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EXTRACTIVE ALKYLATION OF 6-MERCAPTOPURINE AND DETERMINATION IN PLASMA BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY*

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SUMMARY

An analytical procedure was developed for the determination of 6-mercaptopurine in plasma. Owing to the polar character and low plasma concentrations of the compound, extraction and derivatization was carried out directly from the plasma sample by extractive alkylation. Determination was made using gas chromatography—mass spectrometry with multiple-ion detection.

Conditions with respect to the rate of formation and the stability of the derivative formed in the extractive alkylation step were evaluated. The selectivity of the method to azathioprine and to metabolites was thoroughly investigated. No 6-mercaptopurine was formed from azathioprine added to water or plasma and run through the method. The method enables the detection of 2 ng of 6-mercaptopurine in a 1.0-ml plasma sample. Quantitative determinations were done down to 10 ng/ml 6-mercaptopurine in plasma.

INTRODUCTION

6-Mercaptopurine is an immunosuppresive cytostatic agent which is used in the treatment of leukemia in children. This compound is also the primary metabolite of azathioprine [6-(1-methyl-4-nitro-5-imidazolyl)thiopurine], a drug extensively used in autoimmune and other diseases and in organ transplantation [1]. Intracellularly, mercaptopurine is converted by hypoxanthine—guanine phosphoribosyltransferase to thiosinic acid, which is thought to

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be the main active metabolite, and to other thioanalogues of purine derivatives [1].

Until recently, available methods for the determination of 6-mercaptopurine [2—12] have not been sensitive or selective enough to enable studies on the pharmacokinetics of 6-mercaptopurine during immunosuppresive treatment with azathioprine or 6-mercaptopurine. A high sensitivity has been obtained after conversion of 6-mercaptopurine to purine-6-sulphonate and determination of the derivative by fluorimetry [13]. The lower limit of sensitivity was 10 ng/ml. Gas chromatographic analysis has been performed after flash methylation [8, 9] or extractive methylation [12]. However, these methods did not take into account that one of the metabolic pathways of 6-mercaptopurine is an S-methylation and this metabolite would be co-determined in the procedure. The poor stability of the derivative also enhanced the problems in quantitative determinations. Recently, two liquid chromatographic methods for the assay of azathioprine and 6-mercaptopurine with high sensitivity and selectivity were described [14, 15].

From the available pharmacokinetic data, it is obvious that very low plasma concentrations will be seen some hours after a single dose of 6-mercaptopurine. A quantitative extraction of 6-mercaptopurine is difficult to obtain owing to the hydrophilic character of the compound. Furthermore, a derivatization of the acidic groups is also mandatory before gas chromatographic analysis. The difficulties encountered above made it necessary to develop a method which performed extraction and derivatization in a one-step procedure and which used a very sensitive and selective detection device. Therefore, interest was focused on direct extractive alkylation of the plasma sample with pentafluorobenzyl bromide and determination by gas chromatography—mass spectrometry with multiple-ion detection.

EXPERIMENTAL

Gas chromatography and mass spectrometry

Studies on the conditions for extractive alkylation of 6-mercaptopurine (Wellcome, London, Great Britain) were performed in a Pye-Unicam series 204 gas chromatograph equipped with flame ionization detector. The glass column (150 × 0.18 cm) was packed with 3% OV-17 on Gas-Chrom Q (100—120 mesh) and operated at 265°C in the analysis of pentafluorobenzylated mercaptopurine. Injector and detector temperatures were kept at 320°C. Nitrogen (30 ml/min) was used as carrier gas. Identification of the derivatives was done in an LKB 2091 mass spectrometer.

6-Mercaptopurine concentrations in biological samples were determined in a Finnigan 4000 gas chromatograph—mass spectrometer equipped with a multiple-ion monitoring device (Finnigan, Sunnyvale, CA, U.S.A.). The mass spectrometer was operated in an electron impact mode with an ionization energy of 70 eV. The injector of the gas chromatograph was of the Grob capillary type and operated at 275°C in splitless mode. The injector was equipped with valves which were programmed to vent the injector 60 sec after injection of sample. The glass capillary column (15 m × 0.2 mm inner diameter) contained SE-30 as stationary phase. A 20—25 kPa pressure of

helium was applied to the column, which was directly interfaced to the ion source. The temperature of the gas chromatographic oven was programmed from 240 to 280°C at a rate of 10 ml/min. Samples could be injected every 5—6 min.

For the injection, solid sample syringes (SGE, Ringwood, Australia) were used. They were cleaned in a Hamilton syringe cleaner between injections.

The molecular ions, m/z = 512 for S-9-dipentafluorobenzyl-6-mercaptopurine and m/z = 346 for the internal standard derivative (9-pentafluorobenzyl-S-methyl-6-mercaptopurine), were monitored.

Identity of the derivatives

The following mass spectrometric data were obtained from the derivatives after extractive alkylation with pentafluorobenzyl bromide. Derivative of 6-mercaptopurine: m/z (percentage relative abundance) = 119 (18); 163 (14); 181 (100); 331 (73); 332 (11) and 512 (M^+, 73). Derivative of S-methyl-6-mercaptopurine: m/z = 119 (14); 165 (100); 181 (48); 254 (15) and 346 (M^+, 62).

Reagents and chemicals

Tetrabutylammonium and tetrahexylammonium ion solutions were prepared by neutralization of the corresponding hydrogen sulphate (Labkemi, Stockholm, Sweden) and purified by washing with methylene chloride and heptane. Dilution to the desired concentration was made with buffer. Tetrapentylammonium ion solution was prepared by shaking the iodide salt (Eastman Kodak, Rochester, NY, U.S.A.) with an equivalent amount of silver oxide overnight.

Standard samples of 6-mercaptopurine were prepared by dissolving the compound in water and dilution to 100 ng/ml. Aliquots of 0.5, 1.0, 2.0 and 5.0 ml of this solution were diluted to 10 ml with blank plasma. A 1.0-ml aliquot was taken to analysis.

METHODS

Extractive alkylation of 6-mercaptopurine

One millilitre of an aqueous solution of 6-mercaptopurine (1 mg/ml) was mixed with quaternary ammonium ion solution and methylene chloride containing the alkylating reagent. Tetradocosane or triacontane (0.3 mg/ml) dissolved in the organic phase was used as internal marker. The mixture was shaken at room temperature or at 50°C. The alkylation reaction was quenched by the addition of 0.5 ml of 0.1 *M* hydrochloric acid, and after shaking for some minutes a few microlitres of the organic phase were taken for analysis by gas chromatography with flame ionization detection. The height ratio of the peak of the product to that of the internal marker was calculated.

Partition of 6-mercaptopurine as ion pair with tetrabutylammonium ion

The extraction constant, K_{EX}, of the 6-mercaptopurine—tetrabutylammonium ion-pair was determined by shaking equal volumes of aqueous phase

(pH 10) containing 2 mg/ml 6-mercaptopurine 0.005—0.05 *M* tetrabutylammonium ion, and an organic phase of methylene chloride containing *p*-chlorobenzophenone as internal standard. After equilibration for 30 min, the organic phase was separated, methyl iodide was added and the amount of 6-mercaptopurine extracted in the organic phase was determined in comparison to a sample with a known concentration of 6-mercaptopurine and methylated quantitatively by extractive alkylation.

Determination of 6-mercaptopurine in plasma

To a 1.0-ml plasma sample of 6-mercaptopurine, 0.1 ml of internal standard solution (500 ng/ml of S-methyl-6-mercaptopurine), 0.1 ml of 0.1 *M* tetrabutylammonium ion solution and 0.5 ml of 1 *M* phosphate buffer (pH 10) were added. This mixture was shaken for 30 min with 2 ml of methylene chloride containing 2% of pentafluorobenzyl bromide. After centrifugation (500 *g*, 15 min) the organic phase was transferred to another tube and evaporated to dryness at 60°C (Buchler Vortex Evaporator). The residue was dissolved in 20 μl of ethanol. Of this, 1—2 μl were then evaporated on the needle of the solid sample syringe.

RESULTS AND DISCUSSION

Extraction and alkylation conditions for 6-mercaptopurine

In recent studies 6-mercaptopurine was extracted from plasma with a fourfold excess of butanol [8] or with ethyl acetate—isopropanol after addition of a stabilizing agent [9]. It was anticipated that the extraction yield using these procedures was not quantitative, thus decreasing the precision of the analytical procedure. An improved extraction yield of polar organic compounds can be achieved after addition of complexation agents, such an approach being used with 6-mercaptopurine by the addition of phenylmercuric acid before toluene extraction [3].

Ionized compounds can also be extracted as ion-pairs, where the type and concentration of the counter-ion as well as the properties of the organic phase govern the degree of extraction. The extraction constant (K_{EX}) for 6-mercaptopurine with tetrabutylammonium ion was 4.6. Use of a more lipophilic quaternary ammonium ion in the extraction of 6-mercaptopurine would increase the extraction yield and a quantitative extraction should be possible. On the other hand, a more efficient extraction of interfering components from the biological sample would occur, which would decrease the selectivity of the method.

As the possibility for an efficient and selective extraction of 6-mercaptopurine was limited, interest was focused on simultaneous extraction and derivatization to enhance both yield and sensitivity of the analytical procedure.

Recently, 6-mercaptopurine was derivatized before gas chromatographic analysis using extractive alkylation conditions. Tetrahexylammonium ion was used as counter-ion and the pH of the aqueous phase was in the range 13—14 [12]. The yield of dimethyl derivative was reported to be low, most probably explained by a rapid degradation of the product with use of the

more lipophilic quaternary ammonium ions at high pH of the aqueous phase. The extraction of hydroxide ions as ion-pair with the tetrahexylammonium in the organic phase is not negligible. Extractive alkylation of 6-mercaptopurine using a less lipophilic counter-ion would thus be favourable both to the stability of the derivative formed and to selectivity in the analysis of biological samples.

Extractive alkylation with pentafluorobenzyl bromide

Extractive alkylation of metimazol with pentafluorobenzyl bromide and determination by gas chromatography—mass spectrometry with multiple-ion detection was recently demonstrated [16]. The use of pentafluorobenzyl bromide as alkylating reagent was justified by the higher detection selectivity obtained when a higher mass number of the derivative was monitored.

In the analysis of 6-mercaptopurine the use of pentafluorobenzyl bromide was not solely motivated by the increased detection selectivity, but also to avoid the co-determination of an S-methylated metabolite which can be formed in man [2]. From the general considerations above on the extractive alkylation of 6-mercaptopurine with alkyl iodides the counter-ion and pH were chosen. The rapid hydrolysis of pentafluorobenzyl bromide also supports the use of mild reaction conditions (cf. ref. 17). The high reactivity of the reagent made it possible to use only 2% of pentafluorobenzyl bromide in the reaction. A low concentration of tetrabutylammonium ion, 0.005 *M*, was also used to increase the selectivity of the method. Recently, it was emphasized that the use of low concentrations of reagent is necessary to achieve sufficient selectivity and sensitivity in the analytical procedure [18]. The time for complete extractive alkylation using the conditions of the method was 25 min and 30 min for 6-mercaptopurine and the internal standard, respectively. Temperature did not influence the reaction rate, as can be seen in Fig. 1. This observation is contrary to the extractive alkylation of some sulphonamide diuretics where an elevated temperature was essential both for rapid reaction and type of derivative [19, 20].

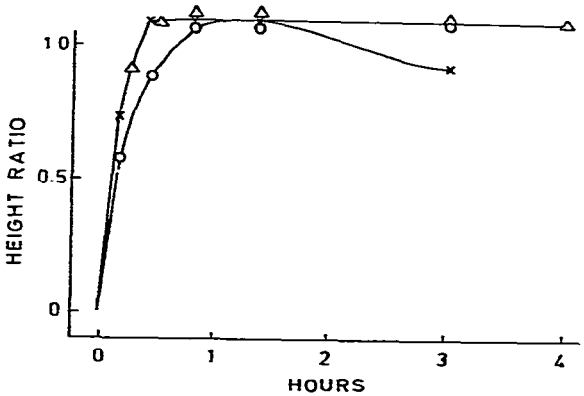

Fig. 1. Extractive alkylation of 6-mercaptopurine with pentafluorobenzyl bromide. Aqueous phase: 2 ml buffer solution (pH 10) containing 6-mercaptopurine (1 mg/ml) and 0.005 *M* tetrabutylammonium ion. Organic phase: methylene chloride (1 ml) with 2% pentafluorobenzyl bromide at 50°C (×) and at 25°C (△), or 1% pentafluorobenzyl bromide at 50°C (○).

An isomerization was observed of the product both with 6-mercaptopurine and with the internal standard when pentafluorobenzyl bromide was used as alkylating reagent. The isomers showed the same mass spectrometric fragmentation pattern as the derivatives from 6-mercaptopurine and the internal standard. The yields of the isomers were 7 and 11%, respectively. Furthermore, the isomers were formed in reproducible yield (± 7% of the main product, n = 20) and were completely resolved from other peaks in the gas chromatographic systems. This means that the extractive alkylation with pentafluorobenzyl bromide can be used in quantitative determinations with good precision. Isomerization in the extractive methylation of 6-mercaptopurine has also been reported [12]. This isomerization could not be observed in the above studies with any alkyl iodide. On the other hand, if methanol was added to the reaction mixture a much higher ratio of the two isomers of S-9-dipentafluorobenzyl-6-mercaptopurine was found.

Identity of pentafluorobenzyl derivatives

Extractive alkylation of 6-mercaptopurine with pentafluorobenzyl bromide resulted in a derivative with the pentafluorobenzyl groups in the 9-position and at the sulphur atom. The prominent peaks in the mass spectrum correspond to: m/z = 181, pentafluorobenzyl group; m/z = 331, molecular ion minus one pentafluorobenzyl group; and m/z = 512, the molecular ion. Mass spectral analysis of the derivative from the internal standard revealed a monopentafluorobenzylated product.

Gas chromatographic and mass spectrometric conditions

After alkylation of 6-mercaptopurine, the derivative showed good gas chromatographic properties. Symmetric peaks with no indication of absorption losses were seen using capillary columns with the non-polar stationary phase SE-30. A symmetrical peak was also obtained using packed columns with OV-17 as stationary phase.

Selectivity of the determination in the presence of azathioprine

6-Mercaptopurine is the major metabolic product of azathioprine. The conversion of azathioprine to 6-mercaptopurine is rapid [14], but nevertheless it is stated that azathioprine exhibits some pharmacologic activity and contributes to the overall effect. It was therefore of importance to establish whether azathioprine was co-determined in the method. Addition of azathioprine to water samples and run through the method using buffer with pH 8, 10 and 13 did not give any 6-mercaptopurine, indicating that azathioprine was stable in the extractive alkylation step. Addition of azathioprine to fresh human blood, however, gave considerable amounts of 6-mercaptopurine, which means a rapid conversion in the presence of red blood cells. This is shown in Fig. 2, which demonstrates that at room temperature about 50% conversion has already occurred within 30 min. This observation is in agreement with previous findings [2, 14]. The conversion was quantitative, and could be used as an indirect method for the determination of azathioprine.

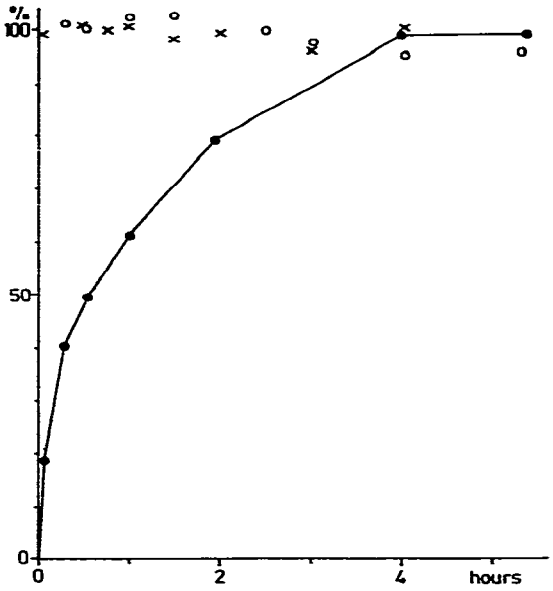

Fig. 2. Stability of 6-mercaptopurine in blood (○) and plasma (×). Yield of 6-mercaptopurine after addition of azathioprine to blood (●). Temperature 25°C.

Cooling the blood sample in an icebath immediately after drawing gave a very small conversion (< 10%) of azathioprine to 6-mercaptopurine.

It follows that this procedure is mandatory when studying the pharmacokinetics of azathioprine and 6-mercaptopurine after azathioprine administration. If this important measure were disregarded, the assay would give falsely high 6-mercaptopurine concentrations and correspondingly low azathioprine concentrations in the plasma.

Formation of 6-mercaptopurine from azathioprine was not observed in plasma. 6-Mercaptopurine was also found to be stable when added to plasma or human blood, as evidenced in Fig. 2.

Capability of the method

The limit of detection of the present method was 2 ng of 6-mercaptopurine per ml of plasma. Quantitative determinations could be done down to 10 ng/ml. The precision of the method at the 10 and 100 ng/ml level was 6 and 10%, respectively (n = 10). If 1,4-dithioerythritol (10 µl of a 1% solution) was added to plasma, a better precision of 7.1% at the 100 ng/ml level of 6-mercaptopurine was obtained (n = 10). 1,4-Dithioerythritol stabilizes 6-mercaptopurine [9], which improves the precision. However, 1,4-dithioerythritol rapidly degrades azathioprine and can not therefore be used for plasma samples from patients given azathioprine.

A typical chromatogram from the analysis of 6-mercaptopurine in plasma samples is shown in Fig. 3. No interfering peaks in the area of the peak of the derivative of 6-mercaptopurine could be seen. Also, standard curves for 6-mercaptopurine in the concentration range 10—100 ng/ml passed through the origin.

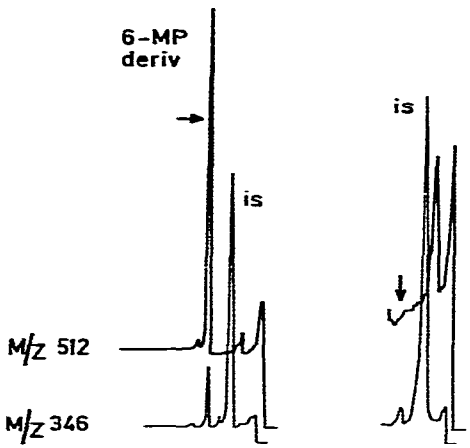

Fig. 3. Chromatogram from analysis of 6-mercaptopurine in plasma samples. *m/z* 512 = S-9-dipentafluorobenzyl-6-mercaptopurine; *m/z* 346 = derivative from internal standard. Right panel: blank plasma sample. Left panel: analysis of a patient plasma sample containing 30 ng/ml 6-mercaptopurine. is = internal standard.

Efforts were made to quantify the absolute recovery of 6-mercaptopurine through the analytical method. However, the derived compound could not be isolated in completely pure form after synthesis. Furthermore, the yield of derivative after addition of 6-mercaptopurine to plasma, 100 ng/ml, was virtually higher than that after preparation of the derivative at the mg/ml level and dilution to the desired concentration with solvent.

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