International Journal of Pharmaceutics, 46 (1988) 89–93
Elsevier

IJP 01532

Determination of trimethoprim in rat blood, plasma, prostate gland and seminal vesicles by high-performance liquid chromatography

Guoman Chen, Yu-Hsing Tu, Loyd V. Allen, Jr. and Da-Peng Wang

University of Oklahoma Health Sciences Center, College of Pharmacy, Oklahoma City, OK 73190 (U.S.A.)

(Received 15 June 1987)
(Modified version received 6 January 1988)
(Accepted 16 February 1988)

Key words: Trimethoprim; High-performance liquid chromatography; Blood; Plasma; Prostate gland; Seminal vesicle

Summary

Trimethoprim is selectively extracted from rat blood (85.48%), plasma (97.82%), prostate gland (91.52%) and seminal vesicles (85.74%) using alkaline conditions with dichloromethane. A high-performance liquid chromatographic (HPLC) method for rapid, accurate, specific and reliable determination of trimethoprim has been developed. It provides excellent linearity of standard solutions over the range of 8.18–40.88 μg/ml in blood and plasma, and 8.18–40.88 μg/g in prostate gland and seminal vesicles. This HPLC method also gave a linear response for trimethoprim from 0.10 to 40.50 μg/ml. The precision of the HPLC method at the detection limit (0.1 μg/ml) was ±0.95%. The within-day and between-day precisions were determined to be ±1.07% ($n = 4$) and ±2.06% ($n = 4$) at a concentration of 20.25 μg/ml. There was no apparent interference for the quantitative determination of trimethoprim. The preliminary results suggest that with this extraction and analytical method, the determination of trimethoprim levels in biological fluids and tissues can be achieved and is applicable for pharmacokinetic studies.

Introduction

Trimethoprim, a commonly used antibacterial substance interferes with folic acid metabolism by inhibiting dihydrofolate reductase and is used extensively in the treatment of a variety of infections in man (Bushby et al., 1968; Grunberg et al., 1966; Hughes et al., 1969). This drug can be administered either alone or in combination with sulfamethoxazole.

Analytical methods for trimethoprim in human body fluids have been reported, including a microbiological assay (Bushby et al., 1968), spectrofluorimetry (Schwartz et al., 1969), radioenzymatic assay (Yoger et al., 1985), autoradiography (Schwartz et al., 1970), differential pulse polarography (Brook et al., 1973), thin-layer chromatography (Siegel et al., 1974) and gas–liquid chromatography (Land et al., 1978). The use of high-performance liquid chromatography in the determination of trimethoprim concentration in biological fluids has also been reported (Ascalone, 1980, 1981; Bury et al., 1979; Bye et al., 1977; Gochin et al., 1981; Watson et al., 1980; Weinfeld et al., 1980). Data are available in the determina-

Correspondence: Loyd V. Allen, Jr., University of Oklahoma Health Sciences Center, College of Pharmacy, 1110 N. Stonewall Ave., Oklahoma City, OK. 73190, U.S.A.

0378-5173/88/$03.50 © 1988 Elsevier Science Publishers B.V. (Biomedical Division)

tion of trimethoprim concentration in human prostate gland by using the spectrofluorometric (Dabhoiwala et al., 1976; Oosterlinck et al., 1975; Wright et al., 1982) and microbiological (Madsen et al., 1976) methods. However, no information is available for the analytical procedure of trimethoprim in the prostate gland and seminal vesicles by high-performance liquid chromatography.

The purposes of this investigation were to: (1) develop optimum extraction procedures for trimethoprim from rat blood, plasma, prostate gland and seminal vesicles; (2) develop a rapid, precise, reliable and specific high-performance liquid chromatographic method that will separate trimethoprim from other sample components during the extraction procedure; and (3) emphasize the importance of analytical methodology in biological tissues or fluids.

Materials and Methods

Experiments were carried out in male rats weighing 200–250 g. An initial loading dose of 25 mg/kg of trimethoprim was given intraperitoneally (i.p. injection). Blood samples were collected in heparinized tubes at 90 min after injection by withdrawing directly from the left ventricle. Prostate gland and seminal vesicles were taken immediately after surgery. A control group was also performed using the same procedure except that no trimethoprim was injected.

Sample preparation

Plasma samples were obtained from the supernatant after centrifuging the heparinized blood at 2000 g-r.c.f. for 15 min (RC2-B Sorvall superspeed centrifuge, Ivan Sorvall, Norwalk, CT). One gram of the epididymal tissue was thoroughly homogenized (Brinkmann Homogenizer, Brinkmann Instruments Co., Westbury, NY) with 1 ml of 0.1 M phosphoric acid. This homogenization process was undertaken in a temperature-controlled ice-bath with successive mincing cycles of less than 30 s each time. The homogenized solution was centrifuged at 11,000 r.c.f. for 15 min and the supernatant transferred to a tube. One ml of distilled water was added to the pellet material and the homogenization process repeated. The supernatant was removed and combined with the previous one.

Extraction procedure

To 1 ml of plasma (or plasma obtained from 1 ml blood sample), or prostate gland or seminal vesicle sample (obtained from 1 g of tissue), 1 ml of 2 N sodium hydroxide solution (lot WEMP, Mallinckrodt, St. Louis, MO) was added followed by 4 ml dichloromethane (lot 720772, Fisher Scientific Co., Fair Lawn, NJ) containing 36 μg/ml chlorphenesin carbamate as the internal standard (lot 209-EX, Upjohn Co., Kalamazoo, MI). This solution was mixed on a mechanical shaker (Model 1105 Adams Nutator, Clay Adams, Parsippany, NJ) for 15 min and centrifuged at 11,000 r.c.f. for 10 min. The organic layer was removed and placed in a tube and another 3 ml dichloromethane added to the residue. The same extraction procedure was repeated and the organic layer combined with the previous one. The organic solvent was evaporated under a stream of air until dry. The residue was reconstituted with 2 ml 0.1 M phosphoric acid (lot 140811, J.T. Baker Chemical Co., Phillipsburg, NJ) and 20 μl of this solution injected into the high-performance liquid chromagraph.

Standard solutions

A stock solution of 160 μg/ml trimethoprim was prepared by accurately weighing the specific amount of pure material and dissolving this in 0.1 M phosphoric acid solution in a volumetric flask. A series of standard solutions of trimethoprim in blood or plasma ranging from 8.18 to 40.88 μg/ml were obtained. Several different concentrations of trimethoprim standard solutions (8.18–40.88 μg/g) in prostate gland or seminal vesicles were also prepared by adding different amounts of trimethoprim stock solution to the tissue samples.

Chromatographic conditions

A reverse-phase high-performance liquid chromatographic system equipped with a dual piston pump (Model 6000A, Waters Associates, Milford MA), a loop injector (Altex 210A, Beckman In-

struments, San Ramon, CA), a variable UV absorbance detector (Model 450, Waters Associates, Milford, MA) set at 201 nm, and a NOVA PAK C_{18} column (3.9 mm × 15 cm with 5 μm packing, Waters Associates, Milford, MA) was employed. The mobile phase was a mixture (v/v) of 40% methanol (lot 744684, Fisher Scientific Co., Fair Lawn, NJ) and 60% 0.005 M heptane sulfonic acid sodium salt (lot 720150, Fisher Scientific Co., Fair Lawn, NJ) solution at pH 3.06. The flow rate was set at 1.3 ml/min. The absorbance of the drug was recorded on a strip-chart recorder (Omniscribe, Houston Instruments) at a chart speed of 1.0 cm/min. A peak height ratio method was used to calculate the concentration of trimethoprim in reference to the internal standard.

Results and Discussion

The analytical and chromatographic conditions developed in this study for the analysis of trimethoprim from rat blood, plasma, prostate gland and seminal vesicles were simple, rapid, sensitive, reliable and sufficiently specific to separate trimethoprim from other components in the extraction procedures. The relative retention times for trimethoprim and chlorphenesin carbamate were 5.4 and 7.4 min, respectively. Representative sets of such chromatograms are shown in Fig. 1. No apparent interference for the quantitative determination of trimethoprim was observed.

The absolute recovery, which was the actual quantity of trimethoprim obtained from this extraction procedure, was determined based on the ratio of concentration obtained from directly injected aqueous standards to that of trimethoprim added to the blood, plasma or tissue samples. The recovery relative to the chlorphenesin carbamate internal standard, as calculated by the ratios of peak height, was assessed from the ratio of trimethoprim/internal standard obtained from the

TABLE 1

Recovery rates [a] for trimethoprim (24.5 μg/ml) from rat blood, plasma, prostate gland and seminal vesicles

Extraction sample	Absolute recovery (%)	Relative recovery (%)
Blood	85.48 ± 2.14	102.84 ± 2.41
Plasma	97.82 ± 0.82	103.94 ± 1.38
Prostate gland	91.52 ± 1.09	103.21 ± 2.77
Seminal vesicles	85.74 ± 1.33	101.45 ± 1.51

[a] Mean ± S.D. ($n = 5$).

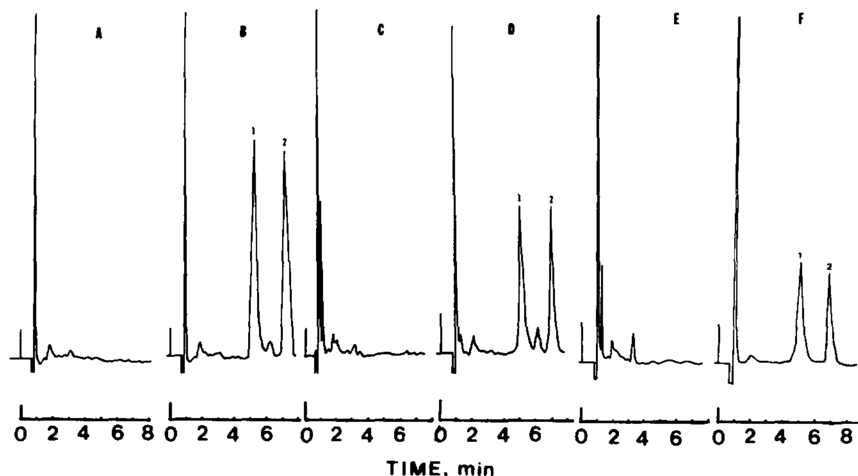

Fig. 1. Chromatograms from extracts of (A) control rat plasma, (B) trimethoprim standard in rat plasma (24.5 μg/ml) with internal standard (35.5 μg/ml), (C) control rat prostate gland, (D) trimethoprim standard in rat prostate gland (24.5 μg/g) with internal standard (35.5 μg/g), (E) control rat seminal vesicles, and (F) trimethoprim standard in rat seminal vesicles (24.5 μg/g) with internal standard (35.5 μg/g). Note: the difference in peak heights is due to different attenuation settings ($AUF = 0.01$ for A and B; $AUF = 0.02$ for C, D, E and F). Key: (1) trimethoprim; (2) chlorphenesin carbamate.

blood, plasma, prostate gland and seminal vesicles to directly injected comparable standard/internal standard. By using the internal standard in the study of relative recovery, variations from the extraction procedures and also the instruments can be avoided. Both absolute and relative recovery rates for trimethoprim from different rat fluids and tissues are shown on Table 1.

The following Eqns. 1 and 2 show the calculation of absolute and relative recoveries:

$$\text{Absolute recovery} = \frac{\dfrac{P_x}{P_{std}} \cdot C_{std}}{C_x} \qquad (1)$$

$$\text{Relative recovery} = \frac{\dfrac{P_x/P_I^1}{P_{std}/P_I^2} \cdot C_{std}}{C_x} \qquad (2)$$

where P_x = peak height of sample from extraction; P_I^1 = peak height of internal standard from extraction; P_I^2 = peak height of directly injected internal standard; P_{std} = peak height of directly injected standard; C_{std} = concentration of directly injected standard; C_x = concentration of sample in body fluids or tissue.

The calibration curve obtained by plotting the ratio of the peak height of trimethoprim to that of the internal standard, chlorphenesin carbamate, versus its respective concentrations ranged from 0.10 to 40.50 μg/ml and was linear ($r > 0.99$). The linearity of trimethoprim was demonstrated over the range of either 8.18–40.88 μg/ml in blood and plasma or 8.18–40.88 μg/g in prostate gland and seminal vesicles throughout the analytical procedures in this study (Table 2). The sensitivity limit of this detection was found to be 0.1 μg/ml and the precision ($n = 10$) at this concentration was found to be ±0.95%. Excellent linear correlation coefficients (> 0.99) between trimethoprim concentrations and peak height ratios for all the situations were observed. The within-day and between-day precision of this HPLC method was established by injecting trimethoprim samples at the concentration of 20.25 μg/ml five times each day for 4 consecutive days. The result for the within-day precision was ±1.07% and the between-day precision was ±2.06%. An application of this analytical method was carried out by the detection of trimethoprim concentrations in rat blood, plasma, prostate gland and seminal vesicles by obtaining samples at 90 min after intraperitoneal injection at the dose of 25 mg/kg. The results obtained utilizing the standard curves were 17.18 μg/ml for blood, 21.48 μg/ml for plasma, 23.69 μg/g for prostate gland and 20.52 μg/g for seminal vesicles. The results showed that trimethoprim appeared to accumulate in the prostate gland and seminal vesicles indicating its potential use in the treatment of bacterial infections in epididymal tissues. Further studies on the pharmacokinetics

TABLE 2

Linearity of spiked trimethoprim in rat blood, plasma, prostate gland and seminal vesicles

Spiked conc. [a]	Peak height ratio [c]			
	Blood	Plasma	Prostate	Seminal vesicles
8.18	0.577 ± 0.016	0.350 ± 0.004	0.363 ± 0.012	0.331 ± 0.006
16.35	0.966 ± 0.016	0.694 ± 0.007	0.774 ± 0.010	0.715 ± 0.004
24.53	1.490 ± 0.001	1.065 ± 0.005	1.407 ± 0.041	1.018 ± 0.005
32.70	2.045 ± 0.013	1.426 ± 0.003	1.914 ± 0.030	1.208 ± 0.016
40.88	2.531 ± 0.014	1.746 ± 0.003	2.478 ± 0.019	1.701 ± 0.009
Slope	0.061	0.043	0.066	0.040
Intercept	0.026	−0.001	−0.224	0.025
r [b]	0.998	1.000	0.998	0.991

[a] μg/ml for blood and plasma and μg/g for prostate gland and seminal vesicles.
[b] Correlation coefficient.
[c] Mean ± S.D. ($n = 3$).

of trimethoprim at different time periods will be conducted.

The extraction and chromatographic procedures described in this study allow the quantitation of trimethoprim concentrations in rat blood, plasma, prostate gland and seminal vesicles. The standard curves obtained were linear with an excellent fit over the required range. The methods developed are rapid, accurate, specific and reliable and can be applicable to further pharmacokinetic studies.

References

Ascalone, V., Assay of trimethoprim, sulfamethoxazole and its N^4-acetyl metabolite in biological fluids by high-pressure liquid chromatography. *J. High Resolution Chromatogr. Commun.,* 3 (1980) 261–264.

Ascalone, V., Assay of trimethoprim, sulfadiazine and its N^4-acetyl metabolite in biological fluids by normal-phase high performance liquid chromatography. *J. Chromatogr.,* 224 (1981) 59–66.

Brook, M.A., de Silva, J.A.F. and D'Arconte, L.M., Determination of trimethoprim and its *N*-oxide metabolites in urine and man, dog and rat by differential pulse polarography. *J. Pharm. Sci.,* 62 (1973) 1395–1399.

Bury, R.W. and Mashford, M.L., Analysis of trimethoprim and sulphamethoxazole in human plasma by high pressure liquid chromatography. *J. Chromatogr.,* 163 (1979) 114–117.

Bushby, S.R.M. and Hitchings, G.H., Trimethoprim, a sulphonamide potentiator. *J. Pharmacol. Chemother.,* 33 (1968) 72–90.

Bye, A. and Brown, M.E., An analytical approach to the quantitation of known drugs in human biological samples by HPLC. *J. Chromatogr. Sci.,* 15 (1977) 365–371.

Dabhoiwala, N.F., Bye, A. and Claridge, M., A study of concentrations of trimethoprim-sulphamethoxazole in the human prostate gland. *Br. J. Urol.,* 48 (1976) 77–81.

Gochin, R., Kanfer, J. and Haigh, J.M., Simultaneous determination of trimethoprim, sulphamethoxazole and N^4-acetylsulphamethoxazole in serum and urine by high performance liquid chromatography. *J. Chromatogr.,* 223 (1981) 139–145.

Grunberg, E. and Delorenzo, W.F., Potentiation of sulfonamides and antibiotics by trimethoprim [2,4-diamino-5-(3,4,5-trimethoxy-benzyl)pyrimidine]. *Antimicrob. Agents Chemother.,* (1966) 430–433.

Hughes, D.T.D., Drew, C.D.M., Johnson, T.B.W. and Jarvis, J.D., Trimethoprim and sulphamethoxazole in the treatment of chronic chest infections. *Chemotherapy,* 14 (1969) 151–157.

Land, G., Dean, K. and Bye, A., The gas–liquid chromatographic analysis of trimethoprim in plasma and urine. *J. Chromatogr.,* 146 (1978) 143–147.

Madsen, P.O., Kjaer, T.B. and Baumueller, A., Prostatic tissue and fluid concentrations of trimethoprim and sulfamethoxazole. *Urology,* 8 (1976) 132–139.

Oosterlinck, W., Defoort, R. and Renders, G., The concentration of sulphamethoxazole and trimethoprim in human prostate gland. *Br. J. Urol.,* 47 (1975) 301–304.

Schwartz, D.E., Koechlin, B.A. and Weinfeld, R.E., Spectrofluorimetric method for the determination of trimethoprim in body fluids. *Chemotherapy,* (Suppl.) 14 (1969) 22–29.

Schwartz, D.W., Vetter, W. and Englert, G., Trimethoprim metabolites in rat, dog and man: qualitative and quantitative studies. *Arzneim-Forsch.,* 20 (1970) 1867–1871.

Sigel, C.W., Grace, M.E., Nichol, C.A. and Hitching, G.H., Specific TLC determinations of trimethoprim and sulfamethoxazole in plasma. *J. Pharm. Sci.,* 63 (8) (Aug 1974) 1202–1205.

Vree, T.B., Hekster, Y.A., Baars, A.M., Damsma, J.E. and Van der Kleijn, V., Determination of trimethoprim and sulfamethoxazole (co-trimoxazole) in body fluids of man by means of high performance liquid chromatography. *J. Chromatogr.,* 146 (1978) 103–112.

Watson, I.D., Shenkin, A., McIntosh, S.J. and Cohen, H.N., Assay for trimethoprim in serum and urine by means of ion-pair chromatography. *Clin. Chem.,* 26 (1980) 1791–1795.

Weinfeld, R.E. and Macasieb, T.C., Determination of trimethoprim in biological fluids by high performance liquid chromatography. *J. Chromatogr.,* 164 (1979) 73–84.

Wright, W.L., Larking, P. and Lovell-Smith, C.J., Concentrations of trimethoprim and sulphamethoxazole in human prostate gland after intramuscular injection. *Br. J. Urol.,* 54 (1982) 550–551.

Yogev, R., Melick, C. and Lolita, T.P., Ratioenzymatic assay for trimethoprim in very small serum samples. *Clin. Microbiol.,* 21 (1985) 249–250.