Biochemical Pharmacology, Vol. 36, No. 20, pp. 3439–3444, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

CONVERSION OF SPIRONOLACTONE TO 7α-THIOMETHYLSPIRONOLACTONE BY HEPATIC AND RENAL MICROSOMES

LYNN B. LACAGNIN, PAT LUTSIE and HOWARD D. COLBY*

Department of Biomedical Sciences, University of Illinois College of Medicine at Rockford, Rockford, IL 61107-1897, U.S.A.

(*Received* 3 *November* 1986; *accepted* 13 *March* 1987)

Abstract—Recent observations indicate that 7α-thiomethylspironolactone is an important circulating metabolite of the mineralocorticoid antagonist spironolactone (SL). Studies were carried out to determine possible sites and pathways of 7α-thiomethyl-SL formation and, in particular, to evaluate SL metabolism by guinea pig hepatic and renal microsomal preparations. In the absence of *S*-adenosylmethionine (SAM), liver and kidney microsomes rapidly converted SL to 7α-thio-SL as the only metabolite. The rate of 7α-thio-SL production was greater in liver than kidney. In the presence of SAM, 7α-thio-SL was further converted to 7α-thiomethyl-SL by liver and kidney microsomes. The rates of methylation with 7α-thio-SL as substrate were three to four times greater for liver than for kidney, but the K_m values were similar (~30 μM) in the two tissues. Maximal enzyme activity was obtained with SAM concentrations of 25–200 μM. NADPH had no effect on SL or 7α-thio-SL metabolism by liver or kidney microsomes. To determine if a pathway involving the C-S lyase enzyme might contribute to circulating 7α-thiomethyl-SL levels *in vivo*, guinea pigs were treated with SL or its dethioacetylated derivative, canrenone, and plasma metabolites were analyzed by HPLC. Both 7α-thiomethyl-SL and canrenone were found to be circulating metabolites in SL-treated animals, but only canrenone was identified in the plasma of canrenone-treated guinea pigs. The results indicate that the liver and kidney are potential sites of 7α-thiomethyl-SL production and that its formation probably does not involve the C-S lyase pathway.

Spironolactone (SL) is a diuretic which is widely used in the treatment of essential hypertension, congestive heart failure, and other edematous states [1, 2]. The drug acts primarily in the kidney by competitively blocking the binding of aldosterone and other mineralocorticoids, resulting in sodium loss, diuresis, and potassium retention [2]. Metabolism of SL is extremely rapid, and very little of the parent compound is detectable in blood or urine of humans or experimental animals treated with SL. Consequently, the actions of SL have been attributed largely to metabolites of the drug.

The results of previous investigations suggested that canrenone, the dethioacetylated derivative of SL (Fig. 1), was the principal circulating metabolite of the drug [3, 4]. In addition, since canrenone has some antimineralocorticoid activity, the biological effects of SL were also attributed to canrenone. However, a relatively non-specific fluorometric assay was used to measure canrenone in many of those early studies. More recent investigations, in which specific HPLC assays for canrenone have been employed, indicate that the fluorometric method overestimates circulating canrenone levels [5–8]. In addition, pharmacokinetic analyses indicate that canrenone can account for only a small part of the biological activity of SL [5–10].

Several of the sulfur-containing metabolites of SL, including 7α-thio-SL and 7α-thiomethyl-SL, have been proposed as potential mediators of SL actions because of their high affinities for the renal aldosterone receptor [11, 12]. Recently, 7α-thiomethyl-SL has been identified as a major plasma metabolite in both humans and animals treated with SL [13, 14]. In addition, 7α-thiomethyl-SL is measured in the fluorometric assay for canrenone, which may account for the overestimates of circulating canrenone levels in earlier studies. Thus, 7α-thiomethyl-SL may be the active metabolite responsible for the antimineralocorticoid effects of SL, but little is known about its site(s) of production or the factors affecting its formation. The studies described in this paper were initiated to address the first of these questions, and the results suggest that both liver and kidneys are potential sites of 7α-thiomethyl-SL formation.

MATERIALS AND METHODS

Spironolactone (SL), 7α-thio-SL (SC-24813), 7α-thiomethyl-SL (SC-26519), and canrenone were provided by G. D. Searle & Co. (Chicago, IL). Progesterone and *S*-adenosylmethionine (SAM) were obtained from the Sigma Chemical Co. (St. Louis, MO). The purity of all steroids was confirmed by high performance liquid chromatography (HPLC). All solvents used were HPLC grade and were obtained from the Fisher Chemical Co. (Pittsburgh, PA).

* Address correspondence to: Howard D. Colby, Ph.D., Department of Biomedical Sciences, University of Illinois College of Medicine at Rockford, Rockford, IL 61107-1897.

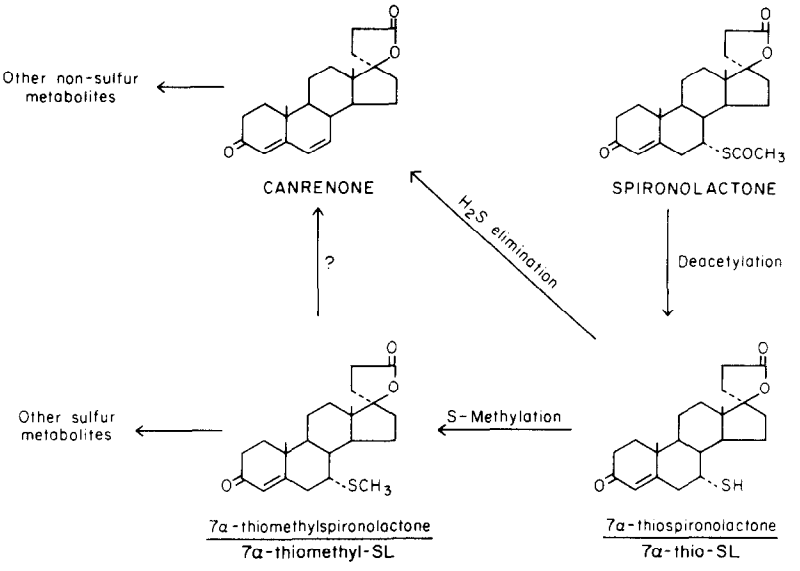

Fig. 1. Proposed pathways involved in the metabolism of spironolactone.

Adult (800–1000 g) male English Short Hair guinea pigs were obtained from Camm Research Institute (Wayne, NJ) and maintained under standardized conditions of lighting (6:00 a.m. to 6:00 p.m.) and temperature (22°) on a diet of Purina Laboratory chow and water *ad lib.* All animals were allowed at least 7 days to become acclimated to the housing conditions prior to use in experiments. In pretreatment experiments, guinea pigs received spironolactone or canrenone (25 mg/kg body weight) as i.p. injections (in saline containing 3 drops Tween 80 per ml) 24 and 4 hr before being killed. Controls received the vehicle alone. Animals were killed by decapitation between 8:00 and 9:00 a.m., and blood was collected in heparinized beakers. Plasma was obtained by centrifugation and stored frozen if not used immediately.

After sacrifice, tissues were quickly removed and placed in cold 1.15% KCl, 0.05 M Tris–HCl buffer (pH 7.4) on ice. All steps in the preparation of microsomal fractions were carried out at 0–4°. Tissues were homogenized, and the homogenates were centrifuged at 900 *g* for 10 min and then at 9000 *g* for 20 min. The post-mitochondrial supernatant fractions were centrifuged at 105,000 *g* for 60 min, and the microsomal pellets were washed once and resuspended in KCl–Tris buffer (pH 7.4). The method of Lowry *et al.* [15] was used for the determination of microsomal protein concentrations.

Incubation medium contained 0.05 M Tris–HCl (pH 7.4), 5.0 mM $MgCl_2$, and various amounts of microsomal protein in a total volume of 2.5 ml. Where indicated, SAM was also included in the incubation medium. Incubations were done in 25-ml Erlenmeyer flasks at 37° under air in a Dubnoff metabolic incubator. The reaction was initiated by the addition of SL or 7α-thio-SL (100 μM) in small volumes (25 μl) of ethanol. After incubation, all flasks received 40 μg of progesterone to serve as an internal standard. One set of flasks, which received the internal standard but was not incubated, served as the 0-time control. Additional control flasks included microsomal preparations incubated without substrate, or substrate incubated in buffer but without microsomal protein. The incubation medium was extracted with 5 ml ethyl acetate, and the extracts were filtered and evaporated to dryness as described previously [14]. Samples were then reconstituted in 400 μl of acetonitrile for subsequent analyses by HPLC.

The analysis of SL and its metabolites was carried out using the HPLC method previously described [14]. A Waters HPLC system (Waters Associates, Milford, MA) equipped with two model 6000A pumps, a model 710B automatic sample injector, a model 730 data module, a model 720 system controller, a model 440 absorbance detector, and a model RMC-100 radial compression system were used. The HPLC analyses were done with a C_{18} radial pak cartridge (5 μm). Compounds were separated using a 30-min concave gradient (curve 9) of 65–100% methanol–water. The solvent flow rate was 1.0 ml/min, and the chromatographic system was operated at ambient temperature. The eluant was monitored by absorbance at 254 nm and the u.v. detector was operated at 0.05 a.u.f.s.

Mass spectral analyses of authentic 7α-thiomethyl-SL, canrenone, and the plasma metabolites obtained from SL- or canrenone-treated guinea pigs were done on a Finnegan model 4021 automated GC/MS equipped with an INCOS automatic data system (Sunnydale, CA). Samples were run at 20 eV by direct probe in the EI model.

RESULTS AND DISCUSSION

Incubation of hepatic microsomes with SL in the absence of *S*-adenosylmethionine (SAM) resulted in the rapid formation of 7α-thio-SL (55.7 ± 7.3 nmol/min × mg protein) as the only metabolite (Fig. 2). Similar results were obtained with renal microsomal preparations, but the rates of 7α-thio-SL production

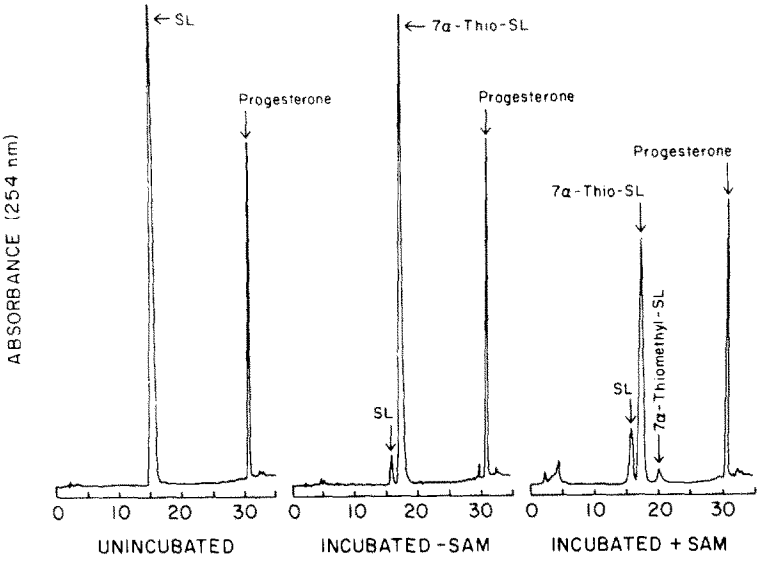

Fig. 2. HPLC analyses of spironolactone (SL) metabolism by guinea pig hepatic microsomes in the presence and absence of *S*-adenosylmethionine (SAM). Incubations and HPLC analyses were done as described in Materials and Methods. Incubation flasks contained 1 mg microsomal protein, SL (100 μM) and SAM (50 μM), and were incubated for 15 min.

were somewhat lower (22.6 ± 3.9 nmol/min × mg protein) in kidney than in liver. The addition of SAM (50 μM) to the incubation medium brought about the formation of a metabolite having a retention time identical to that of 7α-thiomethyl-SL with both hepatic (Fig. 2) and renal microsomes. Identification of the metabolite as 7α-thiomethyl-SL was confirmed by mass spectroscopy, as described previously [14]. As illustrated in Fig. 3, 7α-thio-SL, like SL, was converted to 7α-thiomethyl-SL by hepatic and renal microsomes when SAM was included in the incubation medium. Small amounts of canrenone were also produced, whether or not SAM was present. NADPH had no effects on SL or 7α-thio-SL metabolism, in the presence or absence of SAM, by hepatic or renal microsomes (data not shown).

The effects of various SAM concentrations on the rates of methylation of 7α-thio-SL by hepatic and renal microsomes are illustrated in Fig. 4. Maximal activities were obtained with SAM concentrations of 25–100 μM. Higher concentrations decreased the rates of 7α-thiomethyl-SL production. The decrease

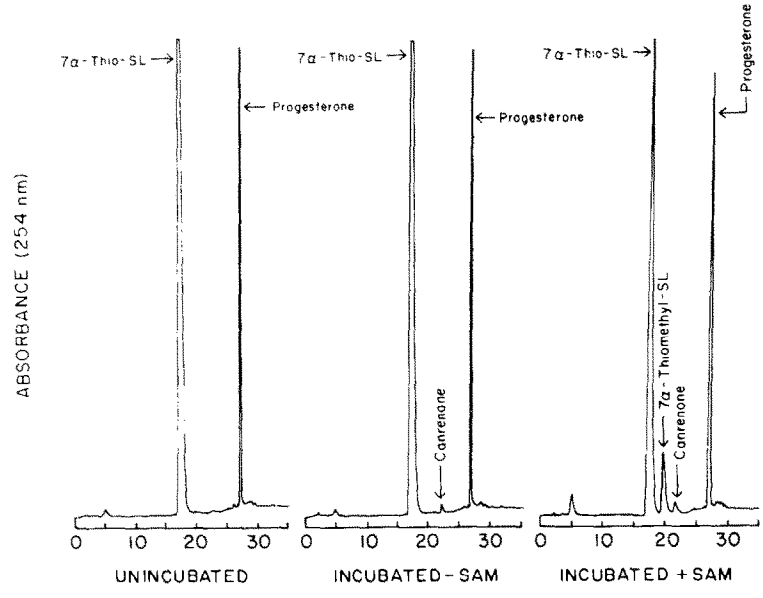

Fig. 3. HPLC analyses of 7α-thiospironolactone (7α-thio-SL) metabolism by hepatic microsomes in the presence and absence of *S*-adenosylmethionine (SAM). Conditions were identical to those described in the legend for Fig. 2 except that the 7α-thio-SL (100 μM) was used as substrate in place of SL.

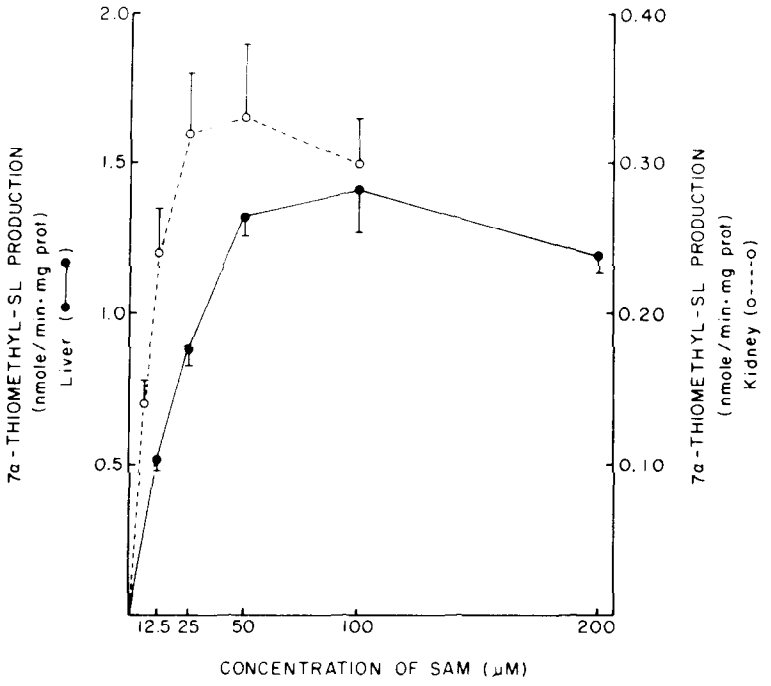

Fig. 4. Effects of various *S*-adenosylmethionine (SAM) concentrations on the rates of conversion of 7α-thiospironolactone to 7α-thiomethylspironolactone (7α-thiomethyl-SL) by hepatic and renal microsomes. Incubation conditions were the same as those indicated in the legend to Fig. 3 except that SAM concentrations were varied. Each value is the mean ± SE of four observations.

in 7α-thiomethyl-SL production at high concentrations of SAM may be caused by *S*-adenosylhomocysteine (SAH), a by-product of the methyltransferase reaction. SAH has been shown to competitively inhibit a wide variety of methyltransferase reactions *in vitro*, including *O*-, *N*-, and *S*-methyltransferases [16–20], but it is not yet clear whether this also occurs *in vivo*.

When incubations were done with 50 μM SAM, 100 μM 7α-thio-SL, and 1 mg microsomal protein, the rates of 7α-thiomethyl-SL formation were linear for up to 60 min for kidney and approximately 20 min

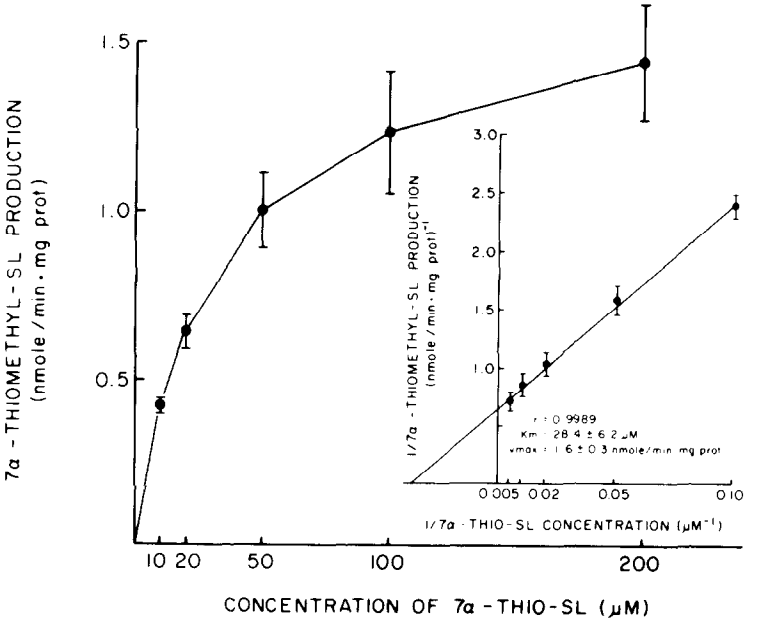

Fig. 5. Kinetic analysis of 7α-thiospironolactone (7α-thio-SL) conversion to 7α-thiomethylspironolactone (7α-thiomethyl-SL) by guinea pig hepatic microsomes. Incubation conditions were identical to those described in the legend for Fig. 3 except that 7α-thio-SL concentrations were varied as indicated. Each value is the mean ± SE of four observations.

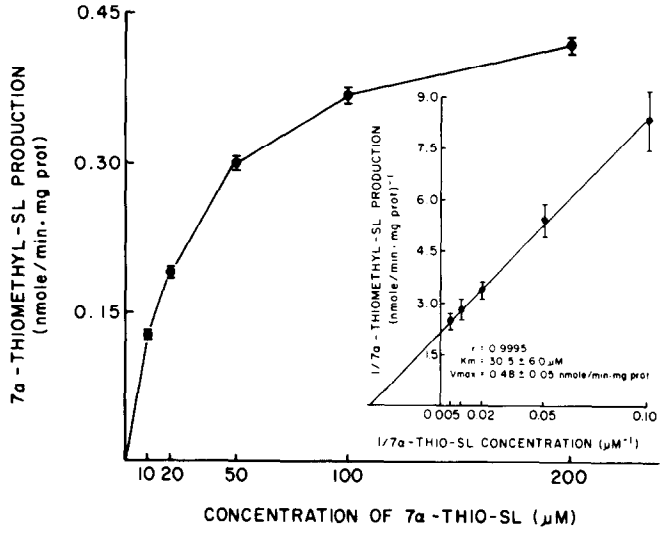

Fig. 6. Kinetic analysis of 7α-thiospironolactone (7α-thio-SL) conversion to 7α-thiomethylspironolactone (7α-thiomethyl-SL) by guinea pig kidney microsomes. Incubation conditions were identical to those described in the legend for Fig. 3 except that 7α-thio-SL concentrations were varied as indicated. Each value is the mean ± SE of four observations.

for liver preparations (data not shown). The kinetic parameters obtained for the methylation reactions in liver and kidney microsomes are indicated in Figs. 5 and 6 respectively. Maximal enzyme activity was three to four times greater in liver than in kidney but the apparent K_m values (~30 μM) were similar in the two tissues.

These results indicate that the liver and kidney are potential sources of 7α-thiomethyl-SL, the major circulating metabolite of SL and probable mediator of its antimineralocorticoid effects [13, 14]. In both tissues, the production of 7α-thiomethyl-SL from SL appears to proceed via deacetylation to 7α-thio-SL followed by S-methylation (Fig. 1). We previously demonstrated that the deacetylation reaction occurs in various tissues, but activity was greatest in hepatic and renal microsomal preparations [21]. The data presented in this paper indicate that methylation of 7α-thio-SL is also catalyzed by hepatic and renal microsomal preparations, probably by a thiol methyltransferase similar to those involved in the detoxication of various xenobiotics [22, 23]. The possibility that methylation occurs in other subcellular fractions as well cannot be excluded at this time. In the case of 7α-thio-SL, S-methylation probably represents an activation process since recent observations suggest that 7α-thiomethyl-SL mediates the therapeutic effects of SL [13, 14]. Hepatic production of 7α-thiomethyl-SL may contribute significantly to circulating metabolite levels, but further studies are

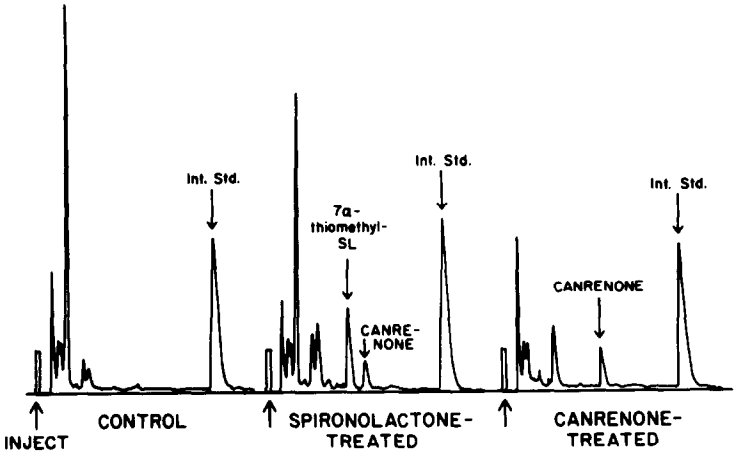

Fig. 7. Representative HPLC analyses of plasma from control, spironolactone-treated, and canrenone-treated guinea pigs. Treatment schedule for animals and analytical techniques are described in Materials and Methods.

needed to test this hypothesis. Since red blood cells have been shown to catalyze the methylation of 7α-thio-SL [24], their contribution to circulating 7α-thiomethyl-SL levels also needs to be examined. It seems less likely that the kidney is a major contributor to plasma 7α-thiomethyl-SL levels, but formation of this putative active metabolite at or near its site of action may be important for the antimineralocorticoid effects of the parent compound, SL.

The production of 7α-thiomethyl-SL *in vivo* may also occur via a pathway involving the C-S lyase enzyme system [25–27]. The C-S lyases cleave S-substituted cysteine conjugates to thiols which can then be further processed by, for example, methylation. Thus, it is possible that canrenone conjugates (derived from SL) could be cleaved by a C-S lyase to generate 7α-thio-SL, which then, in turn, could be S-methylated to 7α-thiomethyl-SL. To test this hypothesis, canrenone or SL was administered to guinea pigs, and the plasma metabolites in each group were evaluated by HPLC. Following the administration of large doses of SL to guinea pigs, the major plasma metabolite was 7α-thiomethyl-SL (Fig. 7), as previously noted [13, 14]. A compound having a retention time similar to that of canrenone was also found in the plasma of SL-treated guinea pigs. The identities of both 7α-thiomethyl-SL and canrenone were confirmed by mass spectroscopy (data not shown). In canrenone-treated animals, by contrast, canrenone was the major circulating metabolite (Fig. 7), and 7α-thiomethyl-SL could not be identified in the plasma. Thus, these preliminary results suggest that plasma 7α-thiomethyl-SL is not the product of a C-S lyase pathway.

In summary, the results indicate that the liver and kidneys have the enzymatic capacity to convert SL to 7α-thiomethyl-SL, the putative active metabolite. Metabolism appears to proceed by deacetylation followed by S-methylation. Enzyme activities were greater in the liver, but renal production of 7α-thiomethyl-SL may be important because of its proximity to the site of action. Thus, both hepatic and renal metabolism of SL may contribute to the antimineralocorticoid effects of the drug.

Acknowledgements—These investigations were supported by USPHS research grant GM-30261 awarded by the National Institute of General Medical Sciences. Spironolactone, canrenone, 7α-thiospironolactone, and 7α-thiomethylspironolactone were provided by G. D. Searle & Co., Chicago, IL.

REFERENCES

1. C. M. Kagawa, *Endocrinology* **67**, 125 (1960).
2. F. J. Saunders and R. L. Alberti, *Aldactone; Spironolactone: A Comprehensive Review*. Searle, Inc., NY (1978).
3. N. Gochman and C. L. Gantt, *J. Pharmac. exp. Ther.* **135**, 312 (1962).
4. W. Sadée, M. Dagcioglu and S. Riegelman, *J. pharm. Sci.* **61**, 1126 (1972).
5. C. G. Dahlöf, P. Lundborg, B. A. Persson and C. G. Regardh, *Drug Metab. Dispos.* **7**, 103 (1979).
6. U. Abshagen, E. Besenfelder, R. Endele, K. Koch and B. Neubert, *Eur. J. clin. Pharmac.* **16**, 255 (1979).
7. F. W. H. M. Merkus, J. W. P. M. Overdiek, J. Cilissen and J. Zuidema, *J. clin. expl. Hyper.—Theory and Practice* **A5**, 239 (1983).
8. P. C. Ho, D. W. A. Bourne, E. G. Triggs and V. Heazlewood, *Eur. J. clin. Pharmac.* **27**, 441 (1984).
9. G. J. Huston, P. Turner and M. Leighton, *Br. J. clin. Pharmac.* **3**, 201 (1976).
10. L. Ramsey, J. Shelton, I. Harrison, M. Tidd and M. Asbury, *Clin. Pharmac. Ther.* **20**, 167 (1976).
11. J. W. Funder, D. Feldman, E. Highland and J. B. Edelman, *Biochem. Pharmac.* **23**, 1493 (1974).
12. C. Sakauye and D. Feldman, *Am. J. Physiol.* **231**, 93 (1976).
13. H. W. P. M. Overdiek, W. A. J. J. Hermens and F. W. H. M. Merkus, *Clin. Pharmac. Ther.* **38**, 469 (1985).
14. J. H. Sherry, J. P. O'Donnell and H. D. Colby, *J. Chromat.* **274**, 183 (1986).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
16. J. K. Coward, M. D'Urso-Scott and W. D. Sweet, *Biochem. Pharmac.* **21**, 1200 (1972).
17. T. Deguchi and J. Barchas, *J. biol. Chem.* **246**, 3175 (1971).
18. R. A. Weisigen and W. B. Jakoby, *Archs Biochem. Biophys.* **196**, 631 (1979).
19. P. A. Pazmino and R. A. Weinshilboum, *Clinica Chim. Acta* **89**, 317 (1978).
20. J. Axelrod, in *Concepts of Biochemical Pharmacology* (Eds. B. B. Brodie and J. R. Gillette), Vol. 2, p. 609. Springer, Berlin (1971).
21. J. H. Sherry, J. P. O'Donnell and H. D. Colby, *Life Sci.* **21**, 2727 (1981).
22. R. M. Weinshilboum, S. Sladek and S. Klumpp, *Clinica Chim. Acta* **97**, 59 (1979).
23. J. Bremer and D. M. Greenberg, *Biochim. biophys. Acta* **46**, 217 (1961).
24. R. A. Keith, I. Jardine, A. Kerremans and R. M. Weinshilboum, *Drug Metab. Dispos.* **12**, 717 (1984).
25. G. L. Larsen and J. E. Bakke, *Xenobiotica* **13**, 115 (1983).
26. S. Suzuki, H. Tomisawa, S. Ichihara, H. Fukazawa and M. Tateishi, *Biochem. Pharmac.* **31**, 2137 (1982).
27. M. Tateishi, S. Suzuki and H. Shimizu, *J. biol. Chem.* **253**, 8854 (1978).