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Note

Assay of 6-mercaptopurine in human plasma

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The cytotoxic drug 6-mercaptopurine (6MP) is extensively used in the treatment of childhood lymphoblastic leukaemia to prolong the duration of remission induced with other drugs, but the response to maintenance therapy varies widely [1]. Inter-individual differences in pharmacokinetics may explain some of these unpredictable responses.

The precise mode of action of 6MP as an antileukaemic drug is not fully understood. In vitro studies have shown that cytotoxicity can be related to the incorporation of 6MP-derived 6-thioguanine nucleotide (6TGN) into DNA [2]. We have shown that the red blood cell concentrations of the active 6MP metabolite 6TGN can be used as an index of the cytotoxic activity of 6MP [3, 4]. Red cell concentrations of 6TGN are a reflection of the cumulative dose of 6MP rather than the daily dose prior to assay [4, 5].

Several methods have now been published for measuring plasma 6MP concentrations [6—12]. Because of the difficulties involved in extracting and quantifying such a polar, light sensitive compound [13] from biological fluids, no one method has achieved universal recognition. For several years we have been assaying the thiopurine drugs 6MP and 6-thioguanine, plus their metabolites, by direct fluorometric assays [14—17]. We have previously quantitated plasma 6MP concentrations in clinical studies [18] by a modification of the method published by Maddocks [14]. This assay, although selective and sensitive, is cumbersome and time-consuming.

This paper reports a reversed-phase high-performance liquid chromatographic (HPLC) assay for 6MP, based on the extraction of the drug as its phenyl mercury derivative coupled to the UV detection of the subsequently

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liberated thiopurine. The assay is sufficiently sensitive and robust for routine clinical use.

EXPERIMENTAL

Materials

6-Mercaptopurine (6MP), 6-thioguanine (TG; 2-amino-6-mercaptopurine), 6-thioxanthine (TX; 2-hydroxy-6-mercaptopurine), 1-heptanesulphonic acid and DL-dithiothreitol (DTT) were obtained from Sigma (London, U.K.). 6-Thiouric acid (TU; 2,8-dihydroxy-6-mercaptopurine) was obtained from Calbiochem (CP Labs, Bishop Stortford, U.K.). Stock solutions (100 μg/ml) of the thiopurines were prepared by dissolving 10 mg in 0.1 *M* sodium hydroxide (4 ml) in a light-protected container at 4°C. Water was then added before the addition of 1 *M* hydrochloric acid to a final concentration of 0.1 *M*. The stability of the thiopurine stocks, at 4°C, was one month, three months, one month and 48 h for TU, 6MP, TG and TX, respectively.

Methanol (HPLC grade) and toluene (glass-distilled) were obtained from Rathburn Chemicals (Walkerburn, U.K.). Amyl alcohol, AnalaR grade (BDH, Poole, U.K.), was added to the toluene at a final concentration of 170 m*M*, before use. Phenyl mercury acetate (organic, analytical standard; BDH), 89 m*M*, was prepared weekly by dissolving the compound in gently boiling water. All stock solutions were protected from light during storage. All other reagents were of analytical-reagent grade and obtained from BDH. With the exception of the mobile phase, in which the water used was distilled, water used for all solutions was distilled and deionised. The glassware used for the analysis of thiopurines was soaked overnight in 30% nitric acid and rinsed in distilled deionised water.

Sample preparation

For the study of plasma concentration versus time profiles 5-ml samples of blood were collected in lithium heparin tubes at 0, 15, 30, 45, 60, 90, 120, 150, 180, 240 and 300 min after an oral dose of 6MP. The whole blood was centrifuged immediately to prevent the cellular metabolism of 6MP to its nucleotide thioinosinic acid [16, 17]. The sample was spun at 400 *g* for 10 min at 10°C and the plasma pipetted into a 5-ml polystyrene tube which was then placed on ice. All plasma samples were stored at −20°C within 2 h of sampling.

Extraction of 6MP

To a sample volume of 1 ml, 0.1 ml of 10 m*M* DTT, 0.1 ml of 2 *M* sodium hydroxide, 2 ml of 89 m*M* phenyl mercury acetate (PMA) and 8 ml of toluene were added in a 10-ml round bottomed graduated test-tube. The tubes were shaken for 10 min and centrifuged at 900 *g* for 5 min at 10°C. The toluene, 7 ml, was transferred to graduated conical test-tubes and back-extracted with 0.2 ml of 0.1 *M* hydrochloric acid and, after shaking and centrifuging as before, the toluene was aspirated to the interface with the aqueous layer. A 50-μl aliquot was injected on to the chromatograph.

HPLC procedure

Samples (50 μl) were injected through a Rheodyne injector (HPLC Technology, Macclesfield, U.K.) fitted with a 0.1-ml sample loop on to a 25 cm × 3.9 mm μBondapak C_{18} column, particle size 10 μm (Waters Assoc., Northwich, U.K.). The analytical column was protected by a Guard-Pak (Waters) pre-column module containing a C_{18} insert. 6MP was detected with a Model 4020 variable-wavelength UV detector (Pye-Unicam, Cambridge, U.K.) set at 322 nm and a sensitivity of 0.02 a.u.f.s. Peaks were traced on a Model 6500 flat-bed potentiometer recorder (LKB, Croydon, U.K.) at 300 mm/h. A Model 6000A chromatography pump (Waters) was used for solvent delivery. The mobile phase of methanol—water (3:97, v/v) contained 0.2% glacial acetic acid and 0.1% 1-heptanesulphonic acid. The column was flushed daily and left standing in methanol—water (20:80, v/v).

Calibration

Calibration graphs were constructed by spiking control plasma with 6MP aqueous standard in the range 10—500 ng ml^{-1}. No suitable internal standard could be found for this assay. Therefore, 6MP plasma standards were prepared and treated in parallel with the patients' plasma samples.

Statistics

Mean values are presented ± standard deviation (S.D.). Coefficients of variation (C.V., mean ± S.D.) are expressed as a percentage. The extraction efficiency of plasma 6MP, as a function of concentration, was assessed for statistical significance using two-way analysis of variance.

RESULTS AND DISCUSSION

Plasma spiked with 6MP

The retention time of 6MP was 7.4 min and the sensitivity of the assay was 10 ng ml^{-1} of plasma for a 50-μl injection. The lower limit of the standard curve was defined as the reading twice the blank.

The calibration curves for 6MP were linear and passed through the origin (Table I). The mean extraction efficiency for twelve plasma 6MP standard

TABLE I

ASSAY REPRODUCIBILITY IN PLASMA

Mean of twelve 6-mercaptopurine curves over a three-month period.

Concentration (ng ml^{-1})	Peak height (mean ± S.D.) (cm)	Coefficient of variation (%)
500	45.95 ± 2.79	3.9
200	16.95 ± 0.91	5.4
100	8.68 ± 0.38	4.4
50	4.15 ± 0.26	6.3
20	1.30 ± 0.13	8.8
10	0.66 ± 0.05	7.3

curves, over a three-month period was 68 ± 3.3%. There was no statistical difference in the recovery as a function of concentration.

When protected from light the 6MP back-extract, in 200 μl of 0.1 *M* hydrochloric acid, was stable for 48 h at 4°C and at least 10 h at 20°C. This enables the storage of samples at 4°C until sufficient are processed to load an autoinjector. When loaded the autoinjector should be situated so that the samples are not exposed to direct sunlight and the ambient temperature should not exceed 20°C.

PMA extraction

When phenyl mercury—thiopurine adduct formation occurs at an alkaline pH, optimal for 6MP and TG, TU and TX are poorly extracted from plasma. Hydroxy group substituents increase the acidic strength of thiopurines [19], optimal complex formation with PMA occurring at a lower pH.

The purity of the water used to prepare reagents can influence the formation of the phenyl mercury—6MP adduct [17]. Decreased extraction efficiencies (45—55%) are observed with distilled deionised water of a conductivity < 4 mΩ. 6MP is susceptible to oxidative degradation in aqueous solutions. The presence of contaminant ions and UV light promotes this process [13, 20]. The extraction efficiency is restored to 68% by the addition of DTT, which prevents the oxidation of thiol groups [21], before the formation of the phenyl mercury derivative. The addition of DTT produced no apparent interference with the formation of phenyl mercury—6MP.

Analysis of other thiopurines

The retention times of TU, 6MP, TX and TG aqueous standards were 5.2, 7.4, 8.3 and 25 min, respectively (Fig. 1). Sharp symmetrical peaks are shown for TU, 6MP and TX. Due to its long retention time the peak for TG was broad and small. With the mobile phase composition and UV detection optimised for 6MP the detection limit for TG (λ_{max} 342 nm) was 200 ng ml^{-1}

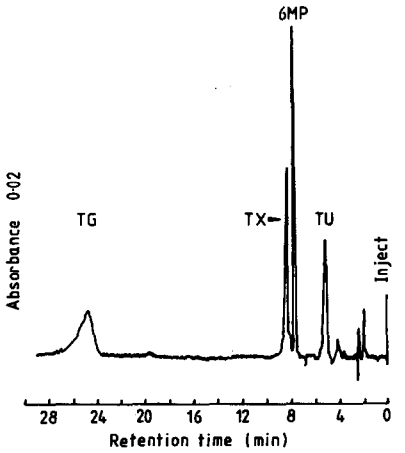


Fig. 1. Typical chromatogram of TU, 6MP, TX and TG aqueous standards. The retention times are 5.2, 7.4, 8.3 and 25 min, respectively. The injection of 50 μl of 0.1 *M* hydrochloric acid contained 100 ng TU, 20 ng 6MP, 20 ng TX, 100 ng TG and 0.05 m*M* DTT.

of plasma. Likewise, optimisation of extraction and detection for 6MP means that TU and TX are not detected when added to plasma at concentrations < 500 ng ml^{-1}.

Selectivity

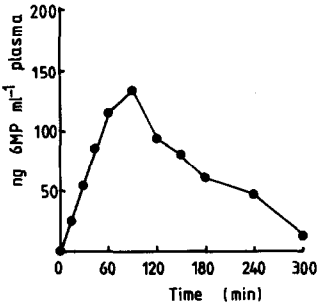
Several drugs that might be administered concurrently with 6MP were chromatographed to check for possible interference with the assay procedure. No interference was observed with methotrexate, prednisone, cytosine arabinoside, co-trimoxazole or caffeine. The following metabolites of 6MP did not interfere with the assay: 6-mercaptopurine riboside, 6-mercaptopurine riboside 5′-phosphate (thioinosinic acid), 6-thioguanine riboside and 6-thioguanine riboside 5′-phosphate.

Analysis of patient samples

The assay reported here was used to quantitate the plasma 6MP concentrations in a child with lymphoblastic leukaemia on remission maintenance chemotherapy. An oral dose of 6MP, 75 mg m^{-2}, was taken after an overnight fast. Fig. 2 illustrates the plasma 6MP concentration versus time profile. The 0-min patient sample was identical to the patient blank (Fig. 3), no interfering peaks were observed in the chromatograms.

Following an oral dose 6MP undergoes extensive intestinal and first-pass hepatic metabolism. The intestinal mucosa and the liver are the primary sites of the enzyme xanthine oxidase [22] which forms the inactive metabolites TU and TX which are excreted in the urine. Intestinal and hepatic anabolism, initiated by the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT), results in the formation of the intracellular nucleotide metabolites of 6MP. These metabolites are responsible for the cytotoxic effects of 6MP. Patients with the Lesch—Nyhan syndrome, who lack the enzyme HGPRT, do not develop 6MP mediated bone marrow toxicity [23].

The plasma concentration versus time profile gives a measure of the amount



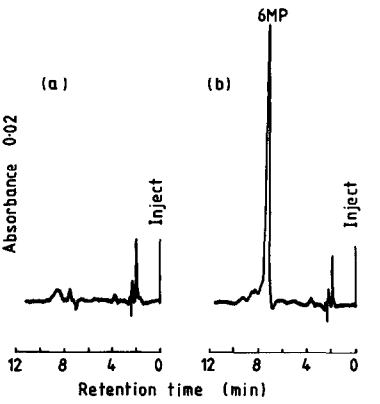


Fig. 2. Plasma concentration versus time profile after an oral dose of 6MP, 75 mg m^{-2}, during the remission maintenance treatment of acute lymphoblastic leukaemia. Area under the curve = 19928 ng/ml min.

Fig. 3. HPLC tracings after an oral dose of 6MP. (a) 0-min and (b) 60-min patient sample.

of drug available to the systemic circulation after an oral dose. Not surprisingly the observed plasma concentrations of 6MP in this study patient are low. This is in agreement with the limited bioavailability of 6MP reported in other studies [24].

Using this assay we are investigating the plasma concentrations of unmetabolised 6MP and the intracellular concentrations of the active metabolite 6-thioguanine nucleotide [17] with respect to the biological effectiveness of 6MP oral remission maintenance chemotherapy in childhood lymphoblastic leukaemia.

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