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Note

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

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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

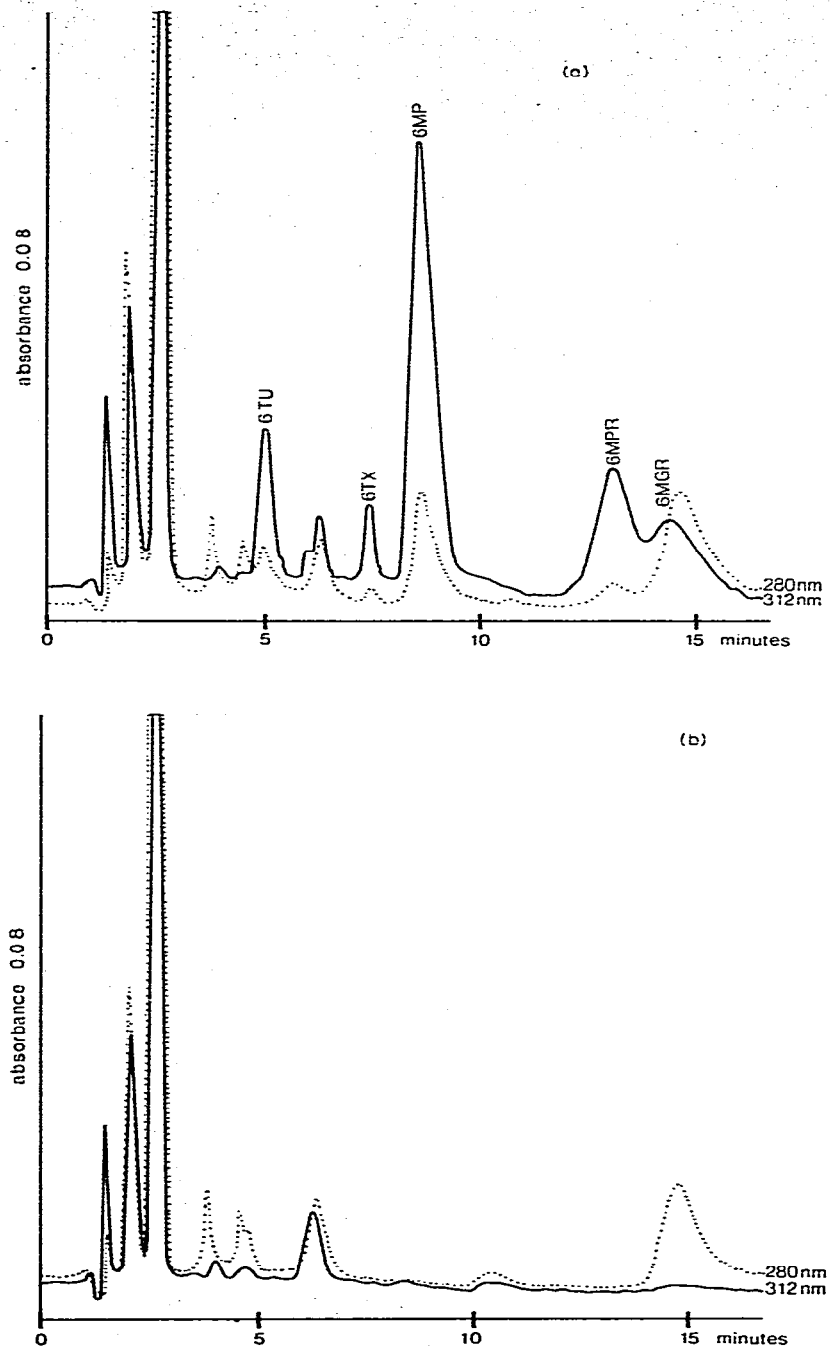
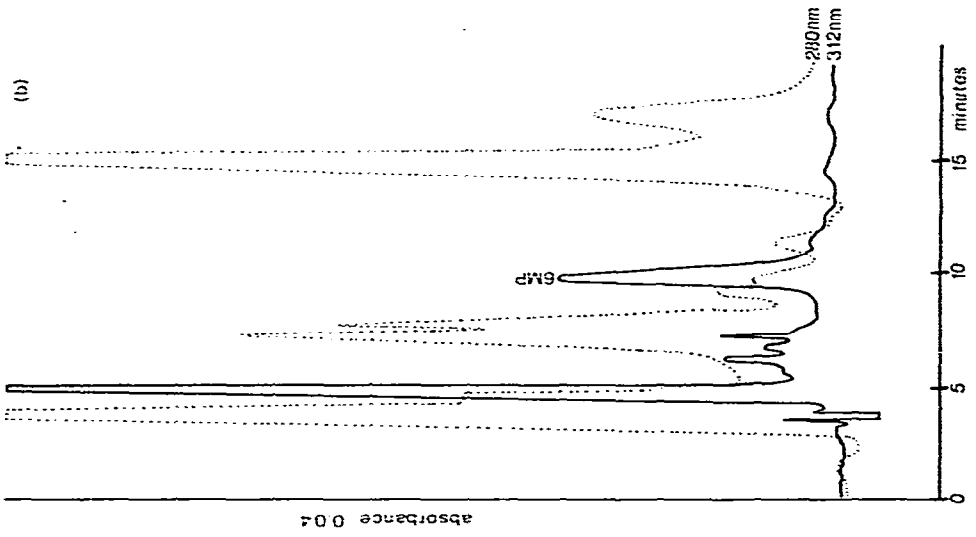
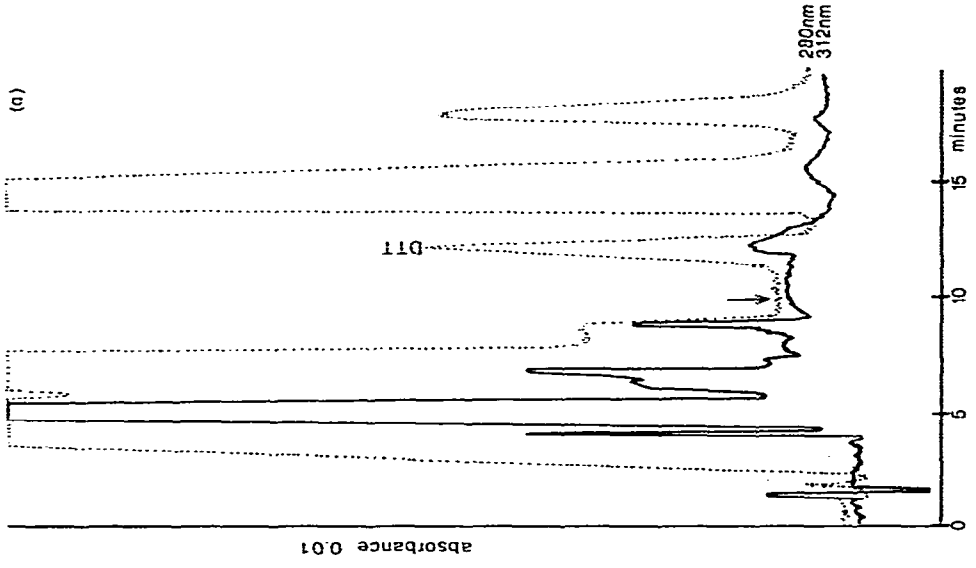


Fig. 1. Elution profiles of the separation of 6MP, 6MPR and 6MGR on Spherisorb-ODS columns (for separation conditions, see Experimental). (a) Chromatogram of human plasma sample with added 6-thiouric acid (6TU), 6-thioxanthine (6TX), 6MP, 6MPR and 6MGR. (b) Chromatogram of human plasma sample without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


(b)
280nm
312nm
6MP
0
5
10
15
minutes
absorbance 0.04


(a)
280nm
312nm
DTT
0
5
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15
minutes
absorbance 0.01

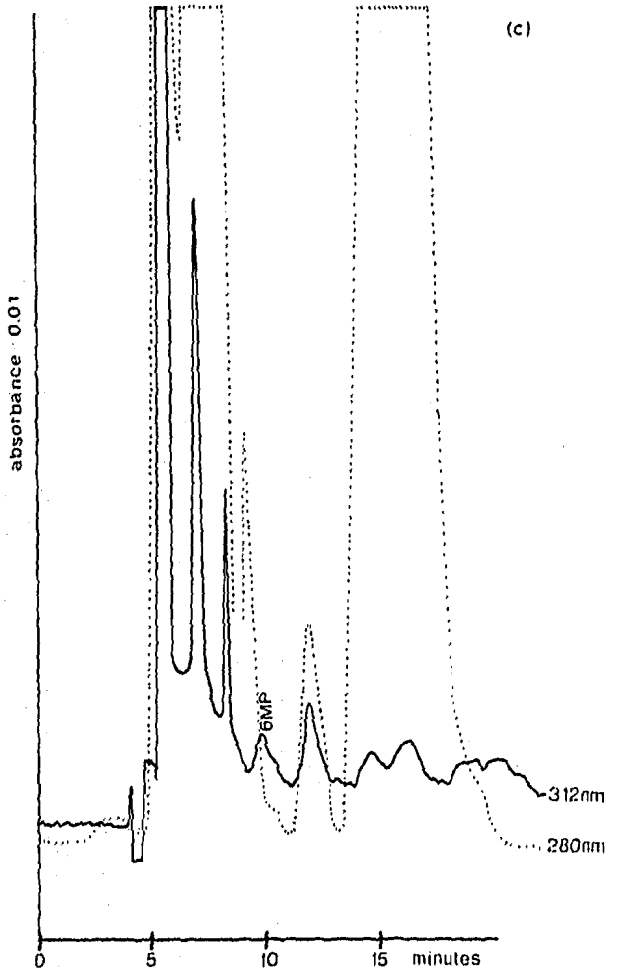

Fig. 2. HPLC of 6MP in plasma of a 28-kg Labrador dog. (a) Plasma sample collected before 6MP administration. (b) Plasma sample collected 10 min after intravenous injection of 50 mg of 6MP. (c) Plasma sample collected 5 h after intravenous injection of 50 mg of 6MP.

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.

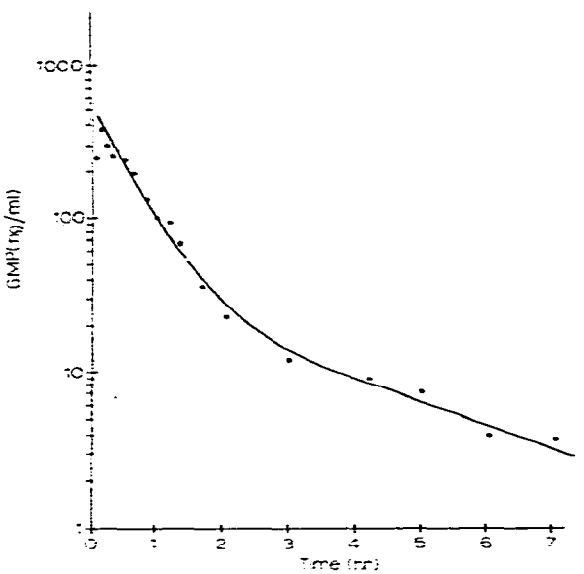

Fig. 3. Plasma levels of 6MP as a function of time after intravenous injection of a 28-kg Labrador dog with 50 mg of 6MP.

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.

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