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A GAS CHROMATOGRAPHIC METHOD FOR MEASURING 6-MERCAPTOPURINE IN SERUM

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SUMMARY

A method is presented for the measurement of 6-mercaptopurine (6-MP) in serum. Serum samples containing the drug were treated with dithioerythritol and saturated with ammonium carbonate. 6-MP was then extracted from the serum with a mixture of isopropanol–ethyl acetate (1:1, v/v) containing 0.1% ethanethiol. The solvent was evaporated, the residue dissolved in dilute hydrochloric acid, washed with chloroform, and extracted with ethyl acetate. 6-MP was derivatized with trimethylanilinium hydroxide and measured gas chromatographically. The sulfhydryl-protecting reagents, dithioerythritol and ethanethiol, were added to prevent the decomposition of 6-MP. The extraction and clean-up method recovered 78 ± 2% (S.E.) of the 6-MP present. Tracer amounts of [8-^{14}C]-6-MP served as the internal standard during the extraction part of the method. Theophylline was used as the internal standard during the gas chromatographic analysis. The standard curve obtained from the gas chromatograph was linear between 0.5 and 20 μg/ml of 6-MP. Serum samples were stored in the freezer for two weeks without significant loss of drug. No interference was encountered from normal serum constituents or xanthines, such as caffeine or theophylline added to serum.

INTRODUCTION

The value of measuring steady-state serum concentrations of many types of drugs has been demonstrated[1]. While serum levels of antineoplastic agents are not routinely monitored there is evidence that antitumor activity may be predicted from such knowledge. Arabinosylcytosine, methotrexate and cyclophosphamide plasma concentrations appear to correlate with their antitumor effect[2]. Such information is not available for the widely used agent 6-mercaptopurine (6-MP).

Several methods exist for the measurement of 6-MP in plasma. Hamilton and Elion[3] measured radioactivity after injection of [6-^{35}S]-6-MP. Loo *et al.*[4] formed an azo dye with Bratton–Marshall reagent and quantitated 6-MP colorimetically. Finkel[5] oxidized 6-MP to purine-6-sulphonate with potassium permanganate and

measured the latter fluorimetically. Coffey *et al.*[6] assayed 6-MP with a UV spectrophotometer after column chromatography. These methods lack specificity and in some cases sensitivity.

This communication describes a new method for measuring 6-MP in serum by utilizing the high sensitivity and selectivity of gas chromatography.

MATERIALS AND METHODS

Reagents

6-MP was generously donated by Burroughs, Wellcome and Company (La-Salle, Canada). [8-^{14}C]-6-MP hydrate with a specific activity of 2.55 mCi/mmole was purchased from New England Nuclear (Boston, Mass., U.S.A.). Theophylline and dithioerythritol (DTE) were obtained from Sigma (St. Louis, Mo., U.S.A.) and ethanethiol and Methelute (0.2 *M* trimethylanilinium hydroxide in methanol) from Pierce (Rockford, Ill., U.S.A.) and used without further purification. All other reagents employed in the assay were of the highest available purity.

Solutions

Ethanethiol-containing solutions. Ethanethiol was stored in well sealed containers at −20°. Sufficient ethanethiol to produce a 0.1% solution was transferred to the organic solvent. The process took place in a fume-hood. These solutions were then stored at −20° and were stable for several weeks.

DTE solution. DTE, stored as a powder at −20°, was dissolved in distilled water to make a 1% solution. This was freshly prepared prior to each analysis.

Stock solutions. Stock solutions of theophylline and 6-MP of 10 μg/ml were prepared in methanol and stored at 5°. 6-MP and theophylline were stable under these conditions for several months.

Radioactive 6-MP solution. [8-^{14}C]-6-MP, found to be 98% pure in our laboratory by thin-layer chromatography, was diluted in methanol to give a ^{14}C concentration of 2.5×10^4 dpm/ml (about 0.75 μg/ml). The solution was stored at −20° and was stable for several months.

Apparatus

A Nuclear Chicago (Des Plaines, Ill., U.S.A.) Mark II Model 6847 liquid scintillation system was used for all radioactivity counting.

A Packard (Downers Grove, Ill., U.S.A.) Model 417 Becker gas chromatograph with a flame ionization detector was used for all determinations.

One-ml reacti-vials with PTFE laminated discs and a reacti-therm heating module used in the preparation of derivatives for gas chromatography were purchased from Pierce.

Instrumental and chromatographic conditions

The chromatographic column was 10% (w/w) SE-30 on Chromosorb W HP, 100–120 mesh (Chromatographic Specialties, Brockville, Canada). The column material was packed in a glass column, 5 ft. × 0.25 in. O.D., previously treated with 5% dimethyldichlorosilane (Pierce) in toluene overnight. The column was held in the oven with graphite ferrules, "Graphloks" (Supelco., Bellefonte, Pa., U.S.A.), and

normal Swagelok fittings. Microsep F-138 septa (Hamilton, Whittier, Calif., U.S.A.) were used.

The operating conditions were as follows: oven temperature 135°, injector port temperature 330° and detector temperature 250°. The nitrogen and hydrogen flow-rates were 100 ml/min and the air flow-rate was about 1 l/min.

Column preparation and conditioning

The preparation of the column packing was modified after that of Gehrke and Lakings[7]. The desired amount of SE-30 was dissolved in excess chloroform and added to a round-bottomed flask containing Chromosorb W HP covered with chloroform. The flask was attached to a rotary evaporator and mixed for 15 min. The chloroform was evaporated under vacuum with heating to 60°. The column packing remained under high vacuum for an additional 30 min.

A glass column, the detector end plugged with ¼ in. glass-wool, was filled to the level where the column would enter the injector heater. A second plug of glass-wool was inserted there. The remaining space was filled with uncoated Chromosorb W HP and a third plug held it in place. This procedure permitted on-column injection of the sample with no apparent bleeding of SE-30 from the injector port.

The column was conditioned by heating at 300° for 2 h under no-flow conditions followed by 24–48 h at 250° with a nitrogen flow-rate of 40 ml/min.

Solvent extraction of 6-MP from serum

(1) A 2-ml volume of serum was placed in a 15-ml centrifuge tube, to which 100 μl of radioactive 6-MP solution and 0.2 ml of DTE solution were added. The mixture was saturated with ammonium carbonate.

(2) An 8-ml volume of isopropanol–ethylacetate (1:1, v/v) with ethanethiol were added, the centrifuge tube sealed with a PTFE stopper and shaken for 5 min. The sample was centrifuged for 5 min, the supernatant transferred to a 15-ml centrifuge tube and evaporated to dryness in a water-bath at 60° under dry nitrogen. After the supernatant of a second serum extraction was added to the centrifuge tube, the sides were washed with 0.5 ml of ethanethiol in methanol and 0.5 ml of ethanethiol in chloroform and the sample evaporated to dryness.

(3) To the residue were added 0.4 ml of 1 *M* sodium chloride, 0.05 ml of 0.2 *M* hydrochloric acid and 1 ml of ethanethiol in chloroform. The sample was gently vortexed for 30 sec. It was sealed and centrifuged for 5 min. Any insoluble material that gathered at the interface of these two solutions was forced into the chloroform layer by heating while gently turning the tube. The chloroform layer was then removed and discarded. This washing procedure was repeated twice.

(4) The aqueous solution was neutralized by the addition of 0.2 ml of 0.5 *M* sodium phosphate buffer pH 6.0. 5 ml of ethanethiol in ethylacetate were added and the mixture was vortexed for 20 sec. The tube was sealed and centrifuged for 5 min. The ethyl acetate was transferred to a test tube containing 2 μg of theophylline and the aqueous solution was re-extracted. The second extract was added to the first and the ethyl acetate dried by addition of about 500 mg of anhydrous sodium sulphate and transferred to a 15-ml graduated centrifuge tube and the volume measured. 1 ml was removed for radioactivity counting. The sample was evaporated to dryness.

(5) The residue was taken up in 0.2 ml of methanol and transferred to a 1-ml

reacti-vial containing 10 μl of Methelute. The sample was heated at 135° for 30 min, allowed to cool, evaporated under a nitrogen stream and redissolved in 20 μl of methanol. 1–2 μl were injected into the gas chromatograph.

Because ethanethiol was unpleasant to use, much of the extraction procedure was carried out in a fume-hood. When this was not possible the samples were well sealed.

Thin-layer chromatography

All samples were chromatographed on 1.0 mm thick cellulose plates with fluorescent indicator (Macherey, Nagel and Co., Düren, G.F.R.) in butanol–acetic acid–water (25:4:10). 6-MP was detected as a dark blue quenching spot against the fluorescent background under ultraviolet light. Sections of the plates, 0.5 cm in width, were scraped into counting vials containing 3 ml of methanol and 10 ml of toluene–Triton X-100 based counting solution and counted directly.

Calculations

The concentration of 6-MP in plasma was calculated using the following equation:

$$\text{Concentration in serum } (\mu\text{g/ml}) = \frac{\text{Concentration from the standard curve } (\mu\text{g/ml})}{\text{Fractional recovery of radioactivity}}$$

The standard curve was determined from plasma and corrected for recovery.

RESULTS

Linearity of response and sensitivity

Serum samples spiked with 0.5–20 μg/ml of non-radioactive 6-MP plus trace amounts of [^{14}C]-6-MP were assayed. The standard curve is shown in Fig. 1. The peak height ratios were corrected to 100% recovery as determined by the amount of radioactivity extracted. The curve was linear in this range. The lower limit of sensitivity was 0.2 μg/ml.

Precision and accuracy

The precision and accuracy of the method were determined by assaying six 2-ml serum samples to which 10 μg of 6-MP had been added. The mean value was 4.9 $\pm$ 0.1 (S.E.) μg/ml.

Importance of DTE and ethanethiol

The sulfhydryl-protecting reagents, DTE and ethanethiol, were required in the method. Fig. 2 shows two gas chromatograms of 6-MP measured in serum. In both analyses the initial concentration was 5.0 μg/ml. In the absence of ethanethiol and DTE the peak height ratio was markedly reduced. The reason for the loss of 6-MP was due to decomposition during the extraction. In Fig. 3A, 98% of the radioactivity extracted from serum in the presence of ethanethiol and DTE was in the form of 6-MP. Without the protective reagents considerable decomposition of 6-MP occurred (Fig. 3B).

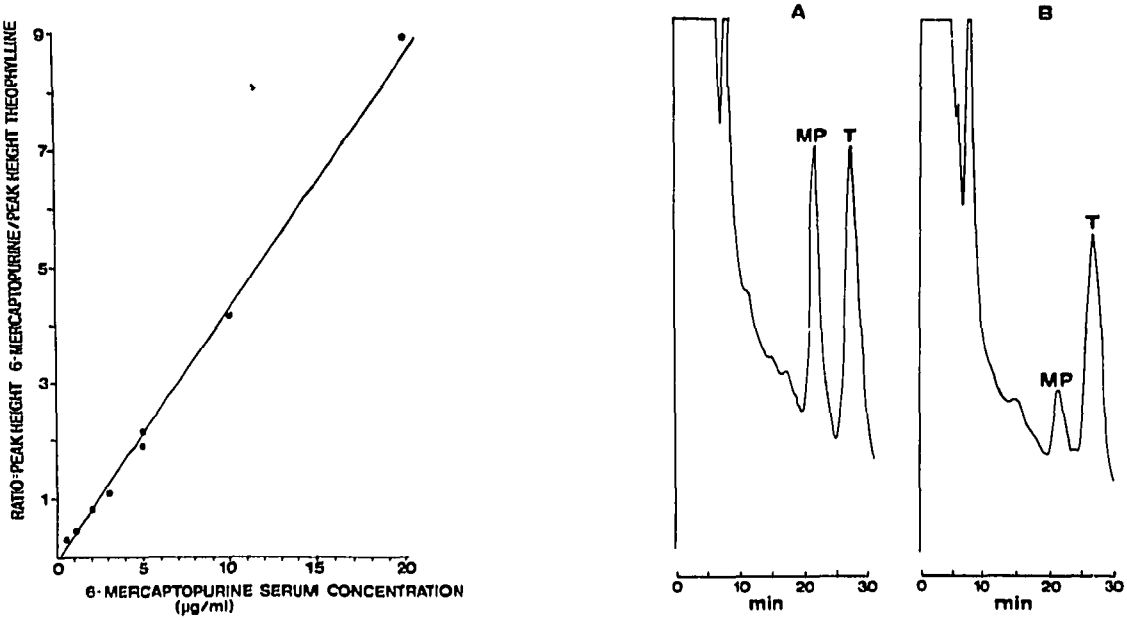

Fig. 1. Standard curve for the quantitative analysis of 6-MP in serum. Peak height ratio was corrected for recovery.

Fig. 2. Gas chromatographic tracings of 6-MP (MP) and the internal standard, theophylline (T) analyzed in serum. (A) DTE and ethanethiol added; (B) neither DTE nor ethanethiol added.

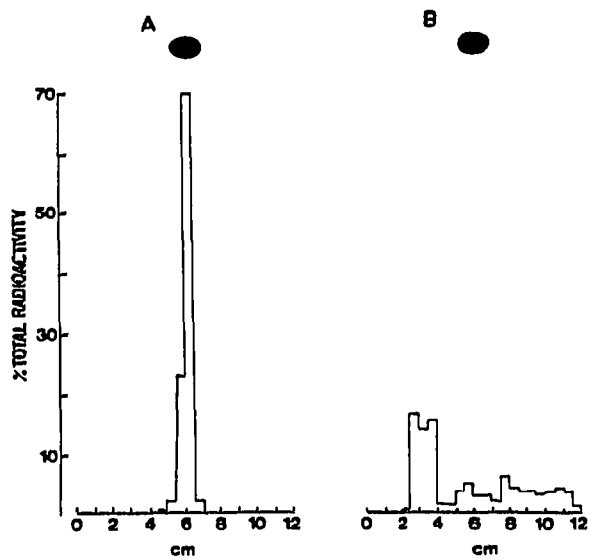

Fig. 3. Thin-layer radiochromatograms of serum extracts of [8-^{14}C]-6-MP. The spots correspond to non-radioactive 6-MP on the plate. (A) DTE and ethanethiol added; (B) neither DTE nor ethanethiol added.

Recovery of 6-MP from serum

The recovery of non-radioactive 6-MP was determined by comparing the peak height ratios of five samples at the level of 5 μg/ml of 6-MP assayed directly from stock solutions against six samples of the same concentration prepared from serum. The recovery of 6-MP was 77.5 $\pm$ 2.0% (S.E.).

Stability

The stability in serum of 6-MP was evaluated by assaying 7 samples of 5 μg/ml, stored in the freezer at —20°, over a two-week period. Table I shows that there is no significant loss during storage.

TABLE I

STABILITY OF 6-MP IN SERUM

Samples were stored at —20°.

Days stored	*Serum level (μg/ml)*
0	5.0
1	5.0
2	4.8
3	4.8
4	5.0
7	4.9
8	5.0
15	5.0

Interference

Possible interference by endogenous substances, *e.g.* caffeine and theophylline, was evaluated. Blank samples and samples spiked with caffeine (100 μg/ml) or theophylline (100 μg/ml) contained no interfering peaks.

DISCUSSION

This paper presents a gas chromatographic method for the analysis of 6-MP in serum. One problem encountered initially was the instability of the drug in serum and in the extraction solvents. To a major degree this problem has been overcome by the use of DTE and ethanethiol. The addition of DTE alone has been shown to prevent the decomposition of 6-MP both in serum and in the organic solvents. Ethanethiol used alone had little effect on the stability of 6-MP in serum but did offer protection in ethyl acetate. Ethanethiol was employed in this method because its presence reduced the amount of DTE required and thereby diminished the amount of residue remaining at the end of the extraction procedure. The use of these materials also facilitated the analysis of frozen serum samples containing 6-MP. It is apparent from the results that samples can be frozen for periods of up to two weeks without influencing the reliability of the assay.

It was not possible to find an ideal internal standard, *i.e.* a substance extracted as well as 6-MP with a retention time close to 6-MP on the gas chromatograph.

Purine, 2-mercaptopurine, 6-thioguanine, 6-methylpurine, 2,6-dithiopurine, 6-chloropurine, 8-bromopurine, 6-methylmercaptopurine, 6-methoxypurine, 8-chloroxanthine and 6-chloro-9-methoxy-methylpurine were tried. All proved to be unsatisfactory. As a result of this, two internal standards were employed: one for the solvent extraction phase and one for the gas–liquid chromatography phase. Radioactive 6-MP was used to determine the recovery of the non-radioactive drug from plasma. The total amount of radioactivity added to the plasma was 75 ng and did not influence the quantitative aspects of the assay. The internal standard for the gas chromatography portion of the assay was theophylline. It was introduced before an aliquot was taken for radioactivity counting. Treatment of patients with theophylline or caffeine would not invalidate this assay. These substances, if initially present in plasma, are eliminated during the extraction procedure, probably in the chloroform wash.

Because 6-MP can not be chromatographed directly, a more volatile derivative was prepared. Gehrke and Lakings[7] have prepared silyl derivatives of naturally occurring purine and pyrimidine bases. This was not possible for 6-MP. Other derivatizing agents as boron trifluoride in methanol, heptafluorobutyrylimidazole, N-trifluoroacetylimidazole, boron trichloride in 2,2,2-trifluoroethanol, trifluoroacetic anhydride and heptafluorobutyric anhydride were tried and found unsatisfactory. The only agent capable of producing a suitable derivative was trimethylanilinium hydroxide (Methelute). On-column methylation with Methelute was compared with derivatization at 135° for 30 min prior to injection. The latter method was found to reduce the variability in peak heights and produce a smaller solvent front.

An attempt was made to measure steady-state serum levels in patients on oral maintenance doses of 6-MP. Twelve subjects were sampled. In serum taken from eleven subjects no 6-MP could be found. In the single serum sample in which 6-MP was detected, the concentration approximated 0.3 μg/ml. The failure to find 6-MP in sera of patients receiving the drug could reflect poor absorption, rapid disappearance of the drug from sera, and/or degradation en route to the laboratory. Results from animal experiments suggest that degradation was unlikely. Rabbits were administered i.v. the usual human therapeutic dose of 6-MP, 2.5 mg/kg, and serum samples were drawn at regular intervals. Under these conditions it was possible to measure the drug 5 and 10 min after injection. 15 min after treatment the concentrations in the serum had fallen below the sensitivity level of the method.

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