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Journal of Chromatography B, 807 (2004) 301–305
JOURNAL OF CHROMATOGRAPHY B
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Determination of trimethoprim in low-volume human plasma by liquid chromatography

Matthew W. Hruska [a,b], Reginald F. Frye [b,*]

[a] Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15261, USA
[b] Department of Pharmacy Practice, University of Florida, College of Pharmacy, P.O. Box 100486, Gainesville, FL 32610, USA

Received 10 October 2003; received in revised form 20 April 2004; accepted 23 April 2004

Available online 25 May 2004

Abstract

Trimethoprim is an anti-infective agent used in the treatment of urinary and respiratory tract infections and mild to moderate pneumocystis carinii pneumonia. Trimethoprim is also a selective in vitro inhibitor of cytochrome P450 2C8 and may have utility as an in vivo inhibitor of this enzyme. A simplified high performance liquid chromatography (HPLC) method was developed to determine trimethoprim in human plasma. Samples are processed by protein precipitation with perchloric acid and chromatographic separation is achieved on a Synergi Polar-RP column (4 micron, 150 mm × 4.6 mm) using a mobile phase consisting of 50 mM ammonium formate-acetonitrile-methanol (pH = 3.0; 90:6:4 (v/v/v)). Detection is monitored at 280 nm. Intra- and inter-day precision ranged from 1.1 to 1.9 and 0.9 to 4.1%, respectively. The assay is simple, economical, precise, and is directly applicable to human studies involving steady state trimethoprim pharmacokinetics.
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Keyword: Trimethoprim

1. Introduction

Trimethoprim (Fig. 1), a dihydrofolate reductase inhibitor, is an anti-infective agent used predominantly in the treatment of urinary and respiratory tract infections [1]. Trimethoprim is also used in combination with sulfamethoxazole or dapsone to treat mild to moderate cases of pneumocystis carinii pneumonia (PCP) [2]. Treatment of PCP requires trimethoprim doses of 15–23 mg/kg/day, which yields mean plasma concentrations of 6.7–18.4 μg/ml (23.1–63.4 μM) [3,4]. The frequency of severe adverse effects including neutropenia and thrombocytopenia increases when trimethoprim plasma concentrations exceed 8 μg/ml [4]. Trimethoprim is well-absorbed after oral administration (bioavailability ∼95%) and achieves peak plasma concentrations 1–4 h after single dose administration. It has a half-life of ∼10 h, protein binding of 44%, a volume of distribution ranging from 70 to 100 l, and it is extensively eliminated in the urine as unchanged drug [1].

In addition to its therapeutic applications, trimethoprim is a selective in vitro inhibitor of cytochrome P450 (CYP) 2C8 (K_i = 32 μM; 1 μM = 290.3 μg/l) [5], and may be useful as an in vivo inhibitor of this enzyme. Peak plasma concentrations after trimethoprim 200 mg given twice daily are approximately 20 μM [6]. Wen et al., [5] predicted based on expected plasma concentrations, that in vivo inhibition of CYP2C8 would be approximately 80% assuming that hepatic trimethoprim concentrations would be approximately 130 μM, which is based on the liver to plasma partitioning ratio of 6.5:1 observed in monkeys [7]. These observations suggest that trimethoprim may be useful as a selective enzyme inhibitor in vivo, but further study is required.

There is a need to measure trimethoprim in plasma to ensure effective concentrations are achieved in patients being treated for PCP and also to aid in the evaluation of trimethoprim as an in vivo inhibitor of CYP2C8. Thus, a simple, efficient method for determination of trimethoprim in plasma is desired. Several methods for the determination of trimethoprim in human plasma have been reported [8–13]. In general they require large (1 ml) sample volumes [8–10], involve a multi-step, lengthy extraction process or solid-phase extraction [11,13] or require the use of an ion-pairing agent for

* Corresponding author. Tel.: +1-352-392-8551; fax: +1-352-392-9388.
E-mail address: frye@cop.ufl.edu (R.F. Frye).

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doi:10.1016/j.jchromb.2004.04.021

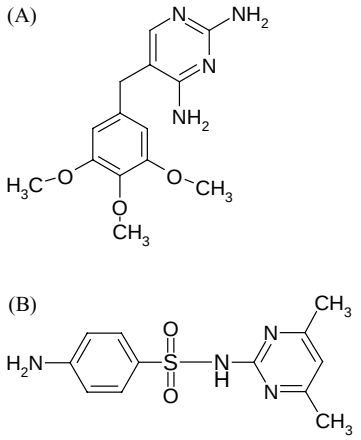

Fig. 1. (A) Trimethoprim and (B) sulfamethazine (internal standard).

suitable chromatography with a C_{18} column [12]. A large sample volume (e.g., 1 ml) is a problem in certain populations such as pediatric AIDS patients requiring trimethoprim therapy for PCP [11]. Ronn et al. improved the applicability to special populations by decreasing the sample volume to 125 μl, but the sample preparation involved multiple steps including protein precipitation, transfer of supernatant and subsequent dilution with mobile phase [11]. Therefore, the goal of this work was to establish a method that uses a small sample volume and an efficient, economical extraction method. In this paper, we introduce an HPLC method with simplified sample processing and ultraviolet wavelength detection to determine trimethoprim in human plasma. The method was used to determine steady-state trimethoprim plasma concentrations in healthy human subjects.

2. Experimental

2.1. Chemicals

Trimethoprim (≥98%) reference standard and sulfamethazine (≥99%) (internal standard, IS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile and methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). Perchloric Acid was purchased from J.T. Baker (Phillipsburg, NJ, USA). Ammonium formate and formic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). Blank human plasma was purchased from the Central Blood Bank of Pittsburgh (Pittsburgh, PA, USA). Deionized water was obtained from an in-house Millipore (Billerica, MA, USA) water system.

2.2. HPLC separation

The HPLC system consisted of a Waters 2695 separation module and a Waters 486 Tunable Absorbance Detector (Waters, Corp., Milford, MA, USA). Detection and quantification were performed using Millenium^{32} software version 3.1 (Waters, Corp., Milford, MA, USA). Separation was achieved with a Synergi® Polar-RP 4μ 150 mm × 4.6 mm column (Phenomenex, Torrance, CA, USA) heated to 30 °C and an isocratic mobile phase of ammonium formate (pH 3.0; 50 mM)-acetonitrile (ACN)-methanol (MeOH) (90:6:4 (v/v/v)), delivered at a flow rate of 1 ml/min. The monitoring wavelength was set at 280 nm, with a run time of 14 min.

2.3. Standard preparation

Trimethoprim stock solution was prepared at a concentration of 1 mg/ml in methanol. Dilutions prepared in methanol at concentrations of 250 and 50 μg/ml were used to prepare calibration standards and quality control (QC) samples. The internal standard (IS) sulfamethazine was dissolved in methanol to prepare a 50 μg/ml stock solution. Standards and QC samples were prepared at the beginning of the validation experiment by spiking batches of blank human plasma and dispensing into 200 μl aliquots. Aliquots were stored at −20 °C until analysis was performed.

2.4. Plasma sample preparation

Aliquots of plasma (200 μl) were placed in microcentrifuge tubes, IS (10 μl or 500 ng) was added, and the tubes were briefly vortexed. Perchloric acid (25 μl) was added to each sample, which was then vortexed for 2 min, and centrifuged at 3000 × *g* for 10 min. The supernatant was transferred to HPLC vials, and capped. A 75 μl aliquot was injected onto the HPLC system for analysis.

2.5. Calibration and linearity

Calibration curves consisted of seven non-zero standard concentrations of trimethoprim in human plasma: 0.5, 1, 2.5, 5, 7.5, 10, 20 μg/ml. Duplicate calibration curves were assayed daily for 3 days, with the lowest concentration (0.5 μg/ml) prepared in triplicate. For each curve, the trimethoprim to IS peak height ratios were calculated and plotted against the nominal trimethoprim concentration. Calibration curves for trimethoprim were constructed by weighted ($1/y^2$) linear regression analysis.

2.6. Precision and accuracy

Precision and accuracy were determined by the analysis of trimethoprim QC samples spiked at concentrations of 2, 8, and 16 μg/ml. Replicate QC samples ($n = 12$) were analyzed on Day 1 to determine intra-day precision and accuracy. Inter-day precision and accuracy was determined from replicate QC samples analyzed on Day 1 ($n = 12$), Day 2 ($n = 6$), and Day 3 ($n = 6$) for a total of 24 QC samples at each concentration. Mean, standard deviation and relative standard deviation (R.S.D.) were calculated from the QC values and used in the estimation of intra- and inter-day precision. Accuracy (bias) is expressed as the percent devia-

tion between the mean concentration relative to the nominal concentration.

2.7. Selectivity and stability

Selectivity was analyzed by processing six different sources of blank plasma. Blank plasma samples were processed in duplicate and compared to plasma spiked with the lowest trimethoprim standard. Sample carryover was determined by inserting vials of blank mobile phase in various positions throughout the third validation run. Batches of high and low QC samples were prepared and subjected to three freeze-thaw cycles prior to processing and analysis. After each freeze thaw cycle, aliquots were extracted and analyzed and determined acceptable at >95% peak height of control samples not exposed to the freeze-thaw cycles. The stability of processed samples was determined by repeated analysis of high and low QC samples for over 24 h post-extraction. The limit of quantitation (LOQ) was defined as the lowest standard value having a signal to noise (S:N) ratio of at least 10:1 and acceptable precision and accuracy (i.e., within 15%). The limit of detection (LOD) was defined as the smallest detectable peak having a S:N ratio of at least 3:1.

2.8. Extraction efficiency

Extraction recovery of trimethoprim was calculated by comparing the response obtained from extracted QC samples versus the response observed after direct injection of reference samples. Reference samples consisted of water spiked with appropriate amounts of trimethoprim standards. Responses obtained from the reference samples were defined as 100%.

2.9. Application to plasma sampling

Trimethoprim pharmacokinetics were determined in eight healthy volunteers. The protocol was approved by the University of Pittsburgh Institutional Review Board and signed informed consent was obtained from each subject. Trimethoprim, 2 × 100 mg tablets (Watson Pharmaceuticals, Corona, California, USA), were administered every 12 h for a total of 5 days. Multiple plasma samples were collected for 48 h after reaching steady state on Day 4 and stored at −20 °C until analyzed. Plasma trimethoprim concentrations were determined as described above.

3. Results

3.1. Chromatographic separation

Representative chromatograms of plasma samples are shown in Fig. 2. Fig. 2A depicts a double-blank sample (no IS) and a sample spiked with trimethoprim 0.5 μg/ml (LOQ) and IS. Fig. 2B shows a subject plasma sample obtained at steady-state during trimethoprim 200 mg twice daily administration. Retention times were approximately 7.1 and 12.2 min for trimethoprim and IS, respectively. The peaks of interest were well separated and free from interference with endogenous peaks. Sulfamethoxazole eluted at approximately 9 min (data not shown) and did not interfere with either peak of interest.

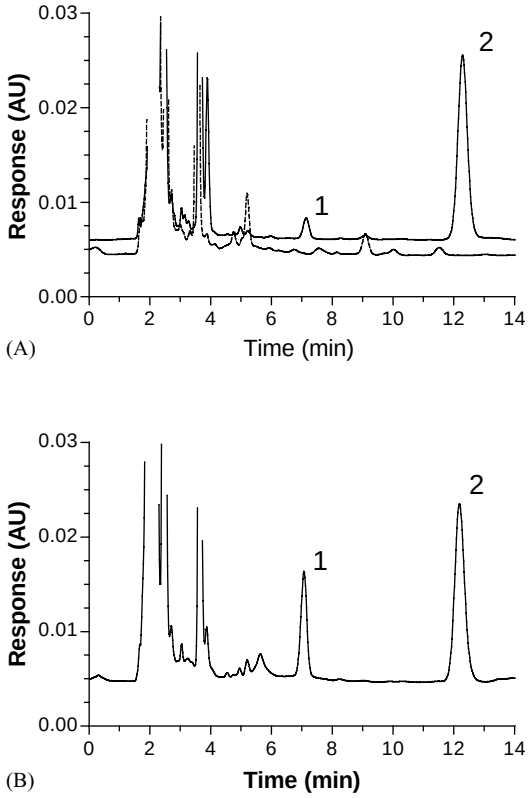

Fig. 2. Representative chromatograms of (A) extracted double blank plasma (- - -) and plasma sample spiked (—) with trimethoprim 0.5 μg/ml (LOQ) and IS. (B) Subject plasma sample after administration of trimethoprim 200 mg twice daily at steady state (concentration of 2.89 μg/ml). Peaks are (1) trimethoprim and (2) IS.

3.2. Precision, linearity, and accuracy

Linear calibration curves were obtained for trimethoprim over the concentration range of 0.5–20 μg/ml; the mean regression equation was: $Y = 0.2083 \pm 0.0013X + 0.0033 \pm 0.0002$. The correlation coefficients calculated for each run were >0.999. Response remained linear at all concentrations with no saturation of signal. Intra- and inter-day precision were within±5% and accuracy was within±6.25% (Table 1).

3.3. Selectivity, stability, and recovery

No endogenous interference with trimethoprim or IS was observed in six extracted blank plasma samples and there was no evidence of sample carryover. The signal to noise

Table 1
Intra- and inter-day precision and accuracy of trimethoprim in human plasma

Concentration (μg/ml)		R.S.D. (%)	Deviation (%)
Spiked	Observed (mean ± S.D.)		
Intra-assay precision quality controls			
2.00	2.15 ± 0.04	1.7	7.5
8.00	7.52 ± 0.14	1.9	−6.0
16.0	15.3 ± 0.2	1.1	−4.4
Inter-assay precision quality controls			
2.00	2.12 ± 0.04	1.9	6.0
8.00	7.64 ± 0.19	2.4	−4.5
16.0	15.3 ± 0.4	2.7	−4.4
Standards			
0.50	0.50 ± 0.01	1.4	−0.0
1.00	1.01 ± 0.02	2.0	1.0
2.50	2.55 ± 0.04	1.6	2.0
5.00	4.99 ± 0.08	1.6	−0.2
7.50	7.48 ± 0.07	0.9	−0.3
10.0	10.2 ± 0.3	2.9	2.0
20.0	19.5 ± 0.8	4.1	−3.0

Table 2
Extraction recovery of trimethoprim in human plasma ($n = 3$)

Spiked concentration (μg/ml)	Recovery (%)	R.S.D. (%)
2.0	80.5 ± 0.9	1.14
8.0	76.9 ± 0.7	0.96
16.0	79.6 ± 1.1	1.41

ratio of trimethoprim at 0.5 μg/ml and IS at 50 μg/ml were greater than 10:1. The LOQ was 0.5 μg/ml and the LOD was 0.1 μg/ml with a S:N ratio of 5:1. Trimethoprim and the IS were stable in processed samples at room temperature for at least 24 h prior to analysis. Samples subjected to three freeze-thaw cycles showed no sign of degradation. Extraction efficiency determined at the three QC concentrations was approximately 76–80% (Table 2).

3.4. Application to plasma sampling

Eight healthy subjects received trimethoprim 200 mg twice daily for 5 days. The mean (S.D.) steady-state concentration versus time profile for all eight subjects on Day 4 is depicted in Fig. 3. The concentrations observed were similar to those reported previously in HIV-infected subjects receiving trimethoprim 200 mg twice daily [14].

4. Discussion

The method presented provides simplified and economical detection of trimethoprim in small volumes of human plasma, using protein precipitation coupled with ultraviolet detection. We used protein precipitation to extract trimethoprim, which is less time consuming than liquid–liquid extraction [8] and less expensive than solid phase extraction

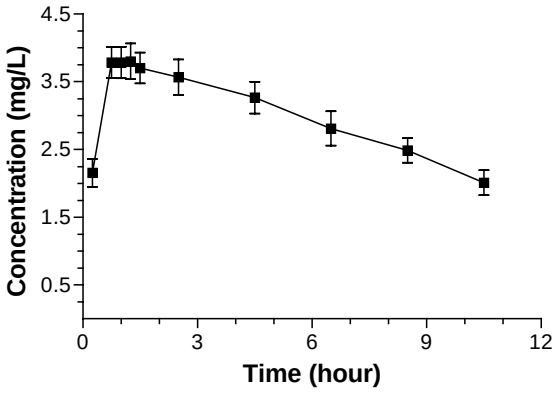

Fig. 3. Mean ± S.D. concentration-time data at steady-state (Day 4) from eight subjects after administration of trimethoprim 200 mg twice daily for 5 days.

[12,13]. Use of the phenyl Polar-RP column with a mobile phase containing a mixture of organic solvents yielded separation from endogenous interference and symmetrical peaks without the need for an ion-pairing agent. Further, sulfamethoxazole, an agent commonly co-administered with trimethoprim, does not interfere with either peak of interest, so with modification this method could be used for pharmacokinetic studies involving the combination product. This method allows unbiased detection of trimethoprim and is directly applicable to trimethoprim steady-state pharmacokinetic studies.

Trimethoprim is most commonly administered in combination with sulfamethoxazole (e.g., Bactrim), but the single drug formulation is used for selected applications. Trimethoprim and sulfamethoxazole have been shown in vitro to selectively inhibit CYP2C8 and CYP2C9 activity, respectively; however, sulfamethoxazole loses selectivity and inhibits CYP2C8 activity at relatively high concentrations [5]. Therefore, to evaluate trimethoprim as a CYP2C8 inhibitor in vivo, the trimethoprim only formulation must be administered. The trimethoprim only formulation is also used in combination with dapsone as an alternative treatment for PCP in patients who can not tolerate sulfamethoxazole. Monitoring plasma concentrations of trimethoprim during PCP treatment may be helpful to verify therapeutic concentrations or to help avoid severe concentration-related adverse events, since increased neutropenia, thrombocytopenia, and severe adverse events has been associated with plasma concentrations greater than 8 μg/ml [4].

In summary, the method presented here uses a simple extraction method, has consistent recovery efficiency, and is applicable for analyzing trimethoprim plasma concentrations in steady-state pharmacokinetic studies or patients receiving high dose trimethoprim therapy.

Acknowledgements

This project was supported in part by NIH Research Grant R01 MH63458, funded by the National Institute of

Mental Health and the Office of Dietary Supplements, and NIH/NCRR/GCRC#5M01RR00056.

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