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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF TRIMETHOPRIM AND SULFAMETHOXAZOLE IN MICROLITER VOLUMES OF CHINCHILLA MIDDLE EAR EFFUSION AND SERUM

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SUMMARY

A reversed-phase high-performance liquid chromatographic procedure was developed to analyze 25-μl volumes of chinchilla middle ear effusion and 50-μl volumes of serum for trimethoprim and sulfamethoxazole. The small sample volumes were dictated by the chinchilla model of otitis media and our need to collect multiple samples over a 12-h drug dosing interval. The drugs were separated on a cyanopropylsilane column using acetonitrile–40 m*M* sodium phosphate, (16:84, v/v), pH 4.8. Trimethoprim and the internal standard were detected at 230 nm while sulfamethoxazole was detected at 250 nm. Middle ear effusion and serum samples were extracted with ethyl acetate–dichloromethane (25:75, v/v). The limit of quantitation was 0.5 μg/ml for sulfamethoxazole and 0.1 μg/ml for trimethoprim (coefficient of variation $<20\%$), the limit of detection 0.25 and 0.05 μg/ml, respectively. Middle ear and serum samples of a chinchilla with experimentally induced otitis media receiving 10 mg/kg trimethoprim and 50 mg/kg sulfamethoxazole intramuscularly were collected over a 12-h period and analyzed. All statistics that validate the analytic method are reported

INTRODUCTION

Co-trimoxazole, an antibacterial preparation containing trimethoprim (TMP) and sulfamethoxazole (SMX), is effective in treating infections due to various aerobic gram-negative and gram-positive bacteria (1). The need to correlate pharmacotherapeutic effects of TMP and SMX with serum concentrations has produced numerous studies directed toward quantifying serum and urine levels of TMP and SMX using high-performance liquid chromatographic (HPLC)

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methods [2]. Many studies also include the analysis of certain *n*-acetyl sulfonamide metabolites which are primarily indicated in crystalluric conditions [2–5]. Most of these HPLC procedures have comparable limits of detection, however, the sample volume may range between 50 μl [6] and 1 ml [7], and methods of sample preparation vary from organic solvent extractions [2,3,7] to simple protein precipitation [2,5,6,8]. The literature also contains numerous descriptions of HPLC conditions (normal and reversed phase, ion pairing) and methods of detection (ultraviolet, electrochemical) for analyzing TMP and SMX [2].

For purposes of human TMP/SMX therapeutic drug monitoring, large amounts of serum and urine are available for analysis, therefore, quantification of TMP and SMX has become commonplace in many laboratories. As a result, most susceptible upper respiratory and urinary tract infections are successfully treated with SMX/TMP.

The chinchilla has provided a useful model for studying acute otitis media since middle ear effusion (MEE) can be easily induced [9,10]. Information pertaining to antibiotic levels in MEE have been obtained primarily using antibiotic sensitivity assays, which are less specific and require more time than HPLC analyses [9,10]. In determining levels of TMP and SMX in the middle ear of the chinchilla [11], we have developed a reversed-phase HPLC method for detecting small concentrations of TMP and SMX in 25-μl volumes of MEE. Limits of detection and method validation parameters are reported. The method also accommodates the analysis of TMP and SMX in 50-μl volumes of chinchilla serum.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of an HP 1090L liquid chromatograph equipped with a filter photometric UV detector, automatic sample injector and HP 3393 integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.). The separations were performed on a cyanopropylsilane column (Zorbax CN, 5 μm particle size, 150 mm×4.6 mm I.D.; DuPont, Wilmington, DE, U.S.A.). Acetonitrile, ethyl acetate, dichloromethane, methanol and water were all HPLC grade (Fischer Scientific, Pittsburgh, PA, U.S.A.). All other chemicals were analytical grade.

Drug solution

A 100 μg/ml solution of TMP was prepared in water. SMX (1 mg/ml) was prepared in methanol. Storage of SMX at 4°C resulted in precipitation of the drug at a 1 mg/ml concentration, therefore, both drugs were prepared fresh each day.

Internal standard (I.S.) and extraction solution

A methanolic solution of 3 mg/ml cimetidine was used as the I.S. A volume was added to the extraction solvent (ethyl acetate–dichloromethane 25:75, v/v) to a concentration of 2.5 μg/ml.

Chromatography

The mobile phase consisted of acetonitrile–40 m*M* monobasic sodium phosphate (16:84, v/v) adjusted to pH 4.8 with hydrochloric acid and was pumped through the column at 1.8 ml/min. The heated column compartment was maintained at 45°C. Ultraviolet detection of analytes was programmed: the earlier eluting TMP and I.S. were monitored at 230 nm, SMX was detected at 250 nm (100 ma.u.f.s.). A 12-μl sample was injected onto the column.

Standard curves

A large standard calibration curve ($n = 5$ for each concentration point) was prepared in MEE and serum on each of three separate days for computing linearity, precision and accuracy. The standard curves were constructed by spiking MEE and serum with aliquots of TMP and SMX to achieve concentration ranges of 0.1–15 and 0.5–200 μg/ml, respectively.

Quantification

Calibration curves were plotted using measured peak-height ratios of drug to I.S. versus known concentrations of TMP and SMX. The concentrations of TMP and SMX in unknown samples were subsequently determined from the calibration curves.

Sample preparation

MEE and serum samples were obtained according to previously published methods [9–11]. MEE samples were collected at 2, 5, 8 and 12 h while serum was collected at 1, 2, 5, 8 and 12 h.

MEE samples were prepared by placing 25 μl into a clean 10 mm × 75 mm glass tube. A 250-μl aliquot of 200 m*M* sodium phosphate, pH 6.2, was added followed by 1.5 ml of the extraction solution containing I.S. The tubes were vortexed, centrifuged and the aqueous layer was discarded. The remaining organic fraction was transferred to a clean glass tube and evaporated to dryness under nitrogen at 40°C.

Serum samples were prepared by placing 50 μl into a clean glass tube. A 250-μl volume of 200 m*M* sodium phosphate, pH 7.4, was added along with 1.5 ml of the extraction solution. The samples were vortexed, centrifuged and the organic fraction was evaporated. The dried residue from MEE and serum extraction was reconstituted in 100 μl methanol.

Recovery

Samples containing known concentrations of TMP and SMX (0.1–15 and 0.5–200 μg/ml, respectively) in MEE and serum were compared with corresponding concentrations of drug added to 1.5 ml of extraction solution to simulate 100% recovery. The recovery solutions were dried and reconstituted in 100 μl methanol. The two standard curves were analyzed together. The recovery was determined by calculating the drug concentrations found in MEE and serum using the regression equation obtained from the 100% recovery curve, dividing the serum or MEE

concentration by the recovery curve concentration and recording the difference as a percentage.

Quality controls

Quality controls (QC) were analyzed in quadruplicate over three days to determine accuracy and between-run precision of TMP and SMX. Drug-free serum and MEE were spiked with known concentrations of TMP and SMX. Three QC levels were prepared: 0.1, 2.5 and 15 μg/ml for TMP and 0.5, 50 and 200 μg/ml for SMX. The solutions were mixed, separated into 100-μl aliquots and stored at −20°C. Prior to analysis, the QC samples were brought to room temperature, then carried through the appropriate sample preparation along with the standard curve samples.

Statistics

Linearity was calculated by linear regression analysis and reported as the correlation coefficient (r). The intercept and slope determinations included standard deviation estimates. All standard deviation measurements used in calculating between-run and within-run precision were sample standard deviations ($n-1$). The coefficient of variation (C.V.) was determined by dividing sample standard deviation by the mean ($\bar{y}$) and expressing the quotient as a percentage.

RESULTS

Linearity and within-run precision

Data describing the linearity and within-run precision of TMP and SMX in serum and MEE are displayed in Table I. An excellent linear response was observed for TMP and SMX over the concentration ranges studied. The correlation coefficients of TMP and SMX in MEE were 0.99981 and 0.99950, respectively, while in serum the values were 0.99980 and 0.99965.

Within-run precision for MEE samples ranged from 16.9% (0.1 μg/ml) to 0.9% (15 μg/ml) for TMP and 4.1% (0.5 μg/ml) to 1.2% (200 μg/ml) for SMX. Within-run precision in serum was 3.8% (0.1 μg/ml) to 0.8% (15 μg/ml) for TMP and 6.3% (0.5 μg/ml) to 1.8% (200 μg/ml) for SMX.

Limits of quantitation and detection

Limit of quantitation was determined directly from within-run precision values. The lowest concentration of drug which resulted in a C.V. $<20\%$ was set as the limit of quantitation. This drug concentration was used as the minimum value in constructing the calibration curves. The limits of quantitation of TMP and SMX in MEE and serum did not differ significantly. The limits of quantitation for TMP and SMX were 0.1 and 0.5 μg/ml, respectively.

The limit of detection was defined as the amount of drug which resulted in a peak-height three times that of the baseline noise. For TMP and SMX the limits of detection were 0.05 and 0.25 μg/ml, respectively.

TABLE I

LINEARITY AND WITHIN-RUN PRECISION OF TMP AND SMX

In all cases, $n = 5$.

TMP			SMX		
Concentration (μg/ml)		C.V. (%)	Concentration (μg/ml)		C.V. (%)
Added	Found (mean ± S.D.)		Added	Found (mean ± S.D.)	
Middle ear effusion					
0.1	0.11 ± 0.02	16 9	0.5	0.44 ± 0.02	4.1
2.5	2.50 ± 0.06	2.2	50	49.74 ± 0.35	0.7
15.0	14.94 ± 0 13	0.9	200	200.12 ± 2.44	1.2
Intercept = 0.00148 ± 0.00517 (mean ± S.D.)			Intercept = 0.00406 ± 0.05554 (mean ± S.D.)		
Slope = 0.0492 ± 0.00019 (mean ± S.D.)			Slope = 0.02306 ± 0.00014 (mean ± S.D.)		
r = 0.99981			r = 0.99950		
Serum					
0.1	0.10 ± 0 003	3.8	0.5	0.80 ± 0.05	6.3
2.5	2.52 ± 0.05	2.0	50	50.35 ± 2.31	4.6
15.0	15.04 ± 0.12	0.8	200	201 99 ± 3.65	1.8
Intercept = 0.00082 ± 0.00207 (mean ± S.D)			Intercept = −0.0008 ± 0.00837 (mean ± S.D.)		
Slope = 0.02449 ± 0.00009 (mean ± S.D.)			Slope = 0.00431 ± 0.00003 (mean ± S.D.)		
r = 0 99988			r = 0.99965		

Between-run precision and accuracy

Between-run precision and accuracy of TMP and SMX in serum and MEE are outlined in Table II. The precision of drug measurements in serum or MEE did not differ greatly. In all cases, the C.V. remained below 20% for the lowest concentration. Accuracy of the drug measurements was good throughout the range studied.

Recovery

Data describing the recovery of TMP and SMX in MEE and serum are shown in Table III. The mean recoveries of TMP and SMX in MEE were 68.9 and 85.5%, respectively. However, the serum extraction conditions favored TMP recovery (81.5%) while SMX recovery decreased to 63.8%. Examination of the individual recovery points for each drug showed a consistent amount of TMP recovered in both MEE and serum. SMX, however, showed an initial high recovery for 0.5 and 1 μg/ml levels, but a drop in recovery starting at 10 μg/ml. This phenomenon was more apparent under serum extraction conditions.

Chinchilla MEE and serum

Chromatograms showing the resolution of TMP, SMX and I.S. in MEE and serum are shown in Figs. 1 and 2, respectively. Blank MEE and serum showed no interfering peaks.

TABLE II

BETWEEN-RUN PRECISION AND ACCURACY OF TMP AND SMX

In all cases, $n = 12$.

TMP			SMX		
Concentration (μg/ml)		C.V. (%)	Concentration (μg/ml)		C.V. (%)
Added	Found (mean ± S.D.)		Added	Found (mean ± S.D.)	
Serum					
0.1	0.10 ± 0.02	16.1	0.5	0 65 ± 0.12	18.6
2.5	2.50 ± 0.06	2.3	50	50.27 ± 1.37	2.7
15	14.99 ± 0.14	0.9	200	204.54 ± 3.89	1.9
Middle ear effusion					
0 1	0.11 ± 0.01	11.9	0.5	0.52 ± 0.09	18.3
2.5	2.50 + 0.07	2.9	50	49.53 ± 0.35	0.7
15	14.90 ± 0.20	1.4	200	200.04 ± 3.46	1.7

TABLE III

RECOVERY OF TMP AND SMX

Concentration (μg/ml)	Recovery curve concentration (mean ± S.D.) (μg/ml)	Amount recovered (mean ± S D.) (μg/ml)	Recovery (%)
Middle ear effusion. TMP			
0.1	0.18 ± 0.01	0.11 ± 0.01	60
2.5	2.43 ± 0.10	1.75 ± 0.04	72
15	15.05 ± 0.02	10.44 ± 0.12	69
			Mean recovery: 68 9
Middle ear effusion· SMX			
0.5	0.98 ± 0.08	1.02 ± 0.02	104
50	49.59 ± 0.33	38.81 ± 0.14	78
200	200.39 ± 0.75	155.00 ± 1.77	78
			Mean recovery: 85.5
Serum. TMP			
0.1	0.12 + 0.01	0.10 ± 0.003	79
2.5	2.54 ± 0.02	2.03 ± 0.01	80
15	15.11 ± 0.11	12.08 ± 0.08	80
			Mean recovery: 81.5
Serum· SMX			
0.5	1.10 ± 0.01	1.08 ± 0.05	98
50	49 61 ± 0.10	26.73 ± 0.46	54
200	201.94 ± 0.47	101.19 ± 11.58	50
			Mean recovery: 63 8

Chinchilla MEE and serum samples were analyzed over a 12-h period for TMP and SMX after administration of 10 mg TMP and 50 mg SMX per kg intramuscularly (Fig. 3A and B, respectively). In serum, both SMX and TMP reached

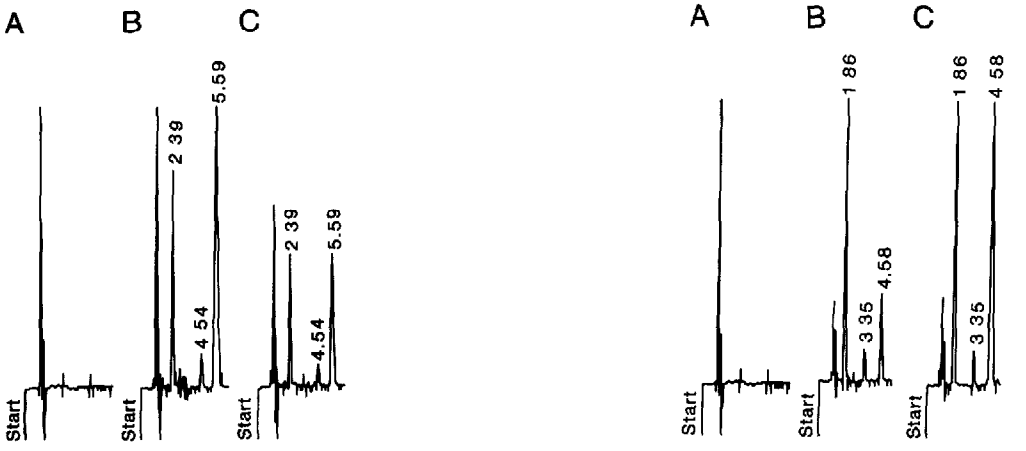

Fig. 1. Chromatograms of chinchilla middle ear effusion. (A) Blank MEE; (B) 50 μg SMX and 2.5 μg TMP per ml MEE standard; (C) chinchilla sample; retention times: I S. = 2.39 min; TMP = 4.54 min; SMX = 5.59 min.

Fig. 2. Chromatograms of chinchilla serum. (A) Blank serum; (B) 10 μg SMX and 1 μg TMP per ml serum standard; (C) chinchilla sample; retention times: I.S. = 1.86 min; TMP = 3.35 min; SMX = 4.58 min.

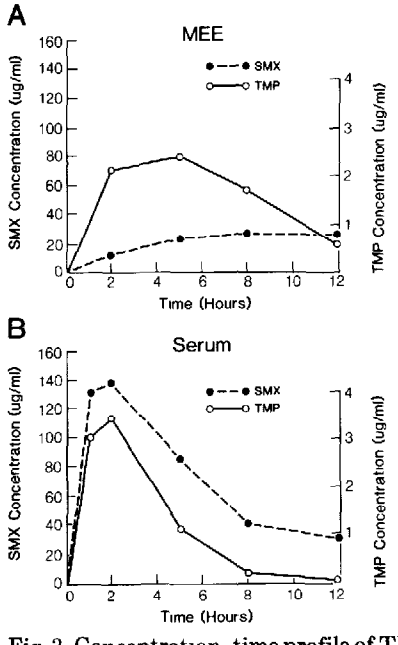

Fig. 3. Concentration-time profile of TMP and SMX levels in chinchilla middle ear effusion (A) and serum (B).

maximum concentrations at 2 h (3.3 and 140.0 μg/ml, respectively) and showed a similar elimination profile up to 12 h. Concentrations of both drugs in MEE were significantly lower than levels seen in serum. The time needed to achieve

maximum concentrations was also prolonged. The peak concentration of TMP was 2.5 μg/ml which occurred at 5 h. SMX, however, showed a very slow increase with a peak concentration of 57.0 μg/ml occurring between 8 and 12 h.

DISCUSSION

Treatment of compartmentalized infections such as otitis media presents a difficult problem since the antibiotic must penetrate out of the serum into a different body compartment to be effective [9]. The treatment of acute otitis media in children is not always successful and failure to achieve appropriate serum SMX/TMP levels may be partly responsible. Sampling directly from the infected compartment would give specific evidence of effective or ineffective antibiotic levels. To study this hypothesis in otitis media results in a dramatic reduction in sample volume available for analysis. Therefore, new methods of quantifying agents such as TMP and SMX in compartments like the middle ear must be developed to satisfy the minute sample volume available.

We report here an HPLC method capable of quantifying TMP and SMX, simultaneously, in 25 μl of ear fluid with only a 12-μl injection volume. The analytic procedure was linear for both drugs over a concentration range which adequately covers levels commonly achieved during routine therapy [12,13]. The method is simple and fast and does not require elaborate HPLC instrumentation or sample preparation.

Both SMX and TMP were readily extracted from MEE and serum using ethyl acetate–dichloromethane (25:75, v/v). The HPLC conditions for serum and MEE analysis were identical, however, the pH of the extractions had to be changed slightly to eliminate interference. It was reported in an earlier study [7] that an ethyl acetate–chloroform (25:75, v/v) mixture was most successful in extracting both drugs simultaneously. The use of dichloromethane over chloroform did not appear to influence these results. However, as indicated by the recovery data, the pH of the extraction did have an effect. TMP is slightly basic and therefore shows better recovery at pH 7.4. SMX is slightly acidic and favors the more acidic extraction. The results are in line with other published data [7].

The difference in ionic character also aided in the separation process. Resolution of TMP and SMX during chromatography was easily controlled by adjusting the pH of the mobile phase. A lower pH forced TMP to elute earlier while SMX was better retained. The I.S. was not dramatically affected by the change. The conditions of the analysis allowed 300–400 injections before column performance was affected. However, as the column showed deterioration the mobile phase was made more acidic (down to pH 4) to obtain adequate separation. The pH change did not cause other MEE or serum constituents to interfere in the chromatography.

The extraction conditions were selective for TMP and SMX, yet other serum or MEE components were not extracted. The use of a precolumn was not necessary since several hundred samples could be chromatographed without dramatic column deterioration.

TMP and SMX levels were determined in chinchilla MEE and serum over a 12-h period after receiving 10 mg TMP and 50 mg SMX per kg body weight. It

was evident from this study that MEE levels of TMP and SMX were below those obtained in serum. The beneficial TMP/SMX ratio of 1:20 also was not achieved in the middle ear during therapy.

The HPLC method described shows excellent linearity, sensitivity and precision for determining subtherapeutic levels of SMX and TMP in 25-μl volumes of ear fluid and may be used to quantify these agents in other body compartments.

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REFERENCES

1 G.P. Wormser and G.T. Keusch, Ann. Int. Med., 91 (1979) 420.
2 D.J Miner, in S.H.Y. Wong (Editor), Therapeutic Drug Monitoring and Toxicology by Liquid Chromatography, Vol 32, Chromatographic Sciences Series, Marcel Dekker, New York, 1985, pp. 269–307.
3 L. Nordholm and L. Dalgaard, J Chromatogr., 305 (1984) 391.
4 L. Essers and H. Korte, Chemotherapy, 28 (1982) 247.
5 D. Jung and S. Oie, Clin. Chem., 26 (1980) 51.
6 A. Weber, K.E. Opheim, G.R. Siber, J.F. Ericson and A.L. Smith, J Chromatogr., 278 (1983) 337.
7 O. Spreux-Varoquaux, J.P. Chapalain, P. Cordonnier, C. Advenier, M. Pays and L. Lamine, J. Chromatogr , 274 (1983) 187.
8 L. Nordholm and L. Dalgaard, J. Chromatogr , 233 (1982) 427.
9 S.K. Juhn, J Edlin, T.T.K. Jung and G.S. Giebink, Arch. Otorhinolaryngol., 243 (1986) 183.
10 S.K. Juhn, P. Sipila, J. Jung and J. Edlin, Acta Otolaryngol. Suppl., 14 (1984) 45.
11 D.M. Canafax, G.S. Giebink, G.R Erdmann, R.J. Cipolle and S.K. Juhn, in D.J. Lim (Editor), Recent Advances in Otitis Media, B.C. Decker, Philadelphia, PA, 1988, in press.
12 A. Kucers and N. Bennett, in The Use of Antibiotics, William Heinemann Medical Books, London, 3rd ed., 1979, p. 687.
13 R.B. Patel and P.G. Welling, Clin. Pharmacokin., 5 (1980) 405