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Note

Improved chromatographic method for the determination of trimetrexate in urine

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The antifolate trimetrexate, 3,4-diamino-5-methyl-6-[(3,4,5-trimethoxy-anilino)methyl]quinazoline (TMTX) has demonstrated antitumor activity [1,2] and has been evaluated in several phase I clinical trials [3–6]. TMTX can be analyzed by radioassay methods utilizing TMTX inhibition of dihydrofolate reductase (DHFR) [7–9] and by high-performance liquid chromatography (HPLC) [10]. Both analytical approaches have pharmacological relevance; the DHFR inhibition assays can quantitate the total activity produced by TMTX and any active metabolites, while HPLC can quantitate the parent drug TMTX with the further possibility of isolating, identifying and quantitating TMTX metabolites.

While using the HPLC method of Ackerly et al. [10] for analysis of patient samples from a phase I pharmacokinetic study, it was found that the urine extraction method yielded a high level of background noise in the chromatogram, consisting of substantial interfering chromatographic peaks. Thus, a more selective extraction method was developed utilizing CN Bond Elut instead of the C_{18} Bond Elut extraction columns used by Ackerly et al. [10]. A gradient elution HPLC method was also developed to aid in further isolating the TMTX peak from potential interfering peaks and to accommodate the introduction of an internal standard, trimethoprim (TMP), into the methodology.

Thus, in this study an improved extraction procedure and gradient elution

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reversed-phase HPLC method for the analysis of TMTX in human urine will be presented.

EXPERIMENTAL

Chemicals

TMTX glucuronate was obtained from Warner-Lambert/Parke Davis Pharmaceutical Research (Ann Arbor, MI, U.S.A.). TMP, triethylamine, sodium citrate and sodium acetate were purchased from Sigma (St. Louis, MO, U.S.A.). HPLC-grade orthophosphoric acid (85%) was purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). The extraction and chromatography solvents (water, acetonitrile and methanol) were all HPLC grade and were purchased from Burdick and Jackson (Muskegon, MI, U.S.A.).

Sample preparation

A urine blank using the extraction method of Ackerly et al. [10] consisted of applying human urine (1.0 ml) to a preconditioned (9 ml methanol followed by 9 ml water) 500 mg/2.8 ml C_{18} Bond Elut disposable column from Analytichem International (Harbor City, CA, U.S.A.). The column was washed sequentially with water (6 ml), acetonitrile (1 ml) and 75% 0.02 *M* sodium acetate (pH 4.5) with 25% methanol (1 ml). The column was eluted with methanol–0.08 *M* sodium citrate (95:5; 1.25 ml). The eluate was evaporated to dryness at room temperature and reconstituted in 1.0 ml of CN elution buffer in order to directly compare the C_{18} blank with the urine blank prepared using the CN extraction method.

For the CN extraction, 50 μl of the appropriate TMTX standard (in water) or water (for the blank urine) and 100 μl of the internal standard TMP (20 μg/ml in water) were added to human urine (1.0 ml) and then further diluted with water (1.0 ml). The urine sample was applied to a preconditioned (3 ml acetonitrile followed by 3 ml of water) 100 mg/ml CN Bond Elut disposable column from Analytichem International. The column was washed with water (3 ml) and eluted with 1.0 ml buffer [acetonitrile–water (15:85) containing 0.75% (v/v) triethylamine and 0.375% (v/v) orthophosphoric acid (85%)].

The urine blank from the C_{18} column (reconstituted in 1.0 ml of CN elution buffer) and the CN urine blank or urine plus standards were transferred to injection vials, and 200 μl were injected.

High-performance liquid chromatography

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 HP 1040A diode-array UV detector. The column effluent was monitored at 241 nm, the absorbance maximum of TMTX. The chromatograph was operated with a Hewlett Packard HP-85B personal com-

puter, 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$\times$4.6 mm I.D.) preceded by a 15 mm$\times$3.2 mm I.D., 7-μm Aquapore C_{18} guard column (Brownlee Labs., Santa Clara, CA, U.S.A.). TMP and TMTX were eluted, with retention times of 5.8 and 11.0 min, respectively, by a gradient mobile phase at a flow-rate of 1.5 ml/min. The starting mobile phase was 91% water containing 0.16% triethylamine, 0.08% orthophosphoric acid (85%) and 9% acetonitrile (pH 4.2). The acetonitrile was linearly increased from 9 to 35% over 15 min, and was followed by a 5-min reequilibration at 9% acetonitrile prior to the next injection. Standard curves consisting of four points (0.1, 0.5, 1, and 5.0 μg/ml) were plotted as the peak-height ratio of TMTX to TMP versus concentration of TMTX. The linear regression lines were calculated by the method of least squares.

RESULTS AND DISCUSSION

Aliquots of the same urine sample were extracted through a CN Bond Elut and a C_{18} Bond Elut. Under identical gradient HPLC conditions the CN extraction (Fig. 1A) yielded a baseline with no slope and very few chromatographic peaks, while the C_{18} extraction (Fig. 1B) showed an ascending slope

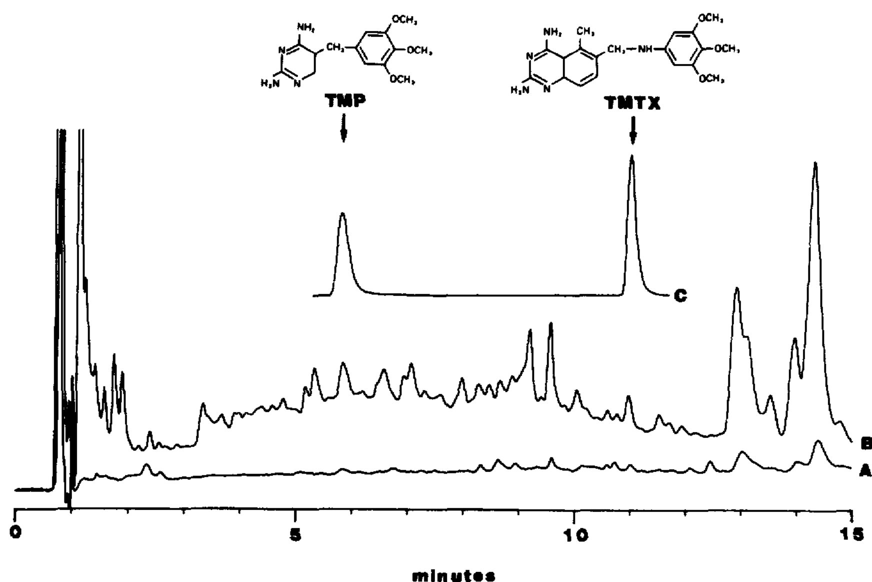

Fig. 1. Chromatograms of blank urine samples and a chromatogram of standard TMP and TMTX. (A) Blank urine extracted with a CN Bond Elut. (B) Blank urine extracted with a C_{18} Bond Elut. (C) Superimposed chromatogram of TMP (2 μg/ml) and TMTX (0.5 μg/ml). Injection volume, 200 μl; detection, 50 mA.U. for all three chromatograms.

with significant interfering peaks eluting at the retention times for both TMP and TMTX. When using the isocratic HPLC method described by Ackerly et al. [10] both the ascending slope and interfering peaks from the C_{18} extraction appeared to be even more severe. Upon analysis of urine samples from certain patients on this study, several sizable, unique interfering peaks were observed. Because the size of these peaks decreased with time they probably represent concomitant medications or their metabolites. These observations prompted the development and use of the gradient HPLC method.

The introduction and selection of TMP 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 reasonably well met then analysis precision error can be introduced [11]. TMP has a similar chemical structure to TMTX (Fig. 1) especially with respect to the trimethoxyphenyl group and the diamino heterocyclic ring moiety in both drugs. The amines in the heterocyclic ring probably play a major role in the retention mechanism of TMTX and TMP on the CN Bond Elut, while the quinalozine makes TMTX more hydrophobic than TMP and therefore aids in efficiently separating the two by reversed-phase chromatography.

There was a linear relationship between the peak-height ratio of TMTX to TMP and the concentration of TMTX in the standards extracted from urine with the CN Bond Elut. The equation obtained from triplicate standard curves after CN extraction of urine spiked with TMTX and TMP was $y=3.73x-0.079$ ($r=1.0000$). The average recovery from the CN Bond Elut for TMTX was $93.6\pm2.3\%$ (mean$\pm$S.D.) and for TMP it was $94.2\pm1.8\%$, over the linear range. The equation obtained over five months of standard curves was $y=3.23x-0.036$ ($r=1.0000$) with a standard error of the mean for the slope of 0.093.

The CN Bond Elut extraction of TMTX from urine was more selective than the C_{18} Bond Elut extraction method as judged by the appearance of the obtained chromatogram (Fig. 1). Furthermore, the CN Bond Elut method was less expensive because the 100 mg/ml CN Bond Elut costs about half that of the 500 mg/2.8 ml C_{18} Bond Elut.

TMTX concentrations in patient urines collected for 48 h following drug administration were found to be in the range 0.1–0.3 μg/ml, thus the lower limit of linearity had to be at least 0.1 μg/ml. This level of sensitivity was obtainable using both extraction methods but the CN procedure further enhanced the overall sensitivity because (1) the final elution buffer allowed for the direct injection of 200 μl without the steps of evaporation and resuspension needed with the C_{18} method and (2) the absence of interfering peaks improved the baseline resolution of the peaks of interest.

The CN Bond Elut cartridge extraction of TMTX proved to be a rapid, cost-effective and efficient method for the determination of TMTX in human urine.

The overall chromatographic method was also improved using gradient elution, while the assay precision was increased with the introduction of the internal standard, TMP.

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