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Note

A novel method for the quantitation of 6-mercaptopurine in human plasma using high-performance liquid chromatography with fluorescence detection

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6-Mercaptopurine (6-MP) has been used since 1953 to induce and maintain remissions in both acute and chronic leukaemias [1, 2]. Most regimens utilise 6-MP in combination with other cytotoxic drugs such as methotrexate and vincristine (e.g. POMP regimen) [3]. Dosages in these regimens have remained fairly constant, usually 75 mg/m^2/day, although individuals may have their doses reduced to avoid toxic effects. Herber et al. [4] have shown that there is no correlation between drug dose and biological effects although the same laboratory has reported a relationship between the red cell 6-thioguanine nucleotide concentration and response in childhood leukaemia [5]. To evaluate whether there is a relationship between 6-MP plasma levels and response pharmacokinetic studies are needed. Confirmation of a relationship would allow "tailoring" of the drug dose to the individual patient to produce optimum response to treatment.

In the past, various methods have been used to quantitate 6-MP levels in plasma including colorimetric and fluorometric assays [6, 7], gas chromatography (GC) [8, 9] and high-performance liquid chromatography (HPLC) [10—12]. The colorimetric and fluorometric assays lacked the sensitivity needed for accurate pharmacokinetic studies and therefore GC and HPLC methods are preferred as they can combine both sensitivity and selectivity, although some methods have a long sample turnover time which produces problems when manual injection is the only method available [13, 14]. Jonkers et al. [15] have developed a fluorescent method but this method needs post-column derivatisation which requires specialised equipment. This laboratory has used various published methods [10—12] but we have found that plasma samples tend to produce interfering peaks which could not be removed by changing the extraction process and a different technique was sought.

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In 1981, Newton et al. [16] reported a method for selectively measuring biological thiols by their selective reaction with monobromobimane (mBBr) and subsequent separation on reversed-phase HPLC. On reaction with thiols mBBr produced highly fluorescent products.

This paper describes how the selective reaction of mBBr with thiols can be utilised to selectively and sensitively measure 6-MP levels in plasma using reversed-phase HPLC with fluorescence detection.

EXPERIMENTAL

Materials

6-MP, 2-mercaptopurine (2-MP), 6-mercaptopurine riboside (MPR), 6-mercaptopurine riboside-5^1-phosphate (MPRP), 6-methylmercaptopurine (6MMP), adenine (Ad), xanthine (Xan) and uric acid (UA) were all obtained from Sigma (Poole, U.K.). Cysteine (Cys), glutathione (GSH) and guanine (Gua) were purchased from BDH (Eastleigh, U.K.). Hypoxanthine (Hx) was procured from Nutritional Biochemicals (Cleveland, OH, U.S.A.). Methotrexate (MTX) was kindly supplied by Lederle (Gosport, U.K.) and mBBr was obtained from C.P. Labs. (Bishops Stortford, U.K.). Methanol was HPLC grade from Fahrenheit Laboratory Supplies (Rotherham, U.K.). Chloroform, acetone, glacial acetic acid and sodium hydroxide were all analytical grade obtained from May and Baker (Dagenham, U.K.)

Stock solutions of 1 mg/ml of MPR and MPRP were made up in methanol and stored at 4°C. 6-MP and possible cross-reactants were stored at 4°C as 1 mg/ml stock solutions in 0.02 *M* sodium hydroxide, mBBr was stored at −20°C as 100 μg/ml stock solution in acetonitrile.

All stock solutions were protected from light during storage.

Methods

Plasma (1 ml) and 2 ml of methanol were placed in a 5-ml screw-top vial, vortex-mixed for 30 sec and centrifuged for 10 min at 2750 *g* on a Beckman Model J-6 centrifuge (Beckman-RIIC, High Wycombe, U.K.). The supernatant was decanted into a 10-ml screw-top test tube and 6 ml of chloroform were added and mixed on a rotating mixer. The phases were then separated by centrifuging at 2750 *g* for 10 min. The chloroform layer was discarded and the aqueous layer was again washed with 6 ml of chloroform as above. After the second wash 500 μl of the aqueous phase was transferred to a 5-ml screw-top glass vial which had been precooled on ice for 10 min. A 25-μl aliquot of a 100-μg/ml solution of monobromobimane was added and the mixture incubated in the dark at 4°C overnight. After incubation the reaction mixture was put on ice and 25 μl of glacial acetic acid was added and vortex-mixed. The mixture was then centrifuged at 9950 *g* for 2 min in an Eppendorf 5412 bench centrifuge (Anderman & Co., East Molesey, U.K.) to remove any precipitate. A 50-μl aliquot of the mixture was injected into the HPLC system.

The chromatographic procedure was performed using a Kipp Analytica 9208 pump (MSE Scientific Instruments, Crawley, U.K.) connected to a Waters 420 AC fluorescence detector (Waters Assoc., Harrow, U.K.) equipped with a 395-nm band-pass excitation filter and a 455-nm long-pass emission filter.

A 150 × 5 mm, 5-μm Hypersil-ODS column (HETP, Macclesfield, U.K.) was used for the separation. The mobile phase consisted of acetone—water (20:80) with 1% glacial acetic acid added and buffered to pH 4.0 with 1 *M* sodium hydroxide. The flow-rate was 1 ml/min with a back pressure of 15 MPa. 6-MP standards in plasma underwent the extraction and analytical procedure in parallel with the quality controls and samples. The standard curve was obtained by plotting peak height of the 6-MP—mBBr adduct against concentration of 6-MP in each standard.

RESULTS AND DISCUSSION

Typical chromatograms obtained from blank plasma and plasma spiked with 6-MP are shown in Fig. 1. The 6-MP adduct has a retention time of 7.2 min which allows a good separation from other peaks. The sample turnover time of approximately 12 min allows rapid quantitation of samples.

Previously published methods for the quantitation of 6-MP in plasma using reversed-phase HPLC and UV detection tend to suffer from a long sample turnover time or a high background interference close to the peak of interest.

The procedure uses a similar sample preparation to Narang et al. [12] utilising the low solubility of 6-MP in organic solvents. Methanol is used to precipitate protein in the sample and is later selectively removed by washes in chloroform.

Plasma standards give a linear response over the range 20—500 ng/ml (equation of line $y = 0.221x + 3.73$, correlation coefficient = 0.999) with coefficients of variation being given in Table I. A quadratic function may also be

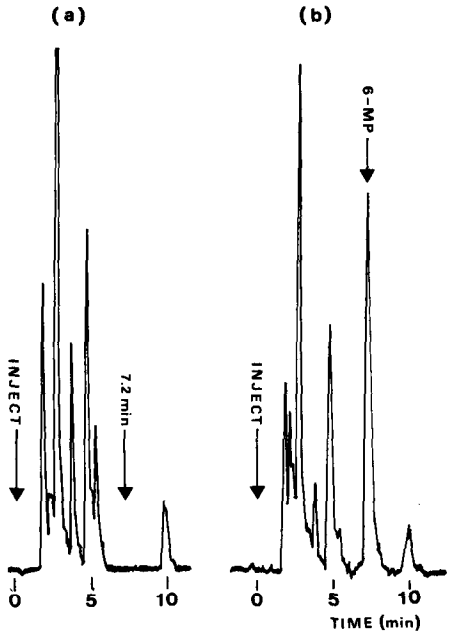

Fig. 1. Typical HPLC tracings of (a) blank plasma showing retention time of 6-MP peak; (b) plasma spiked with 500 ng/ml 6-MP.

TABLE I

MEAN OF FIVE STANDARD CURVES SHOWING STANDARD DEVIATIONS (S.D.) AND COEFFICIENTS OF VARIATION (C.V. %)

Concentrations of standards (ng/ml)	Mean peak height (mm)	S.D.	C.V. (%)
500	118.0	5.15	4.36
200	53.4	3.02	5.66
100	25.7	1.64	6.40
50	14.6	0.79	5.39
20	7.6	0.42	5.47

fitted to the data shown (equation: $y = 1.373 + 0.269x - 7.057 \cdot 10^{-5} x^2$). There is no significant difference between the standard curve fitted to this quadratic function and that fitted by linear regression as shown by a paired *t*-test. Also a paired *t*-test comparison of plasma samples calculated by both methods showed no significant difference. Within the limits of the standard curve specified, a linear response may be assumed, although beyond these bounds this correlation breaks down. We have found that the standard range is satisfactory for the measurement of therapeutic levels of 6-MP in patients and suggest that any samples above this range should be diluted until within the standard curve. Dilutions should be made with normal human plasma. The sensitivity of the assay, defined as the concentration of 6-MP giving a signal-to-noise ratio of 2:1, was approximately 20 ng/ml for a 50-μl injection. Adapting a theoretical definition [17], i.e. sensitivity equals 2 S.D. of the intercept at zero divided by the slope of the standard curve, the sensitivity was 18 ng/ml. Although a number of possible internal standards were considered no suitable compound was found. However, an internal standard, though desirable, is not essential in the method as described, since the standards employed are prepared in a matrix, i.e. plasma, identical to that of the sample, and are subsequently treated in parallel with them. Inter-assay and intra-assay coefficients of variation are shown in Table II for two quality controls.

No interference was found with the following compounds: 2-MP, MPR, MPRP, 6MMP, Ad, Xan, UA, Cys, GSH, Gua, Hx or MTX. Thiols in plasma, such as cysteine, do react with bimanes producing peaks near the solvent front (i.e. 1—4 min after injection). With the standard range quoted above, endogenous thiols do not affect the reaction with 6-MP since addition of more bimane after incubation does not increase the 6-MP peak suggesting that this reaction has gone to completion.

TABLE II

INTER-ASSAY AND INTRA-ASSAY VARIATION OF HIGH AND LOW QUALITY CONTROLS (HQC AND LQC) MADE FROM POOLS OF PATIENTS PLASMA

	Inter-assay (n = 4)			Intra-assay (n = 5)		
	Mean concentration (ng/ml)	S.D.	C.V. (%)	Mean concentration (ng/ml)	S.D.	C.V. (%)
HQC	160.0	6.63	4.14	162.1	6.20	3.82
LQC	34.6	3.29	9.48	33.1	3.14	9.46

We have found that the reaction of bimane with 6-MP will proceed to completion in 2 h at room temperature. However, a problem arose with this incubation method since a small interfering peak was seen in blank plasma which effectively decreased the sensitivity of measurement to 50 ng/ml. This interfering peak is not seen in the 4°C incubation and is probably a breakdown product of the bimane. This method was used to measure the pharmacokinetics of 6-MP in two acute lymphoblastic leukaemia (ALL) patients on maintenance therapy. The results are shown in Fig. 2. The patients were given their normal oral dose of 6-MP after an overnight fast and on a separate occasion after a standard breakfast. It can be seen from the results that there is a substantial decrease in absorption and bioavailability of the drug, as measured by AUC, when the dose was administered after a meal. The peak plasma levels obtained in the two subjects after an overnight fast correlate with levels published by Zimm et al. [18]. In their paper Zimm et al. suggest that the presence of food in the stomach during oral administration of 6-MP may cause variations in plasma levels. Our preliminary results confirm this suggestion and further studies are now underway to investigate this observation more closely.

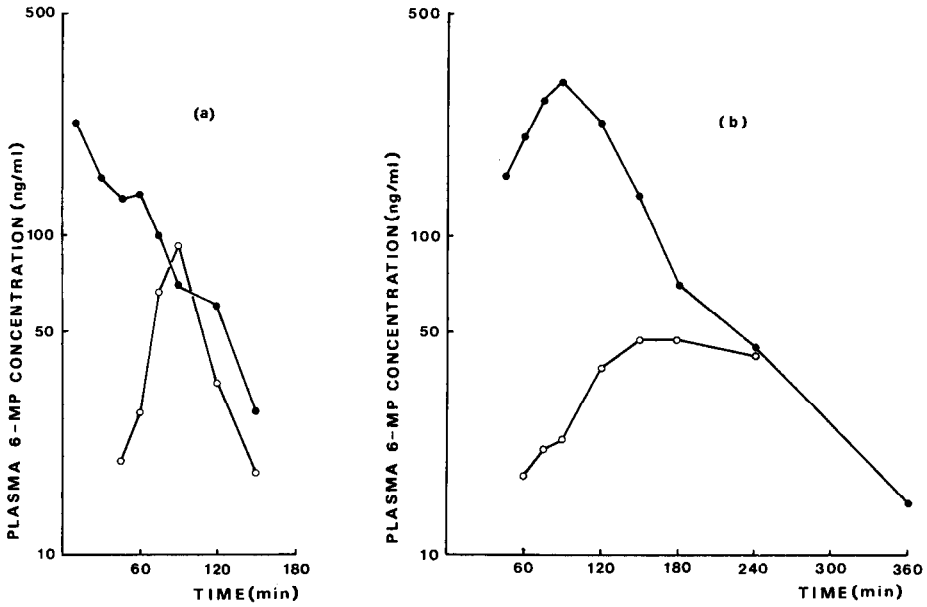

Fig. 2. Pharmacokinetics following oral administration of 6-MP in two patients with ALL. (a) Patient JR: age 10 years, dose 60 mg; (b) patient MB: age 63 years, dose 125 mg. •, Dose administered after overnight fast; ○, dose administered 15 min after standard breakfast. Area under curve (AUC) (ng/ml min) patient JR: post fast: 13255, post breakfast: 4693; patient MB: post fast: 33772, post breakfast: 6952.

Although we have only used the procedure for the measurement of pharmacokinetic samples, by decreasing incubation time to 1 or 2 h it could be used with decreased sensitivity for therapeutic drug monitoring.

The method described allows a specific and sensitive assay for the measurement of 6-MP in human plasma, which because of the rapid separation of fluorescent adduct by HPLC will facilitate further pharmacokinetic studies on this important anti-leukaemic drug.

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