Assay for Trimethoprim in Serum and Urine by Means of Ion-Pair Chromatography

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We describe a rapid, precise, and reliable procedure for assay of trimethoprim in serum and urine by ion-pair chromatography. Trimethoprim concentrations in urine are determined by an externally standardized, direct-injection procedure; assay in serum involves a simple preliminary extraction and internal standardization. The assays are suitable for pharmacokinetic studies and have been applied to determination of trimethoprim concentrations in serum and urine during therapy with Co-trimoxazole, a sulfonamide/trimethoprim preparation.

Additional Keyphrases: drug assay • monitoring therapy

Trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidineline] inhibits dihydrofolate reductase (EC 1.5.1.3) and therefore interferes with folic acid metabolism. This is the mechanism of the antimicrobial effect of trimethoprim (1).

In Great Britain, trimethoprim was, until recently, available pharmaceutically only in combination with a sulfonamide (e.g., Co-trimoxazole), but now preparations containing only trimethoprim have been introduced. In antimicrobial therapy the minimum inhibitory concentration of the drug must be achieved rapidly and maintained during all or most of the course of therapy. In steady-state therapy, the concentration is most likely to be below the minimum inhibitory concentration just before the maintenance dose is ingested. If this concentration or greater is not consistently maintained, the probability of drug resistance developing increases. Knowing the concentration of the drug in serum, urine, or other fluids will help the clinician avoid such a situation.

Concentrations of antimicrobials in serum are frequently determined microbiologically; the degree of inhibition of growth of a test organism by a given amount of body fluid from a subject dosed with the drug is used as an index of the effective antimicrobial concentration (2). Because such methods are relatively imprecise, chemical analyses have also been used. Trimethoprim can be estimated fluorometrically in body fluids (3), but the assay is lengthy and its precision may be poor. We found that the use of a method designed for determination of trimethoprim in pharmaceutical formulations by reversed-phase liquid chromatography (4) lacked sensitivity and chromatographic efficiency. Recently “high-performance” liquid chromatography was used to measure trimethoprim in serum, but trimethoprim was poorly resolved from other sample components (5).

To investigate the pharmacokinetics of a trimethoprim/sulfonamide combination, a rapid, reliable, precise procedure that is applicable to biological fluids is needed. Here we describe an assay involving ion-pair chromatography that fulfills these requirements.

Materials and Methods

Materials

Sodium hydroxide (2 mol/L) and sodium lauryl sulfate (6 g/L, in 50 mmol/L phosphoric acid) were prepared in deionized water from “Analar”-grade reagents (British Drug Houses, Poole, Dorset, U.K.). Diazepam (Roche Products Ltd., Welwyn Garden City, Herta., U.K.), 250 µg/L in dichloromethane (BDH, Analar grade) or 300 µg/L m-aminoacetophenone (Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.) was used as an internal standard. The eluent was a 7/3 (by vol) mixture of methanol and the solution of sodium lauryl sulfate in phosphoric acid. Trimethoprim (Berk Pharmaceuticals, Guildford, Surrey, U.K.) standards for estimation of serum concentrations were prepared in horse serum no. 5 (Wellcome Reagents Ltd., Beckenham, U.K.) over the concentration range 0–6 mg/L; these standards are stable for at least six months at −20 ºC. Standards for use in estimating concentrations of the drug in urine were aqueous and covered the range 0–600 mg/L.

Chromographic Conditions

A Model 8500 chromatograph (Varian Associates, Walton-on-Thames, Middlesex, U.K.) was fitted with a Vari-Chrom (Varian) variable-wavelength detector. We used two prepacked stainless-steel columns (250 X 4.6 mm, i.d.) during this work, one packed with a 10-µm (av. particle diameter) C₁₈-alkyl reversed-phase packing, Partisil-10/ODS-2 (Whatman Ltd., Maidstone, Kent, U.K.), the other with 5-µm C₁₈-alkyl reversed-phase packing SODS (Hi-Chrom, Woodley, Berks., U.K.). A 7120 Rheodyne valve fitted with a 10-µL loop (Magnus Scientific, Alsager, Stoke-on-Trent, U.K.) was used to apply samples to the column. Operating conditions were: temperature, ambient; flow rate, 2 mL/min; band width, 4 nm; wavelength, 230 nm; chart speed, 1 cm/min; absorbance settings, 0.2 (serum extracts), 1.0 (urine injections); and time, constant fast.

Procedures

Serum extraction. To 1 mL of serum in a Quickfit MF24/1/5 tube (Corning Ltd., Stone, Staffs., U.K.) add 1 mL of 2 mol/L sodium hydroxide and 5 mL of the internal standard solution. Stopper the tubes tightly, shake them for 10 min on a mechanical shaker, then centrifuge at 3000 rpm for 5 min. Transfer the organic layer to a conical tube, place the tube in a water bath at 40 ºC, and evaporate the solvent under a stream of air. Dissolve the residues in 25 µL of eluent (see Materials) and apply 10 µL of this solution to the chromatographic column via the sampling loop.
Determination in urine. Using the 10-μL sampling loop, apply 25 μL of either urine or aqueous standard directly to the column.

Linearity, Recovery, Precision, and Accuracy
To determine linearity as a function of on-column sample weight, we chromatographed 10-μL aliquots of aqueous trimethoprim standards, 0–1 g/L (equivalent to 0–10 μg on-column weight). Serum trimethoprim standards covering the range 0–6 mg/L were analyzed; the difference in detector response between these and directly injected aqueous standards represented the absolute percentage loss.

Relative analytical recovery was assessed from the ratio of directly injected trimethoprim/internal standard solution to comparable assayed serum standards.

The within-batch precision was assessed from alternate analyses of trimethoprim standards at two concentrations: serum at 0.45 and 1.25 mg/L and urine at 100 and 200 mg/L. Daily measurements made during six weeks were used to estimate between-batch precision.

Accuracy was judged from the results of recovery studies and from investigation of interference by a number of drugs prepared in aqueous solution. Drugs studied were: acephenyline, amitriptyline, amobarbital, butriptyline, carbamazepine, chloralhydrate, clomipramine, chlorpromazine, chlormethiazole, chlorpromazine, clonazepam, dapsone, desipramine, dextropropoxyphene, diazepam, digoxin, dothiepin, doxepin, fluphenazine, gentamicin, imipramine, lorazepam, miamamide, medazepam, nitrazepam, nortriptyline, oxyprenophenzone, paracetamol, penicillin, percyazine, perphenazine, pheno- barbital, prazepam, primaquine, primidone, promazine, promethazine, prothidion, prothixyline, salicylate, sulfadiazine, sulfamethoxazole, sulfamoxole, temazepam, and trimipramine. Thirdly, we estimated the relative error for both serum and urine procedures, using sample concentrations of 0.45 and 100 mg/L, respectively.

Pharmacokinetics
A volunteer was orally dosed with a sulfonamide/trimethoprim combination; each dose contained the equivalent of 160 mg of trimethoprim and 800 mg of sulfamethoxazole; the dosing interval was 12 h and the regimen was continued for five days. Blood was drawn immediately before and at intervals after dosage up to 96 h; urine was collected before dosing and thereafter 12- or 24-h specimens were collected.

Results
Determination of Chromatographic Conditions
Various eluents were examined to determine the best chromatographic conditions for use with a C₁₈ reversed-phase column. Our results for typical neutral, acidic, basic, and ion-pair solvents in relation to retention, chromatographic efficiency, and resolution from sulfonamides as column capacity factor k', reduced plate height h, and resolution function Rₛ, respectively, are summarized in Table 1.

The retention volume for diazepam was 9 mL ± 0.5 mL; the retentions of m-aminoacetophenone and trimethoprim relative to diazepam were 0.61 and 0.81, respectively. The retention of a small unknown peak relative to diazepam (see Figure 1a) was 0.69.

Analytical Recovery
Serum: Absolute recovery depended on the total amount of extraction solvent transferred and was 80 ± 10%. Recovery relative to the diazepam internal standard, as calculated by the ratios of peak heights, was found to be 98 ± 9.2% (n = 30), and for the m-aminoacetophenone internal standard 101.6 ± 7.7% (n = 30).

Urine: Absolute recovery was 100 ± 1.0% (n = 10).

Linearity and Sensitivity
Serum and urine: Linearity was demonstrated over the range 0–6 μg on-column sample weight for extracts from serum with use of either internal standard and for directly injected aqueous standards (i.e., equivalent to 0–6 mg/L for serum extracts and 0–600 mg/L for directly injected standards). Sensitivity—i.e., the lowest quantifiable concentration—was equivalent to 20 μg/L when 5-μm packing material was used and 70 μg/L when 10-μm packing was used.

Precision
Within-batch: Assay of 30 serum extracts at two concentrations, 0.450 and 1.25 mg/L, with diazepam as internal standard was found to be 98 ± 9.2% (n = 30), and for the m-aminoacetophenone internal standard 101.6 ± 7.7% (n = 30).

Table 1. Comparison of Retention, Efficiency, and Resolution in the Chromatography of Trimethoprim with Neutral, Acidic, Basic, and Ion-pair Solvents

<table>
<thead>
<tr>
<th>Solvent and proportions (by vol)</th>
<th>Column capacity factor k'</th>
<th>Reduced plate height h</th>
<th>Resolution function (Rs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol/water (90/10)</td>
<td>1.1</td>
<td>30</td>
<td>1.5</td>
</tr>
<tr>
<td>Acetonitrile/H₃PO₄ (50 mmol/L) (1/3)</td>
<td>1.3</td>
<td>15</td>
<td>0.3</td>
</tr>
<tr>
<td>Methanol/water/ammonia (rel. dens. 0.88), (90/10/0.1)</td>
<td>1.1</td>
<td>30</td>
<td>1.5</td>
</tr>
<tr>
<td>Methanol/H₃PO₄ (50 mmol/L)/lauryl sulfonic acid (70/30/0.2)</td>
<td>3.5</td>
<td>5</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Fig. 1. Chromatograms of serum extract from subject on 160 mg trimethoprim and 800 mg sulfonamide, 12 hourly (10-μL injection) (a, left), and from drug-free subject (20-μL injection) (b, right).

T = trimethoprim, D = diazepam internal std., U = unidentified peak.
standard yielded the following data: 0.45 mg/L standard, mean 0.442 mg/L (CV, 6.1%) and for the 1.25 mg/L standard, mean 1.229 mg/L (CV, 4.6%). For the urine procedure the observed mean and CV for 30 samples at two concentrations, 100 and 200 mg/L, was 98.3 mg/L (CV, 3.3%) and 205.2 mg/L (CV, 1.5%). Thirty serum extracts with a concentration of 1.0 mg/L, with m-aminoacetophenone as internal standard, gave a mean of 1.022 mg/L (CV, 7.9%).

Between-batch: For 30 serum samples, the mean and CV of between-batch assay of concentrations of 0.45 and 1.25 mg/L was: mean 0.443 mg/L (CV, 6.6%) and 1.229 mg/L (CV, 6.3%), respectively. Thirty serum extracts with a concentration of 1.0 mg/L, with m-aminoacetophenone as internal standard, gave a mean of 1.01 mg/L (CV, 9.0%).

For the urine procedure, 30 injections at concentrations of 100 and 200 mg/L gave a mean of 98.9 mg/L (CV, 3.6%) and 205.0 mg/L (CV, 2.0%), respectively.

Accuracy

Serum: The sulfonamide was not extracted efficiently and was not retained under the chromatographic conditions we used. Some basic drugs, phenothiazines, tricyclic antidepressants, and benzodiazepines were extracted and could be chromatographed; only carbamazepine has so far been demonstrated as having a $k'$ value approximating that of trimethoprim. None of the other drugs tested co-eluted with diazepam, but nitrazepam and lorazepam eluted close to m-aminoacetophenone (retentions relative to diazepam were 0.56 and 0.70, respectively; $R_s \approx 1.0$ for both).

Urine: Although no extraction was used, no interference by drugs other than noted above was found. A small endogenous peak could not be resolved from trimethoprim using the

10-μm packing ($R_s = 0.3, h = 20$) but with the 5-μm packing ($R_s = 1.25, h = 5$) there was good separation. The relative error was calculated as: $t = \frac{\text{observed concentration} - \text{expected (added) concentration}}{\text{standard error and applied to}}$ the serum 0.45 mg/L standard and the urine 100 mg/L standard; $t$ values of 1.4 and 1.65 were obtained, which were not significant at the 5% level.

More than 100 drug-free serum and urine samples have been analyzed and no interference was noted.

Separation

The chromatogram obtained from an extract of serum from a subject taking trimethoprim, with diazepam as internal standard, is shown in Figure 1a; an extract of serum from a patient taking no known medication, with diazepam as internal standard, is shown in Figure 1b; the differences in the internal standard height are due to differences in injected volume (a, 10 μL; b, 20 μL). The chromatograms of the equivalent urines are shown in Figures 2a and 2b, respectively.

Quantitation

Serum: Because linearity has been established and found to be reproducible, unknown serum concentrations were calculated as follows. The ratio trimethoprim peak height/internal standard peak height was compared with the ratio obtained for a 1.5 mg/L serum trimethoprim standard assayed in triplicate, and the unknown concentrations were calculated by proportion.

Urine: External standardization was used; concentrations

Minimum inhibitory concentration for trimethoprim in serum is ≤2 mg/L.
ranged from 25 to 400 mg/L. To allow for any variation in the chromatography while performing a batch analysis, a 100 mg/L standard was injected after every fourth sample; the values obtained from this “correction” standard were referred to the value for the 100 mg/L standard used in the calibration line. This was the “target” value. Any variation in a drift standard was corrected, and the correction was applied to the two unknown samples on either side of that standard.

Application

The initial absorption and distribution curve (first 12 h) is illustrated in Figure 3. The peak absorption is seen at about 2 h, with a concentration in serum of 2.1 mg/L; using data on the post-peak concentrations after the initial dose, we calculated the elimination half-life (as a plot of time vs log concentration) to be 10.8 h for this subject. Continued dosing at 12-h intervals (i.e., a time approximating the elimination half-life of the drug) leads to “steady state” conditions. Figure 4 shows the concentrations of trimethoprim in serum during the full course of treatment. The mean peak and trough concentrations after the first 24 h were 3.8 and 2.0 mg/L, respectively, with ranges of 3.3–4.3 and 1.5–2.4 mg/L. Figure 5 shows the concentrations of trimethoprim determined in the urine. The percentage of the dose excreted in the urine as unmetabolized (i.e., pharmacologically active) trimethoprim after the initial dose was 36.7% (i.e., 58.7 mg); a total of 56% of the administered drug (i.e., 586 mg) was excreted during the five-day period; the concentrations ranged between 126 and 235 mg/24 h.

Discussion

The procedures described here allow quantitation of trimethoprim in the concentrations found in human body fluids during the course of antimicrobial therapy. The standard curve is linear over the required range, and the methods are precise, accurate, and reliable. They have been used by a variety of operators to process over 600 samples without any difficulty.

Initially, diazepam was used as the internal standard because the method was required for assay of samples from volunteers whose medication was known. However, for application of this procedure to the general population, an alternative internal standard may be necessary because diazepam is so frequently prescribed. Compounds containing amines were strongly retained in this chromatographic system and m-aminoacetophenone proved to be a good alternative internal standard, eluting before trimethoprim. m-Aminoacetophenone therefore saves analysis time; an efficient column will resolve the benzodiazepines that elute close to this standard.

The assays are rapid: 30 urine samples can be analyzed in about 2 h and the same number of serum samples with diazepam as internal standard in a little less than 4 h. With m-aminoacetophenone as internal standard, the time required for chromatography is similar to that for chromatography of urine samples.

For accuracy, an efficient column, capable of resolving an endogenous urinary constituent, must be used. Because the drug is not extracted, no internal standard is required for the urine assay; the time required between injections is less than for the initial serum assay. Such an approach is possible owing to the excellent precision obtained when a sampling loop is used for injection of the sample only.

As may be seen from Table 1, the use of reversed-phase chromatography with protonation of the amine groups of trimethoprim and formation of an ion pair gave the greatest efficiency and optimum retention times; this is consistent with the work of Knox and Jurand (6).

As illustrated by the results shown in Figures 3–5 these analytical procedures are applicable to drug-disposition studies of trimethoprim in humans. The dosage frequency was 12 h, approximating the biological half-life of trimethoprim. Such a frequency should result in cumulation being more than 95% complete by the fifth dose (7), i.e., about 60 h after the initial dose. This is not reflected in Figure 4, where the steady state appears to have been reached in 36 h. This may be the result of individual variation in drug-disposition kinetics or some other cause. However, in a larger series of subjects we have found the expected cumulation (unpublished results).

The calculated elimination half-life is within the range quoted by other workers (8). The serum “steady-state” and values for urine are sufficiently high to ensure an adequate antimicrobial effect for most common pathogens.

Dosage intervals and amounts may require adjustment and, to confirm that the minimum inhibitory concentration is achieved, it is necessary to measure the drug concentrations in body fluids (9). Brumfitt and Hamilton-Miller (10) established the minimum inhibitory concentrations for trimethoprim in serum and urine to be of the order of \( \leq 2 \) mg/L. It is clearly seen from Figures 4 and 5 that this value is consistently exceeded, with particularly high concentrations appearing in urine. In patients with impaired renal function who are receiving combined sulfonamide/trimethoprim therapy it is advisable to maintain serum and urinary concentrations of both drugs equivalent to those found in persons with normal renal function (9).

We thank Dr. H. Townsend (Berk Pharmaceuticals, Guildford, Surrey, U.K.) for the supply of drug standards and some items of equipment to facilitate the development of this assay.

References