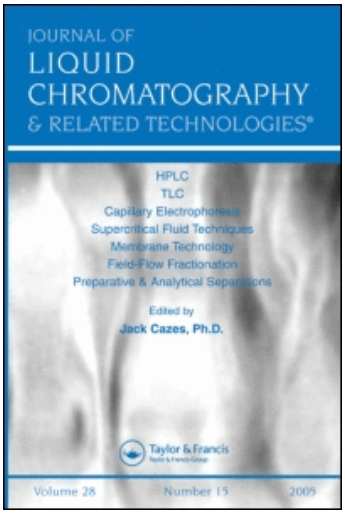

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Trimethoprim Analysis by LC

Joseph E. Svirbely[a]; Amadeo J. Pesce[a]
[a] Department of Laboratory Medicine, University of Cincinnati Hospital, Cincinnati, Ohio

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TRIMETHOPRIM ANALYSIS BY LC

Joseph E. Svirbely and Amadeo J. Pesce
Department of Laboratory Medicine
University of Cincinnati Hospital
Cincinnati, Ohio 45267

ABSTRACT

A method is described for measurement of the antimicrobial agent trimethoprim in biologic fluids by reverse phase LC. Preliminary isolation uses a simple rapid sorbent extraction. The assay is linear from 0.2 - 100 ug/mL (r = 0.99), sensitive (0.05 ug/mL), reproducible (p less than 0.96), and relatively free of interferences from metabolites and commonly administered drugs. Only atenolol, procainamide, and oxazepam interfered. The assay is suitable for therapeutic monitoring and for pharmacokinetic assays.

INTRODUCTION

In order to study the pharmacokinetic behavior of trimethoprim across the peritoneal barrier in human subjects, a quick and sensitive sensitive assay for trimethoprim was necessary. A review of the literature yielded a single adequate LC method for trimethoprim (1). This assay measured trimethoprim by LC following time-consuming solvent extraction. Other assays (2-4) measured sulfamethoxazole and trimethoprim following a single solvent extraction

The problems of extracting these two chemically dissimilar drugs by a single extraction have been reviewed (5).

The authors of the current study were interested in determining whether or not it was possible to extend sorbent extraction techniques, which had proved successful in their laboratory for the measurement of cyclosporin, tricyclic antidepressants, and barbiturates to the measurement of an antimicrobial agent. Advantages of the sorbent extraction include high efficiency and selectivity, and rapid extraction without toxic solvents or time-consuming shaking. The principal disadvantage is the cost of sorbent extraction columns, which is offset by the decreased technician time required for extraction and savings in solvent cost.

METHODS AND MATERIALS

Chromatographic System

The chromatographic system (Waters Associates, Milford, MA 01757) consisted of a model U6K injection port, a model 501 pump, μBondapack C18 reverse-phase column (15 cm) at room temperature (25°C), a model 440 detector (wavelength fixed at 254 nm), and a model 740 recorder. Chart speed was 1 cm/min. Samples were injected manually through the injection port onto the column with an injection volume of 5-40 μL of extracted sample. The samples can also be injected on column with an automated sample injector such as the WISP 710B (Waters Associates) used in this laboratory. Flow rate was 2.0 mL/minute. The column was stored in methanol between runs, and equilibrated with mobile phase for 15 minutes before each run. No column regeneration was used, and the column

was used daily for 180 days. An average run during the trimethoprim kinetics project consisted of 36 samples plus standards.

Reagents

The mobile phase consisted of a mixture (70:30 v/v) of sodium acetate (0.01 mol/L) (Fisher Scientific, Fair Lawn, NJ 07410) in deionized water and acetonitrile (HPLC grade, J.T. Baker Chemical Co., Phillipsburg, NJ 08865). The mobile phase was filtered through a 0.2 μ membrane and degassed prior to use. The mobile phase was stable for 2 weeks with recycling. The buffer used for sample preparation consisted of sodium carbonate (0.1 mol/L) (HPLC grade, Fisher Scientific) in deionized water.

Trimethoprim stock standard solution (1 mg/mL) was made from trimethoprim (gift of Hoffmann-LaRoche, Nutley, NJ 07110) dissolved in methanol (stable 6 months at 4°C) (HPLC grade, Fisher Scientific). Working trimethoprim solution (100 μg/mL) was made from stock using deionized water as diluent. Standard curves were made by pipetting 0, 2, 5, 10, 20, 50, and 100 μL of working trimethoprim standard into 1 mL aliquots of pooled plasma, mixing, and freezing (stable for 6 months at -20°C).

The internal standard was 2,4-diamino-5-(3,5-dimethoxy-4-methylbenzyl)-pyrimidine (gift, Hoffmann-LaRoche). A stock solution (1 mg/mL) in methanol (HPLC grade, Fisher Scientific) (stable 6 months at 4°C) was diluted with deionized water to make a working solution (1 μg/mL) (made fresh daily).

Procedures

The sample was prepared for extraction by combining 500 μL standard, control, or patient sample (serum, plasma, dialysate,

urine, cerebrospinal fluid) with 500 µL of working internal standard (1 µg/mL) and 200 µL of carbonate buffer. The mixture was agitated for 30 sec. and stored (capped).

Sorbent extraction columns (Bond-Elut 1 mL C18 columns, Analytichem International, Harbor City, CA 90710) were inserted into the vacuum apparatus (Bond-Elut System, Analytichem International). Columns were solvated with two washes of methanol, followed by two rinses with deionized water.

The prepared extraction mixtures were applied to the columns under vacuum and the solution drawn through was discarded. The columns were washed with deionized water. The extracting solvent (100 µL methanol) (HPLC grade, Fisher Scientific) was applied and allowed to percolate without vacuum, through the sorbent phase for 30 seconds. The vacuum was applied and the extracting solvent collected in 12 x 75 mm glass tubes. A second bolus of methanol (100 µL) was applied to the column, allowed to percolate without vacuum for 30 seconds, and the vacuum reapplied. Approximately 120-130 µL of methanolic extract is obtained by this procedure. The entire extraction procedure, including preparation of extraction mixtures and extraction, took 25 minutes for 20 batched samples.

RESULTS AND DISCUSSION

Calculation

Runs lasted approximately 7 minutes (Figure 1) with trimethoprim eluting at 2 minutes and the internal standard eluting at 6 minutes. The height of the trimethoprim peak was divided by the

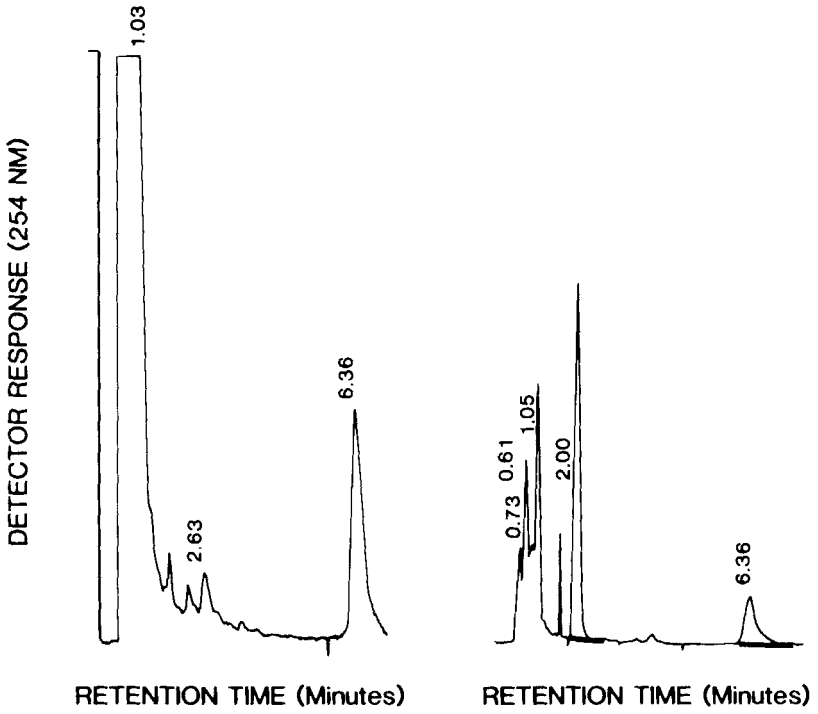

FIGURE 1: Typical chromatograms from analytical run. Chromatogram at left is zero standard at a detector response of AUFS 0.01. Chromatogram at right is 5.0 ug/mL spiked standard obtained from same run at a detector response of AUFS 0.05. Trimethoprim eluted at 2.0 minutes (right panel) and the internal standard eluted at 6.36 minutes for both.

height of the internal standard peak for each standard, and the peak height ratios obtained were plotted against concentration to obtain a standard curve (Figure 2). The peak height ratios of spiked controls and patient samples were obtained in the same way and compared to the standard curve to obtain concentration values.

Linearity

A standard curve was run for six standards (2, 5, 10, 20, 50, and 100 µg/mL) using a more concentrated internal standard (10

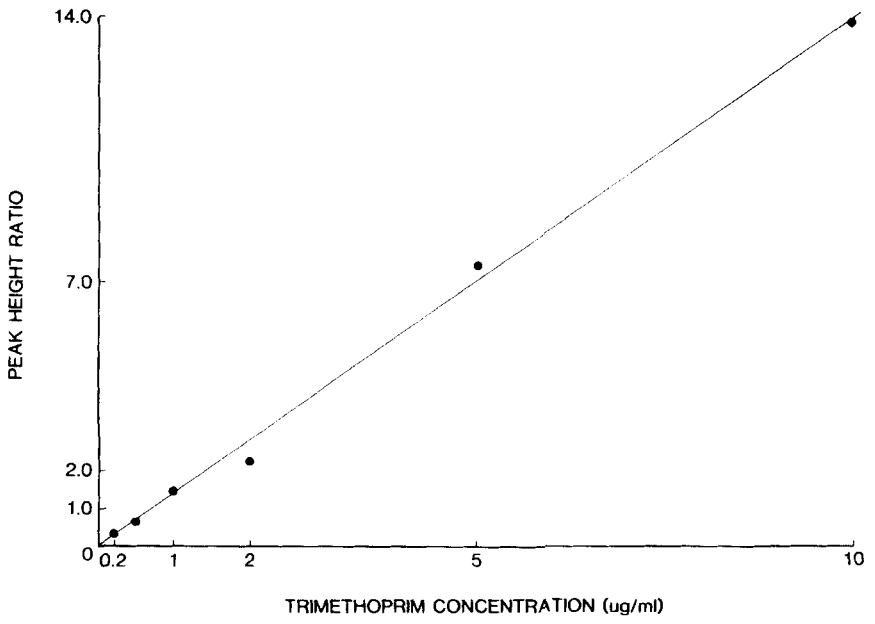

FIGURE 2: Typical standard curve obtained for trimethoprim.

µg/mL) to ascertain linearity. Linear regression performed on the curve gave an r value of 0.99.

Reproducibility

To ascertain reproducibility, serum from nine subjects given trimethoprim orally was aliquoted into four samples each. Four separate runs involving four separate standard curves, each run a week apart, were performed, and four values for each patient were obtained. To determine goodness of fit of replication, variation between columns was studied using a one-way Analysis of Variance measuring replication as a treatment. The computed F value was significant ($p < 0.96$) for reproducibility.

Recovery

To determine recovery, the peak heights of the extract of plasma samples spiked with trimethoprim were compared with the same amount of drug as aqueous solution injected directly onto the column without extraction. Recovery was calculated as 82% for 4 µg/mL standards and 73% of 40 µg/mL standards, each run three times.

Interferences

Possible interferences were also studied. The common trimethoprim metabolites (1- and 3-oxides, and 3'- and 4'-hydroxy compounds) eluted in the solvent front. Commonly administered drugs were made up as 1 µg/mL standards and shot on column. Compounds which gave a response were spiked in plasma at physiologic concentrations, extracted, and shot on column with trimethoprim and the internal standard. Atenolol and procainamide interfered with the trimethoprim peak. Oxazepam interfered with the internal standard peak. Diazepam, though it did not interfere, is metabolized to oxazepam, and in this way may interfere. The results of the interference study are summarized in Table 1. Lipemia interfered with extraction but hemolysis did not interfere. The elevated BUN, creatinine, and glucose found in peritoneal dialysis fluid did not interfere with extraction or analysis.

Sensitivity

Concerning sensitivity, a 0.05 µg/mL trimethoprim-spiked standard, extracted as above, yielded a peak height of 2 cm at a recorder attenuation of 4 and a detector response of 0.01 AUFS.

TABLE I

List of Drugs Tested in Interference Study

Drugs which interfered with trimethoprim	RRT
Atenolol	0.44
Procainamide	0.52
(Trimethoprim)	(0.39)
Drugs which interfered with internal standard	
Oxazepam	1.0

Drugs which gave detector response but did not interfere:

Theophylline, Acetaminophen, Caffeine, Chlordiazepoxide, Prazosin Hydrochloride, Diazepam, Thioridazine

Drugs which did not give detector response:

Amitriptyline, Ampicillin, Cefitoxin, Chlorpheniramine, Chlorpromazine, Cimetidine, Clonidine, Desipramine, Digoxin, Diphenhydramine, Doxepin, Doxylamine, Erythromycin, Flurazepam, Imipramine, Lidocaine, Minoxidil, Nadolol, Nortriptylline, Propranolol, Quinidine

Note: Drugs which gave a detector response were spiked in pooled plasma at physiologic concentrations and extracted before retesting.

DISCUSSION

Sorbent extraction has been shown to be an effective extraction method preparatory to LC for trimethoprim. The sorbent extraction method described is linear, sensitive, reproducible, and free of most interferences.

Trimethoprim is routinely used for treatment of bladder infection, by itself or coupled with sulfamethoxazole. It has also

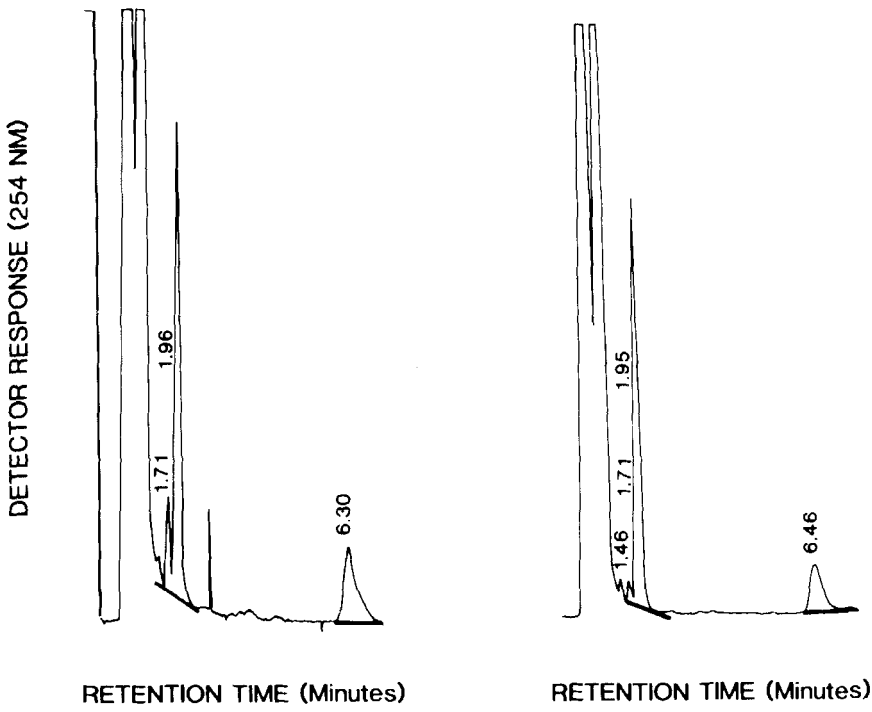

FIGURE 3: Typical chromatograms of patient samples. Chromatogram at left is plasma trough level from a patient with peritonitis. Level calculated as a concentration of 4.4 ug/mL. Chromatogram at right is dialysate fluid from the same patient drawn 2½ hours after i.p. dose of trimethoprim. Level calculated as a concentration of 5.5 ug/mL. Trimethoprim eluted at 1.96 minutes in left chromatogram, and 1.95 minutes in the one at the right. Internal standard eluted at 6.30 minutes and 6.46 minutes, respectively.

recently been used to treat Pneumocystis carinii infection. The authors have used trimethoprim and sulfamethoxazole to treat peritonitis associated with peritoneal dialysis (Figure 3). Singlas et al. have also had success treating peritonitis in peritoneal dialysis patients with sulfamethoxazole and trimethoprim (6). The usual

effective blood level of trimethoprim for most susceptible infections is 4-10 µg/mL (7). For treatment of more resistant organisms, therapeutic monitoring of trimethoprim levels would be indicated in order that effective non-toxic levels could be maintained.

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