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HPLC determination of tetroxoprim and sulphadiazine in pharmaceutical dosage forms and in biological fluids

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Abstract: Two HPLC methods for determination of tetroxoprim and sulphadiazine in pharmaceutical dosage forms and in biological fluid are reported. Both methods show good linearity, precision, accuracy and reproducibility. The serum levels and urinary excretion of tetroxoprim and sulphadiazine in man, after oral administration of two different syrup formulations, are reported. Tetroxoprim embonate, an insoluble salt very useful for obtaining a suspension with good palatability, shows a bioavailability not statistically different from that of tetroxoprim base. Sulphadiazine shows the same bioavailability in the two syrups.

Keywords: *Tetroxoprim; sulphadiazine; pharmaceutical dosage forms analysis; serum and urine analysis; reversed-phase HPLC; UV detection.*

Introduction

The selective and competitive inhibition of bacterial dihydrofolate reductase is a property of some compounds of the 2,4-diamino-5-benzylpyrimidine [1]. Tetroxoprim (2,4-diamino-5-[3,5-dimethoxy-4(2-methoxy ethoxy) benzyl] pyrimidine) is a bacterial dihydrofolic reductase inhibitor more specific than trimethoprim [2]. Tetroxoprim showed marked antibacterial activity; the combination of tetroxoprim (TXP) with sulphadiazine (SDZ) showed *in vitro* synergistic activity against a large number of bacterial pathogens [3, 4]. The combination of TXP and SDZ (Oxosint®) has shown good results in extensive clinical trials [5–8].

Methods currently available for determining tetroxoprim in biological fluids use radioactive (^{14}C) assays [9, 10], microbiological assays [11, 12], spectrofluorimetric assay [13] and high-performance liquid chromatographic assays [14–18]. Sulphadiazine is determined in biological fluids by colorimetric assay [19], by gas-chromatographic assay [15] and by high-performance liquid chromatographic assays [14, 16–18].

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Some of the reported methods are not specific for the two drugs and some require extensive sample work-up; no applications are described for both drugs in pharmaceutical dosage forms.

In this paper methods are reported for quantitative determination by HPLC of both tetroxoprim and sulphadiazine in the Oxosint® pharmaceutical dosage forms and in the biological fluids. Moreover, the serum levels and urinary excretion values of both drugs in man, after oral administration of two different formulations of Oxosint® suspension, are reported.

Experimental

Materials

The solvents used were all of HPLC grade (LiChrosolv, from Merck, Darmstadt, FRG or Carlo Erba, Milan, Italy). The water was previously bidistilled through a glass distiller and filtered on a 0.45 μm membrane (type FH, Millipore). The other reagents were all of analytical grade. Standard tetroxoprim and sulphadiazine were prepared in the authors' laboratories. Oxosint® tablets and syrups were lots from the authors' laboratories. Each tablet contained 100 mg of tetroxoprim, 250 mg of sulphadiazine and excipients to 600 mg. Syrup A contained 2 g tetroxoprim, 5 g sulphadiazine in a suitable vehicle (miglyol) with buffer substances, suspending and stabilizing agents and preservatives to 100 g. Syrup B contained 3.16 g tetroxoprim embonate (equivalent to 2 g tetroxoprim), 5 g sulphadiazine in sucrose syrup with buffer substances, suspending and stabilizing agents and preservatives to 100 g.

Chromatographic conditions

The high-performance liquid chromatograph was a Pye–Unicam LC3, equipped with a variable wavelength detector, an integrator and a Rheodyne injection valve. The column used was packed with LiChrosorb RP-18 reverse phase 10 μm supplied by Brownlee Labs (Santa Clara, CA, USA). For tetroxoprim the mobile phase was methanol–water (50:50) with sodium pentansulphonate (1 mg ml^{-1}); the flow-rate was 1 ml min^{-1}, pressure 60 bar; detector wavelength 235 nm; and the quantity injected was 20 μl. For sulphadiazine the mobile phase was methanol–water–glacial acetic acid (15:84:1, v/v/v); the flow rate was 1 ml min^{-1}, pressure 50 bar; detector wavelength 254 nm; and the quantity injected was 20 μl.

Standards

Pharmaceutical dosage forms. Prepare a standard solution of tetroxoprim in methanol at a concentration of 40 μg ml^{-1}. Prepare a standard solution of sulphadiazine in sodium bicarbonate 0.1 M at a concentration of 100 μg ml^{-1}. Filter both the solutions through a 0.5 μm Millipore membrane.

Serum and urine. Prepare a standard solution of tetroxoprim in methanol at a concentration of 100 μg ml^{-1}; from this solution prepare standard solutions in human serum and urine at 0.5 and 10 μg ml^{-1} respectively. Prepare a standard solution of sulphadiazine in sodium bicarbonate 0.1 M at a concentration of 100 μg ml^{-1}; from this solution prepare standard solutions in human serum and urine at 5 and 25 μg ml^{-1} respectively. Serum and urine standards are treated as described under procedure.

Procedure

Pharmaceutical dosage forms.

(a) Tetroxoprim. Five tablets were pulverized in a mortar and the mixture was quantitatively transferred to a 250 ml tared volumetric flask with methanol up to the mark. The suspension was filtered through a filter paper and 1 ml of the filtrate diluted to 25 ml with methanol. This solution, after filtration on a 0.5 μm Millipore membrane, was injected (20 μl) for tetroxoprim determination. A 4.0 g sample of suspension (A or B) was added with 50 ml of methanol and, after stirring, diluted to 200 ml in a tared volumetric flask with the same solvent. A 5 ml sample of the resulting solution was diluted to 50 ml with methanol, filtered through a 0.5 μm Millipore membrane and injected in the apparatus (20 μl).

(b) Sulphadiazine. Five tablets were pulverized in a mortar and the mixture was quantitatively transferred to a 1000 ml tared volumetric flask with sodium bicarbonate 0.1 M up to the mark. The suspension was filtered through a paper filter and 2 ml of the filtrate diluted to 25 ml with bicarbonate solution. This solution, after filtration on a 0.5 μm Millipore membrane, was injected (20 μl) for sulphadiazine determination.

A 5 g sample of suspension (A or B) was diluted to 250 ml with a mixture constituted by 0.1 M sodium bicarbonate (85 parts) and methanol (15 parts). After stirring for 5 min the solution was heated at 70°C on a water bath for 5 min. The suspension was filtered through a paper filter and from the filtrate 5 ml was taken and diluted in a tared volumetric flask to 50 ml with the mobile phase used for HPLC (see above).

The solution was filtered through a 0.5 μm Millipore membrane and injected (20 μl).

Serum and urine. A 1 ml sample of serum was treated with 1 ml of acetonitrile. After mixing on Vortex for 2 min, the mixture was centrifuged at about 5000 rev min^{-1} for 10 min. The supernatant was filtered on 0.2 μm Millipore membrane and injected for tetroxoprim or sulphadiazine determinations (20 μl).

A 1 ml sample of urine was diluted to 10 ml with water, filtered on 0.2 μm Millipore membrane and injected for tetroxoprim or sulphadiazine determinations (20 μl).

Quantitative evaluation

To calculate the tetroxoprim and sulphadiazine content in dosage forms, sera and urines peak areas were compared with those of a solution of standards. No internal standards were used owing to the very simple procedure.

Human bioavailability

Six male volunteers, weighing from 52 to 75 kg, were admitted into the study to evaluate the serum and urine levels of tetroxoprim and sulphadiazine after crossover administration of syrup A and B at the dosage of 200 mg of tetroxoprim and 500 mg of sulphadiazine per subject. The two treatments were alternated in a crossover in two different sessions so that each subject received both preparations. All subjects gave written consent to the study. Prior to entry into the study any previous medications were discontinued with the observance of a three-day washout period. The subjects had fasted for 12 h prior to medication. Food was allowed only 4 h after administration. Venous blood samples (10 ml) for serum specimens were obtained at 0, ½, 1, 2, 4, 6, 8, 12 and 24 h after oral syrup administration. Urine collection was obtained before the treatment as basal and from 0 to 24 h after the oral syrup administrations. The serum and urine samples were frozen until assayed by HPLC methods.

Results

Linearity, precision, accuracy and reproducibility

Under the reported conditions the linear range of the methods for tetroxoprim and sulphadiazine extended from 0.1 to 200 μg ml^{-1} both for the pharmaceutical dosage forms and for the serum and urine samples (Figs 1–4). Correlation coefficients in linearity studies generally exceeded 0.990. In typical standard addition studies, the recoveries of spiked tetroxoprim were 99.0 ± 1.2% in Oxosint® tablets, 98.7 ± 1.6% in Oxosint® syrup A, 98.7 ± 1.8% in Oxosint® syrup B, 95.0 ± 4.3% in serum and 94.8 ± 5.6% in urine. The recoveries of spiked sulphadiazine were 97.8 ± 1.3% in Oxosint® tablets, 98.1 ± 2.1% in Oxosint® syrup A, 98.2 ± 2.3% in Oxosint® syrup B, 94.7 ± 5.3% in serum and 95.0 ± 5.3% in urine. The precision and accuracy of the methods were tested by standard additions to a placebo and replicate injections of the mixtures. The mean inter-assay and intra-assay variability in serum for tetroxoprim were 6.33% and 5.60% respectively and for sulphadiazine were 5.60% and 5.32% respectively (Table 1). Better results were obtained for urines and pharmaceutical dosage forms. The minimum detectability of tetroxoprim and sulphadiazine in biological fluids was 0.1 μg ml^{-1} for both. The reproducibility of the methods was ascertained by replicate assays of tablets (Table 2).

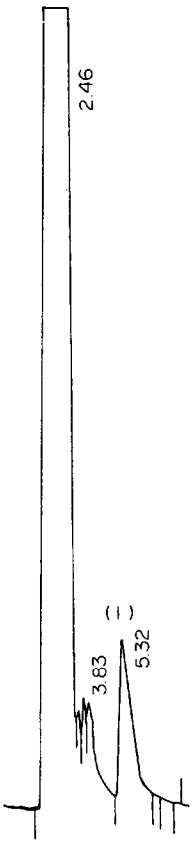

Figure 1
Chromatogram of tetroxoprim (1) from human serum.

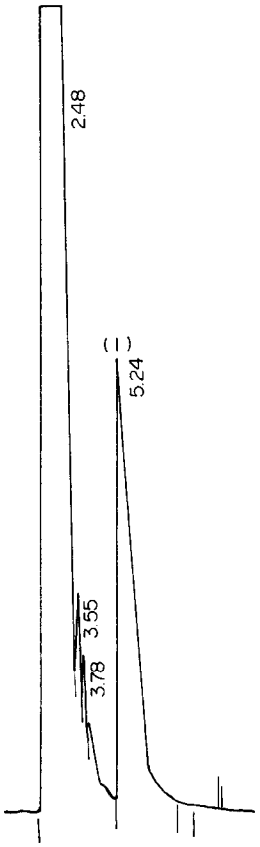

Figure 2
Chromatogram of tetroxoprim (1) from human urine.

Table 1
Intra- and inter-assay variability data

	Inter-assay variability		Intra-assay variability	
Amount added to 1 ml of serum (μg)	Amount found (μg ml^{-1}) Mean ± S.D.	Coefficient of variation (%)	Amount found (μg ml^{-1}) Mean ± S.D.	Coefficient of variation (%)
Tetroxoprim				
0.5	0.50 ± 0.04	8.00	0.50 ± 0.04	8.00
1	1.01 ± 0.08	7.92	1.01 ± 0.06	6.14
2	2.01 ± 0.10	4.97	2.02 ± 0.08	3.96
5	4.98 ± 0.24	4.82	5.02 ± 0.29	5.78
10	9.90 ± 0.54	5.45	9.95 ± 0.41	4.12
Mean ± S.D.		6.23 ± 1.59		5.60 ± 1.66
Sulphadiazine				
1	1.02 ± 0.09	8.82	1.02 ± 0.07	6.86
2	2.01 ± 0.11	5.47	2.00 ± 0.08	4.00
5	5.05 ± 0.29	5.74	5.06 ± 0.38	7.51
10	9.92 ± 0.39	3.93	9.96 ± 0.48	4.82
20	19.86 ± 0.80	4.03	19.78 ± 0.67	3.39
Mean ± S.D.		5.60 ± 1.98		5.32 ± 1.79

Aliquots (1 ml) of control serum were spiked with 100 μl of standards solutions and treated as described in the experimental section. Intra-assay variability was determined from three sets of spiked samples that were extracted and analysed in one day. Inter-assay variability was determined from three sets of spiked samples that were extracted and analysed on three different days.

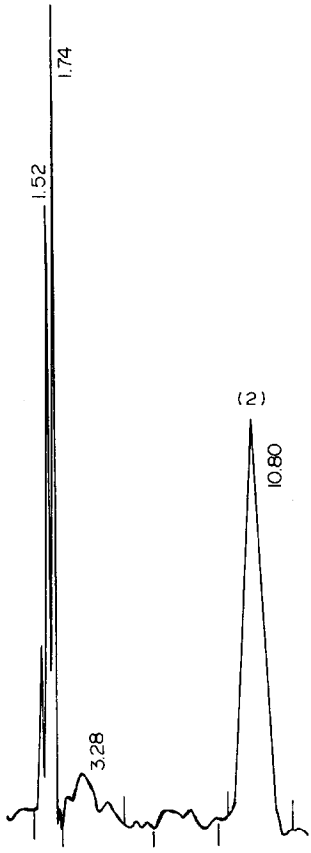

Figure 3
Chromatogram of sulphadiazine (2) from human serum.

Table 2
Reproducibility of HPLC methods using standards containing all tablet excipients

Standards (% added)	Tetroxoprim (% found)	Sulphadiazine (% found)
80	79.4, 81.8, 79.9	78.9, 82.0, 80.1
100*	99.9, 101.3, 99.8	99.5, 101.8, 99.2
120	119.0, 118.7, 117.7	121.8, 120.5, 117.3
$\bar{X}$†	99.8	100.2
R.S.D.†	1.26	1.56

*The 100% standard had a concentration of 100 mg of tetroxoprim and 250 mg of sulphadiazine per tablet.
†Calculated on results expressed as a percentage of target.

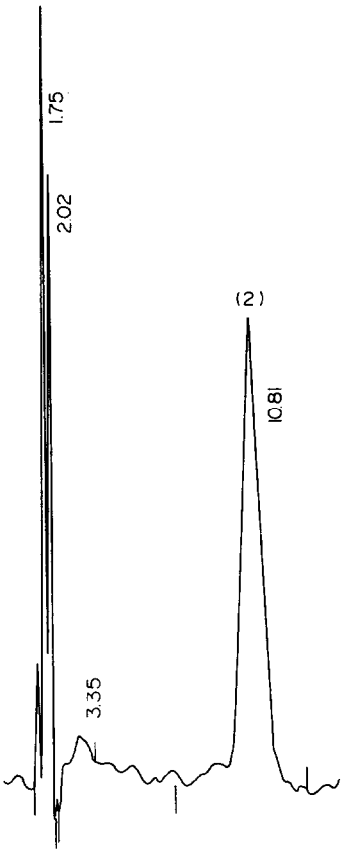

Figure 4
Chromatogram of sulphadiazine (2) from human urine.

Human bioavailability

Tables 3 and 4 report the results of serum levels and urinary excretions of tetroxoprim and sulphadiazine in six volunteers after oral administration of syrups A and B at the dosage of 200 mg of tetroxoprim and 500 mg of sulphadiazine. Variance analysis on serum levels, $AUC_{(0-24h)}$, serum peaks and urinary eliminations shows that there are no significant differences between the two preparations. The tetroxoprim embonate, an insoluble salt very useful for obtaining a better palatable suspension (Syrup B), shows a bioavailability statistically not different from that of tetroxoprim base (Syrup A). Sulphadiazine shows the same bioavailability in the two formulations of Oxosint® suspension.

Discussion

The two HPLC methods for the determination of tetroxoprim and sulphadiazine showed a good linearity in pharmaceutical dosage forms and in biological fluids.

For both drugs the recovery was more than 94%; the inter- and intra-assay variability was not more than 6.33% (coefficient of variation); the minimum detectability in serum and urine was 0.1 $\mu g\ ml^{-1}$; the reproducibility was less than 1.60% (as R.S.D.). These methods are comparable to those of other authors [14, 16–18] for recovery, reproducibility, specificity and minimum detectability.

Table 3
Serum levels and urinary excretion of tetroxoprim after oral administration of syrup A and B, both having 200 mg of tetroxoprim base (mean ± S.E.)

Treatments	Volunteers No.	Serum levels ($\mu g\ ml^{-1}$) after hours ½	1	2	4	6	8	12	24	AUC 0–24 h ($\mu g.h\ ml^{-1}$)	Maximum serum levels ($\mu g\ ml^{-1}$)	Urinary excret. (%)
Syrup A	6	0.610 ±0.067	1.375 ±0.194	1.737 ±0.138	1.997 ±0.197	1.933 ±0.181	1.587 ±0.127	1.072 ±0.127	0.373 ±0.030	27.37 ±2.52	2.003 ±0.195	43.92 ±1.30
Syrup B	6	0.682 ±0.121	1.403 ±0.198	1.755 ±0.133	2.108 ±0.372	1.925 ±0.244	1.662 ±0.208	1.127 ±0.173	0.343 ±0.035	28.08 ±3.50	2.127 ±0.369	45.05 ±0.88

Table 4
Serum levels and urinary excretion of sulphadiazine after oral administration of syrup A and B, both having 500 mg of sulphadiazine (mean ± S.E.)

Treatments	Volunteers No.	Serum levels ($\mu g\ ml^{-1}$) after hours ½	1	2	4	6	8	12	24	AUC 0–24 h ($\mu g.h\ ml^{-1}$)	Maximum serum levels ($\mu g\ ml^{-1}$)	Urinary excret. (%)
Syrup A	6	4.705 ±0.344	8.042 ±0.288	14.987 ±0.668	20.072 ±0.713	18.677 ±0.400	17.615 ±0.476	13.762 ±0.469	6.758 ±0.414	311.85 ±7.92	20.158 ±0.653	46.40 ±1.19
Syrup B	6	4.907 ±0.546	8.187 ±0.457	14.247 ±0.970	20.685 ±1.144	18.155 ±0.787	17.415 ±0.681	15.108 ±1.054	5.970 ±0.566	316.57 ±14.09	20.973 ±0.941	48.32 ±2.90

The human bioavailability studies of the two syrup formulations showed no statistical differences for both drugs. Tetroxoprim embonate, an insoluble salt, showed a bioavailability not statistically different from that of tetroxoprim base. From the present results it was possible to obtain some pharmacokinetic parameters [20] for both drugs. The area under the curve for tetroxoprim ($AUC_{0-\infty}$) was 31.9 mg l^{-1}xh; the absorption rate constant was 0.65 h^{-1}; the half-life of absorption was 1.08 h; the maximum serum concentration was 2.0 μg ml^{-1} at a t_{max} of 4 h; the elimination rate constant was 0.087 h^{-1}; the half-life of elimination was 7.92 h; the volume of distribution was 72.1l and the total clearance was 104.5 ml min^{-1}. The area under the curve for sulphadiazine ($AUC_{0-\infty}$) was 439.7 mg l^{-1}xh; the absorption rate constant was 0.65 h^{-1}; the half-life of absorption was 1.07 h; the maximum serum concentration was 20.0 μg ml^{-1} at a t_{max} of 4.5 h; the elimination rate constant was 0.058 h^{-1}; the half-life of elimination was 12.0 h; the volume of distribution was 19.61 and the total clearance was 18.9 ml min^{-1}. The graphical results of pharmacokinetic parameters reported here are similar to that reported in the literature [10, 12, 13, 15, 21].

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