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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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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.

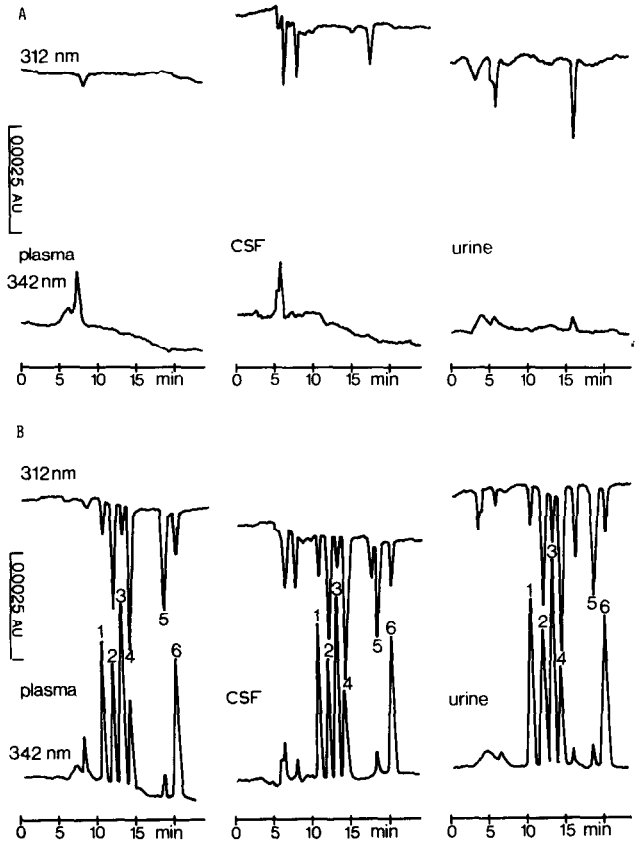

Fig. 1. Scan patterns of separations of 6-thiopurines, following the mobile phase sequence of Table I (injected volume 195 μl). (A) Blanks of plasma, CSF and urine samples; (B) plasma, CSF and urine samples, spiked with 6TU (1), 6TX (2), 0.7 μM 6TG (3), 0.4 μM 6MP (4), 0.4 μM 6MPR (5) and 0.7 μM 6TGR (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

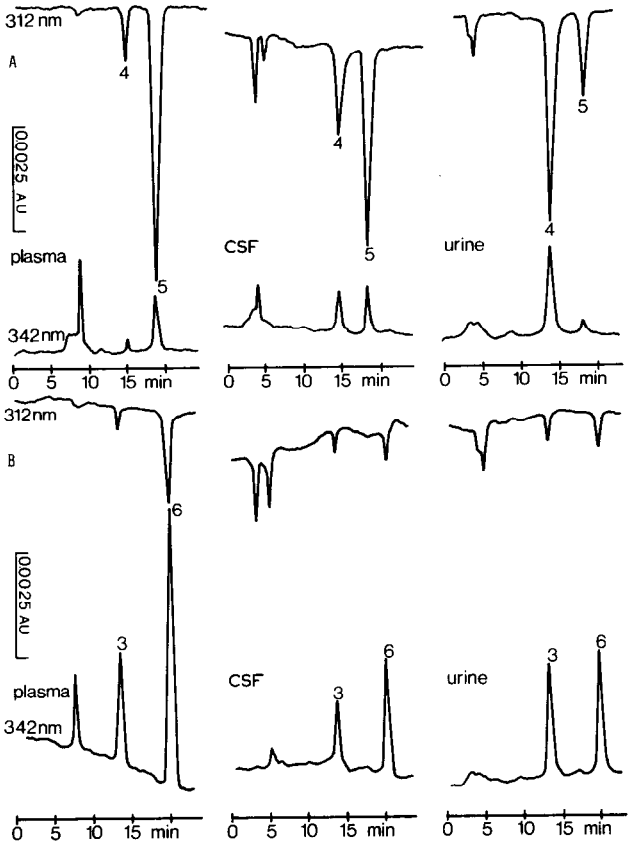

Fig. 2. Scan patterns of plasma, CSF and urine samples (injected volume 195 µl) of a goat, 4 h after an intravenous injection. (A) 6MP (20 mg/kg body weight), urine sample is diluted 5000 times before injection; (B) 6TG (5 mg/kg body weight), urine sample is diluted 25 times before injection. For absorbance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

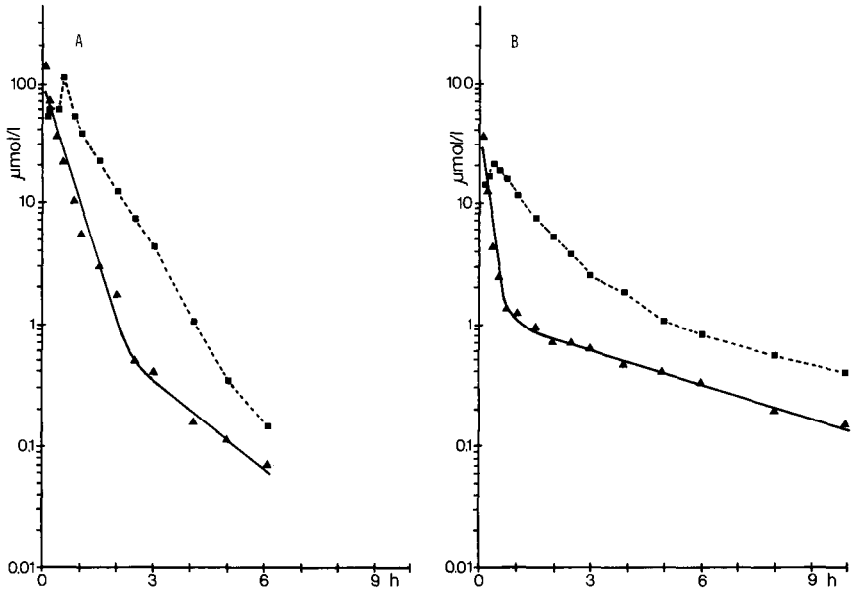

Fig. 3. Concentration—time curves of plasma levels in a goat after an intravenous injection. (A) 6MP (20 mg/kg body weight); (▲) 6MP, (■) 6MPR. (B) 6TG (5 mg/kg body weight); (▲), 6TG; (■), 6TGR.

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.

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