Journal of Chromatography, 224 (1981) 301—309
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 871

DETERMINATION OF 2,4-DIAMINO-5-BENZYLPYRIMIDINES IN COMBINATION WITH SULPHADIAZINE IN BIOLOGICAL FLUIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

GILLIAN B. BISHOP-FREUDLING and HARTMUT VERGIN*

Heumann-Pharma, Biochemical Department, P.O. Box 2260, D-8500 Nürnberg (G.F.R.)

(First received December 18th, 1980; revised manuscript received February 16th, 1981)

SUMMARY

High-performance liquid chromatographic methods for the simultaneous analysis of tetroxoprim (TXP)/sulphadiazine (SDZ) and metioprim (MTP)/SDZ in serum and prostatic secretion (PS) are described. The detection limits in serum and PS were 50 ng TXP per ml and 100 ng SDZ per ml, and 40 ng MTP per ml and 100 ng SDZ per ml, respectively. The intra-assay coefficients of variation were in the range of 2.7—2.19%. Some preliminary data from a pharmacokinetic study in geriatric patients and a distribution study in dogs are presented. These methods enable the investigator to process a large number of TXP/SDZ and MTP/SDZ samples in one working day.

INTRODUCTION

As a consequence of co-trimoxazole (trimethoprim and sulphamethoxazole) favourable therapeutic properties in the selective inhibition of folic acid metabolism [1, 2], numerous diaminobenzyl analogues of trimethoprim were investigated for their antibacterial activity.

Tetroxoprim (TXP; 2,4-diamino-5-[3,5-dimethoxy-4-(2-methoxyethoxy)]-benzylpyrimidine), in combination with sulphadiazine [SDZ; 4-amino-N-(pyrimidyl)-benzenesulfonamide], is the first product of this research to reach the market [manufactured as Sterinor® (Heumann-Pharma) and Tibirox® (Roche)]. Metioprim (MTP; 2,4-diamino-5-[3,5-dimethoxy-4-(methylthio)]-benzylpyrimidine) is at present undergoing thorough clinical investigation. Methods currently available for determining TXP/SDZ concentrations use either microbiological assays [3] or high-performance liquid chromatography (HPLC) [4]. However, methods reported up to date, require extensive sample work-up. We are now in the position to present a new procedure involving only a simple serum-protein precipitation step, and yet allowing one to

0378-4347/81/0000—0000/$02.50

determine the respective benzylpyrimidine and SDZ simultaneously. In order to make distribution studies possible on TXP/SDZ and MTP/SDZ in experimental animals we have expanded the methods to body fluids, i.e. prostatic secretion. The latter can be injected directly onto the column.

EXPERIMENTAL

Materials

All chemicals and solvents were of at least p.a. quality, water glass-distilled; all were prefiltered using a GV 100/1 glass filtration apparatus (Ref. No. 392700) and filter-discs, RC 58, 0.2 μm (Ref. No. 371628) both from Schleicher and Schüll (Dassel, G.F.R.). Acetic acid, ethyl acetate and KH_2PO_4 were obtained from E. Merck (Darmstadt, G.F.R.) and acetonitrile HPLC Grade S from Rathburn Chemicals (Walkerburn, Great Britain). For preparing standard curves, TXP charge No. 79-04923, MTP charge no. Gu 2409790 and SDZ charge No. 3268800 were used (Heumann-Pharma, analytical department).

Instrumentation

All investigations were carried out on a Waters ALC/GPC 204 high-performance liquid chromatograph (Waters, Königstein, G.F.R.), which was equipped with an UV absorbance detector (254 nm), Model 440 and a Waters Data Module 730 integrator. Injections were made with a 10-μl Hamilton syringe through a Waters U6K injector. A 120 × 4.6 mm reversed-phase column packed with 5 μm ODS-Hypersil (Shandon Southern Products, Ashmoor, Great Britain) was used. The packing of the column was performed principally according to Bristow et al. [5].

Sample preparation

Serum was collected by venipuncture from healthy volunteers (MTP/SDZ) and geriatric patients (TXP/SDZ) who had received a single oral dose of 200 mg MTP and 500 mg SDZ and of 200 mg TXP and 500 mg SDZ, respectively. Acetonitrile (1 ml) is added to 1 ml serum. After the sample was shaken for 30—60 sec (Reax 1 DR, Heidolph) it was centrifuged for 15 min at 2700 *g* using a Hettich EBA 3 S centrifuge.

Prostatic secretion (PS) was collected from anaesthetized mongrel dogs, which had been dosed with 5 mg/kg of the respective benzylpyrimidine and 12.5 mg/kg SDZ by an intravenous bolus and followed by constant infusion of 0.5 mg/kg/h TXP (MTP) and 1.25 mg/kg/h SDZ [6]. Aliquots (10 μl) of PS were then injected onto the column in the untreated state.

Method 1: Measurement of TXP and SDZ in serum and PS

Serum standard curves were prepared as follows. A 62 μg/ml TXP and 155 μg/ml SDZ solution in water was prepared from a tenfold concentrated methanolic stock solution. A 0.5-ml aliquot of this standard solution was added to 2.5 ml of drug-free human serum to make up a 12.4 μg/ml TXP and a 31.0 μg/ml SDZ standard. It was then serially diluted with drug-free serum to yield concentrations of 6.20/15.5, 3.10/7.75, 1.55/3.88, 0.78/1.94, 0.39/0.97 and 0.195/0.48 μg/ml TXP/SDZ. After protein precipitation with

acetonitrile and centrifuging as described above, 10-µl aliquots of the clear supernatant were injected. The mobile phase was composed of 800 ml 0.1 *M* KH_2PO_4 (containing 1% acetic acid and 1% ethyl acetate) and of 200 ml acetonitrile. The flow-rate was 1.3 ml/min. The UV detector setting for both drugs was 254 nm. For determining TXP and SDZ concentrations in PS without extraction the same system conditions as described for serum were used. Standard curves with drug-free PS were prepared with the same stock solution and the same serial dilution steps as serum.

Method 2: Measurement of MTP and SDZ in serum and PS

Serum standard curves were prepared from a 62 µg/ml MTP and 155 µg/ml SDZ aqueous solution by serial dilution with drug-free human serum exactly in the same way as described in Method 1. After protein precipitation with acetonitrile and centrifuging at 2700 *g*, 10-µl aliquots of the clear supernatant were injected into the chromatograph. The mobile phase was as used in Method 1. In addition, a flow programme consisting of 1.3 ml/min for the first 3 min and thereafter directly increasing to 2.3 ml/min was employed. Both drugs were detected at 254 nm.

The simultaneous determination of MTP and SDZ from PS was achieved under the conditions given for serum. For details on preparing standard curves with drug-free PS see Method 1.

RESULTS AND DISCUSSION

Fig. 1 includes chromatograms of blank serum and representative serum from a patient, who had received 200 mg TXP and 500 mg SDZ by oral administration. The total elution time per assay is 8 min. Unknown serum constituent peaks from the blank (Fig. 1A) can be in no way whatever seen to interfere with those of either SDZ and TXP. The limit of detection using 1-ml serum samples is 50 ng TXP per ml and 100 ng SDZ per ml respectively. The standard curves for TXP and SDZ were linear from the limits of detection up to 12.4 µg/ml TXP and 31.0 µg/ml SDZ with correlation coefficients (*r*) greater than 0.99.

The precision and accuracy of the assay are shown in Table I. Mean recoveries of 92% with mean intra-assay coefficients of variation (C.V.) of 2.7% were obtained for TXP as well as for SDZ over the concentration range of 0.195—12.4 µg/ml (TXP) and of 0.48—31.0 µg/ml (SDZ) respectively. The chromatographic procedure is reproducible with retention times of 2.22—2.24 min for SDZ and 4.02—4.04 min for TXP. This, and the fact that the compounds are not destroyed in the column, make it possible to measure TXP and SDZ without the use of internal standards.

A pharmacokinetic study of TXP and SDZ in geriatric patients who had received a single dose of 200 mg TXP and 500 mg SDZ has recently been accomplished with this method [7]. Some results are shown in Fig. 2. The TXP and SDZ serum levels found in this controlled group of patients and the main pharmacokinetic parameters, half-life of elimination and volume of distribution, were in correlation with the literature values [8, 9], i.e. they compared well with the results obtained from the double extraction technique pre-

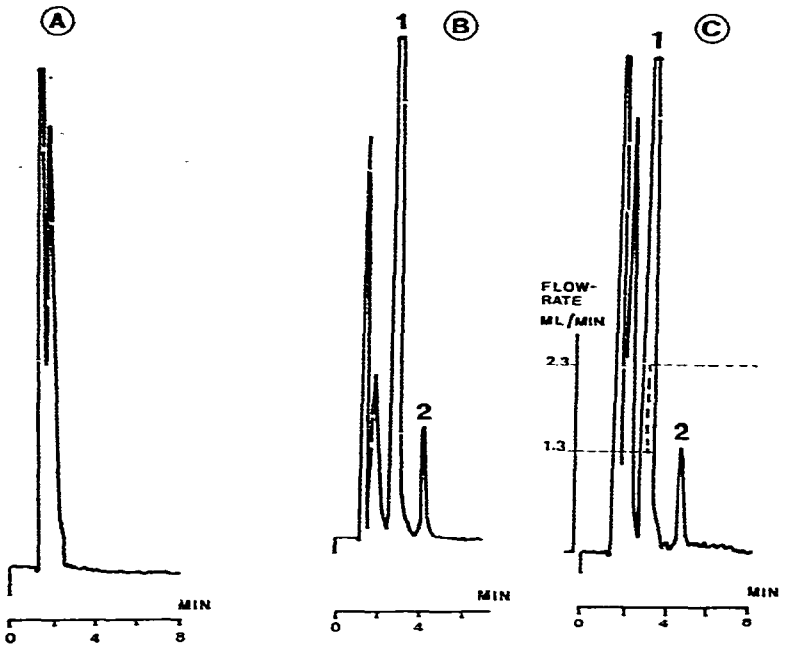

Fig. 1. Assay of tetroxoprim (TXP) and sulphadiazine (SDZ) and metioprim (MTP) and SDZ in serum (see Methods 1 and 2). (A) Drug-free human serum; (B) 3-h serum sample obtained from a geriatric patient taking a single dose of 200 mg TXP and 500 mg SDZ; (C) 3-h serum sample obtained from a healthy volunteer taking a single dose of 200 mg MTP and 500 mg SDZ. Peaks (B): 1 = SDZ, 2 = TXP; (C) 1 = SDZ, 2 = MTP, UV detector setting: 254 nm.

TABLE I

INTRA-ASSAY VARIATION OF TXP AND SDZ IN SERUM

Tetroxoprim (TXP)		Sulphadiazine (SDZ)	
Spiked concentration (μg/ml)	Intra assay C.V. (%) (n = 5)	Spiked concentration (μg/ml)	Intra assay C.V. (%) (n = 5)
0.195	3.1	0.48	3.4
0.39	n.d.*	0.97	n.d.*
0.78	1.9	1.94	1.7
1.55	2.8	3.88	3.2
3.10	2.6	7.75	2.7
6.20	2.7	15.50	2.1
12.40	3.0	31.0	2.9
Mean $\bar{x}$	2.7	Mean $\bar{x}$	2.7

*Not determined.

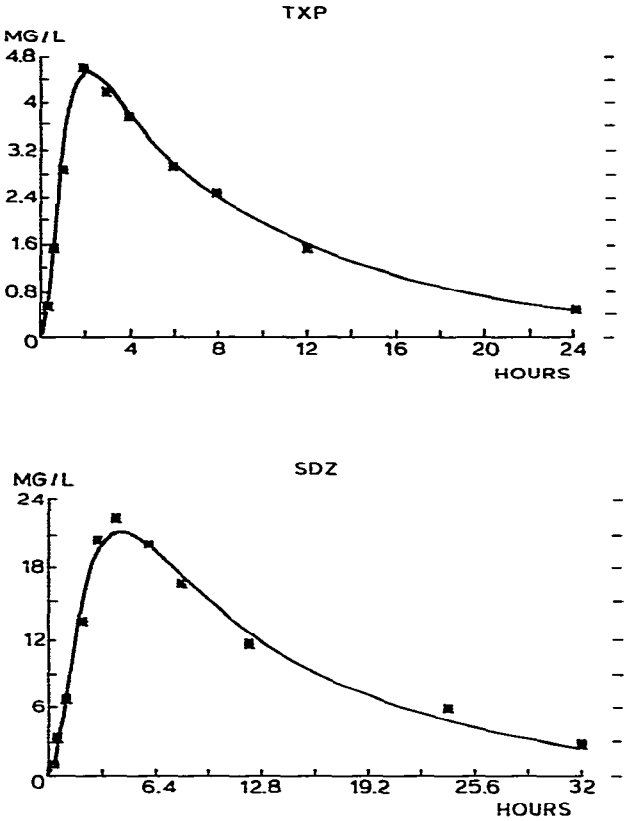

Fig. 2. TXP and SDZ concentrations in serum from 6 geriatric patients after oral administration of 200 mg TXP and 500 mg SDZ. Computer fits carried out on a Wang MVP-system with the aid of the Heinzel Topfit program [12].

viously developed in our laboratories [4]. This consisted of alkalinizing a serum sample, TXP extraction with chloroform (C.V. = 2.8%), lyophilisation of the remaining aqueous phase and hence SDZ extraction (C.V. = 2.2%) with methanol. Comparison of the values obtained using the two techniques to assay five samples from urinary tract infections patients differed from one another by 4.9—1.8%.

The detection limits for TXP and SDZ in PS of dogs were the same as in human serum. In the PS samples no interfering peaks were observed, as shown in Fig. 3. The recoveries of TXP and SDZ added to drug-free PS of dogs were quantitative (Table II). For both compounds standardisation can be carried out externally and PS can be measured by direct injection.

Peak areas shown in Fig. 3 demonstrate a reciprocal concentration relation of SDZ and TXP in PS compared to serum. Thus, under steady-state conditions in the dog (experimental set-up as previously described) approximately 20 μg/ml TXP and 4 μg/ml SDZ can be found in PS. These results are as would be expected [10, 11] and can be attributed to the different pK_a values of TXP and SDZ, and to the weakly acidic nature of PS (pH = 6.5).

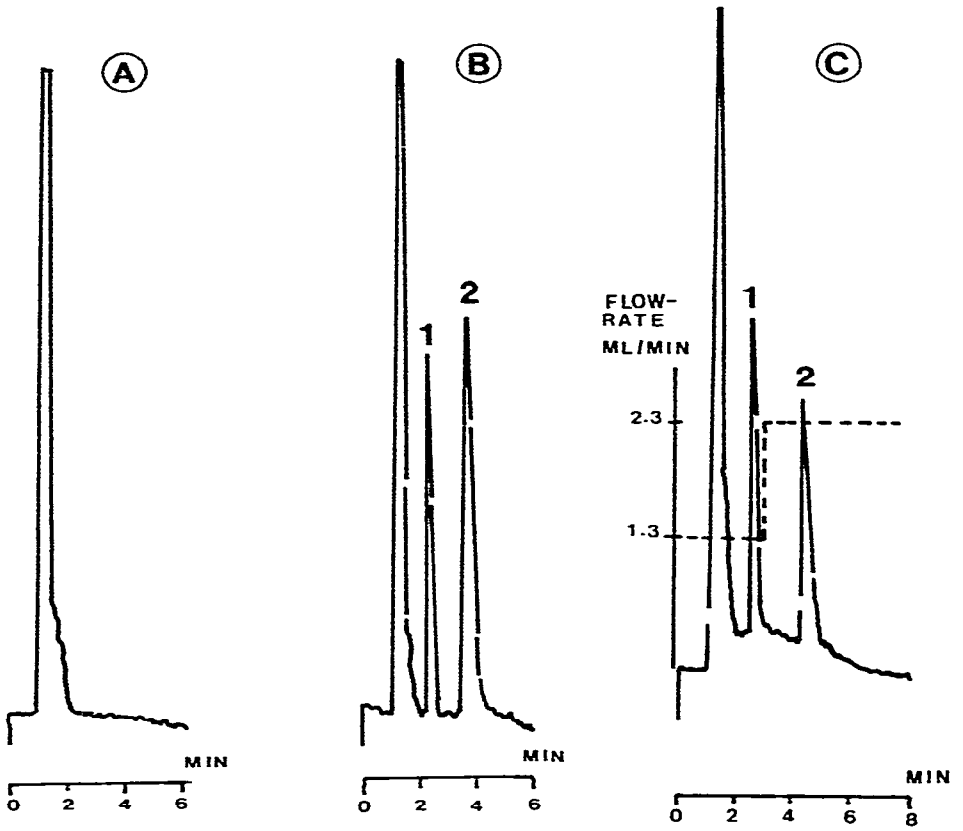

Fig. 3. Assay of tetroxoprim (TXP) and sulphadiazine (SDZ) and metioprim (MTP) and SDZ in prostatic secretion (see Methods 1 and 2). (A) Drug-free canine prostatic secretion (PS); (B) 1-h PS sample obtained from an anaesthetized mongrel dog, dosed with 5 mg TXP per kg + 12.5 mg SDZ per kg by intravenous bolus injection followed by constant infusion of 0.5 mg/kg/h TXP and 1.25 mg/kg/h SDZ; (C) = 1-h PS sample obtained from an anaesthetized mongrel dog, dosed with 5 mg MTP per kg + 12.5 mg SDZ per kg by intravenous bolus injection followed by constant infusion of 0.5 mg/kg/h MTP and 1.25 mg/kg/h SDZ. Peaks (B): 1 = SDZ, 2 = TXP; (C) 1 = SDZ, 2 = MTP. UV detector setting: 254 nm.

TABLE II

RECOVERIES OF TXP AND SDZ FROM PROSTATIC SECRETION (PS) WITHOUT EXTRACTION

Amount of TXP added to 1 ml PS (μg)	Recovery (%)	Amount of SDZ added to 1 ml PS (μg)	Recovery (%)
0.78	102	1.94	103
3.10	96	7.75	96
12.40	101	31.00	102
Mean $\bar{x}$	99.7	Mean $\bar{x}$	100.3

Fig. 1 shows chromatograms of blank serum and a typical serum from a volunteer who had received a single oral dose of 200 mg MTP and 500 mg SDZ. The limit of detection using 1-ml serum samples is 40 ng MTP per ml and 100 ng SDZ per ml respectively. Standard curves prepared for both drugs were linear from the limits of detection up to 12.4 μg/ml MTP and 31.0 μg/ml SDZ ($r > 0.99$). The mean recovery of MTP added in a wide concentration range to drug-free human serum was almost 96% (intra-assay C.V. = 2.19%), whereas recoveries of simultaneously added SDZ of 92% (intra-assay C.V. = 2.7%) were of the same magnitude as obtained for SDZ in the TXP/SDZ assay. Reproducible retention times were found to be 2.22—2.24 min for SDZ and 4.39—4.41 min for MTP. Table III shows precision and accuracy of the MTP/SDZ assay. Due to the high degree of reproducibility for both drugs, standardisation for routine analysis can be carried out externally. Preliminary pharmacokinetic results in human volunteers show maximum serum levels of 2.5—3 μg/ml MTP and 20—24 μg/ml SDZ after administration of a single oral dose consisting of 200 mg MTP and 500 mg SDZ.

TABLE III

INTRA-ASSAY VARIATION OF MTP AND SDZ IN SERUM

Metioprim (MTP)		Sulphadiazine (SDZ)	
Spiked concentration (μg/ml)	Intra-assay C.V. (%) ($n = 5$)	Spiked concentration (μg/ml)	Intra-assay C.V. (%) ($n = 5$)
0.195	2.2	0.48	3.0
0.39	2.5	0.97	3.2
0.78	1.4	1.94	1.9
1.55	2.9	3.88	3.0
3.10	1.8	7.75	2.4
6.20	n.d.*	15.50	n.d.*
12.40	2.3	31.00	2.7
Mean $\bar{x}$	2.19	Mean $\bar{x}$	2.7

*Not determined.

The simultaneous determination of MTP and SDZ from canine PS can be achieved by direct injection of the latter. Analogous to the TXP/SDZ system, recovery values were found to be quantitative for both drugs (Table IV). There was also no interference by endogenous substances (Fig. 3).

A distribution study of MTP and SDZ in dogs, which had received an intravenous bolus followed by constant infusion of both drugs has recently been initiated with this method [6]. Some results are shown in Fig. 4. MTP shows a specific affinity to the prostatic secretion, whereas SDZ, as expected, demonstrates lower values in PS than in serum.

The present HPLC methods allow simultaneous determination of the synergisticly acting chemotherapeutic agents TXP/SDZ and MTP/SDZ. By using these assays an extremely rapid sample clean-up and quantification of the two partner drugs can be achieved, without forgoing the high reproducibility. The methods will be used in further pharmacokinetic and clinical studies.

TABLE IV

RECOVERIES OF MTP AND SDZ FROM PROSTATIC SECRETION (PS) WITHOUT EXTRACTION

Amount of MTP added to 1 ml PS (μg)	Recovery (%)	Amount of SDZ added to 1 ml PS (μg)	Recovery (%)
0.78	101	1.84	101
3.10	96	7.75	96
12.40	103	31.00	103
Mean $\bar{x}$	100	Mean $\bar{x}$	100

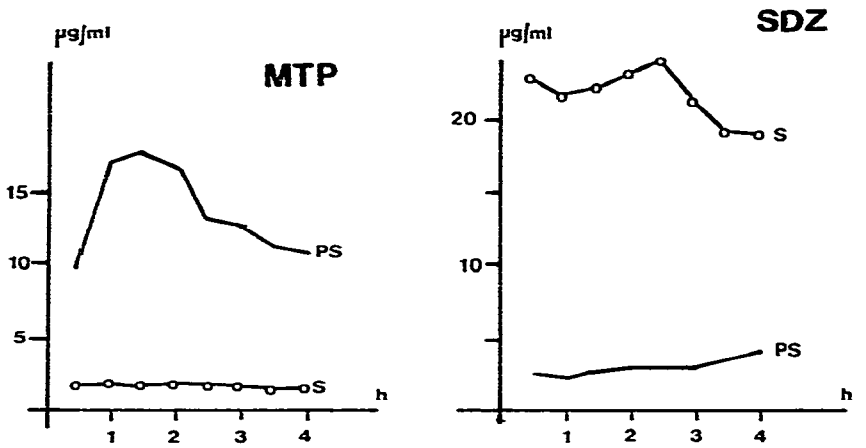

Fig. 4. Metioprim (MTP) and sulphadiazine (SDZ) concentrations in prostatic secretion (PS) and serum (S) in dogs (n = 4), dosed with 5 mg MTP per kg + 12.5 mg SDZ per kg by intravenous bolus injection followed by constant infusion of 0.5 mg/kg/h MTP and 1.25 mg/kg/h SDZ.

The correlation between serum and body fluid and tissue levels of the drugs and the relationships to microbiological and clinical variables will also be investigated.

ACKNOWLEDGEMENT

We are indebted to Miss Heike Joch for her valuable technical assistance.

REFERENCES

1 G.W. Csonka and G.J. Knight, Brit. J. Vener. Dis., 43 (1967) 161.
2 Hoigné, J. Bartholomé-Pato and U. Müller, Schweiz. Med. Wochenschr., 98 (1968) 1939.
3 H. Vergin and E. Fritschi, J. Antimicrob. Chemother., 5, Suppl. B (1979) 103.
4 H. Vergin and G.B. Bishop, Arzneim.-Forsch., 30 (1980) 317.
5 P.A. Bristow, P.N. Brittain, C.M. Riley and B.F. Williamson, J. Chromatogr., 131 (1977) 57.

6 O.S. Nielsen, P. Iverson, H. Vergin and P.O. Madsen, J. Antimicrob. Chemother., 7 (1981) in press.
7 K. Naber, H. Vergin and W. Weigand, Infection, 3 (1981) in press.
8 F.W. Reutter, H. Vergin, R. Sieber and H. Ferber, J. Antimicrob. Chemother., 5, Suppl. B (1979) 149.
9 D.S. Reeves, J.M. Broughall, M.J. Bywater, H.A. Holt and H. Vergin, J. Antimicrob. Chemother., 5, Suppl. B (1979) 119.
10 T.A. Stamey, Urinary Infections, Williams and Wilkins, Baltimore, MD, 1972, p. 191 ff.
11 P.O. Madsen, T.B. Kjaer and A. Baumüller, Urology, 8 (1976) 129.
12 G. Heinzel, in Bozler and Van Rossum, Pharmacokinetics during Drug Development. Data Analysis and Evaluation Techniques. An International Titisee Conference, October 1978, G. Fischer, Stuttgart, New York, 1981.