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Short Communication

Chromatographic analysis of methylmercaptopurine riboside in human plasma and urine

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ABSTRACT

An isocratic reversed-phase high-performance liquid chromatographic (HPLC) method for the determination of methylmercaptopurine riboside (MMPR) in human plasma and urine is reported. Plasma samples were prepared for analysis by addition of internal standard (6-dimethylaminopurine 9-riboside) followed by extraction using disposable C_{18} cartridges. Urine samples were filtered through a 0.22-μm membrane prior to HPLC separation. The column effluent was monitored at 289 nm and quantitation performed using peak heights. The linear range for MMPR determination was from 10 to 500 ng/ml in plasma and from 0.25 to 50 μg/ml in urine. The reported method is convenient, sensitive, and reproducible, illustrating its usefulness for application in pharmacokinetic studies.

INTRODUCTION

The anticancer drug 6-methylmercaptopurine riboside (MMPR) has been clinically tested as a single agent [1,2] and as a biochemical modulator for 5-fluorouracil [3,4]. Pertinent *in vivo* pharmacologic parameters such as plasma concentrations and urinary excretion after MMPR administration in mice [5] and in humans [6] have been studied using thin-layer chromatography of the radiolabelled ([^{35}S]MMPR) drug. Likewise, *in vitro* formation of 5-nucleotide metabolites of MMPR has been examined with anion-exchange HPLC [7–9]. However,

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no pharmacokinetic data have been reported for MMPR either given alone or in combination with 5-fluorouracil.

Thus, in this study a method for the efficient extraction of MMPR from plasma followed by a sensitive HPLC method for the analysis of MMPR from plasma and urine will be presented. Samples from Phase I cancer patients will be analyzed to demonstrate the usefulness of the method towards generating valuable pharmacokinetic data.

EXPERIMENTAL

Chemicals

MMPR was supplied by the National Cancer Institute. 6-Dimethylaminopurine 9-riboside (DMAPR) and monobasic potassium phosphate (KH_2PO_4) were purchased from Sigma (St. Louis, MO, U.S.A.). The extraction and chromatography solvents (water and methanol) were all HPLC grade and were purchased from Burdick and Jackson (Muskegon, MI, U.S.A). Control plasma was purchased from Interstate Blood Bank, (Philadelphia, PA, U.S.A.).

Patients samples

Patient blood samples (5 ml) were obtained at selected times following drug infusion. Samples were centrifuged at 4°C and 1200 *g* for 10 min and the plasma was removed and stored at −70°C until analysis.

Patient urine was collected for 24 h after drug infusion. The urine collection was pooled as 4-h aliquots, the volume was recorded, and a smaller aliquot (4 ml) was stored at −70°C until analysis.

Sample preparation

To prepare the plasma for extraction, the following was added to the appropriate plasma sample (1.0 ml): (1) for plasma blanks, 75 μl of water; (2) for patient samples, 50 μl of water plus 25 μl of the internal standard DMAPR (40 μg/ml in water); or (3) for standard curve samples, 50 μl of the appropriate standard plus 25 μl of DMAPR. All 1.075 ml were then applied to a preconditioned (10 ml methanol followed by 10 ml water) C_{18} Bond Elut disposable cartridge from Analytichem International (Harbor City, CA, U.S.A.). The column was washed with water (10 ml), and MMPR and DMAPR were eluted with 100% methanol (1.0 ml plus 0.5 ml). The solvent was evaporated to dryness with a stream of nitrogen at 40°C. The residue was reconstituted in water (400 μl) and sonicated for 3 min. The HPLC injector volume was 200 μl.

To the urine samples (1.0 ml) either 50 μl of water (urine blanks or patient samples) or 50 μl of the appropriate standard (urine standard curve samples) were added. An aliquot (400 μl) of the prepared urine was filtered through a Millipore (Bedford, MA, U.S.A.) centrifuge filter (Ultrafree-MC, 0.22-μm Durapore). The HPLC injector volume was 25 μl.

High-performance liquid chromatography (HPLC)

The chromatographic system consisted of a Hewlett-Packard (Palo Alto, CA, U.S.A.) HP-1090 Series A liquid chromatograph equipped with an autoinjector/autosampler and an HP1040A diode-array UV detector. The column effluent was monitored at 289 nm, the absorbance maximum of MMPR (Fig. 1A). The chromatograph was operated with a Hewlett-Packard HP-85B personal computer, and data were interpreted with a DPU multi-channel integrator. Chromatography was performed on a Hewlett-Packard reversed-phase C_{18} analytical column (Hypersil ODS, 5 μm, 100 mm × 4.6 mm I.D.) preceded by a 15 mm × 3.2 mm, 7-μm Aquapore C_{18} guard column (Brownlee Labs., Santa Clara, CA, U.S.A.).

For the plasma extracts, MMPR and DMAPR were eluted isocratically, with retention times of 6.9 min ($k' = 18.8$) and 7.9 min ($k' = 21.7$), respectively, at a flow-rate of 2.0 ml/min. The isocratic mobile phase consisted of 82% water containing 0.5 m*M* KH_2PO_4 (pH 5.40) and 18% methanol. MMPR from filtered urine was eluted with a retention time of 6.7 min ($k' = 18.3$) with a flow-rate of 2.0 ml/min. The mobile phase consisted of 82% water containing 0.75 m*M* KH_2PO_4 (pH 5.29) and 18% methanol. Plasma standard curves consisting of six points (10, 25, 50, 100, 250, and 500 ng/ml) were plotted as the peak-height ratio of MMPR to DMAPR *versus* concentration of MMPR. Urine standard curves consisting of eight points (0.25, 0.50, 1.0, 2.5, 5.0, 10.0, 25.0, and 50.0 μg/ml) were plotted as the peak-height of MMPR *versus* the concentration of MMPR. The linear regression lines were calculated by the method of least squares and were weighted by $1/y$.

RESULTS AND DISCUSSION

After C_{18} cartridge extraction, HPLC of control plasma (Fig. 1A) yielded a chromatogram clear of interfering peaks at the retention times of MMPR and DMAPR, while the chromatogram of control plasma spiked with MMPR and DMAPR (Fig. 1B) demonstrated a complete separation of MMPR from DMAPR. Also illustrated in Fig. 1B, the final concentration of DMAPR (1.0 μg/ml) was chosen to yield a peak-height ratio of approximately 1 with the highest MMPR standard (500 ng/ml).

The lowest MMPR dose administered in the Phase I protocol was 75 mg/m^2, thus patient samples from this dose were extracted and chromatographed (Fig. 1C and D) to determine if the lower linear limit of this analytical method would be adequate for the MMPR protocol. As seen in Fig. 1C, the pre-treatment patient plasma was void of interfering peaks and while the post-treatment plasma sample (Fig. 1D) approached the lower linear limit, all the patient samples analyzed were greater than the lowest linear limit of 10 ng/ml of plasma.

The introduction of DMAPR as an internal standard was done to improve the precision of analysis. A suitable internal standard should exhibit similar physical and chemical properties to the compound of interest; if these criteria are not

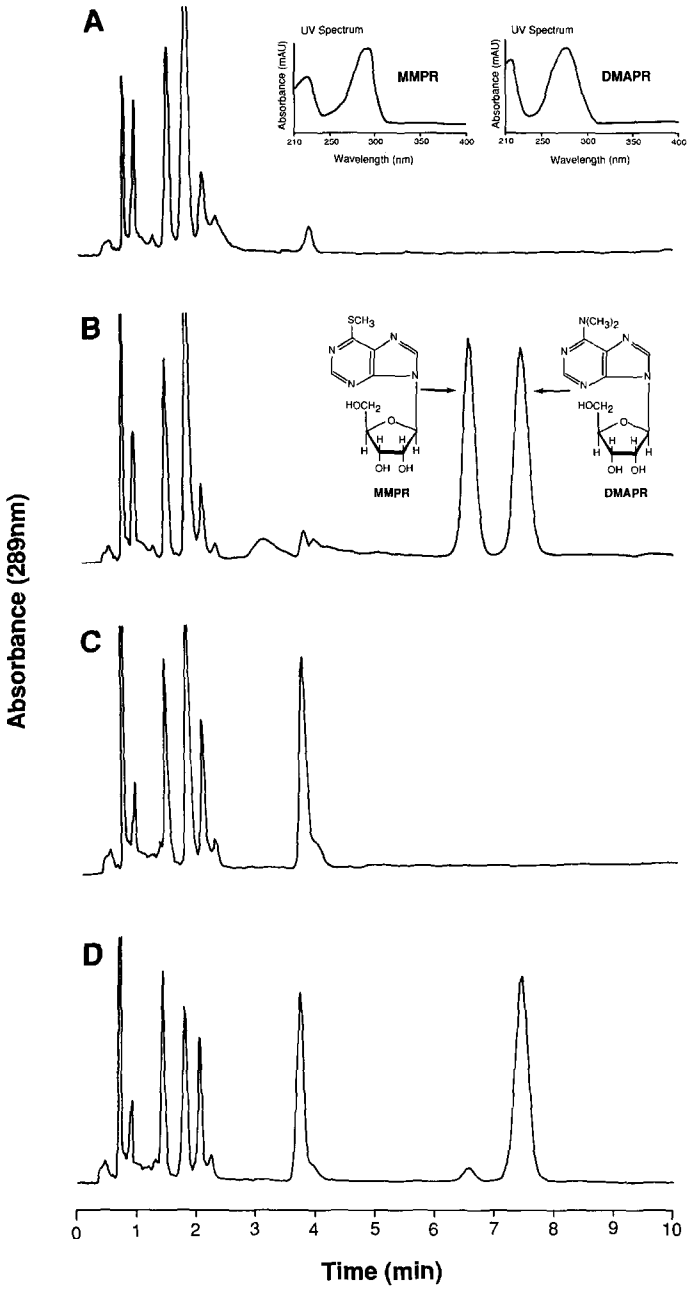

Fig. 1. Chromatograms after C_{18} cartridge extraction of control and MMPR-containing plasma samples. (A) Control plasma; inserts of MMPR and DMAPR UV spectrums; (B) control plasma spiked with MMPR (500 ng/ml) and DMAPR (1.0 μg/ml); (C) pre-treatment patient plasma; (D) post-treatment patient plasma containing MMPR (32.0 ng/ml) and spiked with DMAPR (1.0 μg/ml). For all chromatograms, 200 μl injected and detector at 25 mAU.

reasonably well met then analysis precision error can be introduced [10]. MMPR and DMAPR have very similar chemical structures (Fig. 1B) and differ only at position 6 of the purine ring. As deduced by their respective retention times, the dimethylamino group at position 6 confers a slightly more hydrophobic nature to DMAPR than does the methylmercapto group on MMPR, therefore allowing sufficient separation of MMPR from the internal standard DMAPR using reversed-phase HPLC.

There was a linear relationship between the peak-height ratio of MMPR to DMAPR and the concentration of MMPR from duplicate plasma standards extracted with the C_{18} cartridge. The linear range was 10–500 ng/ml, with a lower limit of detection of 5 ng/ml. The average equation obtained from standard curves, extracted daily with patient samples, was $y = 2.07\ x - 0.00305$ ($r = 0.9999$) with a coefficient of variation (C.V.) for the slope of 0.87%. As calculated from the standard curve for that day, the error for each standard was less than ± 10%, with an average (of the absolute value) for all the errors being 4.5 ± 4.0% (mean ± S.D.). Over the linear range, the average MMPR recovery from the C_{18} cartridge was 100.0 ± 5.8% and for DMAPR it was 110.3 ± 0.3%.

The urine HPLC was similar to the plasma HPLC, the only differences being a slight increase in KH_2PO_4 concentration (from 0.5 to 0.75 m*M*), the smaller injection volume (from 200 to 25 μl) of filtered urine, and no need for an internal standard due to the lack of an extraction step. As seen in Fig. 2A, the pretreatment patient urine was void of interfering peaks and the first 4-h aliquot (Fig. 2B), as well as the last 4-h aliquot (Fig. 2C) had sufficient MMPR present for analysis. Change in urine concentration from one sample collection period to another was illustrated (Fig. 2) by change in the appearance of the chromatograms.

There was a linear relationship between the MMPR peak height and the MMPR concentration from triplicate urine standards. The linear range was 0.25–50.0 μg/ml, with a lower limit of detection of 0.10 μg/ml. The average equation obtained from standard curves, ran daily with patient samples, was $y = 1.71\ x - 0.00194$ ($r = 1.0000$) with a C.V. for the slope of 1.7%. As calculated from the standard curve for that day, the error for each standard was less than ± 7%, with an average (of the absolute value) for all the errors being 1.7 ± 1.7%. The average MMPR recovery from filtered urine was 98.7 ± 6.8%, thus filtering the urine did not significantly effect the MMPR concentration.

Two noteworthy problems were encountered during the development and application of this analytical method. First, the chromatographic system required approximately 3 h to equilibrate prior to the first sample injection which might be related to the relatively low concentration of KH_2PO_4 (0.5 or 0.75 m*M*). The KH_2PO_4 concentration could not be increased without shifting MMPR into interfering urine peaks or into the DMAPR peak, thus the 3-h equilibration was incorporated into the daily methodology and in essence introduced no practical hindrance since the time required for sample preparation entailed much of the 3

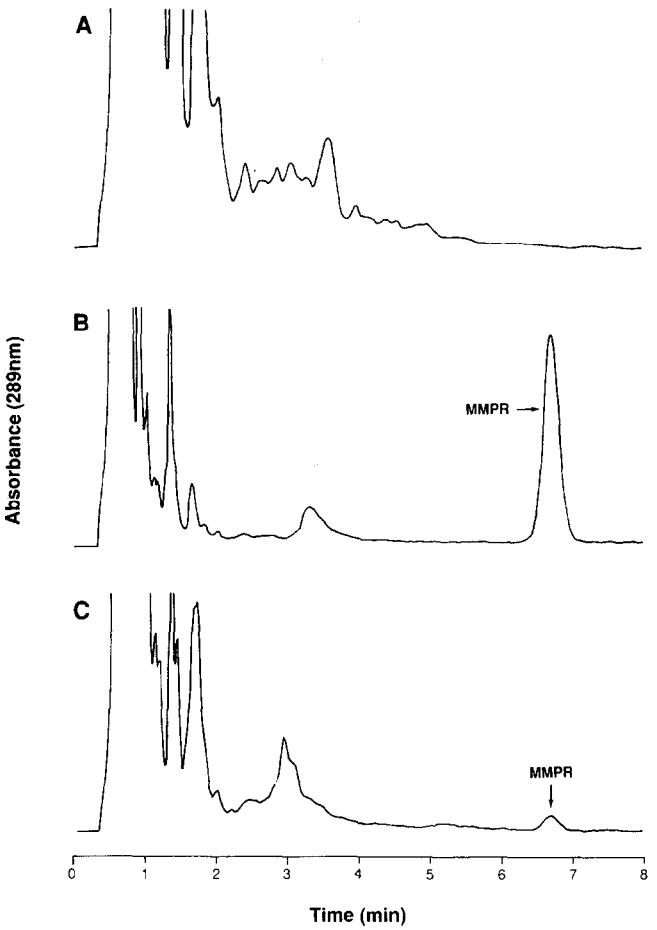

Fig. 2. Chromatograms of patient urine samples. (A) Pre-treatment patient urine from a total volume of 175 ml; (B) 0–4 h patient urine from a total volume of 1275 ml; MMPR = 6.9 μg/ml; (C) 20–24 h patient urine from a total volume of 350 ml; MMPR = 0.49 μg/ml. For all chromatograms, 25 μl injected and detector at 15 mAU.

h. Secondly, the occasional presence of late-eluting compounds, especially from very concentrated urine samples, required an 11-min run time. Thus, peaks from late-eluting compounds would appear in the following chromatogram prior to MMPR elution, keeping analysis time to a minimum while achieving automation.

The C_{18} cartridge extraction of human plasma or the filtration of human urine followed by isocratic reversed-phase HPLC, monitored at 289 nm, proved to be a convenient, sensitive, and effective method for the detection of therapeutic levels of MMPR. The method sensitivity of 10 ng/ml for plasma and 250 ng/ml for urine was more than adequate for the analysis of MMPR in patient samples, thus demonstrating the usefulness of the method towards obtaining meaningful pharmacokinetic results.

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