measured immediately, extracts of all samples were stored refrigerated and analysed within two weeks, since with older solutions additional absorption peaks were observed.

Thiopurine solutions for intravenous injection

Solutions for intravenous injections were freshly prepared; 6MP or 6TG was dissolved in 0.178 *M* sodium bicarbonate to which sodium hydroxide was added until the thiopurine had dissolved completely. The pH of the final solution was about 10. Intravenous injection was performed through a sterile Millipore membrane filter, type Millex GS (pore size 0.22 μm).

RESULTS AND DISCUSSION

Stability and recovery

As discussed previously [1] thiopurines are poorly soluble at low pH. At basic pH solubility is much better, but oxidation of the SH group has to be prevented by addition of, for example, DTT [1, 8]. With the extraction procedure used, the recovery of thiopurines added to plasma is 94% [1].

Quantitation and calibration

Calibration curves were made using standard solutions of known concentrations. Peak areas of 6MP and 6MPR were integrated at 312 nm, peak areas of 6TG and 6TGR were integrated at 342 nm. Peak areas were calculated in mm^2 : peak area = peak height × peak width at half height. The relationships we found between concentrations and integrated areas and the corresponding

TABLE II

CALCULATION FACTORS FOR CALIBRATION CURVES OF 6-THIOPURINES

Concentrations are given in μ*M*, areas in mm^2, optical scale = 0.01 absorbance units at 10 mV, and injected volume is 195 μl.

Thiopurine	Concentration factor	Correlation coefficient
6MP	Conc. = (area × 0.006938) + 0.002873	0.9999
6MPR	Conc. = (area × 0.009792) − 0.028761	0.9989
6TG	Conc. = (area × 0.008236) − 0.001824	0.9997
6TGR	Conc. = (area × 0.009714) + 0.021745	0.9995

correlation coefficients are given in Table II. With an injected volume of 500 µl, we found a lower limit of detection for 6MP, 6TG, 6MPR and 6TGR of 20 n*M*, 25 n*M*, 65 n*M* and 60 n*M*, respectively.

For 6TX and 6TU, no calibration curves have been made because of the lack of purified 6TX and 6TU.

Accuracy, precision and reproducibility

The accuracy of the method was evaluated by analysing plasma samples containing known amounts of 6MP, 6MPR, 6TG and 6TGR in a concentration range of 100–5000 n*M*. The 95% confidence intervals for single determinations of the thiopurines in plasma were calculated, using the *t*-value from a one-tailed Student's *t*-distribution table and the variance of absolute differences between the actual concentrations (100, 250, 500, 1000, 2000 and 5000 n*M*, respectively) and the concentrations found. The results indicate that any found value would fall within approx. 17.3%, 18.6%, 21.4% and 25.9% of its true value in experiments with 6MP, 6TG, 6MPR and 6TGR, respectively. The validation of

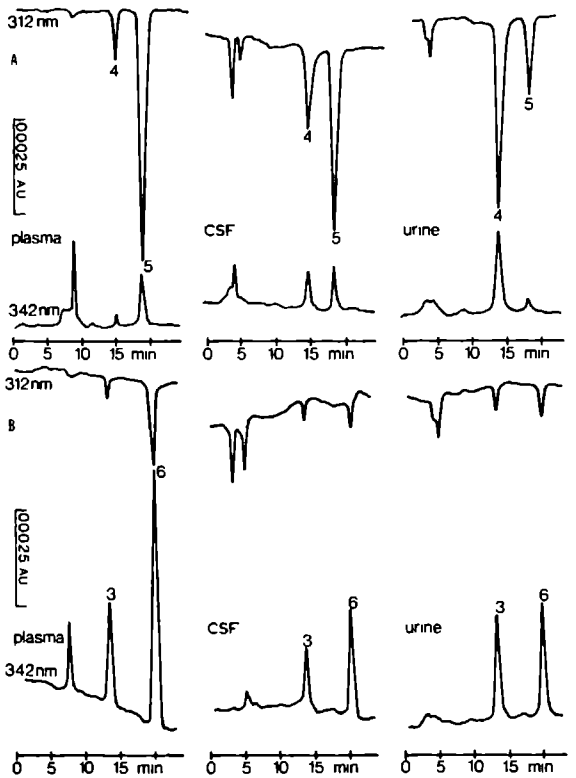

Fig. 2. Scan patterns of plasma, CSF and urine samples (injected volume 195 µl) of a goat, 4 h after an intravenous injection. (A) 6MP (20 mg/kg body weight), urine sample is diluted 5000 times before injection; (B) 6TG (5 mg/kg body weight), urine sample is diluted 25 times before injection. For absorbance units and for identification of the peaks, see legend of Fig. 1.

the method was applied to plasma at the concentrations mentioned above, by determination of the precision (within-day variability) and of the reproducibility (day-to-day variability).

The coefficient of variation for the within-day variation (n = 5) at any concentration of 6MP, 6TG, 6MPR and 6TGR was in the range 1.4—1.7%, 2.1—8.7%, 2.8—10.6% and 3.2—12.5%, respectively. The day-to-day variation (days 1—3) for the same set of data was in the range 2.3—6.0%, 2.8—7.3%, 4.5—9.4% and 4.9—11.8% for 6MP, 6TG, 6MPR and 6TGR, respectively.

Analysis in biological fluids

The method has been applied to the measurement of concentrations of thiopurines in several body fluids after administration of 6MP or 6TG to a goat by an intravenous bolus injection. Scan patterns of analysis in plasma, CSF and urine show the presence of 6MP and 6MPR (Fig. 2A). Chromatographic plots of samples taken after administration of 6TG are given in Fig. 2B. In Fig. 3A and B, time-dependent concentration curves can be seen after push injection of 6MP and 6TG, respectively.

The concentrations of the ribosides of both 6MP and 6TG are higher than the concentrations of the parent compounds. The concentration—time curves of the parent drugs only are far short of representing the total biologically active compounds, at least in goats. In man, it was demonstrated that 6MP and 6MPR are equitoxic at equimolar doses, when given intravenously [12].

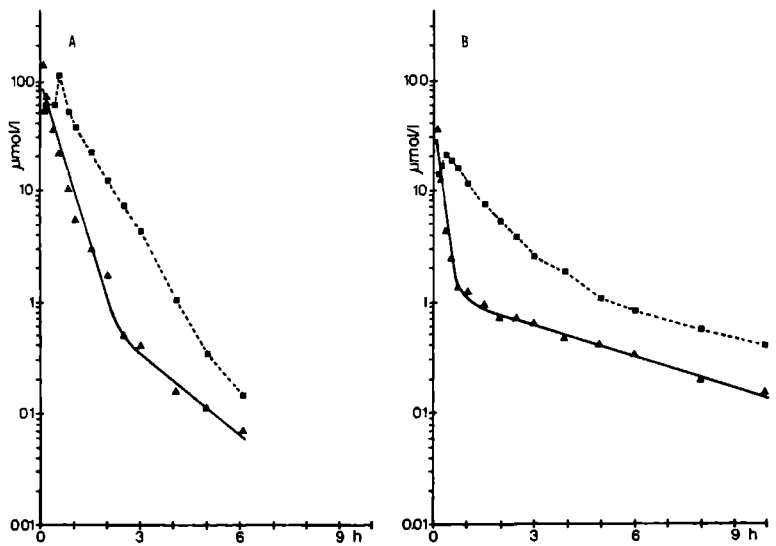

Fig. 3. Concentration—time curves of plasma levels in a goat after an intravenous injection. (A) 6MP (20 mg/kg body weight); (▲) 6MP, (■) 6MPR. (B) 6TG (5 mg/kg body weight); (▲), 6TG; (■), 6TGR.

CONCLUSIONS

In this paper we describe an HPLC procedure to measure 6MP, 6TG, 6MPR

and 6TGR in one single run. The sensitivity of the method enables the pharmacokinetic behaviour of 6-thiopurines in plasma, CSF and urine to be followed for several hours. Since the introduction of HPLC many applications for thiopurines have been reported [2—10]. Several extraction methods do not seem optimal since low recoveries are found [8, 9]. To prevent oxidation of the mercapto (SH—) group of the thiopurines, DTT is added to standards and samples [1, 8]. Other measures have to be taken against oxidation of the thiopurines. Therefore the extraction procedure should be kept short and simple, and should be performed in the cold. The stabilizing effect of DTT increases recovery to 94% [1], and together with all other optimized conditions of sample preparation and chromatography a sensitive and reliable method has been created to measure 6-thiopurines. From our study it may be concluded that all studies on pharmacokinetics of 6MP and 6TG in all kinds of species, including man, should be completed by involving all biologically active derivatives of 6-thiopurines.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. M.J.M. Oosterbaan from the Division of Clinical Pharmacy, who performed the computer analysis of the plasma concentration—time curves, and Dr. J.A.J.M. Bakkeren for critically reading the manuscript.

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CHAPTER IV

6-MERCAPTOPURINE: PLASMA AND CSF CONCENTRATIONS DURING MAINTENANCE TREATMENT OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKAEMIA

Schouten TJ, Abreu RA De, Bruyn CHMM de, Kleijn E van der, Oosterbaan MJM, Schretlen EDAM, Vaan GAM de. Published in essence in: Adv Exp Med Biol 1984;165B:367-370

6-Mercaptopurine, an antimetabolite and an S-phase specific agent, is used extensively in the maintenance treatment of childhood ALL (1, 2). In essence, the dose, way and schedule of administration have remained unchanged from the time the drug was introduced in clinical practice (1, 2, 3). The low oral dose necessitates the availability of a very sensitive and specific method of measurement when studying plasma concentration-time curves. De Abreu et al have developed such a method based on high-performance liquid chromatography (Chapter II, 4). The lower detection limit is approximately 3 ng/ml or 0.016 μM. The addition of dithiothreitol (DTT) remarkably increased the stability of 6MP, and the average recovery of standards added to plasma increased from 20% (without DTT) to 94% (with 60 μg DTT per ml plasma).

In an earlier and orientating phase of our studies, we used this method to determine 6MP plasma and CSF concentrations in patients receiving maintenance treatment of ALL. The schedule of administration of the maintenance therapy was in accordance with the operative protocol V of the "Stichting Nederlandse Werkgroep Leukemie bij Kinderen"(Dutch Childhood Leukaemia Study Group) (Fig.1). In this protocol maintenance treatment is administered in cycles of seven weeks. During the first five weeks 6MP is given daily in an oral dose of 50 mg/m^2 in combination with methotrexate (MTX) 30 mg/m^2, that is also given orally. On the first day of the cycle a first dose of MTX is administered and subsequently this is repeated weekly. During the last two weeks of the seven-week cycle, prednisone is given daily in an oral dose of 40 mg/m^2. In those two weeks vincristin in a dose of 2.0 mg/m^2 is given weekly instead of MTX ("consolidation treatment"). Subsequently the seven-week cycle is repeated. On the first day of a new cycle one starts with both 6MP and MTX and then continues as described above.

Plasma and CSF samples were obtained during routine clinical investigations, performed according to protocol V of the "SNWLK". In this protocol lumbar punctures are required every 14 weeks on the first day of the two weeks of prednisone and vincristin consolidation (fig.1). To study 6MP concentrations in CSF an amount of ± 1.5 ml of CSF was withdrawn. On the same occasion venapunctures were performed to inject vincristin and to obtain blood for routine clinical chemistry. An amount of blood, ± 6 ml, was taken to quantitate 6MP in plasma. The patients were not additionally burdened, for the punctures had to be done anyhow, and the volume of the samples was limited. All samples were obtained approximately 24 hours after the last daily dose of 6MP.
Both CSF and blood samples were withdrawn into heparinized tubes, containing DTT, and chilled immediately at 0°C. Samples were deproteinized and stored at 4°C until analysis. The analysis was performed as described in Chapter II (4).

SNWLK

SCHEMA ALL-PROTOCOL V A

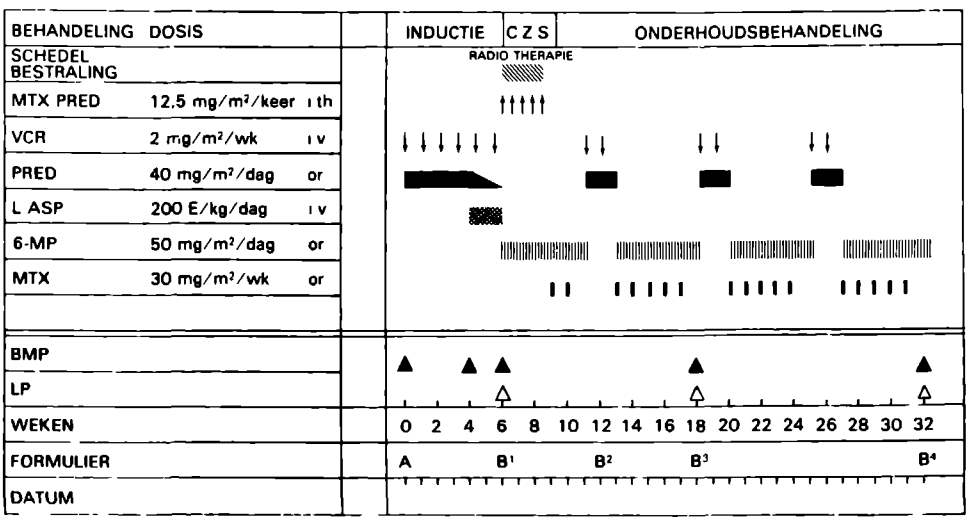

Fig. 1. Treatment scheme of arm A from the protocol ALL V (SNWLK).

The concentrations of 6MP in plasma and CFS obtained on day 35 of a maintenance treatment cycle, are depicted graphically in fig. 2. The mean plasma 6MP concentration was 0.064 µM, with a range of 0.020-0.140 µM. The mean CSF concentration was 0.029 µM with a range of 0.018-0.050 µM. The ratio of the mean 6MP concentration in CSF over the mean 6MP concentration in plasma is approximately 0.40. Plasma and CSF concentrations simultaneously obtained from individual patients are shown in fig.3.

The plasma and CSF concentrations measured and depicted in the figures are close to the limit of detection, and several other samples gave negative results.

In plasma samples obtained during interruption of the administration of 6MP for prednisone-vincristin consolidation, or after stopping maintenance treatment, 6MP was often still present 1-4 weeks after the last dose of 6MP.

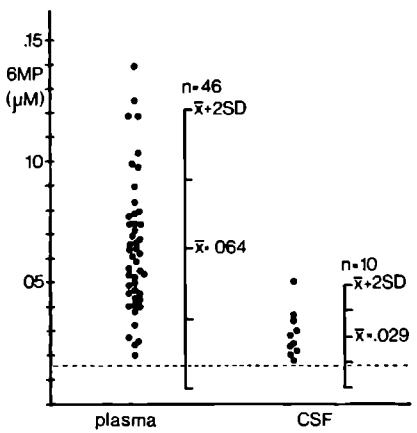

Fig. 2. Graphic representation of 6MP concentrations in plasma and CSF from ALL patients receiving maintenance treatment. Samples were obtained ± 24 hours after the last of 35 daily oral doses of 6MP.

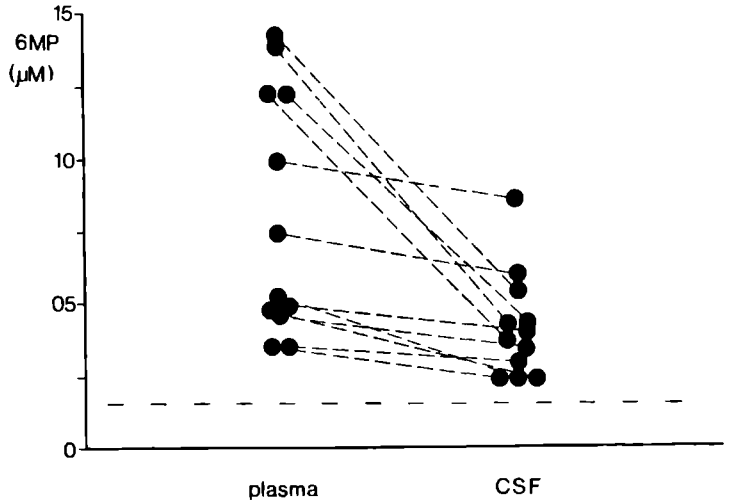

Fig. 3. The simultaneously obtained 6MP concentrations in plasma and CSF in individual patients are connected by broken lines. See also legend to Fig.2.

DISCUSSION

The concentrations measured in plasma and CSF were relatively low, as compared with the concentrations, i.e. 100-200 μM, used in in vitro studies (5, 6, 7). The actual presence of 6MP in CSF in a concentration ratio of approximately 0.40 to simultaneously measured plasma concentrations was an important finding. In older studies by Loo et al and Nelson et al penetration of 6MP into CSF could not be demonstrated (8, 9). On physicochemical grounds however, one would expect reasonable penetration of 6MP (10). Our finding of penetration of 6MP into CSF was only possible because of the sensitivity and specificity of our method.

It was impossible for us to study the intravenous administration of 6MP in patients. There was no formulation of 6MP available to us which was suitable for intravenous administration to humans. It is for this reason, that we performed our studies on intravenous administration in experimental animals (Chapters V, VI, VII, VIII).

By increasing the dose rate and prolonging the duration of administration we tried to simulate the high concentrations during prolonged periods of time, which were found necessary in in vitro studies (5, 6, 7). These latter studies have shown also that 6TG is a more potent antimetabolite than 6MP, and more important, 6TG is maximally effective at a much lower concentration, i.e. 1 to 2 μM for 6TG vs. 100 to 200 μM for 6MP (7). As these in vitro cytolytic concentrations of 6TG seem to be attainable in patients treated with moderate doses of 6TG (11), this drug was included in the second part of our studies (Chapter III, VII).

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CHAPTER V

6-MERCAPTOPURINE: ORAL ADMINISTRATION AND I.V. BOLUS INJECTIONS IN DOGS

Schouten TJ, Abreu RA De, Schretlen EDAM, Bruyn CHMM de, Kleijn E van der, Oosterbaan MJM, Vaan GAM de. Accepted for publication in: Eur Paediatr Haematol Oncol 1985

ABSTRACT

6-Mercaptopurine (6MP) levels in serum of dogs were studied after long-term oral administration and intravenous injection.
During long-term daily oral administration of 50 mg of 6MP more or less constant levels of 6MP have been found after 1-3 weeks. After stopping the administration of the drug, 6MP concentrations remained constant for a further period of 3-6 weeks and then declined within 1-2 weeks below the limit of detection.
I.v. bolus injections of 50 mg of 6MP resulted in concentration versus time curves which fitted into an open two-compartment pharmacokinetic model. Half-life times were in a range of 13-21 and 125-151 minutes, respectively. Peak concentrations were from 2,500 to 10,500 nmol/l.
Our data on i.v. push injections with a customary clinical dose revealed peak concentrations which were low in comparison to the concentrations used in in vitro studies by Tidd and Paterson to achieve maximal cell-kill. Moreover, the half-life times of the 6MP after i.v. bolus injections are short, so prolonged high plasma concentrations are not obtained by i.v. bolus injections.
We suggest that prolonged infusions of 6MP, at relatively high dose rates should result in higher and persisting plasma concentrations and so should promise a more potent antitumor activity.

Keywords: 6-Mercaptopurine

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INTRODUCTION

Nowadays 6-mercaptopurine (6MP) is used mainly for maintenance treatment of acute lymphoblastic leukaemia (ALL) (1). The first study on the pharmacokinetics has been published in 1954 (2). However, such studies have remained rather scanty (3), because radioactively labeled 6MP is not suitable for widespread use. Other methods to quantitate 6MP were also not readily applicable because large amounts of plasma were necessary (4, 5). Moreover, relatively high i.v. doses had to be used in order to obtain measurable concentrations, although daily oral administration at much lower doses is clinical practice (1).

In recent years several specific and sensitive methods for the determination of 6MP and related drugs have been reported (6, 7, 8, 9). We developed a method (9) to study the pharmacokinetic behaviour of 6MP at the customary oral and low dose levels. Along with initial observations on children with ALL (10), we started to explore the pharmacokinetics of 6MP in laboratory animals.

In the present study we report on observations in dogs; we adapted our experiments to the clinical practice in ALL. We determined plasma 6MP concentrations during and after long-term daily oral administration of the drug. Also concentration-time curves were obtained after i.v. bolus injections of the same dose as used in the long-term experiments. We varied the schedules of drug administration because of the explorative nature of the study.

From our findings, and after re-interpretation of data from the literature, we conclude that the therapeutic use of 6MP, and obviously also of other thiopurines in man, is based on tradition rather than on solid experimental grounds. Suggestions are made to develop a more rational dosing and administration of thiopurines.

MATERIALS AND METHODS

Experimental animals

Labrador dogs were selected for reasons of availability, cost, and number and volume of blood samples to be obtained during short- and long-term experiments. The dogs used in our experiments were bred by the Cen-

tral Animal Laboratory of the Catholic University of Nijmegen. All dogs were fathered by the same animal. At the time of the experiments the dogs, of either sex, were between 10-26 months of age and 20.5-29.0 kilograms of weight.

Dose and ways of 6MP administration

To avoid problems in pharmaceutical formulation and in oral administration of the drug tablets of 50 mg, which are commercially available, were used. Tablets are easily swallowed by dogs. For reasons of uniformity it was decided to use the same 50 mg dose for determining concentration-time curves after i.v. bolus injections.

Chemicals

Standard tablets of 50 mg (Purinethol^{R}, Wellcome) were used, when 6MP was given orally . For i.v. injections 6MP was obtained from Fluka AG (Buchs SG, Switzerland). The 6MP solutions for i.v. administration were prepared freshly; 60 mg of 6MP were dissolved in 0.25 ml of a 5.0% solution of sodium hydroxide and diluted with a sterile 0.9% sodium chloride solution as described previously (9). Before 6MP was added, helium was flushed through the solutions in order to prevent oxidation of 6MP. A dose of 50 mg of 6MP was injected through a Millipore membrane filter, type Millex-GS (pore size 0.22 µm).

Preparation of samples for 6MP determination

Blood samples were drawn from a catheter inserted into a jugular vein or by repeated venapuncture, depending on the number of samples to be taken on the day of the experiment and the feasibility of insertion. Samples of 5 to 6 ml were collected in tubes containing heparin and 300 µg dithiothreitol (DTT) to stabilize 6MP. After mixing, the blood sample was centrifuged (5 min, 2,000 x g). One ml of plasma was transferred in duplicate with a calibrated syringe into microtest tubes (type 3810, Eppendorf, Hamburg, GFR) and kept on ice for 5 minutes. Protein was precipitated by the addition of 0.1 ml of ice-cold trichloroacetic acid (TCA) 50% w/v. After shaking vigorously, the tubes were kept on ice for another 10 minutes and centrifuged (10 min, 2,000 x g). 6MP was quantitated with a Spectra Physics SP-8000 high pressure liquid chromatograph

as described previously (9). The concentrations of 6MP are expressed in nmol/l.

Analysis of pharmacokinetic data

After an i.v. bolus injection, as the very first dose of 50 mg of 6MP, plasma concentration profiles were fitted into an open two-compartment pharmacokinetic model by standard procedures (11). Pharmacokinetic parameters were calculated on an HP 9810 calculator (Hewlett Packard).

RESULTS

A concentration-time curve obtained after a first oral dose of 6MP showed a "peak" plasma concentration some 4 to 5 hours after administration of a 50 mg tablet (Fig. 1).

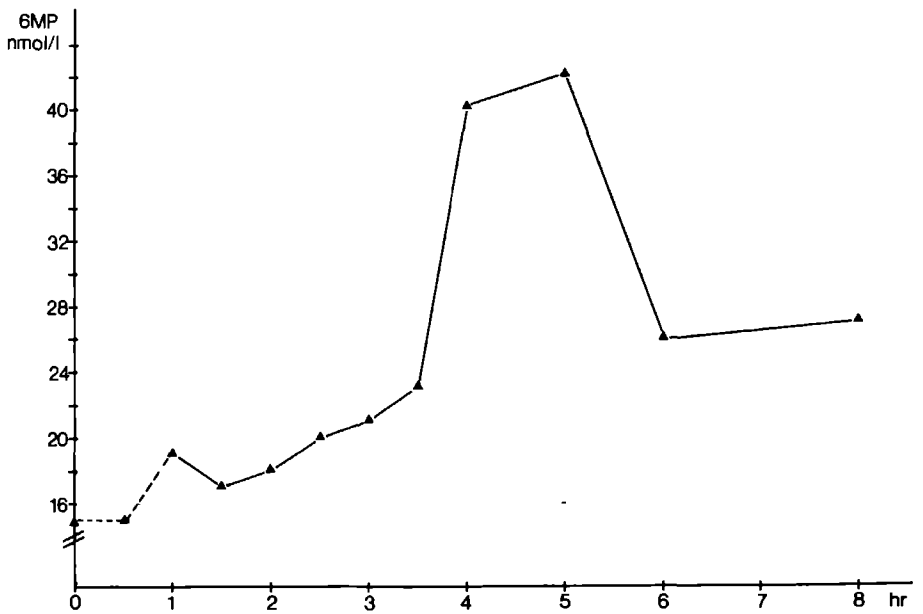

Fig. 1. Concentration versus time curve after a first oral dose of 6MP; 50 mg as a standard tablet. Lower limit of detection is 16 nmol/l (broken line).

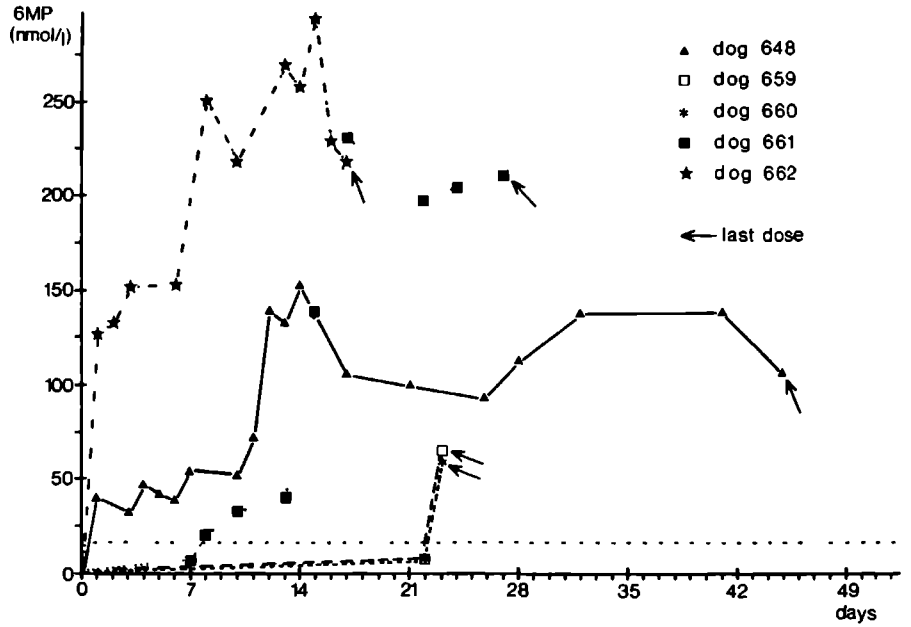

Fig. 2. Concentrations of 6MP in plasma during long-term daily administration. Blood was drawn just before the dogs received another daily dose of 50 mg. Lines start on the first day of administration and stop at the day of the last dose of 6MP. (For exact treatment schedule see table 1; lower limit of detection, 16 nmol/l, is represented by the broken horizontal line).

The results after long-term daily administration of 6MP were somewhat surprising. Due to the explorative nature of the study, treatment schedules were diverse. (table 1). After 1-2 weeks of daily oral 6MP, a "residual" concentration of 6MP could be measured in plasma before the dogs received another daily dose of 6MP (fig. 2; dogs 648, 661, 662). Daily i.v. bolus injections of 6MP during 10 days did not lead to accumulation of the drug, but subsequent oral administration did (dogs 659, 660). The steady state concentrations varied in individual dogs. Moreover, after stopping the prolonged daily administration of 6MP, the level in plasma remained more or less constant for 3-6 weeks. After that period a rapid decline in the concentrations of 6MP to below the limit of detection occurred (fig.3).

TABLE 1.

DURATION AND WAY OF DAILY ADMINISTRATION OF 50 MG 6-MERCAPTOPURINE

dog number	6MP in mg/kg body weight	duration and way of administration
648	1.7	45 days; oral except day 27, when an i.v. injection was given
659	2.2	28 days; i.v. days 1 - 11 and day 24, oral days 12 - 23 and 25-28
660	2.2	25 days; i.v. days 1 - 11 and day 22, oral days 12 - 21 and 23 - 25
661	1.8	30 days; oral except days 1 and 29, when i.v. injections were given
662	2.2	18 days; oral except days 1 and 15, when i.v. injections were given

TABLE 2.

6-MERCAPTOPURINE KINETICS FOLLOWING A FIRST DOSE OF 50 MG INTRAVENOUSLY

dog number	6MP in mg/kg body weight	$t_{\frac{1}{2}}$,1 (corr) (min)	$t_{\frac{1}{2}}$,2 (corr) (min)	V_1 (l)	V_f (l)	A.U.C. (nmol.h/l)
647	1.8	20.9 (0.99)	130.4 (0.97)	83.8	154.7	2512
659	2.2	17.0 (0.99)	--	17.2	--	7799
660	2.2	18.0 (0.99)	125.2 (0.98)	21.0	34.8	7658
661	1.8	13.1 (0.99)	135.6 (0.95)	74.0	169.1	1681
662	2.2	19.9 (0.99)	151.1 (0.99)	65.2	138.5	3141

$T_{\frac{1}{2}}$, 1 and $t_{\frac{1}{2}}$, 2: first and second half-life time according to an open two-compartment model. V_1 and V_f: volume of the central compartment and apparent volume of distribution, respectively; AUC indicates area under the curve. (corr): correlation coefficient.

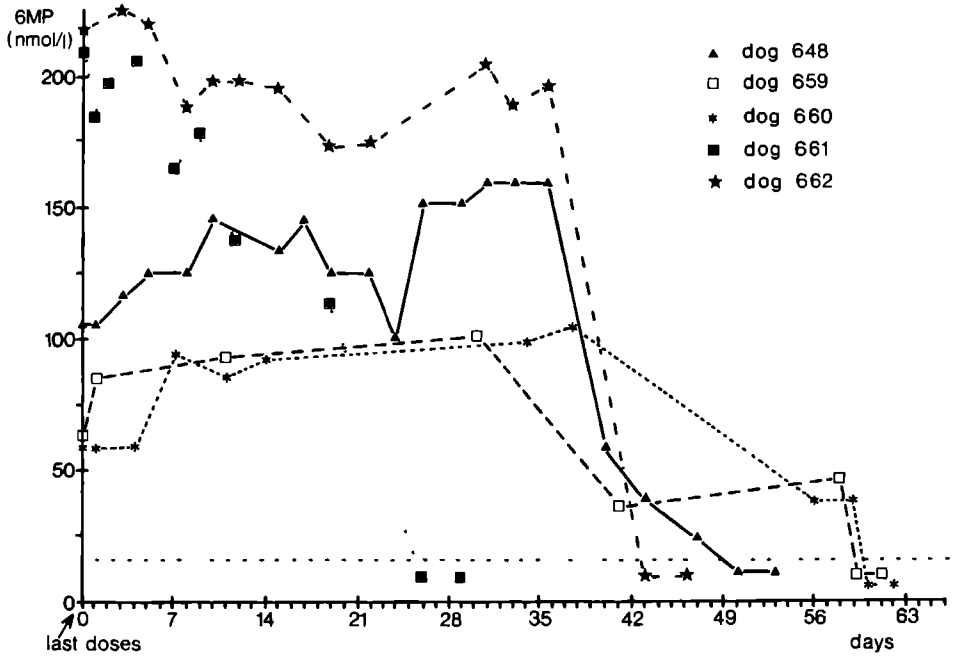

Fig. 3. Disappearance curves of 6MP from plasma. The curves start at the moment of stopping the administration of the drug. (For exact treatment schedule see table 1; lower limit of detection, 16 nmol/l, is represented by the broken horizontal line).

The pharmacokinetic data obtained in 5 dogs, after a first dose of 50 mg of 6MP by i.v. injection, are summarized in table 2. A typical concentration-time profile is depicted in fig. 4A. Plasma levels of 6MP peak at values of 2,500-10,500 nmol/l. This concentration of 6MP declines to less than 100 nmol/l within 3 to 4 hours. A first half-life time ($t_{\frac{1}{2}}$, 1) was calculated with a range of 13-21 minutes. The second half-life time ($t_{\frac{1}{2}}$, 2) has a range of 125-151 minutes. The volumes of the central compartment (V_1), the apparent volumes of distribution (V_f), and the areas under the curve (AUC) have a rather wide range.

Concentration-time curves after i.v. bolus injection, obtained during long-term daily oral application of 6MP (fig. 4B,C) are quite different from the curve after a very first dose i.v. (fig. 4A).

During long-term administration we could not demonstrate any pronounced hematological toxicity. The observed tendency to a slight decrease in hemoglobin concentrations could be due either to the effect of frequent blood sampling, or to an effect of 6MP. White blood cell and thrombocyte counts did not change appreciably. In most dogs a rise in alanine aminotransferase (ALAT) activity became apparent, whereas aspartate aminotransferase (ASAT) and alkaline phosphatase activities did not change. Serum urea and creatinine levels remained constant. After stopping 6MP administration ALAT activity returned to normal values. It was remarkable that ALAT activity normalized before plasma 6MP levels declined. Administration of 6MP did not influence the low to undetectable blood levels of uric acid.

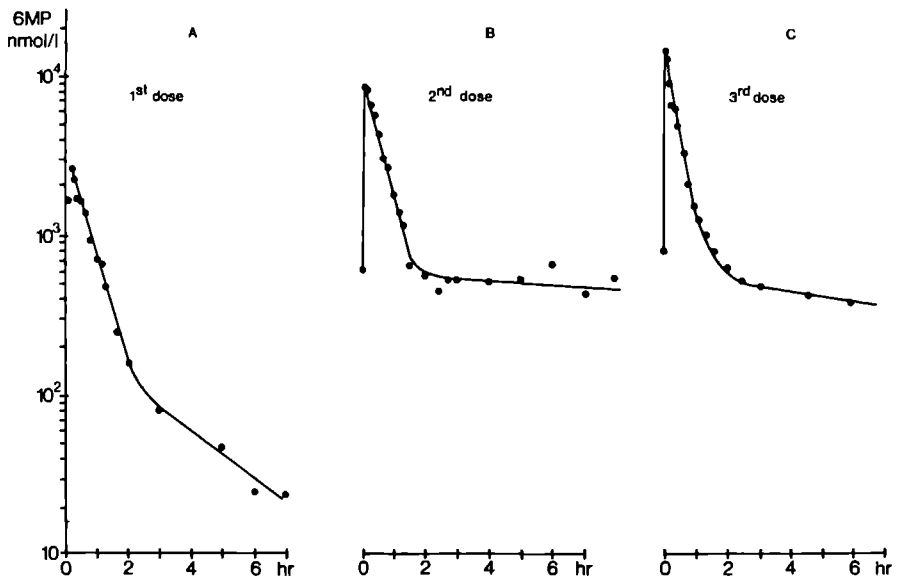

Fig. 4. Concentration versus time curves after 50 mg of 6MP i.v. by bolus injection.

A. Very first dose

B. Second i.v. dose; between the first and second i.v. bolus injection 50 mg of 6MP was given orally and daily for ten days.

C. Third i.v. dose; between the second and the third i.v. bolus injection 50 mg of 6 MP was given once daily orally for ten days again.

This study of 6MP in dogs has revealed a rather peculiar finding i.e. the persistence of low concentrations of 6MP in plasma for 3-6 weeks after stopping the long-term oral administration of the drug (fig. 3). A similar phenomenon has been observed in children, who had received 6MP for treatment of ALL (own unpublished observations).

This implicates a kind of deposit of the drug, the nature of which is not clear. The tight binding of 6MP to proteins, either in plasma or liver cells, may be of importance (12, 13, 14). This binding could also explain why not all of the 6MP is catabolized to 6-thioxanthine and 6-thio-uric acid. The steady state concentrations of 6MP after oral administration are much lower than the concentrations which have a cytolytic effect in vitro (15).

The concentration-time curve after a first oral dose of 6MP in tablet form, revealed a low and late appearing peak of 6MP in plasma (fig. 1). Because of the peculiar shape of the curve and the low concentrations obtained in comparison with in vitro cytotoxic concentrations (15), we did not explore this further in dogs. Moreover, it had been reported already, that the bioavailability of 6MP after oral administration is low in Rhesus monkeys (16), and this was confirmed in patients with ALL recently (17).

After a very first dose of 50 mg of 6MP by i.v. bolus injection, the concentration-time curves fitted best into a standard two-compartment open model (11).The doses were slightly different on a mg/kg body weight basis, making calculation of means difficult. The broad variation in pharmacokinetic parameters is apparent from the data (table 2).

Concentration-time curves after i.v. bolus injections, obtained during long-term oral use of 6MP, were quite different from curves obtained after a first i.v. dose of 6MP (fig. 4B,C versus fig. 4A). The exact impact of this change in pharmacokinetic behaviour after pretreatment with 6MP is unclear. Future studies in humans should consider the influence of previous exposure to 6MP on the pharmacokinetic behaviour.

This and other studies on the pharmacokinetics of 6MP have shown that plasma concentrations of 6MP are short-lived after i.v. bolus injections

(2, 4, 5, 16, 17, 18). Moreover, at conventional doses of 50 to 100 mg/m^2, peak concentrations are rather low in comparison with in vitro maximally effective concentrations (15). In vitro data on thiopurines have clearly demonstrated that, in order to obtain a maximal cell killing effect, cells should be exposed to relatively high concentrations, and this for prolonged periods of time (15, 19).
Considering the low bioavailability of 6MP after oral administration and the rather low and short-lived plasma concentrations after i.v. bolus injections in conventional doses, we suggest that prolonged infusion of 6MP in higher doses should be more effective. The feasibility of this approach has already been demonstrated by us in further animal experiments (20). Moreover, this new way of administration of 6MP may lead to new indications for the drug. Good penetration of 6MP into CSF has been predicted (21) and demonstrated (10, 18, 20). Thus 6MP might play a role in the prophylaxis and treatment of CNS leukaemia and CNS tumours.

ACKNOWLEDGEMENTS

We thank the following persons for their help: J.M. van Baal, M.B. van Leeuwen (technicians); L.J.H.M. Viering, J. Palthe (manuscript preparation); J.A.J.M. Bakkeren (critical reading).

This study was supported by "Koningin Wilhelmina Fonds" (Dutch Cancer Society); grant NUKC KG 81-2).

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CHAPTER VI

6-MERCAPTOPURINE: TOTAL BODY AUTORADIOGRAMS AND PLASMA CONCENTRATION-TIME CURVES OF 6MP AND METABOLITES FROM MARMOSET MONKEYS

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ABSTRACT

In order to study the body distribution of 6-mercaptopurine (6MP), [8-^{14}C]-6MP was given by infusion to Marmoset monkeys at a dose rate of 5 mg/kg body weight/hour for one and four hours, respectively. Both experimental animals were sacrificed two hours after the end of the drug infusion and instantly frozen at -70°C. Whole body sagital sections were made later. During the experiments blood samples were obtained regularly to quantitate 6MP, 6MP riboside (6MPR), 6-thio-xanthine and 6-thio-uric acid in plasma.

The autoradiograms revealed extensive distribution of the ^{14}C label. High levels of radio activity are seen in liver, bile and intestinal contents. Labelling of the central nervous system and bone marrow is obvious. The plasma concentration-time curves of 6MP and 6MPR attain steady state concentrations of 30-40 μM and 6-12 μM respectively. After stopping the infusion of the drug, the concentrations of 6MP and 6MPR get equal. 6MPR contributes to the biological effect of 6MP, as degradation of 6MPR results in 6MP.

In studies on the pharmacokinetics and dynamics one should include all relevant metabolites of 6MP, both in plasma and in the cells.

Acknowledgements: supported by "Queen Wilhelmina Foundation" (Dutch Cancer Society) grant NUKC KG 81-2.

The expert technical assistance of the Gerrie Grutters, John van Baal, and Marinella van Leeuwen, and the secreterial help by Loes Viering and Kitty van den Tempel is gratefully acknowledged, as is the critical review of the manuscript by JAJM Bakkeren, Ph.D.

INTRODUCTION

The purine antagonist, 6-mercaptopurine (6MP) is still used in the treatment of acute lymphoblastic leukaemia in essentially the same way as was initially described by Burchenal in 1953 (1, 2). The rationale of the customary oral administration has been questioned recently, because pharmacokinetic studies revealed a very low bioavailability of the drug after oral administration (3, 4, 5). In each individual target cell 6MP has to be metabolized before a cytotoxic effect can be exerted (6, 7). Some of the various metabolites produced are present only intracellularly like the nucleotides of 6MP, while others like 6MP riboside (6MPR), 6-thio-xanthine (6TX) and 6-thio-uric acid (6TU) can also be found in plasma.

In order to gain insight into the distribution of 6MP throughout the body, $\left[8\text{-}^{14}C\right]$-6MP was given to two Marmoset monkeys by infusion. The arguments for i.v. infusions have been discussed extensively by us. These experiments are part of a series of investigations on 6MP (8, 9, 10, 11, 12). In this paper we present whole body autoradiograms, and concentration-time curves of 6MP, 6MPR, 6TX and 6TU in plasma. These metabolites of 6MP were not considered in other recent studies in monkeys and humans (3, 4, 5, 13, 17).

MATERIALS AND METHODS

Experimental animals

Two male adult Marmoset monkeys (Callithrix jacchus) were used with a body weight of 340 and 315 grams, respectively. The animals were anaesthesized by ketamine HCl (40 mg/kg body weight i.m.) and fluothane. Intravenous drug administration and arterial blood sampling was accomplished through surgically implanted catheters in the femoral vessels. The monkeys were artificially ventilated during the total experimental period. A tracheal canule, placed by tracheotomy was connected to an Amsterdam Infant Ventilator^{R} (Loosco, Amsterdam, the Netherlands). A mixture of oxygen and dinitrogen oxide, admixed with 0.5-1.0% of fluothane was administered. Supplemental ketamine HCl has been given i.m. in amounts of 0.1 ml, equivalent to a dose of 10 mg. At the end of the ex-

periments the animals were sacrificed by an overdose of fluothane. The corpses were immersed immediately after sacrifice in cyclopentane, chilled to -70°C by the preceding addition of solid CO_2.
Whole body sections for autoradiography were made according to a method described previously (14).

Dose and schedule of 6MP administration

A dose of 5mg/kg/hour was given in each experiment. Monkey I received a one hour infusion and monkey II a four hours infusion. Both experiments were terminated two hours after the end of the infusion of the drug.
$[8\text{-}^{14}C]$-6MP, with a specific activity of 1.7 mCi/mmol was used (Amersham Nederland BV, Utrecht, the Netherlands). In the second experiment the specific activity was lowered to 0.8 mCi/mmol by addition of unlabelled 6MP.

Determination of 6MP and metabolites

During and two hours after infusion of the drug, blood samples of 0.3 ml were collected in micro-test tubes containing heparin and dithiotreitol. After chilling on ice, plasma was separated by centrifugation. The plasma samples were stored at 4°C; aliquots of 10 or 20 µl were injected onto a reverse phase HPLC column and separation was performed according to a method described previously (8).
Column effluents were collected in small fractions. The radioactivity of the various fractions was quantitated by scintillation counting (Searle Mark III system, Searle Analytic Inc. DesPlaines, Il, USA). The calculated 6MP, 6MPR, 6TX and 6TU concentrations are based on scintillation counting and corrected for dilution with unlabelled 6MP, counting efficiency and quenching.

RESULTS

Autoradiographic findings

The whole body autoradiograms show wide distribution of the ^{14}C labelled 6MP and 6MP metabolites throughout the body (figs.1 and 2). Especially the activity found in the liver, gall bladder and intestinal lumen is intense. Other secretory organs, like salivary and lachrymal glands, al-

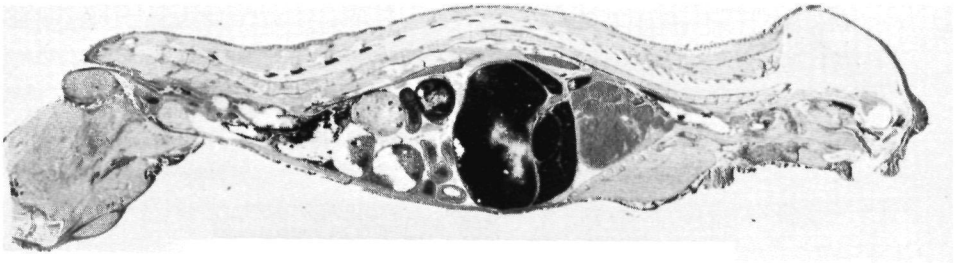

Fig. 1. Autoradiogram of a 30 µm sagittal section of a Marmoset monkey. In this experiment 6-mercaptopurine at a dose rate of 5 mg/kg/hour was given during one hour. Two hours later the animal was sacrificed and frozen instantly. Magnification: 0.50.

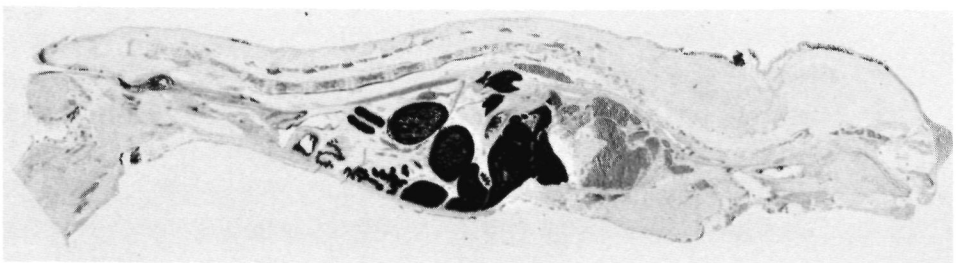

Fig. 2. Autoradiogram of a 30 µm sagittal section of a Marmoset monkey. In this experiment 6-mercaptopurine at a dose rate of 5 mg/kg/hour was given during four hours. Two hours later the animal was sacrificed and frozen instantly. Magnification: 0.50.

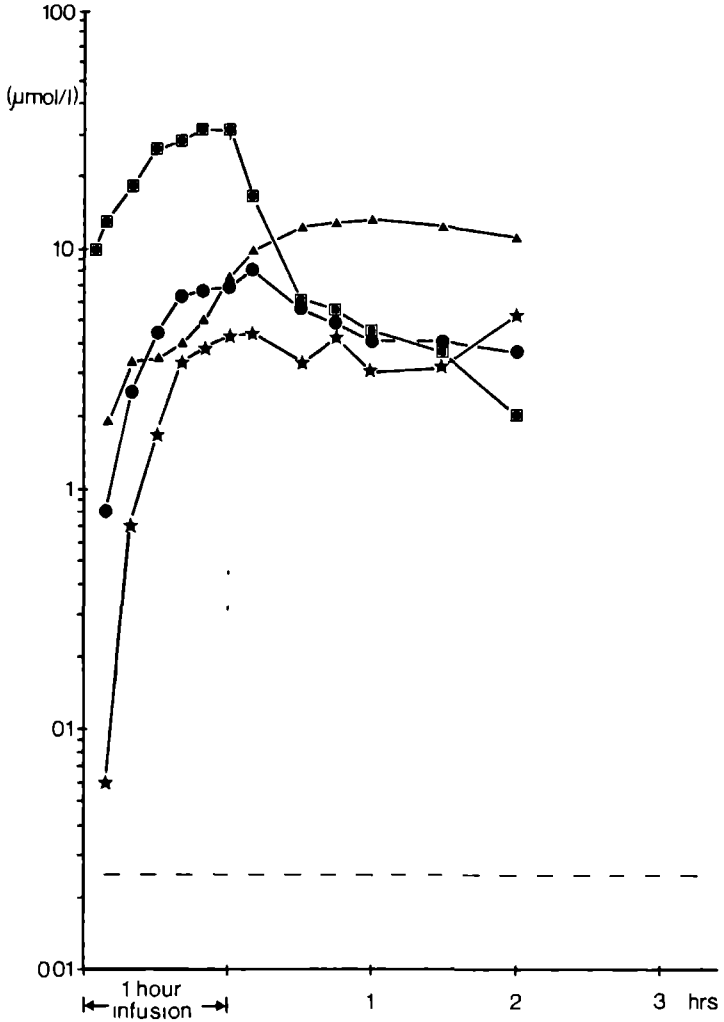

Fig. 3. Concentration-time curves of 6MP(▣), 6MPR (●), 6TX (▲) and 6TU (★). An infusion with a dose rate of 5 mg/kg/hour was given during one hour.

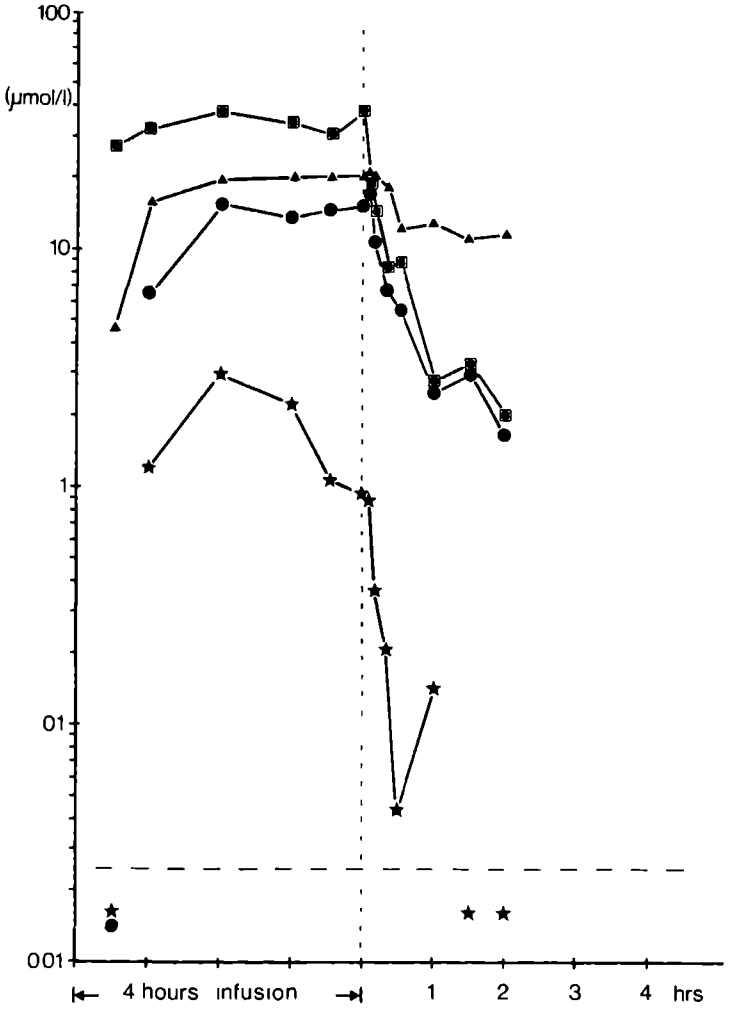

Fig. 4. Concentration-time curves of 6MP (▣), 6MPR (●), 6TX (▲) and 6TU (★). An infusion with a dose rate of 5 mg/kg/hour was given during four hours.

so show a good deal of activity. The appearance of the label in the central nervous system is obvious, with a slightly higher labelling of the gray matter. Also in the bone marrow the label appears as can be seen in the vertebrae. In both experiments the radioactivity is more evident in the marrow in comparison with the bony structures. In the heart, activity is present in the muscular wall as well as in the blood pool. Sections containing testicular tissue reveal good accumulation of activity in these organs.
In the longer experiment the accumulation of activity is higher in the bone marrow. Other differences between the two experiments are not obvious.

Concentration-time curves of 6MP, 6MPR, 6TX, 6TU in plasma
Steady state concentrations of 6MP, in the order of 30-40 μM, are reached after half an hour of infusion (figs.3 and 4). Shortly after starting the 6MP infusion, 6MPR could be demonstrated. The catabolic products of 6MP, 6TX and 6TU, also appear rapidly. There is an apparent difference in disappearance of 6MP, 6MPR and 6TU from plasma between experiment I (fig.3) and II (fig.4). However a more liberal diuresis was present in the second experiment, while total doses are different too. During infusion of 6MP the plasma concentration of 6MPR is 20-35% of the 6MP concentration, but after termination of the infusion, 6MP and 6MPR get nearly the same concentrations.

DISCUSSION

The whole body autoradiograms disclosed extensive distribution of activity throughout the body (figs.1 and 2). The presence of the label in the central nervous system (CNS) is of special interest. Older studies have denied that 6MP penetrates into the CNS (15). However the physicochemical properties of 6MP are such, that one would expect this drug to pass the blood brain barrier (16). Recently we have demonstrated in goats that 6MP and 6MPR reach the cerebrospinal fluid in fair amounts (12), and others have demonstrated this in Rhesus monkeys (13). Also in CSF of ALL patients 6MP has been demonstrated in fair amounts as compared with simultaneously measured plasma concentrations (9, 17).

The extensive labelling of the liver, bile and intestinal lumen is a reflection of the pivotal role of the liver in purine metabolism. (18). It points to a route of elimination of 6MP which is not well-known. Analysis of bile contents after 6MP infusion may clarify the role of the liver in the clearance of thiopurines. Both liver and intestinal mucosa are rich in xanthine oxidase, so 6MP itself will probably be catabolized to 6TX and 6TU. 6MPR, if present in bile, could give rise to an enterohepatic circulation. 6MP is intracellularly anabolized to 6-thio-IMP and either further metabolized to other thionucleotides by kinases or degraded to 6MPR by 5'nucleotidase. 6MPR, when released from the cells, can only be converted to 6MP.

The concentration of 6MPR equals the concentration of 6MP after stopping the infusion (figs. 3 and 4). The biological importance of 6MPR was demonstrated emphatically during high-dose 24 hours infusion in goats (12). Ignorance of the presence of 6MPR may have resulted in an underestimation of the bioavailability of 6MP (3, 4, 5).

The accumulation of radioactivity in bone marrow is especially relevant. The bone marrow is the main site of cytotoxic action of 6MP in leukaemia. Whether the label is intracellular in the haematopoietic cells of the bone marrow or mainly in the peripheral blood pool can only be concluded after further investigations. Ideally one should try to correlate intracellular concentrations of cytotoxic products of 6MP with concentration-time phenomena in plasma.

The ultimate cytotoxic action of 6MP and metabolites is exerted intracellularly, but "an otherwise effective drug will not kill a drugsensitive tumor cell that it cannot (or does not) reach in lethal concentrations" (19).

So determination of plasma concentration-time curves of all relevant metabolites of 6MP will contribute to the design of rational dosage, schedules and ways of administration of this drug.

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CHAPTER VII

6-THIOGUANINE: HIGH-DOSE TWO HOURS INFUSIONS IN GOATS

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SUMMARY

6-Thioguanine (6TG) is poorly absorbed after oral administration. Bolus injections of 6TG result in high peak concentrations with relatively short-lived plasma concentrations. In vitro studies have shown the importance of prolonged exposure to 6TG. Therefore we administered 6TG by infusion at a dose rate of 2 mg/hour during 2 hours. In three goats we determined the plasma concentration-time curves of 6TG and its riboside (6TGR).

A steady state was reached for 6TG and was almost reached for 6TGR within the two hours of infusion. In one experiment we obtained several samples of CSF and we observed good penetration of 6TG and 6TGR into CSF. Urinary excretion of 6TG and 6TGR was also quantitated. The amount of drug and metabolite excreted more than 4 hours after stopping the infusion was negligible. By infusing 6TG, the problems of both erratic absorption after oral administration and acute renal toxicity after bolus injection, can be averted. In our opinion prolonged infusions of 6TG may be of advantage in humans suffering from actively proliferating malignant diseases, and thus should be studied.

FOOT NOTES

Supported by the "Queen Wilhelmina Fund" (Dutch Cancer Society); Grant NUKC KG 81-2.

Abbreviations 6TG: 6-thioguanine; 6TGR: 6TG riboside (6-thioguanosine); 6MP: 6-mercaptopurine; 6MPR: 6MP riboside; CSF: cerebrospinal fluid.

KEY WORDS

6-thioguanine, 6-thioguanosine, 6-thioguanine riboside, 6-mercaptopurine, thiopurines.

INTRODUCTION

The purine analog and antimetabolite 6-thioguanine (6TG) is used mainly in the treatment of acute myeloid leukaemia. The drug itself has no direct cytotoxic effect, but has to be phosphorylated initially by the enzyme hypoxanthine guanine phosphoribosyl transferase to 6-thio-GMP.
The cytotoxic effect is exerted through incorporation into DNA as 6-thio-dGTP (Paterson and Tidd 1975, Nelson et al 1975, Wotring and Roti Roti 1980, Lee and Sartorelli 1981). The influence of 6-thio-GMP, and methylated analogs, on the purine de novo synthesis is probably of minor importance in the mechanism of cell kill (Nelson et al 1975, Wotring and Roti Roti 1980). Under special circumstances however it may be of significance (Lee and Sartorelli 1981).
Pharmacological studies of 6-thioguanine are relatively scarce (LePage and Whitecar 1971, Loo et al 1981, Brox et al 1981, Lu et al 1982, Konits et al 1982). Only recently sensitive and specific analytical methods have been described to quantitate 6TG in biological fluids. We developed an HPLC method to determine 6-thioguanine, 6-mercaptopurine and their respective ribosides (6TGR and 6MPR) (Van Baal et al 1984). The first pharmacological study in humans, using HPLC to detect 6TG in plasma after oral administration, revealed a 30-fold range in peak plasma concentrations. Moreover peak concentrations were seldom as high as the concentration with optimal cell killing effects in vitro. As a result of their findings Brox and coworkers seriously question the rationale of the customary oral administration of the drug (Brox et al 1981). Recent in vitro studies demonstrated a predominant S-phase specificity of 6TG (Wotring and Roti Roti 1980, Lee and Sartorelli 1981). Thus duration of exposure is a factor as important as concentration of the drug. The administration of drugs by i.v. bolus injections frequently results in high peak levels without sustained concentrations of drug in plasma. Therefore, in the present study we explored the administration of 6TG by infusion.

MATERIALS AND METHODS

Experimental animals

African midget goats were obtained from commercial sources and maintain-

ed in the Central Animal Laboratory of our University. During a short thiopental-induced anaesthesia, catheters were introduced percutaneously into both jugular veins; one catheter to administer the drug and one to obtain blood samples. Urine catheters were used to collect urine.
Attempts at inserting a thin catheter in the lumbar space by percutaneous puncture were unsuccessful, except in experiment B in which a catheter functioned, suboptimally, during part of the experiment.

Dose and administration of 6TG

In all three experiments 6TG in a dose of 2 mg/kg/hour was given during 2 hours. The drug, of analytical grade, was obtained from Fluka, Rotterdam, The Netherlands. The desired amount was weighed out in a sterile flacon and dissolved by addition of sterile sodium bicarbonate (1.4%) and sodium hydroxide. After solution the pH was lowered to 9.8-10.2 by addition of chloric acid. The final concentration of 6TG was between 2 and 3 mg/ml. The solution was infused through Millipore membrane filters, type Millex GS (pore size 0.22 μm). A liberal and alkaline diuresis was established before the infusion of 6TG and maintained untill at least two hours after the end of the 6TG infusion.

Preparation of samples and determination of 6TG and 6TGR

Blood samples of ± 6 ml were collected in tubes containing heparin and dithiothreitol. Sample preparation of blood, urine and cerebrospinal fluid (CSF) and analysis was performed according to our HPLC method described recently (Van Baal et al 1984).

RESULTS

The plasma concentration-time curves obtained during and after the infusion of 6TG in the three experimental animals are depicted in fig.1. In all three experiments steady state concentrations of 6TG are present within one hour after the start of the infusion. 6-Thioguanosine, the riboside of 6TG formed by intracellular metabolism, is present from the first sample on. The concentration of 6TGR exceeds that of 6TG within

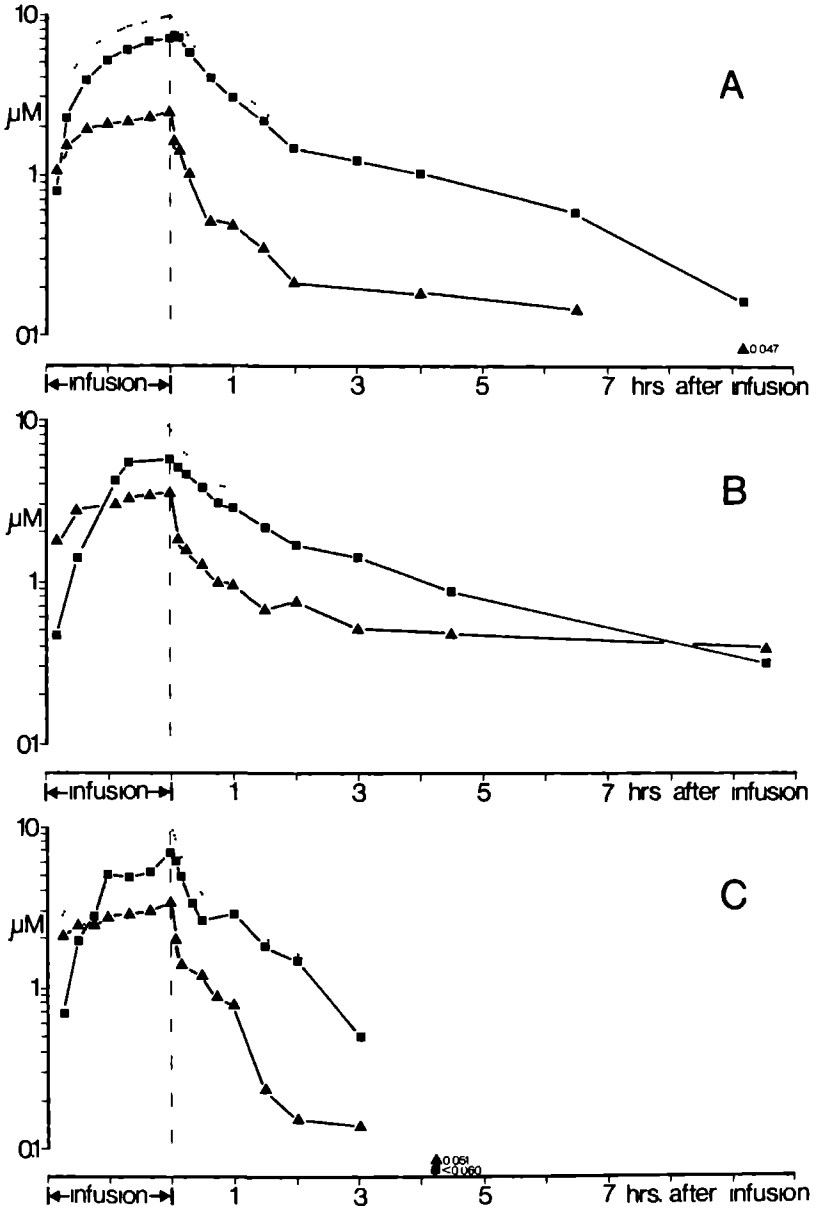

Fig. 1. Concentration-time curves of 6-thioguanine (▲) and 6-thioguanine riboside (=6-thioguanosine) (■) during and after a two hours infusion at a dose rate of 2 mg/kg/hour in three different goats. The dotted lines represent the added concentrations of 6TG and 6TGR.

the first hour and tends to a steady state towards the end of the two hours infusion.

In experiment B several CSF samples, 5 during and 2 shortly after drug infusion, could be obtained. The CSF concentration of 6TG is about 25% of the plasma 6TG concentration towards the end of the infusion. The concentration of 6TGR in CSF is then approximately 40%. While 6TGR CSF concentrations are still increasing, the 6TGR plasma concentration tends to a steady state (data not shown).

Urine excretion of 6TG and 6TGR was determined in all three animals. In fig. 2 the excretion of 6TG in relation to the creatinin excretion is shown during and after the infusions. The amounts of 6TG and 6TGR excreted more than 4 hours after stopping the infusion of 6TG, are negligible. In urine the cumulative combined excretion of 6TG and 6TGR was 49% in experiment A, 26% in B and 27% in C. The overall ratio of 6TG/6TGR in urine was 3.1 in A, 5.2 in B and 2.3 in C.

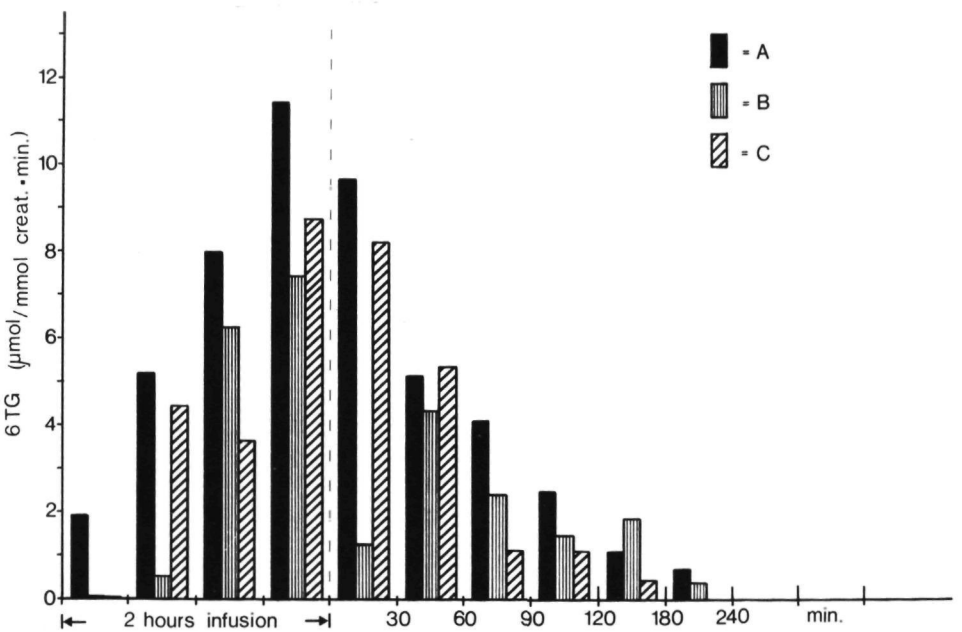

Fig. 2. Urinary excretion of 6TG relative to creatinine excretion, during and after a two hours infusion in three different goats.

Acute side-effects, like crystalluria, were not observed, because a liberal diuresis was maintained. Long-term side-effects could not be observed by routine clinical chemistry and blood counts, performed 2-3 times a week for 3 weeks after the experiments.

DISCUSSION

In a series of investigations we explored the feasibility of parenteral administration of 6-mercaptopurine (Schouten et al 1984, 1985a, 1985b, 1985c), because the customary oral administration is less reliable (Ding and Benet 1979, Zimm et al 1983a, 1983b). We concluded that prolonged infusions at relatively high doses are more rational. The feasibility was established in goats using 24 hours infusions of 6MP (Schouten et al 1985c). We demonstrated excellent penetration of 6MP and 6MPR into CSF in goats. Preliminary results of an on-going study in humans have already been reported and confirm our findings in goats (Zimm et al 1984). In the present study we investigated the administration of 6TG by infusion in goats. We found steady state plasma concentrations of 6TG, with a range of 2.4-3.5 μM, which were reached within the relative short duration of the infusion (fig.1). However the concentration of 6TGR exceeded that of 6TG by a factor 1.5-3.0. This is in accordance with the findings in our study on 6MP in goats (Schouten et al 1985c). 6TGR (6-thioguanosine) has been found to be equiactive to 6TG on a molar basis in humans when given by the intravenous route (Krakoff et al 1961). Thus the total biologically available amount of 6TG may be represented by the sum of the concentrations of 6TG and 6TGR (fig. 1 dotted lines). It can not be excluded, that the riboside concentrations exceeding the concentrations of the parent thiopurine is a typical phenomenon for goats. However we have also found considerable amounts of 6MPR in monkeys receiving 6MP (Schouten et al 1985b) and others have found 6TGR in plasma of humans after very high dose 30 minutes infusions (Konits et al 1982). In experiment B we could obtain a number of CSF samples from the lumbar space. The concentrations measured, are probably lower than the actual concentrations because of admixture of 0.9% NaCl solution used to flush the catheter. Therefore we have not depicted the concentrationtime curve of 6TG and 6TGR in CSF. Notwithstanding the admixture of 0.9% NaCl the

measured concentration of 6TG in CSF was ± 25% and of 6TGR ± 40% of the simultaneously obtained plasma concentration at the end of the infusion. The actually found penetration of 6TG into the CSF is in accordance with what one could expect on physicochemical grounds, like molecular size and partition coefficient in octanol-water (Mellett 1977).

By providing a liberal and alkaline diuresis and extending the duration of infusion, we could prevent crystalluria and the ensuing risk of renal damage, which has been reported in humans (Konits et al 1982). Prolonged infusion of 6TG, resulting in steady state plasma concentrations for longer periods, may lead to a greater cell kill. In vitro studies by Wotring and Roti Roti have shown greater cell kill by 6TG at a concentration of 10 μM during 12 hours than by 100 μM for 3 hours (Wotring and Roti Roti 1980). The 12 hour period equals the doubling time for the L1210 mouse leukaemic cells studied. One might infer that in humans the exposure time should be 24 hours or more, because the cell cycle of human cells is of that order. The administered dose in goats, 2 mg/kg/hour, resulted in a total concentration of 6TG and 6TGR of ± 10 μM, which is identical to the optimal concentration found by Wotring and Roti Roti during prolonged exposure (Wotring and Roti Roti 1980). 6TG and 6TGR are essentially S-phase specific drugs (Wotring and Roti Roti 1980, Lee and Sartorelli 1981). So one can predict that the activity of 6TG in slowly proliferating tumours is low, just as was found in patients with colorectal carcinoma (Konits et al 1982, Britell et al 1981). In actively proliferating malignancies, e.g. acute leukaemia, 6TG is used extensively (Paterson and Tidd 1975). The absorption of 6TG after oral administration is variable (Brox et al 1981), and theoretically one could expect a better activity of 6TG after parenteral administration. It is our opinion that prolonged infusions of 6TG should be explored in humans. A dose of 2 mg/kg/hour, as we have used in goats, seems reasonable to start with. We advocate that pharmacological studies in humans should not only take into account 6TG concentrations, but also the metabolites of 6TG in plasma (Konits et al 1982). Moreover intracellular metabolites, preferably in the target cells, should be studied. For erythrocytes some data are available after 6MP administration (Lilleyman et al 1984). As has been found for 6MP and 6MPR (Schouten et al 1985c, Zimm et al 1984), we also demonstrated the penetration of 6TG and 6TGR into CSF.

Therefore, if this penetration of 6TG into CSF is confirmed in humans, 6TG may contribute in the prophylaxis and treatment of CNS leukaemia and in the treatment of brain tumours. 6TG may have some advantage over 6MP because a greater cell kill at a much lower, and in vivo probably easily attainable, concentration has been reported by Tidd and Paterson (Tidd and Paterson 1974).

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CHAPTER VIII

6-MERCAPTOPURINE: HIGH-DOSE 24 HOURS INFUSIONS IN GOATS[1,4]

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ABSTRACT

In vitro investigations have pointed to the need of both a prolonged exposure to 6-mercaptopurine (6MP) and the use of high concentrations to archieve maximal cell kill. After the customary oral administration the bioavailability appeared to be low. I.v. bolus injections resulted in short-lived high concentrations of 6MP, so prolonged infusions seemed rational. To test the feasibility of this approach 24-hours infusions were given to goats.

We used our improved HPLC method to quantitate 6MP and 6MP riboside (6MPR) in plasma, CSF and urine. The concentrations of 6MPR were in excess to those of 6MP. Since 6 MPR can easily be converted to 6MP, 6MPR acts as a depot for 6MP. Penetration of both 6MP and 6MPR into CSF was excellent. Of the total dose administered, 38% to 68% could be accounted for in the urine, with about equal amounts of 6MP and 6MPR. At doses of 20 and 10 mg. kg^{-1}. hr^{-1}, total concentrations of 6MP and 6MPR in excess of 100 µM were reached during 24 hours infusions. However, all three experimental animals died due to toxicity. A dose of 2 mg. kg^{-1}. hr^{-1} was tolerable; the total steady state concentration of 6MP and 6MPR in two experiments was about 10 µM.

We conclude that the prolonged infusion of 6MP is feasible and especially in view of the excellent penetration of 6MP and 6MPR into CSF, studies using prolonged infusions of 6MP are warranted in man.

Footnotes:

1. Supported by grant SNUKC 81-2 from the Queen Wilhelmina Fund (Dutch Cancer Society).

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3. The abbreviations used are: 6MP(R), 6-Mercaptopurine (riboside); 6TG(R), 6-thioguanine (riboside); CSF, cerebrospinal fluid; DTT, dithiothreitol.

4. Presented in part at the 16th Annual Meeting of the International Society of Pediatric Oncology (SIOP), Barcelona, Spain, September 1984.

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For three decades the thiopurines, 6-mercaptopurine and 6-thioguanine, have been used in the treatment of malignant diseases, in particular the leukemias. The route of administration and the dose have been based on empirical grounds and clinical experience.

The recent availability of sensitive and specific analytical methods has made it possible to perform sophisticated pharmacokinetic studies on these antimetabolites (1,2). The results of these studies led many investigators to question the rationale of the customary oral administration (3,4,26).

Metabolic activation of 6MP[3] and 6TG is necessary in each individual target cell. This involves the transfer of ribose phosphate to 6MP or 6TG with phosphoribosylpyrophosphate as a co-substrate and hypoxanthine guanine phosphoribosyl transferase as the enzyme. 6MP, an analog of hypoxanthine, is transformed to 6-thio-IMP, which can be further metabolised to 6-thio-GMP by two rate-limiting key enzymes. On the other hand 6-thio-IMP can be dephosphorylated to 6MPR by 5'-nucleotidase. 6TG, an analog of guanine, is transformed directly to 6-thio-GMP. Ultimately 6-thio-GMP can be incorporated into DNA and RNA. 6-Thio-IMP and especially its methylated analog 6-methyl-thio-IMP have profound inhibitory effects on the purine de novo synthesis. Which of these biochemical pathways, either incorporation into DNA and RNA or blocking the purine de novo synthesis, is responsible for cell kill is still a point of discussion after so many years (7,14). It cannot be excluded that more than one mechanism is involved and that these mechanisms differ in various tumor (cell) models. Also the method by which a cytotoxic effect is measured may be of importance. For instance, growth inhibition may correlate with the well-known immunosuppressive effects of thiopurines. However, clonogenic assays are the methods of choice for assessing cytotoxic effects of anti-cancer drugs (9,16). In vitro studies, using clonogenic assays, have pointed out the importance of the duration of exposure, in addition to the effect of the concentrations of 6MP and 6TG (21,22,25).

As a rule longer exposure and higher concentrations of 6MP or 6TG lead to greater cell kill. A plateau effect in cell kill becomes apparent at concentrations above 100-200 μM for 6MP and 1-2 μM for 6TG (23).

However, a recent study on 9L rat brain tumor cells continuously exposed to 6MP showed paradoxical behavior of 6MP as a cytotoxic agent i.e. decreasing cell kill with increasing drug dose (10).

The in vitro data on thiopurines and the results of our recent[5] and other pharmacokinetic studies (3,4,18,26) led us to suggest that prolonged infusions of thiopurines should be studied. By prolonged infusion both the problem of the erratic absorption after oral administration (3, 4,26) and the limited period of measurable plasma concentrations after i.v. push injections[5] (3,26) can be circumvented.

Our data on children (18) and other data on monkeys (12) showed good penetration of 6MP into cerebrospinal fluid. An earlier study by Nelson regarding this aspect was negative (13). On the other hand on physicochemical grounds, like molecular weight and lipid solubility, good penetration of thiopurines into the CSF has been suggested by Mellett (11). Therefore it was also of interest to investigate to what extent thiopurines reach the central nervous system during prolonged infusions.

In the present paper we report on the feasibility of prolonged infusions of 6MP in goats, at different dose levels.

MATERIALS AND METHODS

Chemicals

6MP was obtained from Burroughs Wellcome (London, England) and from Fluka (Hicol B.V., Rotterdam, The Netherlands). The 6MP solutions for i.v. infusions were prepared on the day of administration. Helium was flushed through all solutions in which 6MP was dissolved and diluted, in order to prevent oxidation of 6MP. The desired amount of 6MP was dissolved in sodium bicarbonate 1.5% ($^{w}/v$), to which sodium hydroxide was added until 6MP was completely dissolved (final pH 9.6-10.2) (2). The ultimate concentration of the solution was dependent on the limited solubility of 6MP, the dose to be administered, and the characteristics of the dual syringe infusion pump.

Experimental animals

Goats were chosen as experimental animals, because CSF sampling from these animals had been performed previously in our Central Animal Laboratory (8). Moreover, goats are rather docile, a prerequisite condition to maintain i.v. infusions for prolonged periods. During and after the experiments the goats were housed in the laboratory in separate stables. Initially catheters for i.v. infusion and for obtaining blood samples were placed percutaneously in both jugular veins on the day of the study. Later on catheters were inserted by operation 4 to 5 days before the date of the infusion. To prevent catheter damage and to minimize the risk of systemic infection the catheters were tunnelled subcutaneously to the posterior neck. Heparine locks were used to preserve catheter patency. Care was taken to insert the catheter for infusion some 5 to 10 cm deeper than the catheter for sampling purposes.

On the day of the experiments attempts were made to insert a thin catheter by percutaneous puncture into the lumbar spinal canal. An indwelling balloon catheter was used for urine collection.

The insertion of the various catheters was accomplished during a short thiopenthal and ketamine narcosis.

Dose and administration of 6MP

In an initial experiment an i.v. push injection was given to obtain some basic pharmacokinetic parameters. In all other cases infusions were given which were planned to last 24 hours. The starting dose of 6MP was 20 $mg.kg^{-1}.hour^{-1}$. The toxicity of the drug forced us to reduce the dose to 10 and 2 $mg.kg^{-1}.hour^{-1}$, respectively. We started with the higher dose, since our aim was to attain a concentration of 6MP in plasma in the order of 100-200 μM. The drug was infused by a dual syringe infusion pump. The pump was loaded with two 60 milliliter syringes. For practical reasons the volume per hour was chosen in such a way that syringes had to be changed either every four or every eight hours. The total amount of drug was prepared on the day of the experiment and stored in capped syringes at room temperature. The drug was infused through Millipore membrane filters, type Millex GS (pore size 0.22 μm).

Preparation of samples and 6MP determination

Blood samples of 5-6 ml were collected in tubes containing heparin and 300 µg of DTT to prevent oxidation of 6MP (1,2). After thorough mixing, the blood was chilled and then centrifuged (5 min, 2000 x g). One milliliter of plasma was transferred with a calibrated syringe into micro test-tubes (type 3810, Eppendorf, Hamburg, GFR) and kept on ice. Protein was precipitated by adding 0.1 ml recently prepared, ice-cold, trichloroacetic acid (TCA) (50% $^{w}/v$) (2). After vigorous shaking, the tubes were kept on ice and after 10 minutes the precipitate was brought to sedimentation by centrifugation (10 min, 2000 x g).

Cerebrospinal fluid samples of ± 0.5 ml were taken and put in micro test-tubes containing DTT.

Urine samples were taken from measured aliquots collected during specified time periods. Before samples were taken care was given to dissolve deposits in the collecting reservoirs. Urine samples were put in tubes containing DTT. Before analysis NaOH was added to dissolve any deposit.

6MP and 6MPR concentrations were determined on a Spectra Physics SP 8000 high performance liquid chromatograph as previously described (2). The concentrations of 6MP and 6MPR are expressed in µM.

RESULTS

The concentration-time profile after bolus injection of 6MP, in a dose of 20 $mg.kg^{-1}$ (total dose 260 mg = 1.71 mmol) is depicted in Chart 1. Chart 1 illustrates a new and interesting point i.e. the presence of the riboside of 6MP in fairly high concentrations in plasma as well as in CSF. The 6MPR concentrations both in plasma and CSF are even far in excess of concentrations of 6MP itself. In an addendum the data are fitted by pharmacokinetic modeling.

The second experiment, intended to be a 24 hours infusion (dose 20 $mg.kg^{-1}.hour^{-1}$), had to be stopped after 2 hours of infusion, because of excessive stress to the goat from the experimental procedures. Although the experiment could not be completed as planned, the data are of interest (Chart 2). After breaking off the infusion, the 6MP concentration in plasma declined rapidly, but the 6MPR concentrations declined

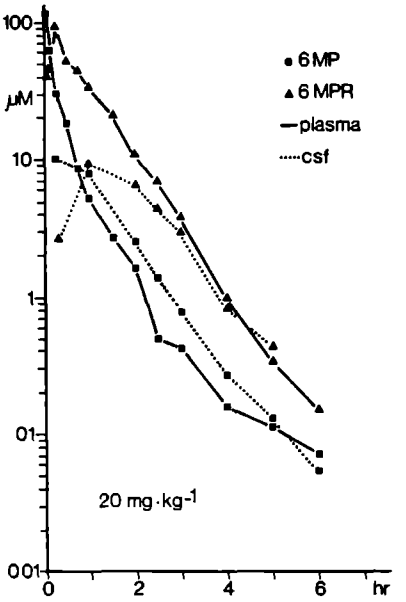

Chart 1. Concentration-time curve after an i.v. bolus injection of 6MP (total dose 260 mg = 1.71 mmol).

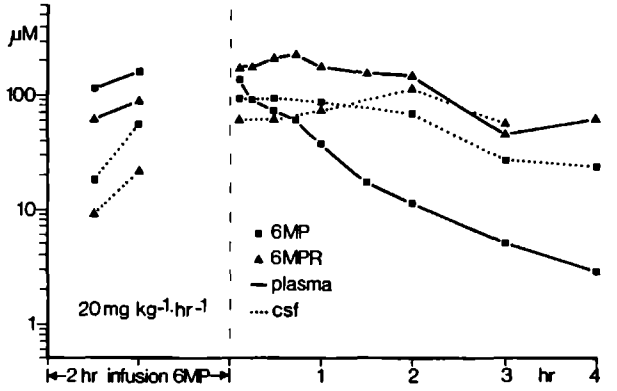

Chart 2. Concentration-time curve during and after a 2 hours infusion of 6MP (total dose 832 mg = 5.46 mmol).

slowly. Penetration of 6MP and 6MPR into the CSF is excellent. The concentration of 6MP in CSF remains rather high and exceeds 6MP concentrations in plasma within half an hour after stopping the infusion of 6MP. In these first two experiments it appeared that the percutaneous introduction of a catheter into the lumbar space is difficult and resulted in paralysis of the hind legs of the goats. To limit the discomfort to the animals no further attempts were undertaken to sample CSF during 24 hours infusions.

In the third experiment, at a dose level of 20 $mg.kg^{-1}.hour^{-1}$, the importance of forced diuresis became apparent; after 6-8 hours of infusion anuria developed due to crystalluria. At the same moment the 6MP concentration in plasma became higher than the 6MPR concentration. This suggests that renal excretion is a major factor in the clearance of 6MP during long-term infusions (Chart 3). Therefore in the following experiments liberal amounts of intravenous fluids (100-150 $ml.kg^{-1}.day^{-1}$) were given and urine pH was kept above 7.0 by intermittent i.v. administration of sodium bicarbonate (8.2%).

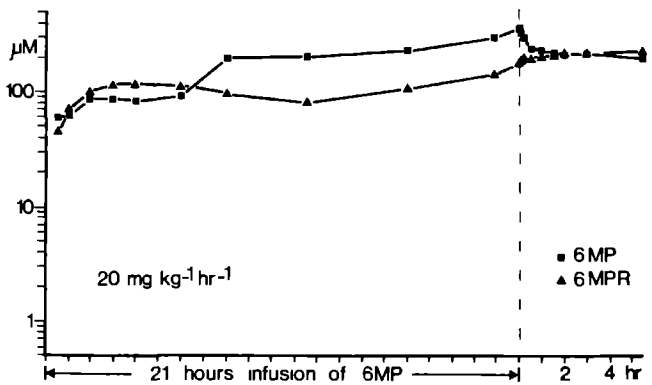

Chart 3. Concentration-time curve during and after a 21 hours infusion of 6MP (total dose 7,000 mg = 45.53 mmol).

With a dose of 10 mg. $kg^{-1}.hour^{-1}$ two very similar concentration-time profiles regarding 6MP and 6MPR concentrations in plasma were obtained. (Chart 4 shows one of them). Unfortunately the goats did not survive for longer than 48 and 96 hours respectively after stopping the infusions. Both goats had shown ill health and severe leucopenia shortly before dying, so a fulminant infection is possible. Routine clinical chemistry showed a tendency to increased values of urea, creatinine and liver transaminases. At autopsy the internal organs were macroscopically unremarkable.

In the last two experiments, with a dose of 2 $mg.kg^{-1}.hr^{-1}$, the feasibility of 24 hours infusion of 6MP was demonstrated (Chart 5). Both goats ultimately survived in good health, although abscesses around the tunnelled catheters appeared in both of them. On conservative management these abscesses cleared. Haematological toxicity was absent, however transient hepatic damage became evident 5-10 days after exposure to 6MP. Values of liver transaminases rose to levels 3-6 times above the pretreatment levels and then returned to normal within a 3 weeks observation period.

The maintenance of properly functioning bladder catheters was difficult because of expression of the catheter by the goat. Sometimes there was loss of urine along the catheter. Furthermore crystalluria occurred at higher dose levels. Therefore incomplete data on the urine excretion are available. After the i.v. push injection 68.5% of the dose administered was recovered from the urine: 36% as 6MP and 32% as 6MPR.

In one of the two goats receiving 6MP in a dose of 10 $mg.kg^{-1}.\ hour^{-1}$ 43% of the total dose administered during 24 hours, could be accounted for in the urine, with about equimolar amounts of 6MP and 6MPR. This is the minimum amount excreted because some urine was lost and also crystals were present in the collecting reservoir.

In the two experiments with a dose of 2 $mg.kg^{-1}.hr^{-1}$ combined urinary excretion of 6MP and 6MPR was 38% and 63% of the total dose. After stopping the infusion of 6MP, the excretion in urine declined very rapidly, so 6MP and 6MPR were hardly detectable 4-6 hours after stopping the administration of 6MP.

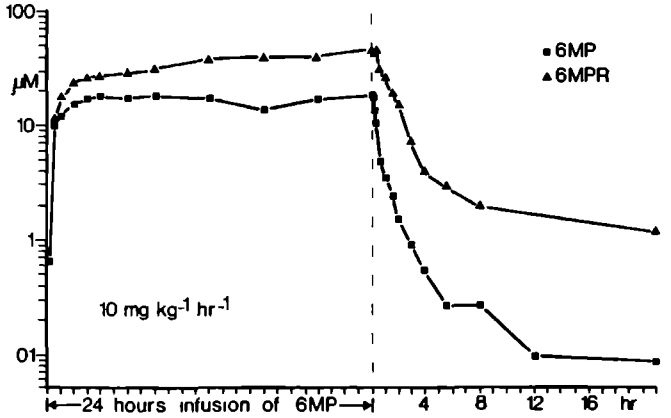

Chart 4. Concentration-time curve during and after a 24 hours infusion of 6MP (total dose 4,080 mg = 23.97 mmol).

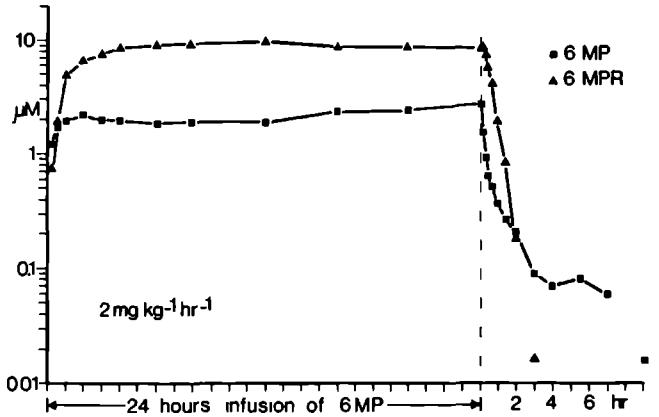

Chart 5. Concentration-time curve during and after a 24 hours infusion of 6MP (total dose 1,008 mg = 5.92 mmol).

In the present study we have shown that 24 hours infusions are feasible without undue toxicity at a dose level of 2 $mg.kg^{-1}.hour^{-1}$.
So the in vitro optimal way of exposure to 6MP (23) can be simulated in vivo by prolonged i.v. infusions.
In this study on goats a metabolite of 6MP, i.e. the riboside of 6MP, was present in high concentrations both absolute and relative to 6MP. 6MPR can only be converted to 6MP, so 6MPR can be regarded as a kind of prodrug of 6MP. In the past it has been demonstrated that on molar basis both substances, 6MP and 6MPR, are equitoxic (15). Therefore the biological activity may better be represented by the sum of the molar concentrations of 6MP and 6MPR.
From charts 3, 4 and 5 it can be seen that steady state concentrations of 6MP are attained in 1-2 hours. 6MP concentrations in plasma remain constant as long as the infusions are maintained (Chart 4 and 5), provided that crystalluria is prevented by forced and alkaline diuresis (Chart 3).
A steady state concentration for 6MPR is also reached after about 2 hours. At a dose level of 10 $mg.kg^{-1}.hour^{-1}$, there is a tendency to an increase in 6MPR concentration with time (Chart 4). This is not apparent at a dose level of 2 $mg.kg^{-1}.hour^{-1}$ (Chart 5).
The sum of the concentrations of 6MP and 6MPR at a dose rate of 20 $mg.kg^{-1}.hour^{-1}$ is in a range of 200-300 µM (Chart 2 and 3). This is probably so high partially because no extra fluids were given in these two experiments. With forced diuresis a combined concentration of 95 µM was attained after 4 hours during a 6 hours infusion at 20 $mg.kg^{-1}$ $hour^{-1}$. Catheter breakdown precluded further study, but this goat did survive in good health (data not shown). At a dose rate of 10 $mg.kg^{-1}$. $hour^{-1}$ the concentration of 6MP plus 6MPR was lower at about 60 µM (Chart 4), unfortunately both goats succumbed to severe myelotoxicity. Both goats, receiving a dose of 2 $mg.kg^{-1}.hour^{-1}$ during 24 hours, did survive. The combined concentration of 6MP and 6MPR was slightly above 10 µM during the infusion. This concentration in vivo is much lower than the in vitro maximal effective concentration of 100-200 µM as reported by Tidd and Paterson for L5178Y mouse lymphoma cells (23). However, actual measured steady state concentrations of 6MP during continuous infusion may not be

compared directly to the concentrations present in vitro at the start of a prolonged incubation period. In our laboratory in vitro experiments on MOLT-4 human leukemic cells with a starting concentration of 2 μM 6MP, pointed out that most of the drug has disappeared from the medium within 3 hours, hence far before the end of a 24 hours incubation period[6]. Moreover, for 6MP the general rule "the higher the dose, the greater the effect", is not necessarily true (10).

We demonstrated that 6MP and 6MPR penetrate well into the CSF (Chart 1 and 2). This provides further evidence that 6MP might be of interest in the treatment of brain tumors and the prevention of meningeal leukemia. Our findings in this and in our earlier study (18), and data in the literature (12) contradict earlier negative findings on the penetration of 6MP and 6MPR into the CSF (13).

Collection of urine from goats during prolonged periods of time is cumbersome. Moreover the metabolism of uric acid as well as of thiouric acid in these animals is different from that in man. Therefore our data on urinary excretion can be regarded as exploratory only. Anyhow a major proportion of the total dose administered was recovered from the urine either as 6MP or 6MPR.

We did not try to define more precisely a maximally tolerated dose per hour during 24 hours infusions in goats. Eventually this has to be done in man using an escalating dose schedule.

A number of problems can be predicted when this kind of administration is applied to man in future studies. The poor solubility of 6MP poses pharmaceutical problems, which probably can be solved by selecting more soluble analogs like 6MPR. The same problem and solution might pertain to 6TG, which in vitro is more potent than 6MP (23).

6MP and catabolites are poorly soluble in urine so a forced diuresis of alkaline urine is a prerequisite condition for the administration of high doses of 6MP, as we learned in this study and as already could be concluded from observations in man (5). 6MP is easily oxidized in biological fluids (1), so urine should be collected in reservoirs containing an anti-oxidant like DTT, when the excretion profile is studied. If necessary, crystals in stored samples of urine can be redissolved by increasing the pH before analysis.

The concentration of 6MP plus 6MPR, about 10 μM at a dose level of 2 mg. $kg^{-1}.hour^{-1}$, is in the range of the concentration of hypoxanthine and

inosine in bone marrow "juice" and CSF (6,20). Hypoxanthine and 6MP are both substrates for the same activating enzyme hypoxanthine guanine phosphoribosyl transferase, with IMP and 6-thio-IMP, respectively as the products. So it is conceivable that physiological variations in hypoxanthine concentrations can influence the effectivity of 6MP. In this way normal bone marrow cells can be protected against the toxic effects of 6MP, but in the case of leukemia the leukemic cells may escape desired cytotoxicity.

Another important problem to circumvent is the relatively high rate of natural and easily acquired resistance of malignant cells to 6MP and 6TG. This is either due to lack of the enzyme hypoxanthine guanine phosphoribosyl transferase or to elevated levels of alkaline phosphatase (17,19). So the thiopurines should be combined with other cytostatic drugs.

The excellent penetration of 6MP and 6MPR into CSF justifies phase I and phase II studies on high dose prolonged i.v. infusions of thiopurines in man.

ACKNOWLEDGMENTS

We thank Theo Arts and all other biotechnicians from the Central Animal Laboratory for skillful experimental assistance, J.A.J.M. Bakkeren for careful reviewing, and L. Viering and J. Palthe for preparing the manuscript.

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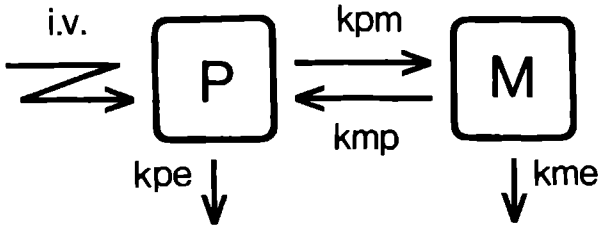
ADDENDUM

The data obtained in the experiment depicted in Chart 1 were fitted by non-linear least squares by means of NONLIN according to the reversible metabolic model:



This resulted in the following parameters:

parameter	estimate	s.d.	
kpm	3.279	0.819	h^{-1}
kpe	2.607	1.416	"
kmp	0.532	0.149	"
kme	0.993	0.183	"
Vp	8.73	2.6	l

s.e. = 0.079 with 23 degrees of freedom.
weighting $1/c^2$
$t_{\frac{1}{2}}$ intrinsic parent drug : 7 min.
$t_{\frac{1}{2}}$ intrinsic metabolite : 27 min.
$t_{\frac{1}{2}}$ elimination-phase : 42 min.

abbreviations:

P = parent drug 6MP

M = metabolite 6MPR

kpm = rate constant of metabolism from P to M in h^{-1}

kmp = rate constant of metabolism from M to P in h^{-1}

kpe = rate constant of total clearance of P in h^{-1}

kme = rate constant of total clearance of M in h^{-1}

Vp = distribution volume of P in l

c = concentration

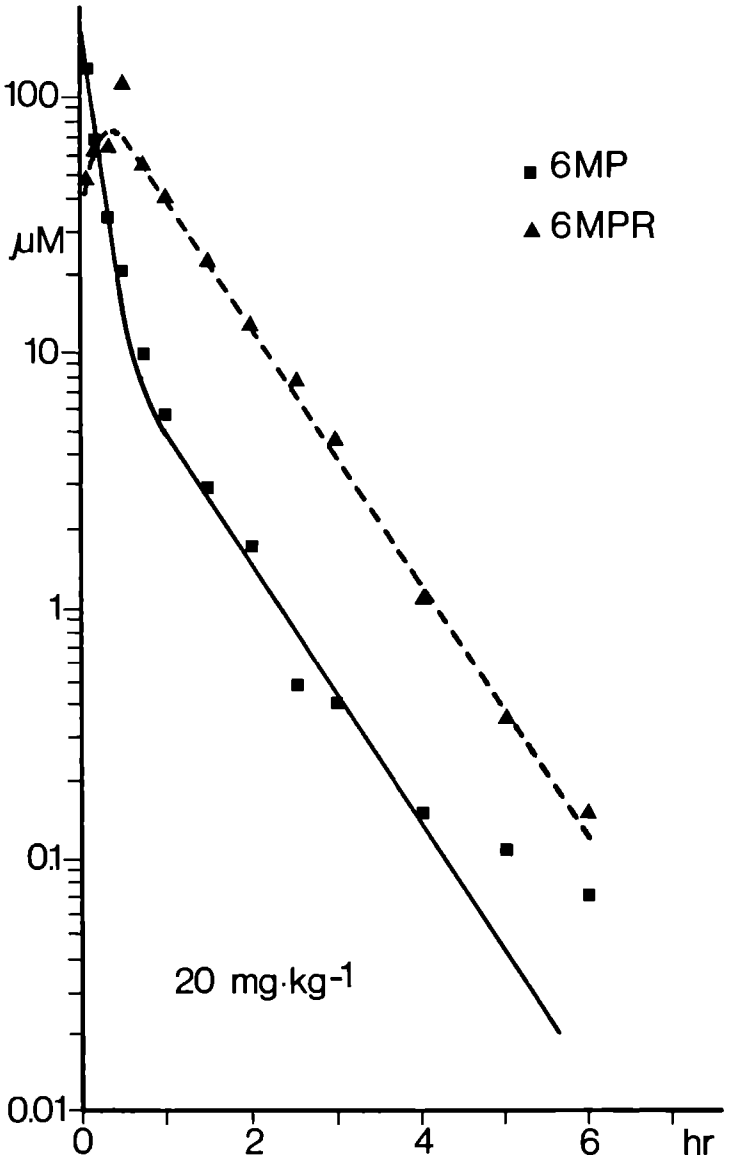
s.d. = standard deviation

s.e. = standard error

The equation ($t_{\frac{1}{2}}$ elimination-phase observed = $\frac{0.693}{kme}$)shows that the $t_{\frac{1}{2}}$ elimination-phase is governed by the total clearance of the metabolite. The clearance of the metabolite is probably mainly renal, so patients with an impaired renal function are prone to a decreased clearance of the metabolite, resulting in a prolongation of $t_{\frac{1}{2}}$ elimination-phase and increased toxicity of parent drug and metabolite.

NONLIN according to: Metzler CM, Elfring GL, McEwen AJ.
A package of computer programs for pharmacokinetic modeling.
Biometrics 1974;30:562.

Acknowledgement: The assistance of E.Termond, T.B. Vree and E. van der Kleijn (Dept. of Clinical Pharmacy) in the calculation and interpretation of the data is greatfully acknowledged.



Calculated plasma concentration-time curves of 6MP and 6MPR after an i.v. bolus injection of 6MP (20 $mg.kg^{-1}$) to a goat. (Same data as in Chart 1, Chapter VIII).

Calculations by means of NONLIN reversible metabolic model.

CHAPTER IX

6-MERCAPTOPURINE AND 6-THIOGUANINE: SURVEY AND SUMMARY

6-Mercaptopurine and 6-thioguanine are mainly and extensively used in the maintenance treatment of ALL and AML. These drugs have been used for decades now and both the dose and way of administration, i.e. daily low doses orally, have hardly changed in this period of time (1, 2, 3, 4, 5, 6). In the sixties the treatment of ALL of childhood was divided into two phases, i.e. the induction and the maintenance treatment. The third phase, which is included at present, is the so-called CNS prophylaxis. This phase is usually spaced between induction and maintenance treatment (5, 6).

In the maintenance treatment of childhood ALL 6MP is the mainstay. The drug is given in a dose of 50-90 mg/m^2, once daily by the oral route, in combination with methotrexate, which is administered once weekly in a dose of 20-30 mg/m^2 orally or eventually by the parenteral route (5, 6). However it should be marked that in 1980 Frei (7), one of the pioneers of maintenance therapy in childhood ALL, rejected the concept of maintenance chemotherapy. Maintenance therapy for leukaemia and lymphomas has been demonstrated to prolong the duration of remission, but there is no clear evidence from any study that maintenance treatment, delivered at low doses, will increase the cure rate. Indeed, the original goal of maintenance treatment was prolonging the time until relapse, and not increasing the cure rate (7).

In vitro studies on 6MP reported in the seventies by Tidd and Paterson made us aware of the importance of high concentrations of the drug during prolonged periods of exposure (8, 9, 10). Their in vitro studies prompted Wotring and Roti Roti to emphasize the importance of extended exposure to 6TG (11). This latter study and also other investigations made clear the S-phase specific cytotoxic activity of 6MP and 6TG (12, 13). Park and coworkers and Lihou and Smith testing the chemosensitivity of human leukaemic cells recently emphasized the importance of prolonged exposure to S-phase specific agents (14, 15).

The original aim of our studies was to gain insight into the clinical pharmacology and pharmacokinetics of 6MP, as applied in the maintenance treatment of childhood ALL. The first results of our studies in man and dogs learned us that we should take a fresh look on 6MP and also on 6TG.

Both drugs are vintage anticancer drugs and used extensively in the maintenance treatment of ALL and AML. The dose, way and schedule of administration of 6MP and 6TG however are based on empirism and not on sound experimental grounds. In exploring other dosages, routes and schedules of administration of 6MP and 6TG we made a start in providing such an experimental basis.
In the following paragraphs we discuss our studies on 6MP and 6TG. We will present a short survey on recent studies on the clinical pharmacology and pharmacokinetics of 6MP and 6TG.

6MP AND 6TG: SURVEY OF OUR STUDIES

Development of sensitive and specific methods to determine 6MP, 6TG, 6MPR and 6TGR

As a first objective we developed a method to determine 6MP in clinically relevant concentrations. An HPLC method was devised which was specific and sensitive. A major step was the use of DTT (dithiothreïtol) as a protective agent throughout the whole analytical procedure. By this step the recovery of 6MP increased from 20% to 94%. Also a relatively large injection volume of 460 µl was used to reduce the limit of detection. We were able to measure concentrations as low as 3 ng/ml, equivalent to 16 nmol/liter or 0.016 µM. The time necessary to perform an analysis was relatively short: in the order of 20-30 minutes per sample (Chapter II). In the latter part of the study we were interested in 6TG, so we adapted our previous HPLC method in such a way that also 6TG and the ribosides of both 6MP and 6TG could be measured by the same analytical procedure. The HPLC columns were prepared and packed in our own laboratory and two columns were used in series. Advantage was taken of the presence of two u.v. detectors and a dual-channel printer-plotter in the HPLC-apparatus. 6MP and 6MPR were monitored at 312 nm, and 6TG and 6TGR at 342 nm. These wavelengths are close to the absorbance maximum of these substances. The relative absorbances at two different wavelengths are of help in characterizing the various peaks. The lower limit of detection was for 6MP found to be at 20 nM, for 6TG at 25 nM, for 6MPR at 65 nM and for 6TGR

at 60 nM. It is possible to apply different injection volumes, depending on whether a 190 or 500 µl injection loop is used. A smaller injection volume gives less deterioration of the HPLC column, while with a larger loop a lower limit of detection is possible. The protective agent, DTT, is of course also used in this new adapted method and degassing of all solutions by helium was also applied again.

Explorative pharmacokinetic studies in ALL patients and dogs

In an orientating study on patients (Chapter IV), receiving maintenance treatment according to the operative protocol V of the "Stichting Nederlandse Werkgroep Leukemie bij Kinderen" (SNWLK), plasma and CSF concentrations of 6MP were determined. The HPLC method described in chapter II was applied. Samples were obtained on the occasion of vena punctures and lumbar punctures scheduled as required by the protocol. All samples were taken about 24 hours after the last of 35 daily doses of 6MP orally. The mean plasma concentration was 0.064 µM. Establishing the presence of 6MP in CSF, with a mean concentration of 0.029 µM, was somewhat surprising because earlier studies could not prove such a penetration of 6MP into CSF (16, 17). The ratio of the mean 6MP concentration in CSF to the mean 6MP concentration in plasma was approximately 0.40. We could not compare oral vs. intravenous administration, as 6MP suitable for i.v. application to humans was not available to us. Concentration-time curves of 6MP after oral administration in the standard dose of 50 mg/m^2 were therefore not obtained.

In parallel with our orientating study in patients with ALL, we started an explorative study in dogs (Chapter V). We applied 6MP of analytical grade for intravenous administration in animals. We could also study some pharmacological aspects of oral administration. A concentration-time curve after a first oral dose of 6MP, as a 50 mg commercially available tablet, revealed a low and late appearing peak of 6MP in plasma. This peak concentration was rather low, 0.040 µM, while concentrations in a range of 20-200 µM are necessary to obtain a cytotoxic effect in vitro (8). On the other hand daily administration of 6MP by the oral route at an usual clinical dose resulted in "steady state" levels of 6MP in plasma after 2-3 weeks. After 10 days of application of 6MP in the

same dose by the i.v. route this was not observed. Rather peculiar was the observation of the persistence of these "steady state" levels after oral administration with a mean of 0.150 μM, during 3-6 weeks after stopping the administration of 6MP. A similar phenomenon has been observed in children off maintenance treatment for ALL after 2 years of continuous complete remission. The persistence of 6MP in plasma of dogs for weeks after discontinuation of 6MP implicates a kind of deposit of the drug. Tight binding of 6MP to plasma and/or liver proteins may play a role. Worth mentioning is the presence of thioguanine nucleotides in erythrocytes of patients with ALL during maintenance treatment (18). Concentration-time curves in dogs after the usual clinical dose of 50 mg i.v. revealed peak plasma concentrations with a range of 2.5-10.5 μM. The concentrations of 6MP declined rapidly to less than 0.10 μM. This is reflected by a short first half-life time of 13-21 minutes. The second half-life time had a range of 125-151 minutes.

From our first explorative pharmacokinetic studies (Chapter IV and V) we realized that the customary low-dose oral administration of 6MP and 6TG was founded only on empirism and had no experimental basis. Our first results gave the direction for further investigations. The finding of the presence of 6MP in CSF, albeit in very low concentrations, encouraged us to continue our study with regard to this aspect. The low 6MP plasma concentrations which we found after the customary dose, either after oral or i.v. bolus administration, brought us to study much higher doses and to explore parenteral administration by prolonged i.v. infusions. This departure from the customary dose and route of administration of 6MP, and 6TG, was sustained by an article of Frei and Canellos (7). These authors drew attention to the critical role of dose in cancer chemotherapy and rejected the concept of maintenance therapy as part of curative intended chemotherapy of disseminated malignant disease.

Infusions of 6MP and 6TG in monkeys and goats

In our experiments on marmoset monkeys we made the step to infusions of 6MP (Chapter VI). Since technical and practical reasons precluded infusion durations of more than a few hours, a one- and a four-hours infusion was given with $\left[8\text{-}\,^{14}C\right]$-6MP. Following the insights gained in our

studies so far, a high dose rate of 5 mg/kg/hour was used. This resulted in steady state levels in plasma in the range of 30-40 μM within half an hour after the start of the infusion. 6MPR could be detected shortly after starting the infusion; the 6MPR level attained was 20-35% of the 6MP concentration during the infusion period. After termination of the infusion 6MP and 6MPR concentrations got equal. 6MPR, which is formed from 6MP intracellularly by phosphoribosylation and subsequent dephosphorylation, can only be degraded to 6MP. So 6MPR acts as a kind of depot form for 6MP.

In order to obtain autoradiograms of the animals $\left[8\text{-}^{14}C\right]$-6MP was used. After infusion of $\left[8\text{-}^{14}C\right]$-6MP a two-hours period was observed before the animals were sacrificed. The autoradiograms revealed extensive distribution of the radiolabel throughout the body. Intense activity was present in liver, gall bladder and intestinal lumen. Other points of interest are the apparent penetration of the label into the central nervous system and the accumulation of activity within the bone marrow. In patients with leukaemia the bulk of the malignant cells resides in the bone marrow, so 6MP should preferably be active at this site of the body. However, the forms in which the label is present at the various sites are not known. We are aware of the fact that in plasma four labeled substances (6MP, 6MPR, 6TX, 6TU) are present, and in concentrations variable over time. Ideally, in further studies one should try to correlate intracellular and intravascular concentrations of 6MP, 6MPR and other metabolites to gain a more thorough understanding of the pharmacological and metabolical fate of 6MP and its derivatives.

In Chapter VII and VIII, we made use of our adapted method for determination of 6MP, 6MPR, 6TG and 6TGR (Chapter III). We had extended our original interest in 6MP as used in maintenance treatment and included the closely related drug 6TG. The arguments for this have been presented in Chapter I. In summary, 6TG might have advantages over 6MP, as the maximal cytotoxic effect was demonstrated to be somewhat greater for 6TG as compared with 6MP (10). The concentrations of 6TG necessary in vitro to attain a maximal cell kill (1-2 μM) are more likely to be applicable in vivo than the concentrations of 6MP which are necessary for an analogous effect (100-200 μM) (10). Moreover, 6TG has a more direct metabolic pathway to the site of the cytotoxic effect, viz. incorporation into DNA as 6-thio-dGTP.

In Chapter VII the results of three experiments in goats, using two-hours infusions of 6TG, are presented. A dose of 2 mg/kg/hour was chosen and this dose quickly resulted in a mean steady state level of 6TG of about 3 μM. 6TGR was even present in concentrations exceeding those of 6TG itself. Although 6TGR is formed intracellularly from 6TG by the addition of a phosphoribosyl group and subsequent dephosphorylation, 6TGR can only be converted back to 6TG by an enzymatic process involving purine nucleoside phosphorylase. Therefore 6TGR can also be regarded as a kind of depot or prodrug for 6TG. The total biological activity of 6TG and 6TGR may be represented by the sum of their concentrations, which is about 10 μM during the second hour of the infusion. This concentration is of the same order as the concentrations which were found to exert a good cytotoxic effect in vitro (10, 11). However, because of the limited duration of the infusions studied, these concentrations were not sustained for as long as is necessary in vitro (11).

In one experiment we obtained several samples of CSF. The concentration of 6TG found in CSF was about 25% of simultaneously measured plasma 6TG. The penetration for 6TGR was even better; in CSF 40% of the simultaneously obtained plasma concentration was present. On physicochemical grounds reviewed by Mellet in 1977 one would expect 6TG to reach the CSF (19), although this was not found in dogs by Loo and coworkers (20). If this penetration of 6TG and 6TGR into CSF is confirmed in humans, these drugs may contribute to the prophylaxis and treatment of CNS leukaemia and to the treatment of brain tumours.

Urinary excretion of 6TG and 6TGR was found during and immediately after the infusion of 6TG. After 4 hours only negligible amounts of 6TG and 6TGR were observed. The combined amounts of 6TG and 6TGR recovered from urine varied from 26 to 49% of the amount of 6TG administered. Catabolites of 6TG were not determined, but it is likely that most of the drug, either in active or inactive form, is excreted quickly. A liberal diuresis is necessary to prevent crystalluria and renal damage, which has been observed in humans (21).

We observed this problem of crystalluria and renal damage also in our studies in goats, using prolonged, i.e. 24-hours infusions of 6MP (Chapter VIII). We started at a really high dose of 6MP, 20 mg/kg/hour, in an attempt to attain and maintain concentrations in plasma in the order of 100-200 μM. These latter concentrations of 6MP must be present in vitro

to obtain a maximal cell killing effect (10). Indeed concentrations in that order, 6MP combined with 6MPR, were demonstrated at dose levels of 10 and 20 mg/kg/hour. The riboside of the parent drug, 6MPR, was again found to be present in amounts far exceeding those of 6MP itself. In the past 6MP and 6MPR have been demonstrated to be equitoxic on a molar basis. So again, as for 6TG and 6TGR, the total biologically active amount of drug may be best represented by the sum of the molar concentrations of 6MP and 6MPR. Acute renal toxicity due to crystalluria, because of the poor solubility of 6MP and its metabolites, could be handled by providing a liberal and alkaline diuresis. However, at a dose rate of 10 mg/kg/hour, severe myelotoxicity became evident. This forced us to reduce the dose rate. A dose rate of 2 mg/kg/hour during 24 hours seemed feasible; no important permanent toxicity was observed. Under these experimental conditions a combined steady state plasma concentration of 6MP and 6MPR was found in the order of 10 μM.
Worth mentioning is the observed excellent penetration of both 6MP and 6MPR into CSF. After a bolus injection of 6MP in a dose of 20 mg/kg concentration-time curves of 6MP and 6MPR in plasma as well as in lumbar CSF could be made. In a two-hours infusion at a dose rate of 20 mg/kg/hour these findings were confirmed and extended.

6MP: EVALUATION OF RECENT PHARMACOLOGICAL AND PHARMACOKINETIC STUDIES FROM THE LITERATURE

The renewed interest in studies on 6MP and 6TG is evident from an increasing number of published methods to quantitate these substances. High-performance liquid chromatography is successfully applied to analyze 6MP, 6TG and metabolites.
In this review we will limit us to the discussion of the recent studies using HPLC methods to determine 6MP and 6TG. For earlier studies one may be referred to the reviews by Paterson and Tidd (1975), LePage (1977), and McCormack (1982) (1, 2, 3).
A new era of pharmacokinetic studies on the thiopurines was started by Ding and Benet in 1979 (22). These studies were extended by a group at the National Cancer Institute, Bethesda, U.S.A. (23, 24, 25, 26). The study by Ding and Benet revealed that on an average only 12% of the oral

6MP dose was available to the systemic circulation in rhesus monkeys. A solution of 6MP was given through surgically implanted stomach tubes and not as a tablet or powder per os as is clinically done. By giving 6MP as an oral solution, dissolution problems are eliminated. The low bioavailability of 6MP in the rhesus monkeys may be caused by a combination of poor absorption and metabolism by xanthine oxidase in the small intestine and in the liver. Using a two-compartment open model a $t_{\frac{1}{2}}$, 1 from 2.7 to 11.2 minutes was found in 4 monkeys. The mean $t_{\frac{1}{2}}$, 2 was 41.6 (range 24.2-51.7) minutes. Concentrations of 6MP in plasma three hours after a bolus injection were about 1% of peak plasma concentrations (22).

In a study, by Narang and coworkers, on the pharmacokinetics of 6MP in the monkey excellent penetration of 6MP into the CSF was showed (23). In three monkeys concentration-time curves of 6MP were obtained simultaneously in plasma and CSF, after an i.v. bolus injection of 4 mg/kg. The mean peak concentration in CSF was about 3 μM and was observed approximately 40 minutes after i.v. bolus injection. The decline of drug concentration in the CSF almost paralleled the plasma concentration decline and appears to suggest the concentration gradient of the plasma compartment to be the driving force behind the partitioning of 6MP between plasma and CSF. The ratio of 6MP concentration in CSF over that in plasma in the three monkeys varied between 0.4 and 1.0. The plasma concentration-time curves could be fitted into a three-compartment open model, in contrast to the two-compartment model used by Ding and Benet (22). In our studies on dogs we could fit our data best into a two-compartment open model (Chapter V).

Further investigations by the same team of investigators from the NCI involved the bioavailability of 6MP in monkeys and in man (24, 25). In monkeys, using doses of 8 mg/kg, equivalent to 100 mg/m^2, there was a marked difference in AUC (area under curve) between the oral and intravenous plasma concentration-time curves, indicating a bioavailability of oral 6MP of 4 per cent (24). In patients, receiving maintenance treatment for ALL, there was also a substantial difference in AUC after oral and intravenous administration, indicating a mean bioavailability of oral mercaptopurine of only 16 per cent (range 5 to 37 per cent). The mean peak plasma concentration of 6MP, after an oral dose of 75 mg/m^2, in 14 fasting children was 0.89 μM (range, 0.29 to 1.82), with a mean

time to peak concentration of 2.2 hours (range, 0.5 to 4). Under normal circumstances, the presence of food in the stomach may act as an additional source of variability (24).

In another report by Zimm and coworkers, the reason for the low bioavailability of 6MP after oral administration was explored (25). Ding and Benet, and Zimm and coworkers had already suggested the first-pass effect to be the cause for the low bioavailability of 6MP (22, 24). The first-pass effect refers to the presystemic metabolism of oral drugs by liver or gut mucosal enzymes. For 6MP the catabolizing enzyme xanthine oxidase is involved and located primarily in intestinal mucosa and liver. By using allopurinol, a well-known inhibitor of xanthine oxidase, the bioavailability in man increased from a mean of 12 to a mean of 59 per cent (25). Allopurinol had no effect on the pharmacokinetics of intravenously administered 6MP. However, allopurinol may deplete intracellular PRPP pools and increase hypoxanthine levels. Both changes may diminish 6MP antileukaemic activity (25). The metabolic effects of allopurinol and the interaction with 6MP are species specific, as was demonstrated by investigations on the pharmacokinetics of 6MP in rabbits (27).

The last study on 6MP to be discussed here, is reported by Zimm and coworkers in abstract only (26). In this preliminary report prolonged intravenous infusions of 6MP during 12-24 hours at a dose rate of 50 mg/m^2/hour are described. Obviously Zimm and coworkers followed the same line of reasoning as we did (Chapter I). The mean steady-state plasma concentration achieved in 9 patients was 6.2 μM, with a less than two-fold difference between the highest and lowest plasma concentrations. In four patients CSF samples were obtained six or more hours after the start of the infusion. The mean CSF level of 6MP at steady-state was 1.9 μM and the mean CSF/plasma ratio for 6MP was 0.39. This latter ratio is nearly identical to the ratio we have found during conventional maintenance treatment for ALL (Chapter IV). The dose rate of 50 mg/m^2/hour is also nearly identical to the dose rate we have found to be feasible in goats (Chapter VIII).

In the studies discussed in this paragraph the presence of 6MPR in plasma or CSF is not mentioned. However, we have demonstrated even higher concentrations of 6MPR than of 6MP itself in plasma and CSF from goats (Chapter VIII). Also 6MPR was found to be present in plasma of marmoset

monkeys in fair concentrations, as compared to 6MP concentrations (Chapter VII). Whether 6MPR was not present in plasma of rhesus monkeys or ALL patients studied by Zimm and coworkers, or was not determined because of limitations in their analytical procedure, is not clear to us. Preliminary data from children, using our improved method of analysis, has demonstrated the presence of 6MPR in plasma and CSF, after administration of 6MP as well as after administration of 6TG.

6TG: EVALUATION OF RECENT PHARMACOLOGICAL AND PHARMACOKINETIC STUDIES FROM THE LITERATURE

The first pharmacological study in which HPLC was used for analysis of 6TG in plasma was reported in 1981 (28). Brox and coworkers state that only one other pharmacological study had been reported earlier. This report by LePage and Whitecar, published in 1971, already drew attention to the very low concentrations of 6TG in plasma after oral administration compared to i.v. dosing (29). The study by Brox and coworkers disclosed a 30-fold range in peak plasma concentration, i.e. from 0.03 to 0.94 μM, after oral 6TG in fasting patients with AML. They correlated the peak plasma concentrations observed in patients to the in vitro concentration of 6TG, i.e. 0.5 μM, required for both complete inhibition of cell growth and cell lysis during continuous exposure. In only 4 out of 25 6TG courses the peak plasma level of 6TG exceeded 0.5 μM and that only for a short period of time. Their study illustrates the erratic oral absorption of 6TG and the frequently low peak plasma levels. Brox and coworkers suggest that the i.v. formulation of 6TG may be superior to the oral gift of the same drug.

In a study, reported by Konits et al in 1982, 6TG was actually given i.v. in high dosage by 30 minutes infusions to patients with colorectal carcinoma (21). Doses of 6TG, ranging from 1000 to 1200 mg/m^2 resulted in peak plasma concentrations of 61 to 118 μM, with an initial $t_{\frac{1}{2}}$ of 3 hours and a terminal $t_{\frac{1}{2}}$ of 5.9 hours. A number of known and other, as yet unidentified, metabolites were found in plasma. In humans 6TGR was demonstrated, although in relatively low concentration in comparison with the values we observed in goats (Chapter VII). Crystals of 6TG and 6TU were observed in the urine of all six patients from whom urine was

collected. The solubility of 6TG and 6TU is poor, and it seems plausible that 6TG and 6TU may precipitate in the renal parenchyma. The finding of renal toxicity, reflected by increases in serum creatinine, in 4 out of 19 patients studied, is in our opinion related to the crystalluria observed by the authors (21).

6MP AND 6TG: A FRESH LOOK ON THEIR APPLICATION

Our study and data from the literature, summarized above, have made it clear that the oral administration of 6MP results in a low bioavailability (22, 24, 25) and that the absorption of 6TG is erratic (28). These findings prompted us to explore other dosages and ways of administration. We demonstrated the feasibility of administering 6MP at a dose rate of 2 mg/kg/hour by 24 hours infusions in goats. In preliminary form similar findings have been presented on patients with ALL (26). The dose rate used in these two investigations, 2 mg/kg/hour and 50 mg/m^2/hour respectively, was similar. In both reports excellent penetration of 6MP into the CSF was established. While we have found higher concentrations of 6MPR than of 6MP itself in plasma and CSF from goats, the presence of 6MPR is not mentioned by Zimm and coworkers (26). It is likely they did not quantitate 6MPR because of limitations in their method of analysis. We observed 6MPR to be present in plasma from marmoset monkeys at 20 to 35% of 6MP concentrations during the infusion of 6MP and in equimolar concentrations after stopping the infusion of 6MP. In goats combined steady state plasma concentrations of 6MP and 6MPR were 10 µM. In patients a mean 6MP concentration of 6.2 µM was observed during steady state by Zimm and coworkers. Moreover, these concentrations were sustained during the infusion, so the prolonged exposure necessary in vitro to achieve an important cytotoxic effect, is simulated by these prolonged infusions.

For 6TG less data are available, however the penetration of 6TG into the CSF is excellent. In goats a steady state 6TG concentration of about 3 µM was attained after a two-hours infusion at a dose rate of 2 mg/kg/hour. However, the concentrations of 6TGR exceeded those of 6TG itself, resulting in a combined 6TG/6TGR steady state concentration of 10 µM.

The prolonged infusion of 6TG at approximately 2 mg/kg/hour should be studied further in humans, because interspecies metabolic differences are an obstacle to transposing data from animals to man.

Some important points should be taken into account, when applying high dose infusions of 6MP and 6TG. Both 6MP and 6TG are S-phase specific agents, showing a delayed cytotoxic action. The incorporation into DNA, for both 6MP and 6TG, as 6-thio-dGTP is a critical step in the cytotoxic mechanism. So it is clear that 6MP and 6TG can only exert an optimal cytotoxic effect on actively proliferating tumours. In human leukaemia this situation is most likely to be present after a haematological remission is induced by other, non-phase specific, anticancer drugs. 6TG and 6MP given as continuous infusions are likely to have their best application in intensive consolidation courses. The excellent penetration of 6TG and 6MP into CSF can be of particular value, for in leukaemia the central nervous system is considered to be a pharmacological sanctuary. In the treatment of micrometastasis, a situation in which tumour cells are actively proliferating, 6TG and 6MP may have some activity too. DeVita (1983) discussed excellently the manifold aspects of the relationship between tumour mass and chemotherapy (30). The predominating problem is that in the phases of treatment as mentioned above, the effect of drugs cannot be determined directly, because the tumour cells to be destroyed are not visible and measurable. The concepts and methods presented eloquently by James Holland (1983), in an important overview entitled: "Breaking the cure barrier", are helpful in understanding and measuring indirectly the effects of chemotherapy on invisible tumour loads (31).

Metabolic effects of 6MP, 6TG and their metabolites may interfere with the cytotoxic effects. This is signalled by in vitro studies, which showed that there may exist a concentration range for an optimal cytotoxic effect, with decreasing cytotoxicity at increasing concentrations (32, 33). The rule of "the higher the dose, the greater the cell kill", which applies to cytotoxic agents in general, may not necessarily be true for 6MP and 6TG.

The interaction of 6MP and 6TG with other drugs needs more investigation. Not so much the well-known interaction of 6MP and allopurinol, but especially interactions which could result in an enhanced incorporation of 6MP and 6TG into DNA and RNA should be considered. The intra-

cellular PRPP concentration is critical for the first anabolic step of 6MP and 6TG. PRPP concentrations can be increased in vitro by pyrroline-5-carboxylate (34), or by previous exposure of cells to methotrexate (35, 36). Such an approach will complicate clinical drug therapy, but may increase the efficacy of 6TG and 6MP as anticancer drugs. Besides time-dependent metabolic and cytotoxicity increasing effects, the combination of MTX and 6MP may have advantages as fas as resistance to thiopurines is concerned. The resistance to 6MP and 6TG is often coupled with an increased sensitivity to inhibitors of the purine de novo synthesis (37), and MTX is one of the more potent of such inhibitors. Another mechanism of resistance to thiopurines, the increasing catabolism of thio-nucleotides by increased alkaline phosphatase activity (38), can be handled by prolonged infusions which lead to continuous delivery of 6MP and 6TG to malignant cells. Of course, thiopurines used either alone or in combination with for instance MTX, are best alternated with other non-cross-resistant chemotherapy (39).

In order to apply infusions of 6MP and 6TG for 24 hours and longer, in the treatment of leukaemias, we first should give up the concept of standard maintenance therapy. The limited value of maintenance treatment in malignant diseases, which are curable by chemotherapy, has been discussed extensively by Frei and Canellos (7). The appreciation that a maintenance approach is inadequate in preventing relapse in adult ALL patients led to the design of intensive, multidrug programs, resulting in remissions persisting for more than 3 years in 40% to 60% of adult patients (40). The essential role of curative consolidation chemotherapy in AML was recently discussed in a leading article in the Lancet. At the end of the paper the following question was asked regarding the treatment of childhood ALL patients: "Might one achieve equally good or even better results with curative consolidation chemotherapy instead by low-dose maintenance therapy?" (41).

CONCLUSION

In our opinion high-dose prolonged infusions of 6MP and 6TG deserve further investigation in man. In this way the in vitro optimal conditions for maximal cell kill can be simulated. Although 6MP infusion has

received somewhat greater attention, there are arguments to included and even favour 6TG (Chapter VII, VIII).

By administering 6MP and 6TG as prolonged infusions, the ability of these drugs to reach the CNS may be exploited best. So 6MP and 6TG can turn out to be effective drugs in the prophylaxis and treatment of CNS leukaemia. Besides 6TG and 6MP, their ribosides are worth considering, because 6TGR and 6MPR are more soluble than their parent drugs. Our findings in goats on the presence of 6TGR and 6MPR after administration of 6TG and 6MP call attention to the fact that also their active metabolites should be taken into account, when considering the total biologically active drug concentration.

Anyhow, we should investigate 6MP and 6TG in a clinical setting, without the prejudice of customary maintenance treatment of ALL. The fact that intracellular processes are ultimately responsible for the cytotoxic action of 6MP and 6TG should not hold us back to perform further pharmacological investigations. The most critical pharmacological principle that must always be recognized in attempting to improve drug treatment of cancer is "an otherwise effective drug will not kill a drug-sensitive tumour cell that it cannot (or does not) reach in lethal concentration" (42).

Ideally one should not only measure all relevant metabolites of 6MP and 6TG in plasma, CSF and urine during pharmacological and pharmacokinetic studies, but also try to quantitate metabolites intracellularly, and preferably in the target cells.

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Bij de behandeling van kinderen met leukemie neemt 6-mercaptopurine (6MP) een centrale plaats in. De antimetaboliet 6MP wordt al meer dan 30 jaar gebruikt op nagenoeg dezelfde wijze, namelijk dagelijks een lage dosis oraal gedurende een lange periode. Na literatuurstudie bleek nog weinig bekend over de relatie van dosering en daaruit voortvloeiende concentraties van 6MP in bloed en andere lichaamsvloeistoffen.

6MP is een analoog van de purine base hypoxanthine. Purine basen zijn natuurlijke stoffen die essentieel zijn voor de vorming van ribo- en deoxyribo-nucleïne zuren. 6MP kan worden ingebouwd in DNA en RNA en dit kan uiteindelijk leiden tot de dood van de (kwaadaardige) cel.

De toepassing van 6MP binnen de behandeling van acute lymfatische leukemie berust tot nu toe op empirische basis. De vraag stelt zich of en in hoeverre de gebruikelijke dosering en wijze van toediening zodanig zijn, dat een optimaal resultaat bereikt wordt met een minimale toxische werking. Voor 6-thioguanine (6TG), een analoog van de purine base guanine, geldt dezelfde vraagstelling. 6TG wordt hoofdzakelijk toegepast bij de behandeling van acute myeloide leukemie.

Binnen de afdelingen kindergeneeskunde en anthropogenetica was reeds een uitgebreide kennis op het gebied van de purine stofwisseling aanwezig en op deze basis werd het onderzoek naar de purine analogen 6MP en 6-thioguanine (6TG) opgezet.

Als eerste stap in het onderzoek is het noodzakelijk de concentratie van 6MP en 6TG in lichaamsvloeistoffen te bepalen. Men dient te beschikken over gevoelige bepalingsmethoden en omdat deze niet beschikbaar waren, werden die door ons ontwikkeld. Deze hogedruk vloeistof chromatografische methoden worden beschreven in de hoofdstukken II en III. De minimale detectiegrens hiervan kon worden verlaagd tot 3 ng/ml = 16 nmol/l.

In hoofdstuk IV wordt een oriënterend onderzoek beschreven bij patienten met acute lymfatische leukemie die het protocol volgen van de SNWLK. Dit houdt ondermeer in dat zij een z.g. onderhoudstherapie krijgen, waarbij dagelijks 6MP toegediend wordt in een dosering van 50 mg/m^2, met daarnaast eenmaal per week een dosis methotrexaat. Dit onderzoek gaf de navolgende resultaten. De gemiddelde concentratie van 6MP na 35 dagen

orale toediening is 0.064 µM in het plasma en 0.029 µM in het hersenvocht (liquor cerebrospinalis). Tevens wordt vastgesteld dat 6MP één tot vier weken na het stoppen van de therapie nog steeds in het plasma aantoonbaar is. Om nader inzicht te krijgen in deze fenomenen diende dierexperimenteel onderzoek te geschieden.

In hoofdstuk V wordt een onderzoek bij de hond beschreven, waarbij 6MP in analogie met de gebruikelijke onderhoudstherapie wordt gegeven. Na eenmalige orale toediening van 6MP aan de hond wordt een lage "piek" concentratie vastgesteld. Na intraveneuze "bolus" injektie wordt een redelijk hoge piek concentratie gevonden, doch vier uur later is 6MP nauwelijks meer in plasma aantoonbaar. Het concentratietijd beloop na bolus injektie past bij een twee compartimenten farmacokinetisch model, met halfwaarde tijden van 13 tot 21 en 125 tot 151 minuten. De bereikte plasma concentraties zijn laag, wanneer men deze vergelijkt met die welke in in vitro studies worden gebruikt. Om een maximaal celdodend effekt te krijgen lijkt het noodzakelijk 6MP intraveneus over een langere periode toe te dienen. Tevens is het van belang na te gaan hoe 6MP zich over het lichaam verdeelt.

In hoofdstuk VI wordt hierop nader ingegaan. 6MP wordt per infuus toegediend. De verdeling van 6MP en metabolieten over het lichaam wordt door middel van autoradiografie bestudeerd. Het verloop van de concentratie van 6MP en metabolieten wordt in plasma bepaald. Hierbij blijkt dat er een metaboliet, namelijk 6-mercaptopurine riboside (6MPR) in het plasma voorkomt en dat de concentratie van deze metaboliet, vooral na het stoppen van de 6MP toediening, relatief hoog is t.o.v. de 6MP concentratie. De metaboliet, 6MPR, kan alleen gemetaboliseerd worden tot 6MP. De biologisch beschikbare concentratie van 6MP kan daarom beter gezien worden als de som van de molaire concentraties van 6MP en 6MPR.

In de hoofdstukken VII en VIII ligt het accent van het onderzoek op langdurige intraveneuze toediening. 6TG, respectievelijk 6MP, worden d.m.v. kontinue infusie gegeven in een bepaalde dosering per tijdseenheid. Voor deze experimenten is de dwerggeit het meest aangewezen proefdier vanwege de ervaring, die wij bij eerder onderzoek met dit model hadden verworven. Zo kan het aantal proefdieren tot het minimaal noodzakelijke beperkt worden. Bij langdurige intraveneuze toediening mag men verwachten dat het plasma concentratie-tijd verloop beter vergelijkbaar is met de concentratie en tijdsduur die in vitro nodig zijn om een maxi-

maal celdodend effekt te bereiken. Bij deze onderzoeken op geiten waren we speciaal geïnteresseerd in de mate waarin 6MP en 6TG in het hersenvocht doordringen.

Bij geiten blijkt de concentratie van het riboside van zowel 6TG als 6MP de concentratie van de oorspronkelijk toegediende verbinding in ruime mate te overtreffen, zowel tijdens als na het stoppen van de infusie. Bij geiten worden dus de biologisch beschikbare concentraties van 6TG en 6MP in belangrijke mate mede bepaald door die van 6TGR en 6MPR. De penetratie van 6TG en 6MP in het hersenvocht is uitstekend. Ook de ribosiden dringen uitstekend door in de liquor cerebrospinalis.

In de urine wordt het grootste gedeelte van de toegediende dosis van 6MP, resp. 6TG terug gevonden, zowel in de vorm van de oorspronkelijke verbinding als in de vorm van het riboside. De concentratie van 6MP en 6MPR, resp. 6TG en 6TGR, in de urine is globaal gelijk.

In hoofdstuk IX geven we een overzicht over de resultaten van onze eigen onderzoeken en we bespreken in het kort recente onderzoeken die in de literatuur gepubliceerd zijn. De gebruikelijke orale toediening van 6MP en 6TG resulteert in nauwelijks meetbare concentraties in plasma, terwijl in in vitro onderzoeken hoge concentraties gedurende een langere periode nodig zijn om een maximaal celdodend effekt te verkrijgen. De toepassing van purine analogen op klinische en empirische basis lijkt achterhaald. Farmakologische en metabole onderzoeken bij proefdieren en mensen moeten alsnog worden verricht om een wetenschappelijke basis te verschaffen voor de toepassing van 6MP en 6TG.

We konkluderen dat onderzoeken, zoals door ons bij proefdieren zijn verricht, voortgezet moeten worden bij de mens. Door 6TG of 6MP per langdurig infuus in vrij hoge dosering toe te dienen, kunnen we zorgdragen voor de aanwezigheid van relatief hoge concentraties van 6MP of 6TG in het bloed gedurende een lange periode. Tevens scheppen we dan de beste voorwaarden waaronder 6MP en 6TG in de hersenen en in het hersenvocht kunnen doordringen. Dit zou er toe kunnen leiden dat 6MP en 6TG een andere rol in de behandeling van kwaadaardige aandoeningen kunnen gaan spelen. Te denken valt aan de zogenaamde profylaxe en de behandeling van leukemie in het centraal zenuwstelsel en de behandeling van hersentumoren.

Het schrijven van een proefschrift mag soms lijken op een eenzame reis door nog niet in kaart gebrachte gebieden; de steun van velen bij de voorbereidingen en de reis zelf is onontbeerlijk. Allereerst wil ik diegenen danken die niet met name genoemd worden of omdat de regels dit verbieden of omdat mijn geheugen te kort schiet. Onder de eerste kategorie vallen mijn promotor en de co-auteurs bij de artikelen. De aktieve bijdrage aan de voltooiing van het proefschrift die de promotor ondanks of juist dankzij zijn emeritaat heeft geleverd, zal ik mij dankbaar blijven herinneren. De tweede kategorie is niet nader te duiden.

Mijn analisten, John van Baal en Marinella van Leeuwen, dank ik voor de essentiële rol die zij speelden in de ontwikkeling van de bepalingsmethoden en de uitvoering van bepalingen en dierexperimenten. John dank ik speciaal voor het in kaart brengen van de gegevens. De schema's en grafieken in het proefschrift werden door hem vervaardigd en samen met hem werden de illustraties in het manuscript verwerkt.

Bij de voorbereiding en uitvoering van de dierexperimenten was de hulp van vele medewerkers van het Centraal Dierenlaboratorium (hoofd Dr.W. J.I. van der Gulden) onmisbaar; speciaal Theo Arts, Gerrie Grutters en Albert Peters wil ik daarvoor danken.

De medewerking van de afdeling Medische Fotografie (hoofd A.T.A. Reijnen) bij het verwerken van de vele illustraties was lofwaardig.

De medewerking van de medische bibliotheek, onder de deskundige leiding van het hoofd E.de Graaff, evenzo.

Het leesbaar maken van de handgeschreven versies van het manuscript is de verdienste van Loes Viering. De latere versies van de artikelen en hoofdstukken werden verzorgd door Janny Palthe en Kitty van den Tempel. Loes, Janny en Kitty excuses voor het drammen dat ik deed, en mijn grote dank; vooral Kitty die de vele "allerlaatste" versies en het drukklaar maken van het manuscript verzorgde.

Behalve de vele co-auteurs heeft met name Dr.J.A.J.M.Bakkeren steeds gewaakt over een respectvol gebruik van de Engelse taal; als ik daarin geslaagd ben is dat voor een groot deel zijn verdienste.

Mijn gezinsleden, Ineke, Lyke en Toke, zijn vaak "lijdende" personen geweest, niet alleen door de spanning die promoveren met zich meebrengt,

maar ook door andere faktoren, die ik hier niet verder wil benoemen. Het gedicht van Toke verwoordt deze spanningen beter dan ik dit kan. Mij leidt dit en de afronding van het proefschrift tot pogingen om inhoud te geven aan de volgende regel uit het lied "enkel en alleen" (R. Chrispijn, Herman van Veen): "Langzaam begin ik te begrijpen waar het allemaal om draait".

CURRICULUM VITAE

Teunis Jan Schouten was born in Meerkerk (Z.H.), the Netherlands, on December 22, 1945. The diploma of secondary school "Hogere Burger School-B", was obtained June 1964 in Utrecht, the Netherlands.
The study of medicine was started in October 1964 and the doctoral examination was passed July 7, 1969 at the State University of Groningen, the Netherlands. During the predoctoral period he participated in an epidemiological field study and in a respiratory function laboratory study on chronic aspecific respiratory afflictions (CARA).
From December 1969 till March 1972 he received his general clinical training in the hospitals of Deventer, the Netherlands. This training was interrupted by a residency in pediatrics for 7 months.
On March 9, 1972 he passed his examination as general physician at the State University of Groningen.
From 1972 till 1977 the training in pediatrics was completed. The first two years at the St.Elisabeth Hospital, Willemstad, Curacao, the Netherlands Antilles (head of the department: Prof.Dr.C.A.Winkel) and for the next three years at the University Hospital of Groningen (head of the department Prof.Dr.J.H.P.Jonxis). During his training in pediatrics the examination of the Educational Commission for Foreign Medical Graduates (USA) was passed (July 23, 1975). From April 1977 till May 1978 he was in private practice in the St.Joseph Hospital, Kerkrade and St.Elisabeth Clinic, School of Midwives Heerlen. Since May 1, 1978 he is at the department of Pediatrics, St.Radboud University Hospital Nijmegen, the Netherlands. He is particularly engaged in the care of children with malignant diseases and in research related to this field. Besides preparing a thesis "6-Mercaptopurine and 6-thioguanine: a fresh look on vintage anti-cancer drugs", several other articles were published. Moreover he is involved in medical education, especially as organizer of postgraduate refresher courses for pediatricians working in private clinical practice.

Teunis Jan Schouten has been married to Ineke Kranenborg since December 23, 1967. They have two lovely daughters, Lyke and Toke.

List of publications authored and co-authored by SCHOUTEN Teunis Jan; December 22, 1945.

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STELLINGEN

Behorende bij het proefschrift

6-MERCAPTOPURINE AND 6-THIOGUANINE: A FRESH LOOK ON VINTAGE ANTICANCER DRUGS

In het openbaar te verdedigen
op donderdag 23 mei 1985
des namiddags te 4.00 uur

door

TEUNIS JAN SCHOUTEN

I

De tot nu toe gebruikelijke toepassing van 6-mercaptopurine en 6-thioguanine, uitsluitend gebaseerd op empirisch-klinische ervaring, is achterhaald.

II

De voorspelbare en o.a. in dit proefschrift aangetoonde goede penetratie van 6-mercaptopurine en 6-thioguanine in de liquor cerebrospinalis is bij de mens, met name wat betreft de therapeutische effecten, onvoldoende onderzocht.

III

De toediening van 6-mercaptopurine en 6-thioguanine per langdurig infuus dient bij de mens nader onderzocht te worden.

IV

Het in vitro aangetoonde metabole synergisme van methotrexaat en 6-mercaptopurine lijkt ook bij patiënten te kunnen worden benut.

(J P.M. Bökkerink, T.J. Schouten, R.A. De Abreu, et al. Tijd Kindergeneesk 1984; 52:118-123, J.P.M. Bökkerink, persoonlijke mededeling)

V

Onderhoudstherapie bij een curatief te behandelen maligniteit is onjuist.

(E. Frei III and G.P. Canellos, Am J Med 1980; 69:585-594)

VI

De kans op ziektevrije overleving van acute lymfatische leukemie is afhankelijk van de initiële therapie.

VII

Restverschijnselen van neurologische, endocriene en psychologische aard, bij een kind dat optimaal behandeld is voor een hersentumor, vereisen een "innige" multidisciplinaire samenwerking en aanpak.

VIII

Bij maligne histiocytose hoort, indien enigszins mogelijk, chromosomaal onderzoek te worden verricht.

(T.J. Schouten, T.W.J. Hustinx, J.M.J.C. Scheres, et al. Cancer 1983; 52:1229-1236)

IX

Interferon is van grote betekenis voor patiënten met ernstige vormen van juveniele papillomatosis van de larynx.

(T.J. Schouten, W Weimar, J H Bos, et al Laryngoscope 1982, 92 686-688)

X

Bij het ernstig benauwde stridoreuze kind hoort d.m.v. een "dwarse hals" röntgenopname een epiglottitis te worden aangetoond c.q. uitgesloten.

XI

Voor het vaststellen van het tijdstip van starten van C.A.P.D. (continue ambulante peritoneaal dialyse) bij het jonge kind is te weinig rekening gehouden met de mogelijkheid van progressieve hersenbeschadiging bij conservatieve behandeling.

(A. Rotundo, T.E Nevins, M. Lipton, et al Kidney Int 1982, 21 486-491)

XII

De optimale concentratie van koper, ijzer en zink van de geadapteerde zuigelingenvoeding is niet eenvoudig af te leiden uit de concentraties van deze stoffen in moedermelk.

(R M Feeley, R R Eitenmiller, J B Jones, et al. Am J Clin Nutrition 1983, 37 443-448)

XIII

Als wetenschappelijke medewerkers worden geacht te publiceren en te promoveren, dan dient de administratieve formatie van een afdeling hierop afgestemd te zijn.

XIV

Ook voor het gezin van de promovendus is promoveren een kwestie van afzien.

XV

In overdrachtelijke zin is het op sloffen promoveren plezierig, maar tijdens het schrijven van het proefschrift zijn klompen boven sloffen te verkiezen

Nijmegen, 23 mei 1985 Teunis J. Schouten