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Note

Ion-paired high-performance liquid chromatographic separation of trimethoprim, sulfamethoxazole and N^4-acetylsulfamethoxazole with solid-phase extraction

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The combination of trimethoprim (TMP) [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] and sulfamethoxazole (SMX) [N^1-(5-methyl-3-isoazoyl)sulfanilamide] is a widely prescribed antibiotic because of its broad-spectrum of antimicrobial activity. Cotrimoxazole (commercially formulated TMP and SMX in a 1:5 weight ratio) has recently obtained significant new importance since it is the drug of choice for both prophylaxis and acute treatment of *Pneumocystis carinii* pneumonia in patients with the acquired immunodeficiency syndrome (AIDS) [1,2]. Subtherapeutic doses of cotrimoxazole are administered for prophylaxis while high-dose regimens are prescribed to treat acute cases of *P. carinii* pneumonia which is the most commonly observed opportunistic infection in AIDS patients [1–3]. Unlike other patient popula-

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tions, adverse reactions to cotrimoxazole in AIDS patients are alarmingly high and often necessitate discontinuation of the drug [4–8]. Several reports [9,10] have described a concentration–toxic effect with sulfonamides and, as such, therapeutic drug monitoring may be indicated in patients receiving high-dose cotrimoxazole therapy.

Factors contributing to the susceptibility to sulfonamide toxicity may, in part, be due to interactions of several competing metabolic pathways regulating N-acetylation of SMX [11]. A decreased rate of acetylation may make more SMX available to be metabolized by other pathways involved in toxicity. As a result, a sensitive and reliable method for the quantification of TMP, SMX and its major metabolite, N^4-acetylsulfamethoxazole (N-SMX), is needed to ascertain the minimal effective concentrations for long-term prophylaxis against *P. carinii* pneumonia and to optimize dosing regimens in patients receiving high-dose cotrimoxazole since the therapeutic window in this latter group is narrowed.

High-performance liquid chromatography (HPLC) analytical techniques have been the only methodologies capable of measuring TMP, SMX and N-SMX utilizing a single sample preparation and separation. However, the structural dissimilarity of TMP and SMX has led to complicated sample preparation procedures. Gochin et al. [12] described a method for the extraction of all three compounds from 1 ml of serum utilizing an extremely complicated extraction procedure involving sixteen steps and more than 2 h to complete. Van der Steuijt and Sonneveld [13] described a greatly simplified extraction process utilizing a simple organic extraction into ethyl acetate, but still an additional protein precipitation step with the addition of methanol–water (1:4) and trichloroacetic acid in hydrochloric acid was required. Additionally they had an excessively long run time of 30 min. Spreux-Varoquaux et al. [14] used a straightforward organic extraction, evaporation and reconstitution procedure without any protein precipitation step. However, they used a relatively toxic organic extraction solvent, chloroform, and required a change in mobile phase during the run to elute all the compounds in under 14 min. Weber et al. [15] avoided the problem of organic extraction by using a protein precipitation step, centrifugation and then direct injection of an aliquot of the supernatant. They were able to achieve nominal results but required dual-wavelength detection at 225 and 254 nm. A further drawback of protein precipitation and direct injection is the dilution of the sample and inability to inject a high percentage of the total drug within the sample onto the column. This results from two aspects: the necessity to avoid protein precipitate when drawing sample into the syringe and the combined requirement for dilution of sample during the protein precipitation step and the necessity of small sample volumes for injection onto an analytical column.

This paper describes an isocratic ion-paired separation with solid-phase ex-

traction which allows rapid sample preparation, a short run time and an excellent lower limit of detection.

EXPERIMENTAL

Materials and equipment

An HPLC pump (Constametric Model III, LDC/Milton Roy, Riviera Beach, FL, U.S.A.) was connected in series with a syringe loading sample injector (Rheodyne Model 7125, Rheodyne, Cotati, CA, U.S.A.), a guard column (2 cm×2 mm I.D.) packed with 40-μm pellicular C_{18} guard column packing material (Upchurch Scientific, Oak Harbor, WA, U.S.A.), a 10-μm μBondapak C_{18} reversed-phase column (30 cm×3.9 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.) and a variable-wavelength UV detector (Kratos Model GM770R, Applied Biosystems, Ramsey, NJ, U.S.A.). A chromatography data acquisition system (Axxiom Chromatography, Model 727, Calabasas, CA, U.S.A.) monitored the detector output and calculated peak heights. TMP, SMX and N-SMX were provided by Hoffman-LaRoche (Nutley, NJ. U.S.A.). Stock solutions of TMP, SMX and N-SMX were prepared in methanol at concentrations of 1 μg/ml and stored at −70°C. A stock solution of the internal standard, *p*-nitrophenol (Sigma, St. Louis, MO, U.S.A.) was prepared in methanol at a concentration of 1 μg/ml and stored at room temperature in an opaque container. The solid-phase extraction procedure utilized 3-ml C_{18} Bond Elut columns (Analytichem International, Harbor City, CA, U.S.A.)

The mobile phase consisted of acetonitrile (Baxter Healthcare, McGaw Park, IL, U.S.A.) and 0.02 *M* citrate buffer (24:76) with 0.8 g of 1-octanesulfonic acid (Sigma). The citrate buffer was made by adding 7 ml of 3.0 *M* hydrochloric acid to 150 ml of 0.1 *M* disodium citrate and diluting to 760 ml with distilled, deionized water (Millipore, Bedford, MA, U.S.A.), then adjusting the pH to 3.00 with 3.0 *M* hydrochloric acid. The final mobile phase solution was filtered through a 0.45-μm filter (Schleicher & Schuell, Keene, NH, U.S.A.) and degassed gently with helium sparging.

The wash solution used for serum sample solid-phase extractions was prepared by adding 7 ml of 3.0 *M* hydrochloric acid and 1.6 g of 1-octanesulfonic acid to 150 ml of 0.1 *M* disodium citrate and diluting to a volume of 800 ml with distilled, deionized water, then adjusting the pH to 3.00 with 3.0 *M* hydrochloric acid. The solution used to elute the compounds of interest from the Bond Elut extraction columns was a 10:1 mixture of methanol (Baxter Healthcare) and triethylamine (obtained through Sigma).

Sample preparation

A 500-μl wash solution was added to a 500-μl serum sample and then vortex-mixed for 3 s; 6 μg of *p*-nitrophenol in 25 μl of methanol were added and the

sample was vortex-mixed for another 3 s. The 3-ml C_{18} Bond Elut solid-phase extraction column was prepared for extraction by wetting column with 500 μl of methanol followed by 1 ml of wash solution. The sample preparation was then decanted onto the Bond Elut column. A vacuum was applied to facilitate elution of sample matrix through the column. The column was then washed with 1 ml of wash solution and allowed to dry for 30 s with vacuum applied. Finally, the compounds of interest were eluted with two 500-μl aliquots of the methanol–triethylamine (10:1) eluting solution. The 1-ml elution was then dried under a stream of nitrogen, reconstituted in 200 μl of mobile phase, and an aliquot was injected onto the analytical column.

Separation and detection

Normally 50–100 μl of reconstituted sample were injected onto the analytical column with a mobile phase flow-rate of 1.5 ml/min operating at ambient temperature. Detection for all three compounds and internal standard was by UV absorbance at a wavelength of 230 nm.

Percentage recovery and variability

Percentage recovery was determined by extracting six to ten samples containing 1, 4 and 8 μg of TMP and N-SMX and 10, 40 and 80 μg of SMX from 500 μl of human serum. The resulting peak heights from the extracted spiked samples were compared with peak heights of direct injections to determine the percentage recovery.

Within-day variability was determined by extracting five to ten samples containing 1.0, 4.0 and 8.0 μg of TMP and N-SMX and 10, 40 and 80 μg of SMX from 500 μl of human serum. For between-day variability 10 ml of serum was spiked with 100 μg of TMP, 50 μg of N-SMX and 1000 μg of SMX. This pool of spiked serum was divided into 500-μl portions (2.5 μg TMP, 5 μg N-SMX and 50 μg SMX per 500 μl sample) and frozen at −70°C. A single between-day variability sample was run with each standard curve.

RESULTS

An extracted blank human serum sample and an extracted serum sample from an AIDS patient receiving cotrimoxazole for *P. carinii* pneumonia are shown in Fig. 1. The solid-phase extraction produces an acceptable blank extraction with no quantifiable peaks appearing. In the AIDS patient receiving cotrimoxazole, TMP, SMX and N-SMX are completely resolved with a run time just under 12 min.

Recoveries from spiked human serum for TMP, SMX and N-SMX ranged from 67 to 94% at the three quantities tested (see Table I). The lowest recoveries were for N-SMX and SMX (73 and 67%, respectively) at the highest concentration tested. The percentage recovery for TMP remained constant over the entire range tested.

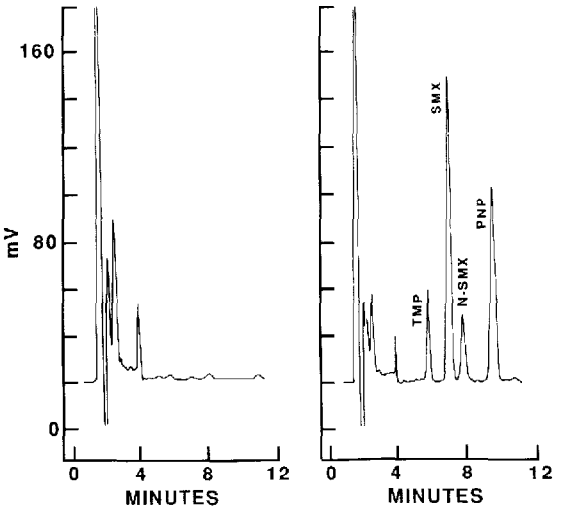

Fig. 1. Chromatogram of extracted human serum samples (250 μl extracted) from a drug-free subject and from an AIDS subject taking cotrimoxazole (sample collected 21 h post-dose, 5 mg/kg TMP and 25 mg/kg SMX) for the treatment of *Pneumocystis carinii* pneumonia. The retention times for trimethoprim (TMP), sulfamethoxazole (SMX), N^4-acetylsulfamethoxazole (N-SMX) and *p*-nitrophenol (PNP) are 5.6, 6.8, 7.6 and 9.3 min, respectively. The serum concentration as determined from standard curve by comparison with the internal standard (PNP) are 5.2, 87 and 17.6 μg/ml for TMP, SMX and N-SMX, respectively.

TABLE I

PERCENTAGE RECOVERY

Human serum spiked with known amounts of each compound were compared with direct injections of compound dissolved in mobile phase. The percentage recovery is calculated by multiplying the ratio of the peak heights of extracted drug or metabolite to a direct injection by 100.

Drug	*n*	Amount (μg)	Recovery (mean ± S.D.) (%)
TMP	10	1	92 ± 5.2
	6	4	94 ± 3.8
	6	8	94 ± 6.2
SMX	10	10	89 ± 4.3
	6	40	91 ± 3.3
	6	80	67 ± 9.7
N-SMX	6	1	94 ± 5.7
	6	4	87 ± 4.2
	6	8	73 ± 22.4

TABLE II

COEFFICIENTS OF VARIATION

Human serum spiked with known concentrations (actual) of each compound were assayed for quantity of each component as determined from a standard curve by the internal standard method (found)

Drug	n	Concentration (μg per 500 μl)		C.V. (%)
		Actual	Found	
Within-day				
TMP	10	1	1.0	5.0
	6	4	4.1	4.6
	5	8	8.1	5.4
SMX	10	10	9.7	3.6
	6	40	43.9	4.9
	5	80	81.6	5.6
N-SMX	6	1	1 0	6.0
	6	4	3 9	5.4
	6	8	7.9	4.8
Between-day				
TMP	6	2 5	2.4	3.4
SMX	6	50	48.4	7.3
N-SMX	6	5	5.1	8.9

The accuracy and coefficient of variation (C.V.) are given in Table II. The C.V. remained within clinically acceptable values over the entire range of the standard curve. The TMP, SMX and N-SMX concentrations determined from the standard curve of peak-height ratios of compound to internal standard (five-point standard curve) was congruous with human serum samples spiked with known concentrations of each compound. The C.V. across standard curves, i.e. the between-day variability, was slightly higher than the within-day variability.

The lowest amount of TMP, N-SMX and SMX which produced quantifiable peaks after solid-phase extraction was 25, 25 and 250 ng, respectively.

DISCUSSION

The ion-paired assay with solid-phase extraction which we have detailed offers several advantages over the previously published methodologies. The extraction method is fast, efficient and allows the entire drug and metabolite quantity within the sample matrix to be injected onto the analytical column. This allows for much lower limits of detection of extracted samples than has been previously reported. Solid-phase extraction methods also can be auto-

mated if large numbers of samples are to be processed. The chromatographic separation using the ion-pairing agent 1-octanesulfonic acid is rapid with a run time under 12 min; yet, it retains the simplicity of an isocratic system operating at room temperature. Detection of the compounds of interest utilizes monitoring UV absorbance at only a single wavelength, 230 nm. This is possible because of the efficiency of the extraction procedure.

One potential concern with the solid-phase extraction is the decrease in percentage recovery and increase in the variability of the percentage recovery for SMX and N-SMX at the highest standard concentration. Most likely this represents saturation of the 3-ml solid-phase extraction columns. To some extent the loss of drug is probably compensated by loss of the internal standard. However, because the internal standard is structurally different from SMX and N-SMX, loss of SMX and N-SMX will probably not be compensated by an equivalent loss of internal standard. Significant loss of SMX and/or N-SMX not compensated by proportional losses of the internal standard would be seen as a convex flattening of the plot of the standard curve (drug amount to peak-height ratio). A more sensitive test of saturation would be to plot drug amount to peak height. This should be non-linear even if internal standard is proportionally lost. By these criteria we were unable to demonstrate saturation at the highest standard concentration (80 μg of SMX and 8 μg of N-SMX). However, it would be prudent to avoid saturation of the solid-phase extraction column by using small serum sample volumes or using a solid-phase extraction column with a higher capacity such as the 5-ml Bond Elut extraction columns.

The increasing clinical use of cotrimoxazole in AIDS patients and the high incidence of adverse drug reactions in this population have stimulated new interest in the pharmacodynamic determinants of clinical efficacy and toxicity. The technical advantages of this ion-paired assay represent a significant advancement in the measurement of TMP, SMX and N-SMX. Additionally, the ability to quantitate TMP, SMX and N-SMX in small serum volumes will facilitate application of this methodology to study populations in which sample volume is limited, such as in pediatric patients and in animal studies utilizing small species such as rats.

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