ASSAY OF AZATHIOPRINE, 6-MERCAPTOPURINE AND A NOVEL THIOPURINE METABOLITE IN HUMAN PLASMA

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- 1 A simple, specific assay for 6-mercaptopurine (6-MP) in human plasma with a sensitivity of 10 ng/ml (66 nmol/l) has been developed.
- 2 6-MP was extracted directly from plasma into toluene using a novel extraction procedure. This involves conversion of 6-MP into a phenyl mercury derivative by its reaction with phenyl mercuric acetate in alkaline plasma and extracting into toluene. Back-extraction of the toluene layer with 0.1N HCl regenerates 6-MP, which is then oxidised to purine-6-sulphonate and assayed fluorimetrically.
- 3 This assay has been modified to measure azathioprine and a new thiopurine metabolite in plasma.
- 4 In a kidney transplant patient given azathioprine, 50 mg i.v., conversion to 6-MP was rapid and the plasma half-life of 6-MP was 36 min.
- 5 These assays are suitable for studying the pharmacokinetics of azathioprine in patients with kidney transplants. The 6-MP assay should also prove useful for studying the pharmacokinetics of the drug in patients with leukaemia.

Introduction

The synthesis of azathioprine by Elion, Callahan, Bieber, Hitchings & Rundles (1961) and its use as an anti-rejection drug by Calne & Murray (1962) have led to major advances in kidney transplantation. The precise mode of action of azathioprine in transplant patients is unknown and there is very little information available on its absorption, distribution, activation and degradation. It is not surprising, therefore, that the clinical use of azathioprine is almost completely empirical. Dosage is very often in the toxic range as it is monitored by observing depression of the peripheral white cell count which is a toxic effect of the drug. As an anti-rejection drug, azathioprine is inconsistent in its effect and transplant rejection and drug toxicity continue to be major problems. Indeed, the results of kidney transplantation have not improved over the last ten years despite greater experience in surgical technique, kidney preservation and tissue typing (Renal Transplant Registry, 1977). Possibly a better understanding of the clinical pharmacology of azathioprine might lead to an improvement in the results of kidney transplantation. For this purpose I have developed assays which are sufficiently sensitive for measuring azathioprine, 6-mercaptopurine (6-MP), which is considered to be an active metabolite, and a new metabolite (X) in plasma. These are described in this paper together with their preliminary clinical applications.

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Methods

Principle of the assay

Finkel (1967) described an assay for 6-MP involving the oxidation of its -SH group to form purine 6-sulphonate which is measured fluorimetrically. This assay is not sensitive enough to measure therapeutic levels of 6-MP in plasma because of very high plasma blank readings, especially with uraemic plasma, and it lacks specificity. In this study it has been modified and a new method of extracting 6-MP from plasma has been developed which overcomes the problems of Finkel's (1967) assay, resulting in increased sensitivity and specificity. The extraction was achieved by exploiting the -SH group of 6-MP as shown in Figure 1. By reacting this group with phenyl-mercuric acetate, two important changes occur. The acidic -SH group is eliminated so that the derivative (6-phenyl-mercury mercaptopurine, 6-PMMP) functions as a base and also 6-PMMP is a less polar compound and has a much greater solubility than 6-MP in organic solvents. After adding phenyl mercuric acetate to alkaline plasma, 6-PMMP is rapidly formed and is readily extracted into toluene. If before adding phenyl mercuric acetate, the plasma is washed with ether or toluene, compounds with -SH groups are more selectively extracted as interfering substances soluble in these solvents are removed. When the toluene extract containing 6-PMMP is shaken with dilute hydrochloric acid, the complex ionises in the acid phase and also splits in the presence of chloride ions to regenerate 6-MP. The 6-MP is then oxidized

Figure 1 Extraction of 6-MP from plasma into toluene, as its phenylmercury derivative, and its back-extraction into 0.1N HCl.

to purine 6-sulphonate which is measured fluorimetrically.

Although this assay was found to be specific for 6-MP when added to normal and uraemic plasma, it was not so when applied to plasma from a patient who had received an intravenous dose of azathioprine owing to the appearance of a new metabolite. This was removed by a buffer wash (McIlvaine's buffer pH 3) of the toluene extract before back-extracting into hydrochloric acid.

Experimental

Instrumentation Relative fluorescence intensity was measured with a spectrophotofluorimeter (Aminco-Bowman) attached to an X-Y recorder (Bryans). The excitation slit width was 0.5 mm and the emission slit width 1 mm; all other slits were at maximum width. An increase in light path length through the sample was produced by inserting stainless steel mirrors in the cell compartment.

Reagents Phenyl mercuric acetate (B.D.H.), 0.3% w/v, was prepared by dissolving the compound in boiling deionised water and kept in a dark bottle. A fresh solution was prepared weekly.

Sodium metabisulphite (B.D.H.), 0.4% w/v, was freshly prepared each week. All other chemicals were Analar grade and solutions of these were replaced monthly.

Volumes of reagents greater than 2 ml were

delivered by Zippette dispensers (Jencons Ltd.) fitted with disposable polythene tips.

A stock solution of 6-MP, 100 µg/ml in 0.1N HCl, did not deteriorate at room temperature for several weeks. This was prepared by dissolving 50 mg 6-MP in 20 ml 0.1N NaOH. Water (300 ml) was then added followed by 52 ml 1N HCl and the solution made up to 500 ml with water. This solution was used to prepare standard solutions of 6-MP in 0.1N HCl. Plasma standard solutions of 6-MP were prepared by adding 50 µl of standard 6-MP solutions in 0.1N HCl to 2 ml plasma and neutralizing with 50 µl 0.1N NaOH.

Extraction and estimation of plasma 6-MP

To each 2 ml of plasma was added 0.2 ml 0.4N NaOH in a 16 ml tube. After mixing, 8 ml ether containing 1.5% amyl alcohol was added. Tubes were shaken (280/min) on a mechanical shaker for 5 min and then centrifuged for 2 min at 800 g. Ether was largely removed by suction and the remainder blown off in a stream of air at room temperature. Phenyl mercuric acetate (2 ml of 0.3% solution) was added to the plasma with mixing followed by 8 ml toluene containing 1.5% amyl alcohol. After shaking and centrifuging, as previously, 7 ml toluene was transferred to a graduated conical tube and washed three times with 5 ml McIlvaine's buffer pH 3.0. The toluene layer (6 ml) was transferred to a graduated conical tube (10 ml), 350 µl 0.1N HCl added and the

tubes shaken and centrifuged. Almost all the toluene layer was then removed by suction.

Oxidation

Potassium chromate, 50 μ l, 2 mM, was added to each tube with mixing. After 2 min 50 μ l 0.4% sodium metabisulphate and 50 μ l 5N NaOH were added, with mixing after each addition, and the tubes centrifuged (800 g for 2 min). The residual toluene was completely removed by aspiration. Relative fluorescence of the solutions was measured at 398 nm with an excitation wave-length of 288 nm.

Estimation of azathioprine in plasma

Azathioprine in plasma (2 ml) was completely hydrolyzed to 6-MP by adding 200 μ l 5N NaOH and incubating at room temperature for 10 min. After neutralization with 200 μ l 5N HCl, 6-MP in plasma was assayed as before. The concentration of azathioprine in plasma can then be calculated from the difference in concentration of 6-MP in hydrolyzed and non-hydrolyzed plasma samples.

Estimation of metabolite X in plasma

This metabolite was extracted from plasma in the same way as 6-MP but the buffer wash of the toluene extract with collected, alkalinized with NaOH to pH 8 and extracted with toluene. This toluene extract was then back-extracted with 350 μ l 0.1N HCl. The back-extract was oxidized as before and fluorescence at 400 nm measured with an excitation wave-length of 325 nm.

Assay of metabolite X has been simplified recently by measuring its natural fluorescence in alkali (without oxidation) at 400 nm with an excitation wave-length of 325 nm. Unoxidized 6-MP is not fluorescent at these wave-lengths. The procedure was the same as for the 6-MP assay except that the oxidation step was omitted and the back-extract was made alkaline by adding 50 μ l 5N NaOH.

Results

6-MP assay

There was a linear relationship between fluorescence and concentration of 6-MP in plasma, over the range 10–1000 ng/ml, as shown in Figure 2. Reproducibility of the assay in replicate analysis was determined at concentrations of 20, 40, 50, 100 and 500 ng/ml of 6-MP. Eight samples at each concentration were measured and the percentage deviation of each result from the mean fluorescence intensity for each

Figure 2 Standard curve for the concentration of 6-MP (ng/ml) in plasma against relative fluorescence intensity. Bars indicate mean $\pm$ s.d. mean ($n=12$).

concentration was calculated. The largest individual deviation was 7.1% and the mean deviation for the whole group was 2.1%.

The extraction efficiency was determined using ^{35}S 6-MP and was 53%. Without the buffer wash the extraction efficiency was 63%. It is much higher using more polar solvents such as ether, but a high extraction efficiency has been sacrificed to obtain greater specificity.

Blank readings for eleven plasma samples from normal subjects had a mean ($\pm$ s.d.) of 0.064 ± 0.012 .

Specificity

Uraemic plasma did not affect the fluorescence readings or the fluorescence scans for 6-MP. Also, assay of 6-MP that had been added to plasma from volunteers who had taken 20 mg prednisone orally showed no interference by prednisone or its metabolites.

Addition of physiological sulphhydryl compounds (glutathione and cysteine) and purines (hypoxanthine, adenine, xanthine and uric acid) to plasma containing 6-MP did not interfere with the assay.

Figure 3 Excitation spectra of oxidised plasma extracts that contained authentic 6-MP (—) and apparent 6-MP (----) from a renal transplant patient 20 min after a dose of azathioprine 50 mg intravenously.

Azathioprine metabolites, 1-methyl-4 nitroimidazole, 1-methyl-4-nitro-5-thioimidazole, and 6-thiouric acid when present in plasma containing 6-MP again did not interfere with the assay.

A patient with a functioning kidney graft was given a dose of 50 mg azathioprine intravenously. Venous blood samples were taken at 0, 10, 20, 40, 60 and 120 min after the dose and the plasma separated and deep frozen. Assay of 6-MP (omitting the buffer pH 3 wash) in each sample was performed together with fluorescent excitation and emission scans as an index of specificity. There was a progressive shift of the peak of the excitation scan with time suggesting interference due to a metabolite, as the zero time plasma sample had a low reading. This metabolite was removed as follows. First the relationship between the efficiency of back-extraction of 6-MP from toluene into the aqueous phase and the pH of the aqueous phase was studied. Using McIlvaine buffers of various pH it was found that very little 6-MP was recovered above pH 3. A buffer wash (pH 3) was introduced into the assay and this was found to remove the interfering metabolite leaving 6-MP, as indicated by fluorescence excitation scans. The plasma concentrations of 6-MP in this patient are shown in Figure 3. Conversion of azathioprine to 6-MP was rapid and the plasma half-life of 6-MP was 36 min.

Plasma samples taken 10 and 20 min after an intravenous dose of azathioprine (100 mg) were taken through the extraction procedure without a buffer wash and back-extracted into 100 μ l 1N HCl. Urine

Table 1 R_F values and colour of spots of thio-metabolites of azathioprine extracted from plasma and urine of kidney transplant patients. Detection in u.v. light at 77°K.

		Solvent system						
		1	2	3	4	5	6	7
Authentic 6-MP		0.38 Green	0.23 Green	0.44 Blue	0.46 Green	0.10 Green	0.47 Blue	—
Apparent 6-MP	b Plasma	0.41 Green	0.24 Green	0.43 Blue	0.45 Green	0.09 Green	0.47 Blue	—
	c Urine	0.41 Green	0.24 Green	—	0.46 Green	0.10 Green	0.46 Blue	—
Metabolite X	b Plasma	a	0.39 Purple	0.31 Purple	—	0.12 Blue	—	0.38 Purple
	c Urine	0.45 Purple	0.41 Purple	0.31 Purple	0.39 Purple	0.13 Purple	0.49 Purple	0.38 Purple

1. HCl (0.1N)

2. Water

3. Isopropanol-methanol-water-ammonia (sp. gr. 0.880), 60:20:20:1, v/v

4. Ethanol-sodium formate (0.5M), 5:2, v/v

5. Isopropanol-HCl (conc.)-water, 130:33:37, v/v

6. Butanol-acetic acid-water, 12:3:5, v/v

7. Isopropanol (5%)—ammonium acetate (5%)

a. 6-MP spot had a purple cap

b. Taken 10 min after azathioprine 100 mg i.v.

c. 6 h collection after azathioprine 50 mg i.v.

— Not seen

Figure 4 Plasma concentrations of 6-MP related to time in a kidney transplant patient given azathioprine 50 mg intravenously.

samples from the same patient were treated in the same way. Chromatography on cellulose thin layers was carried out using seven different solvent systems. Spots were visualized by luminescence at low temperature (-196°C) (Maddocks & Davidson, 1975). The results are shown in Table I; 6-MP together with a new metabolite (X) were detected in both plasma and urine. Further confirmation that 6-MP had been isolated was obtained from fluorescence scans after removing the spots from the chromatogram, dissolving the apparent 6-MP in 0.1N HCl and

oxidizing. Introduction of a buffer wash pH 3 into the extraction procedure, prior to thin layer chromatography, resulted in the isolation of only 6-MP, metabolite X having been removed by the buffer wash.

For additional evidence of specificity, the distribution test of Brodie & Udenfriend (1945) was carried out. In this test the distribution of apparent 6-MP, in plasma from a patient, between toluene and a series of buffers was measured and compared with the distribution of authentic 6-MP. The results show (Table 2) a close agreement for the distribution values of authentic and apparent 6-MP suggesting that the assay is specific.

Metabolic studies using ^{35}S azathioprine in transplant patients, and also mass-spectrometry results suggest that metabolite X is a thiopurine metabolite of azathioprine (Thapliyal & Maddocks, unpublished observations). None of the previously described azathioprine or 6-MP metabolites have its chromatographic and fluorescence characteristics.

Assay of metabolite X

There was a linear relationship between the concentration of metabolite X and its fluorescence intensity after oxidation. The same relationship held for unoxidized metabolite X. The concentration range studied was that normally encountered in the urine and plasma of kidney transplant patients and was prepared by diluting a stock solution of metabolite X isolated by chromatography. Reproducibility was studied by carrying out six replicate measurements at various dilutions of the stock solution. The largest individual deviation was 2.3% and the mean deviation for the whole group was 1.1%. Without oxidation there was no difference in fluorescence readings, the largest individual deviation was 2.3% and the mean deviation for the whole groups was 0.9%.

The specificity of the assay for metabolite X in plasma was checked by determining fluorescence excitation and emission scans which were the same as those obtained with the compound purified by chromatography (325 nm; 400 nm).

Azathioprine assay

Hydrolysis of azathioprine to 6-MP by 0.5N NaOH in plasma was complete after 10 min at room temperature. The linearity, reproducibility and sensitivity of the assay of azathioprine were similar to those of 6-MP. A patient with a kidney transplant was given 50 mg azathioprine by intravenous injection and a blood sample collected 10 min after the dose was administered. The concentration of azathioprine in the plasma was assayed and the specificity of the assay checked by performing

Table 2 Distribution of apparent and authentic 6-MP as 6-phenyl mercury derivatives between toluene and McIlvaine buffers* of various pH.

pH	% of 6-MP remaining in toluene	
	Apparent 6-MP	Authentic 6-MP
2.2	82	79
2.6	91	88
3.0	91	96
4.0	100	96
5.0	100	100

*Buffer volume=5 ml $\times$ 3 Shaking time=5 min.

fluorescence excitation and emission scans. There was a slight shift to the left in the excitation peak to the higher wavelengths compared with the standard 6-MP peak. Thin layer chromatography of this plasma extract with detection of spots using low temperature luminescence resulted in the identification of 6-MP together with a small amount of probably 8-hydroxy-6-MP. The failure to detect 8-hydroxy-6-MP in plasma that had not been hydrolyzed with NaOH suggests that 8-hydroxy-azathioprine was originally present in the plasma sample.

Discussion

There have been several previous attempts at measuring azathioprine or 6-MP in plasma (Hamilton & Elion, 1954; Finkel, 1967; Loo, Luce, Sullivan & Frei, 1968; Coffey, White, Lesk, Rogers & Serpick, 1972; Spreafico, Donelli, Bossi, Wecchi, Standen & Garattini, 1973; Bailey, Wilson & Johnson, 1975) but these assays have either lacked specificity or sensitivity or both and have been of little

value in studying azathioprine pharmacokinetics in patients. The present assay for 6-MP is the first to be developed with sufficient sensitivity and specificity to allow kinetic studies to be done on patients taking therapeutic doses of azathioprine (Maddocks, 1975). Detailed studies of azathioprine pharmacokinetics have now been conducted on renal transplant patients and these results will be reported elsewhere. Recently, Rosenfeld, Taguchi, Hillcoat & Kawal (1977) have developed a gas liquid chromatography-mass spectrometric assay for 6-MP which seems to be specific although less sensitive than the present assay. The cost of instrumentation and personnel however will limit its availability in clinical practice.

The assay for 6-MP in plasma should also be suitable for studying the pharmacokinetics of the drug in patients with leukaemia.

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