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ANALYSIS OF 6-MERCAPTOPURINE IN HUMAN PLASMA WITH A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD INCLUDING POST-COLUMN DERIVATIZATION AND FLUORIMETRIC DETECTION

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SUMMARY

A relatively simple assay with improved reliability and sensitivity for measuring levels of 6-mercaptopurine in human plasma is presented. After extraction of the compound and the added internal standard with phenyl mercury acetate, samples were separated by ion-pair reversed-phase high-performance liquid chromatography. On-line the analytes were oxidized to fluorescent products and detected in a flow-fluorimeter. The within-day coefficient of variation was 3.8% at a concentration of 25 ng/ml. The lower detection limit was 2 ng/ml when 1.0 ml of plasma was used. Mercaptopurine concentration versus time curves of two subjects after a single oral dose of azathioprine are shown.

INTRODUCTION

Since 1953 the 6-thiopurine anti-metabolite 6-mercaptopurine (6-MP) has been in use for the treatment of leukemia. In 1963 azathioprine (AZA), a

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derivative of 6-MP, became available. AZA is used as an immunosuppressive agent in patients who have received organ transplants and for the treatment of autoimmune disorders such as systemic lupus erythematosis, rheumatoid arthritis, idiopathic thrombocytopenic purpura and autoimmune hemolytic anaemia.

Concerning the mode of action of these drugs there is no general agreement. Metabolites of the thiopurines are known to inhibit purine biosynthesis [1—3] and the synthesis of DNA and RNA [1, 3]. The incorporation of thiopurine nucleotides into DNA and RNA of treated mouse lymphoma cells has been shown [4]. It has been suggested that not all the effects of AZA are to be attributed to its major metabolite 6-MP. Other metabolites are thought to have some additional effects on the immune system [5—9].

Little is known about the relationship between the pharmacokinetics of these drugs and their biological effects. Although it is likely that biological effects correlate better with intracellular thiopurine metabolite concentrations than with the plasma concentration of unmetabolized 6-MP [1], it is to be expected that useful information can be obtained from studies of AUC (concentration × time) versus biological effects. The assay of 6-MP plasma concentrations can also be useful for the study of interactions of other drugs with this agent and possibly for dosage adjustments in patients with impaired organ function.

Several methods for measuring plasma levels of 6-MP and AZA have been described of which high-performance liquid chromatography (HPLC) combined with UV detection [10—13] and methods using fluorimetric detection [14, 15] seemed most practical. However, the former lacked sensitivity while the latter were not specific enough. Tidd and Dedhar [4] were the first to combine the advantages of HPLC specificity with the sensitivity of fluorimetric detection in developing a method for determining intracellular levels of thiopurine nucleotides. They performed pre-column oxidation of samples which were separated on an anion-exchange column using a buffer gradient.

The present method is based on separation by ion-pair reversed-phase HPLC, on-line oxidation to fluorescent products and fluorimetric detection of the analytes.

MATERIALS AND METHODS

Chemicals

All aqueous solutions were prepared with double-distilled water. 6-MP and 6-thioguanine (TG; used as internal standard) were obtained from Wellcome (The Wellcome Foundation Ltd., London, Great Britain). Dithioerythritol (DTE) was obtained from Sigma (St. Louis, MO, U.S.A.), sodium octane sulphonate from Serva (Heidelberg, G.F.R.) and phenyl mercury acetate (PMA) from BDH (Poole, Great Britain). All other chemicals were purchased from E. Merck (Darmstadt, G.F.R.). All chemicals were of analytical reagent grade.

Stock solution of 6-MP (1 mg/ml) was prepared in methanol, that of TG by dissolving a 40-mg tablet of TG (Lanvis®) in 250 ml of methanol (160 μg of TG per ml). Standard aqueous solutions of 6-MP (1 μg/ml) and TG (4 μg/ml) were prepared from these stock solutions.

Apparatus

The eluent was delivered by a Kipp 9208 HPLC pump (Kipp Analytica, Emmen, The Netherlands). Samples were injected with a Rheodyne 7120 (Berkeley, CA, U.S.A.) injection valve equipped with a 1-ml loop. Autoanalysis equipment from Technicon Instruments was employed. The detector was an Amin-co-Bowman spectrophotofluorimeter fitted with a 30-μl flow-cell. Excitation and emission wavelengths were 295 nm and 380 nm, respectively. For scanning the fluorescence spectra a standard quartz glass cuvette was used.

Chromatographic system

Separations were performed on LiChrosorb 10 RP-18 obtained as pre-packed columns (250 × 4.6 mm) from Chrompack (Middelburg, The Netherlands). The mobile phase was water–isopropanol (97:3) containing 13.80 g of $NaH_2PO_4 \cdot H_2O$, 0.2 ml of H_3PO_4 (85%), 60 mg of DTE and 500 mg of sodium octane sulphonate per liter. The pH of the eluent was between 3.6 and 3.7. The system was operated at ambient temperature with a flow-rate of 1.5 ml/min. The effluent from the column was fed directly into an automatic system for the oxidation of 6-MP and TG to their fluorescent purine 6-sulphonate derivatives [15] and subsequent detection. Fig. 1 shows the arrangement of this auto-analyzer system.

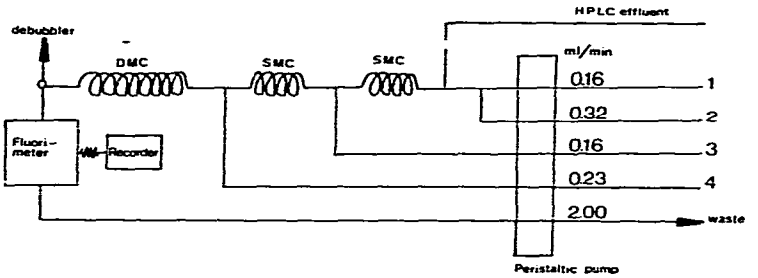

Fig. 1. Diagram of the autoanalyzer system used to produce the purine 6-sulphonates from 6-MP and TG after their separation by HPLC. SMC = single mixing coil; DMC = double mixing coil; 1 = 8 m*M* K_2CrO_4 in 0.5 *N* HCl; 2 = air; 3 = 1.6% $Na_2S_2O_5$; 4 = 4 *M* NH_4OH.

Sample preparation

To 1.0 ml of plasma in a glass-stoppered tube (5 ml) were added 25 μl of the internal standard solution (100 ng of TG), 0.1 ml of a 0.4 *N* sodium hydroxide solution, 1 ml of ethyl acetate containing 0.3% of PMA and 3 ml of diethyl ether. This mixture was shaken on a tumble-mixer for 10 min and centrifuged for 5 min. The overstanding organic layer was then transferred to another tube and 0.5 ml of a 0.1 *N* hydrochloric acid solution was added. This mixture was whirlmixed for 2 min and centrifuged for 5 min. Almost all of the organic layer was then removed by suction, and the remainder was evaporated under a gentle stream of nitrogen at room temperature during 15 min. To the residue 10 μl of an aqueous solution of 3 mg/ml DTE was added, and after mixing it was injected into the chromatographic system.

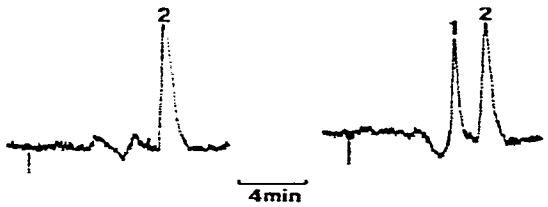

Fig. 2. Chromatograms of plasma samples. Left: blank plasma with internal standard. Right: plasma sample containing 25 ng/ml 6-MP. Peaks: 1 = 6-MP; 2 = internal standard (TG).

RESULTS

Representative chromatograms of plasma samples are shown in Fig. 2. The dip in the baseline just before the 6-MP peak is caused by the rather large volume of dilute hydrochloric acid that is injected into the liquid chromatograph, causing a temporary decrease in the background fluorescence of ammonium hydroxide.

The average of seven calibration plots made during a period of three weeks is described by the equation $y = 0.0231x + 0.0979$, the standard deviation of the slope being 0.0029. The coefficient of correlation ranged from 0.9850 to 0.9994 with a mean of 0.9955. The within-day coefficient of variation, calculated from eleven measurements of a plasma sample spiked with 25 ng of 6-MP, was 3.8%. In the same experiment the recovery was 76.8 ± 3.8% for 6-MP and 63.5 ± 3.2% for the internal standard TG. In view of the peak height to noise ratio (Fig. 2) it can be stated that the lower detection limit of 6-MP in our assay is less than 2 ng/ml when 1 ml of plasma is used.

Fig. 3 presents the 6-MP concentration–time curves of two subjects who received a single oral dose of 275 mg of azathioprine. Plasma samples of 1.0 ml were used for analysis; each dot represents the mean of duplicate determinations.

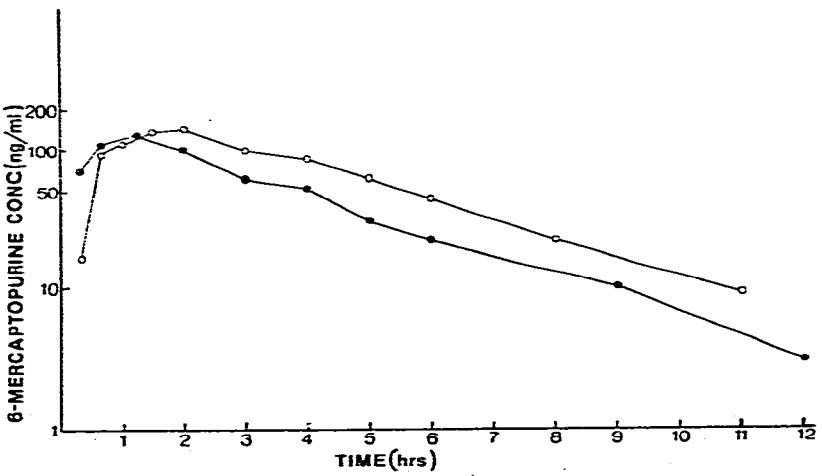

Fig. 3. Semilogarithmic plot of plasma 6-MP concentration vs. time of two subjects after oral administration of 275 mg of azathioprine.

DISCUSSION

The extraction method used is a modification of the method described by Maddocks [15]. Because an HPLC separation is included in our method it was possible to sacrifice some extraction specificity for a higher recovery. In alkalinised plasma the thiol group of thiopurines reacts with the mercury atom of PMA

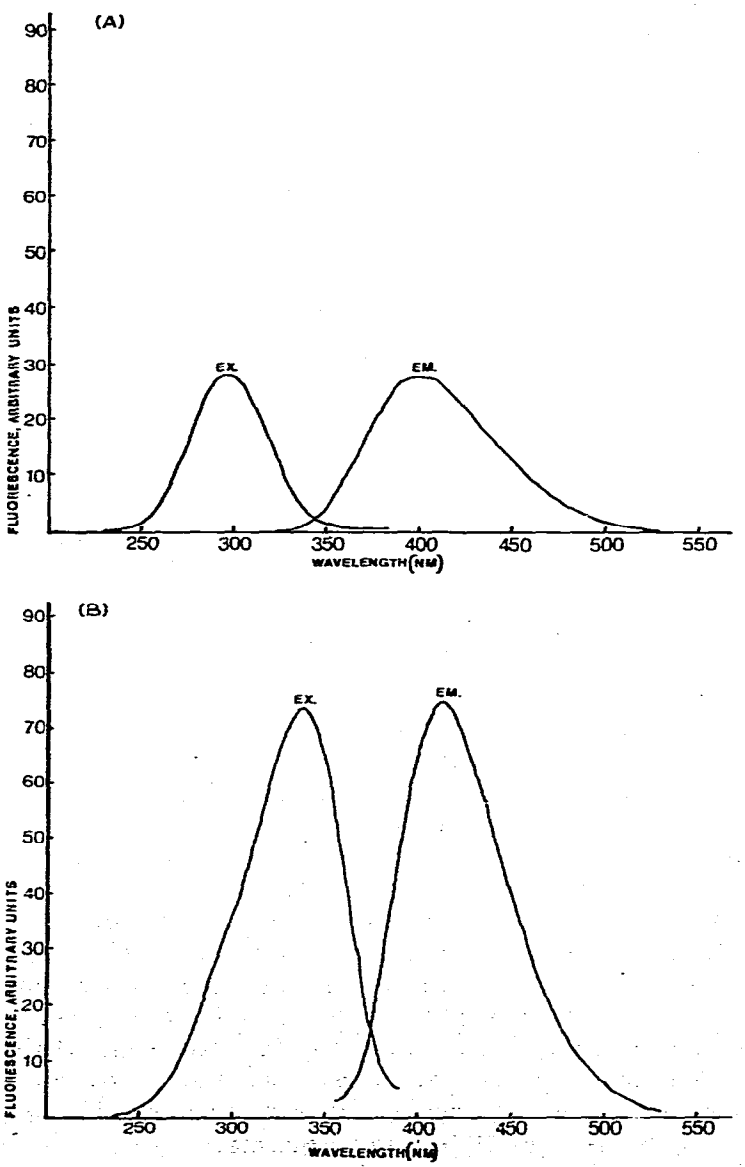

Fig. 4. Fluorescence spectra of oxidized 6-MP (A) and TG (B) in 1 *M* NH_4OH; concentration 5 μg/ml for both compounds and the same instrument setting for both spectra. EX: excitation spectra, maximum at 295 nm for 6-MP and 335 nm for TG. EM: emission spectra, maximum at 405 nm for 6-MP and 410 nm for TG.

to form a complex which can be extracted into organic solvents. When the organic layer is shaken with dilute hydrochloric acid the complex dissociates and the thiopurines are extracted back into the aqueous phase. The method can also be made suitable for the determination of AZA, as this compound can be converted to 6-MP by alkaline hydrolysis. Using this assay, the amount of AZA present in the plasma can be calculated by subtracting the concentration of 6-MP in non-hydrolyzed samples from that of hydrolyzed samples [15].

In order to achieve resolution between 6-MP and TG on the LiChrosorb 10 RP-18 column the addition of octane sulphonate to the eluent was needed. Octane sulphonate increases the capacity factor of TG due to ion-pair formation with the primary amino group of this molecule [16]. Therefore the resolution between 6-MP and TG was also dependent on the pH of the eluent resulting in an increased resolution at a lower pH.

It was found necessary to add DTE to the eluent in order to obtain a linear relationship between injected amount of 6-MP and observed peak height. Furthermore, DTE caused an improvement of peak shape. DTE was synthesized and described for the first time by Cleland [17]. It was presented as a protective agent to keep thiol groups in the reduced state and to reduce disulfides quantitatively. Bailey et al. [18] used DTE during the extraction of 6-MP to prevent decomposition, and Ding and Benet [12] observed improved peak heights when DTE was added during extraction. We never observed any improvement in the recovery of both compounds by adding DTE during the extraction of spiked plasma samples. Therefore we concluded that no significant decomposition of 6-MP or TG occurred during our extraction procedure. Furthermore, DTE was not added during the extraction because it would convert unmetabolized AZA to 6-MP and because DTE could interfere with the PMA extraction. Furthermore, we concluded that an additional effect of DTE in our assay was to prevent adsorption of 6-MP to metal surfaces.

In the post-column manifold the thiopurines were oxidized to their fluorescent purine 6-sulphonates according to the mechanism described by Finkel [14] and modified by Maddocks [15], who applied it in a manual method for 6-MP. By varying all parameters involved in the reaction it was established that the conversion took place quantitatively under our conditions. It appeared that the alkali used was responsible for a background signal. We used ammonium hydroxide instead of an alkali metal hydroxide because, using the same concentration, it reduced the noise level without loss of signal intensity.

The excitation and emission spectra of oxidized 6-MP and TG are shown in Fig. 4A and B, respectively. Because the fluorescence spectrum of the background signal showed an excitation maximum of 325 nm and an emission maximum of 410 nm, the emission wavelength was set at 380 nm instead of 405 nm which is the emission maximum of oxidized 6-MP. Thus some of the response of 6-MP was sacrificed in order to obtain a better signal-to-noise ratio.

In view of the results shown our assay can be considered a reliable and sensitive method for measuring levels of 6-MP in human plasma.

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