S-OXYGENATION OF 7α-THIOMETHYLSPIRONOLACTONE BY THE FLAVIN-CONTAINING MONOOXYGENASE

John R. Cashman and Sandra Pena
Department of Pharmaceutical Chemistry and Liver Center
School of Pharmacy, University of California
San Francisco, California 94143-0446, U.S.A.

CONTENTS

SUMMARY

Liver microsomes and highly purified flavin-containing monooxygenase from uninduced hogs catalyze the NADPH and oxygen-dependent S-oxygenation of 7α-thiomethylspironolactone (7α-TMSL), the major urinary metabolite of spironolactone, an effective antimineralocorticoid in humans. Studies on the biochemical mechanism of S-oxygenation of 7α-TMSL suggests that this reaction is catalyzed exclusively by the flavin-containing monooxygenase and not by cytochrome P-450. This conclusion is based on the effects of selective cytochrome P-450 inhibitors as well as positive effectors and alternate substrates for the flavin-containing monooxygenase. The modest degree of stereoselective S-oxygenation of 7α-TMSL may suggest steric inhibition of oxidation by the flavin-containing monooxygenase.

I. INTRODUCTION

Spironolactone is a clinically effective potassium sparing diuretic used for the treatment of essential hypertension, edematous states and primary aldosteronism /1/. Spironolactone and a major *in vivo* metabolite, canrenone, have major and minor actions, respectively, as competitive inhibitors of aldosterone receptors at the renal distal tubules /2/. Thus, after multiple dosing, canrenone can contribute as much as 25% of the antimineralocorticoid activity as spironolactone in humans /3/.

Previous studies have demonstrated that after oral administration to humans, spironolactone is extensively biotransformed /3-6/. The major metabolic routes involve deacetylation, desulfuration, S-oxidation, and aliphatic hydroxylation /7,8/ (see Figure 1). The metabolic conversion of spironolactone to canrenone has been suggested to occur by others in two distinct steps. In the first step, the thioacetate group of spironolactone is envisioned to be hydrolyzed to 7α-thiospironolactone and in a second step, hydrogen sulphide is eliminated to give canrenone /9/. However, the major metabolites of spironolactone have now been shown to be the sulfur-containing metabolites 7 α-thiomethylspironolactone and 6 β-hydroxy-7 α-thiomethylspironolactone /3,6,10,11/. Although spironolactone is extensively metabolized, it is not clear how canrenone is formed

and/or whether the amount of canrenone excreted is dependent upon enzymatic or nonenzymatic processes. Metabolic oxidations of sulfur-containing compounds usually involve cytochromes P-450 /12/ of the flavin-containing monooxygenase (FMO) /13/. Spironolactone has been observed to interact with cytochrome P-450 /14/ and sulfides like 7α-thiomethylspironolactone have been shown to be oxidized by both cytochrome P-450 and the flavin-containing monooxygenase /12,13/.

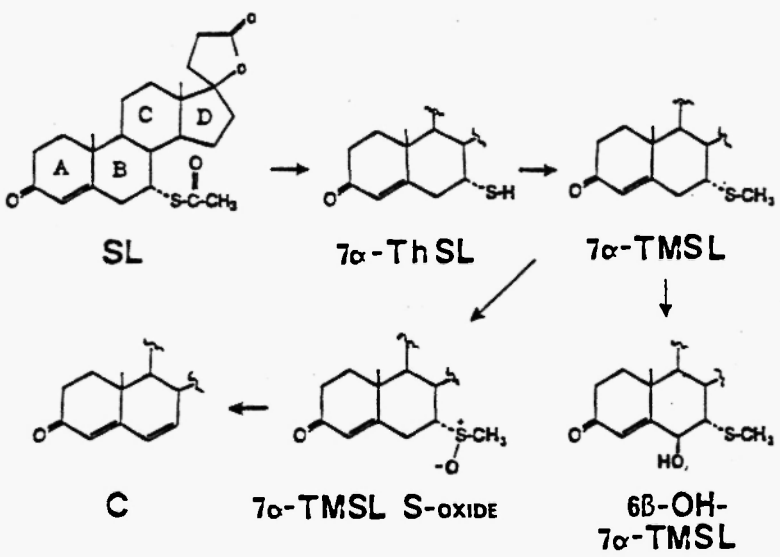

Fig. 1: Overall biotransformation of spironolactone (SL). Abbreviations are: 7α-thiomethylspironolactone (7α-TMSL), 7α-thiospironolactone (7α-ThSL), 7α-thiomethylspironolactone S-oxide (7α-TMSL S-oxide), 6β-hydroxy-7α-thiomethylspironolactone (6β-OH-7 TMSL) and canrenone (C).

In this study we have characterized the *in vitro* oxidative biotransformation of 7α-thiomethylspironolactone, the major metabolite isolated from administration of spironolactone to humans /10,15/. Investigation of the metabolism of 7 α -thiomethylspironolactone could provide information about the nature of the overall (bio)transformation of spironolactone to canrenone.

II. MATERIALS AND METHODS

2.1 Reagents and general procedures

7α-Thiomethylspironolactone and canrenone was obtained from G.D. Searle and Co., Chicago, IL; its diastereomeric S-oxides were synthesized by treating the sulfide with sodium periodate in methanol. The details of the synthetic procedures will be reported separately (Cashman and Pena, submitted). Each 7α-thiomethylspironolactone S-oxide diastereomer was fully characterized by spectroscopic means. ^{1}H-NMR spectra were recorded on a General Electric 500MHz instrument. Ultraviolet spectra of the S-oxides were recorded on a Perkin-Elmer 559A spectrometer. Liquid secondary ion mass spectra were taken on a Kratos MS50 fitted with a cesium gun and gave the correct molecular ion. Thiourea, n-octylamine and sodium periodate were purchased from Aldrich Chemical Company. All chemical reagents and biochemicals used were of the highest quality and were obtained commercially.

2.2 Liver Preparation

Microsomal fractions were isolated by the method described previously /16,17/. Highly purified flavin-containing monooxygenase activity was obtained using the method of Sabourin et al. /18/. Protein concentration was determined by the method of Lowry /19/.

2.3 Metabolic incubation and product analysis

The incubation medium contained 50mM potassium phospate, pH8.4, 0.5mM NADP+, 2.0mM glucose-6-phosphate and 1IU of glucose-6-phosphate-dehydrogenase, and 0.6-2.8mg of microsomes or 10-70μg of purified enzyme. Heat-inactivated microsomes were prepared following the method previously described /13/. At the time intervals indicated, the reaction was stopped and analyzed for products by HPLC analysis of dichloromethane extracts of the reaction mixture. The metabolic products were quantitated by an IBM HPLC model 9533 with a UV detector set at 244nm, fitted with a precolumn and 5μm CRI C_{18} ODS chromosphere (7.7mm x 25cm)

analytical reverse phase column. The mobile phase consisted of methanol/water (65:35, vol/vol) at a flow rate of 1.5ml/min. This system efficiently separates 7α-thiomethylspironolactone S-oxide, 7α-thiomethylspironolactone, canrenone and testosterone which have retention volumes of 3.7-4.0, 11.5, 12.9, and 13.9ml, respectively. For quantitation of diastereomers a mobile phase of methanol/water (50:50 vol/vol) gave the major and minor S-oxide diastereomers with retention volumes of 5.4 and 5.9ml, respectively. The material balance was <90% as judged by comparison of peaks to an authentic internal standard (testosterone).

III. RESULTS

Preliminary studies showed that hog liver microsomes supplemented with NADPH catalyze the oxgenation of 7α-TMSL to two diasteromeric 7α-TMSL S-oxides. The formation of the S-oxide diastereomers was a linear function of protein concentration (0.6-2.8mg of microsomal protein) and with incubation time for at least 10min. Authentic synthetic diastereomeric 7α-TMSL S-oxides added to heat inactivated microsomal protein in the absence of cofactors were recovered quantitatively after 7min incubation.

As shown in Table 1, n-octylamine-stimulated microsomes catalyzed the NADPH-dependent formation of the S-oxides approximately two-thirds as well as in the absence of n-octylamine. These results could suggest that S-oxygenation of 7α-TMSL is catalyzed by the flavin-containing monooxygenase as well as cytochrome P-450 since n-octylamine is a good inhibitor of cytochrome P-450 /20/ and a known positive effector for the flavin-containing monooxygenase /13/. Another possible explanation is that the bulky nature of the steroid nucleus at the active site of the flavin-containing monooxygenase interferes with the stimulatory role of n-octylamine. The molecular basis for the formation of the S-oxide was further examined by evaluating the effects of the various metabolism inhibitors on S-oxide formation, Table 1. Thiourea, an alternate substrate competitive inhibitor for the flavin-containing monooxygenase /21/ markedly decreased S-oxide formation. Aminobenzotriazole, a potent mechanism based inhibitor of cytochrome P-450 /22/ did not inhibit S-oxygenation of 7α-TMSL. Heat inactivation of the microsomal protein employing conditions that

TABLE 1

Effect of Metabolism Inhibitors on S-Oxygenation of 7α-Thiomethylspironolactone by Hog Liver Microsomes

Condition	Product Formed (pmols/min/mg/protein)[1] Minor S-Oxide	Major S-Oxide
Complete System[2]	27 ± 4	67 ± 16
-n-Octylamine	44 ± 6	103 ± 12
-NADPH GS	0.7 ± 1	8 ± 4
+Thiourea[3]	0.7 ± 1	4 ± 1
+Aminobezotriazole[3,4]	-	75 ± 12
+Heat Inactivated Protein	0 ± 0	1 ± 2

1 Determined by HPLC.

2 Complete system contained 2 mg hog liver microsomes, 5.0 mM n-octylamine, NADPH GS, 250 M substrate incubated for 7 min, 33°C, average of 3 determinations ± SD.

3 500μM inhibitor present.

4 Aminobenzotriazole coeluted with the minor S-oxide.

completely destroy flavin-containing monooxygenase activity /13/ but retain 80-85 percent of cytochrome P-450 activity /23/ completely prevented S-oxygenation of 7α-TMSL.

As shown in Table 2, 7α-TMSL is S-oxygenated by the purified hog liver flavin-containing monooxygenase in an NADPH-dependent process. Preliminary studies indicated that the formation of S-oxide was linearly dependent on protein (10-70μg) and with incubation time for 5min. The rate of S-oxygenation is considerably lower than the rate of oxidation of other substrates for the flavin-containing monooxygenase /13,21/. This is consistent with the large bulky nature of the substrate since it has been suggested that steric effects have a pronounced influence on the rate of S-oxygenation of the substrates for the flavin-containing monooxygenase /24/. n-Octylamine stimulates formation of the minor and major S-oxide almost 6 and 7-fold, respectively. In the presence of thiourea, formation of the minor S-oxide could not be observed while the rate of formation of the major S-oxide diastereomer was significantly decreased. Amino-

TABLE 2

S-Oxygenation of 7α-Thiomethylspironolactone
by Purlfied Hog Liver FMO

Condition	(pmols/min/mg protein)[1] Minor S-Oxide	Major S-Oxide
Complete System[2]	225 ± 130	305 ± 94
-n-Octylamine	39 ± 27	45 ± 19
-NADPH GS	10 ± 17	11 ± 4
+Thiourea[3]	0 ± 0	132 ± 73
+Aminobenzotriazole [3,4]	–	302 ± 32
Heat Inactivated Protein	34 ± 34	22 ± 20

1 Determined by HPLC.

2 Complete system contained 56μg FMO, 5.0 mM n-octylamine, the NADPH GS, and 250 M substrate incubated for 5 min, 33°C, average of 3 determinations + SD.

3 500μM inhibitor present.

4 Aminobenzotriazole coeluted with the minor S-Oxide.

benzotriazole did not inhibit the rate of S-oxide formation while heat inactivation of the purified flavin-containing monooxygenase essentially completely inactivated the rate of S-oxygenation. Authentic synthetic 7α-TMSL S-oxides added to the reaction medium were recovered quantitatively after a 5min incubation. Stereochemical analysis of the S-oxygenation products of 7α-TMSL S-oxygenation by the flavin-containing monooxygenase was performed in order to gain some insight into the mechanism of S-oxygenation. Previously, the only published examples of flavin-containing monooxygenase S-oxygenation stereoselectivity demonstrated that the enzyme was extremely stereoselective /25/. Our results suggest that the S-oxygenation of 7α-thiomethylspironolactone is only modestly stereoselective, Table 3.

IV. DISCUSSION

The present study demonstrates that 7α-TMSL is S-oxygenated by the highly purified flavin-containing monooxygenase as well as the

TABLE 3

Stereoselectivity of S-oxygenation of 7α-Thiomethylspironolactone

	Percent S-Oxide Diastereomer Formed			
	Complete[1]		-NOA[2]	
Oxidizing System	Minor	Major	Minor	Major
$NAIO_4$[3]	19.3	80.7	-	-
Pure FMO	25.0	75.0	35.0	65.0
Hog Liver Microsomes	27.0	73.0	30.0	70.0

[1] Complete system contained 2 mg of hog liver microsomes or 56 μg of highly purified hog liver flavin-containing monooxygenase, 5mM n-octylamine, the NADPH generating system, and 250μM substrate incubated for 7 min at 30°C; average of 3 determinations.

[2] Performed in the absence of n-octylamine.

[3] Chemical oxidation in methanol wih one equivalent of $NAIO_4$

microsomal flavin-containing monooxygenase from hogs in a process which probably constitutes a detoxication pathway. Like other sulfides, 7α-TMSL is converted to the S-oxide and from the results reported here, 7α-TMSL S-oxide diastereomers should be significant metabolites *in vivo*. That major quantities of S-oxides are not observed in vivo suggests that other routes of metabolism and/or chemical disposition may occur. Notably, large quantities of canrenone have been detected in the urine of humans administered spironolactone /6,8,10/. Formation of canrenone from 7α-TMSL S-oxides would involve a Cope-type elimination reaction which would benefit from allylic activation (Figure 1). Careful and attentive sample handling is required to prevent this chemical rearrangement. That the flavin-containing monooxygenase does not catalyze this elimination reaction *per se* is apparent from the *in vitro* biotransformation studies outlined herein employing purified enzyme.

7α-TMSL is transformed to diastereomeric S-oxides with purified hog liver flavin-containing monooxygenase. In hog liver microsomes, S-oxygenation occurs in a process solely dependent upon the flavin-containing monooxygenase. In microsome preparations from uninduced hogs, S-oxide formation requires active microsomes and

NADPH. Thiourea, a well documented specific alternate substrate for the flavin-containing monooxygenase /21/ markedly inhibits, while aminobenzotriazole, a mechanism-based inactivator of cytochrome P-450 /22/ does not inhibit the formation of diastereomeric 7α-TMSL S-oxides. Heat inactivation of microsomes under conditions that preserve cytochrome P-450 activity and that destroy flavin-containing monooxygenase activity /23/ completely abolish 7α-TMSL S-oxide formation. This indicates that although n-octylamine does not stimulate microsomal S-oxide formation, 7α-TMSL S-oxygenation is not due to some unusual cytochrome P-450 isozyme, but rather, to the flavin-containing monooxygenase. In the presence of the highly purified hog liver flavin-containing monooxygenase a similar pattern of inhibition of S-oxygenation of 7α-TMSL also holds. We conclude that 7α-TMSL is oxygenated largely, if not exclusively, by the flavin-containing monooxygenase rather than cytochromes P-450.

Hog liver flavin-containing monooxygenase has been shown to S-oxygenate dialkylsulfides with 93-95% stereoselectivity in a process where the minor S-oxide enantiomer arises due to incomplete chiral processing by the enzyme and not to a competing, achiral non-enzymatic sulfoxidation process /25/. The stereoselectivity of 7α -TMSL S-oxygenation by microsomes and highly purified hog liver flavin-containing monooxygenase activity was determined (Table 3). Surprisingly, both hog liver microsomes and purified flavin-containing monooxygenase demonstrated a low degree of stereoselectivity. One possible explanation for the lack of chiral purity for S-oxygenation could be that a concurrent nonenzymatic S-oxygenation contributes to the formation of the minor diastereomer. That this is not the case is apparent since: 1) the ratio of diastereomers remains constant over the time course of the reaction for both microsomes and purified enzyme, and 2) incubations in the presence of catalase did not alter the stereoselectivity of S-oxygenation (data not shown). A second possible explanation for the lack of stereoselectivity could be that the active site of the flavin-containing monooxygenase does not readily accomodate approach of the large steroid nucleus. A third possibility is that steric hindrance between enzyme and substrate allows partial single electron transfer character to the S-oxygenation process /26/ although each diastereomer is stable to inversion /27/.

The lack of significant involvement of cytochrome P-450 in the S-oxygenation of 7α-TMSL makes this compound, or steroids with a similar structure, good substrates to investigate the stereoselectivity

and steric limitations of the hepatic flavin-containing monooxygenase towards S-oxygenation.

V. ACKNOWLEDGEMENTS

We thank Professor D.M. Ziegler for a generous gift of hog liver microsomes and G.D. Searle for a sample of 7α-thiomethylspironolactone and canrenone. The financial support of the National Institutes of Health (GM32426), March of Dimes (J.R.C.) and a National Institutes of Mental Health Summer Research Fellowship Grant Number 1 T35 MH 18910-01 (S.P.) is gratefully acknowledged. The help of the University of California, San Francisco, Bioorganic Biomedical Mass Spectrometry Resource (A.L. Burlingame, Director) is acknowledged (supported by NIH Division of Research Resources Grant RR01614). The excellent typing of Andrea Mazel is gratefully acknowledged.

VI. REFERENCES

1. Kagawa, C.M. Blocking the renal electrolyte effects of mineralocorticoids with an orally active steroidal spirolactone. *Endocrinology* 1960; **67**:125-132.
2. Saunders, F.J. and Alberti, R.L. Aldactone, Spironolactone: a Comprehensive Review. New York: Searly, Inc., 1987.
3. Abshagen, U., Besenfelder, E., Endele, R., Koch, K. and Neubert, B. Kinetics of canrenone after single and multiple doses of spironolactone. *Eur. J. Clin. Pharmacol.* 1979; **16**:255-262.
4. Karim, A. Spironolactone: Disposition, Metabolism, Pharmacodynamics, and Bioavailability. *Drug Metabolism Reviews.* 1978; **8**:151-188.
5. Overdiek, H.W.P.M., Hermens, W.A.J.J. and Merkus, F.W.H.M. Determintaion of the serum concentration of spironolactone and its metabolite by High-Performance Liquid Chromatography. *J. Chromatography.* 1985; **341**:279-285.
6. Merkus, F.W.H.M. Is canrenone the major metabolite of spironolactone? *Clin. Pharm.* 1983; **2**:209-210.
7. Karim, A., Kook, C., Zitzewitz, D.J., Zagarella, J., Doherty, M. and Campion, J. Species differences in the metabolism and disposition of spironolactone. *Drug Metab. Disp.* 1976; **4**:547-555.
8. Karim, A., Hribar, J., Aksamit, T., Doherty, M. and Chinn, L.J. Spironolactone metabolism in man studied by gas chromatography-mass spectrometry. *Drug Metab. Disp.* 1975; **3**:467-478.
9. Sadee, W. Abshagen, U., Finn, C. and Rietbrook, N. Conversion of spironolactone to canrenone and disposition kinetics of spironolactone and canrenoate-potassium in rats. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 1974; **283**:203.
10. Overdiek, H.W.P.M., Hermens, W.A.J.J. and Merkus, F.W.H.M. New insights into the pharmacokinetics of spironolactone. *Clin. Pharmacol. Ther.* 1985; **38**:469-474.
11. Merkus, F.W.H.M., Overdiek, J.W.P.M., Cilissen, J. and Suidema, J. Pharmaco-kinetics of spironolactone after a single dose: evaluation ot the true canrenone serum concentrations during 24 hours. *Clin. Expl. Hypertens.* 1983; **5**:239-248.
12. Huston, D.H. Mechanisms of biotransformation. In: Hathway, D.E., ed., Foreign Compound Metabolism in Mammals, London: The Chemical Society, Burlington House, 1975; **4**:259-274.
13. Ziegler, D.M. Microsomal flavin-containing monooxygenase. Oxygenation of nucleophilic nitrogen and sulfur componds. In: Jacoby, W.B., ed., Enzymatic Basis of Detoxication. New-York: Academic Press, 1980; 201-227.
14. Decker, C., Sugiyama, K., Underwood, M. and Correia, M.A. Inactivation of rat hepatic cytochrome P-450 by spironolactone. *Biochem. Biophys. Res. Commun.* 1986; **136**:1162-1169.
15. LaCagnin, L.B., Lutsie, P. and Colby, H.D. Conversion of spironolactone to 7α-thiomethylspironolactone by hepatic and renal microsomes. *Biochem. Pharmacol.* 1987; **36**:3439-3444.

16. Cashman, J.R. and Hanzlik, R.P. Microsomal oxidation of thiobenzamide. A photometric assay for the flavin-containing monooxygenase. *Biochem. Biophys. Res. Commun.* 1981; **98**:147-153.
17. Ziegler, D.M. and Mitchell, C.H. Microsomal oxidases. IV. Properties of the mixed-function amine oxidase isolated from pig liver microsomes. *Arch. Biochem. Biophys.* 1972; **150**:116-125.
18. Sabourin, P.J., Smyser, B.P., and Hodgson, E. Purification of the flavin-containing monooxygenase from mouse and pig liver microsomes. *Int. J. Biochem.* 1984; **16**:713-720.
19. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randal, R.J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 1951; **193**:265-275.
20. Jeffcoate, C.R.E., Gaylor, J.L., and Calabreese, R.L. Ligand interactions with cytochrome P-450. Binding of primary amines. *Biochemistry* 1969; **8**:3455-3465.
21. Poulsen, L.L. Organic sulfur substrates for the microsomal flavin-containing monooxygenase. *Rev. Biochem. Toxicol.* 1981; **3**:33-49.
22. Ortiz de Montellano, P.R. and Mathews, J.M. Autocatalytic alkylation of the cytochrome P-450 prothetic haem group by 1-aminobenzotriazole. *Biochem. J.* 1981; **195**:761-764.
23. Cashman, J.R., Proudfoot, J., Pate, D.W. and Hogberg, T. Stereoselective N-oxygenation of zimeldine and homozimeldine by the flavin-containing monooxygenase. *Drug Metabl. Disp.* 1988; **16**:616-622.
24. Hajjar, N.P. and Hodgson, E. Flavin-adenine dinucleotide-dependent monooxygenase: its role in the sulfoxidation of pesticides in mammals. *Science,* 1980; **209**:1134-1136.
25. Light, D.R., Waxman, D.J. and Walsh, C. Studies on the chirality of sulfoxidation catalyzed by bacterial flavoenzyme cyclooxygenase and hog liver flavin adenine dinucleotide containing monooxygenase. *Biochemistry,* 1982; **21**:2491-2498.
26. Pryor, W.A. and Hendrickson, W.H., Jr. Reactions of nucleophiles with electron acceptors SN2 or electron transfer (ET) mechanisms: tert-butyl peroxybenzoate/dimethyl sulfide and benzoyl peroxide/N,N-dimethylaniline systems. *J. Amer. Chem. Soc.* 1983; **105**:7114-7122.
27. Cashman, J.R. and Peña, S. Canrenone formation via General-Base-Catalyzed Elimination of 7α-(Methylthio) spironolactone S-oxide. *Chem. Res. Toxicol.* 1989; 2:109-113.