Biochemical Pharmacology, Vol. 21, pp. 1449–1455. Pergamon Press, 1972. Printed in Great Britain.

EFFECT OF PRETREATMENT WITH SPIRONOLACTONE, PHENOBARBITAL OR β-DIETHYLAMINOETHYL DIPHENYLPROPYL-ACETATE (SKF 525-A) ON TRITIUM LEVELS IN BLOOD, HEART AND LIVER OF RATS AT VARIOUS TIMES AFTER ADMINISTRATION OF [^{3}H]DIGITOXIN*

M. C. CASTLE and G. L. LAGE

Department of Pharmacology and Toxicology, School of Pharmacy, University of Kansas, Lawrence, Kan. 66044, U.S.A.

(*Received* 10 *September* 1971; *accepted* 19 *November* 1971)

Abstract—The influence of spironolactone, phenobarbital and β-diethylaminoethyl diphenylpropylacetate (SKF 525-A) pretreatment on the tritium levels in blood, liver and heart of rats given [^{3}H]digitoxin was investigated at various time periods. Blood tritium levels of spironolactone-pretreated rats were significantly lower than control animals 1 hr (38 per cent of control), 2 hr (26 per cent of control), 4 hr (38 per cent of control) and 8 hr (6 per cent of control) after i.p. injection of [^{3}H]digitoxin. Tritium levels in the blood of phenobarbital-pretreated animals were not different from control animals at any of the time periods. SKF 525-A-pretreated animals had blood levels of tritium less than control animals at 1 hr (64 per cent of control) and greater than control animals at 8 hr (174 per cent of control), but were not different from control animals at either 2 or 4 hr. Radioactivity in the heart of spironolactone-pretreated animals was less than that in control animals at all but the 1-hr time interval, while radioactivity in phenobarbital-pretreated animals was not different from that of control animals at any time period and in SKF 525-A-pretreated animals was different only at the 8-hr period (172 per cent of control). Tritium levels in the liver of rats pretreated with spironolactone were greater than control animals at 1 hr (140 per cent of control) and less at 4 hr (66 per cent of control), but were not different at 2 or 8 hr. Phenobarbital pretreatment altered liver levels of tritium only at 1 hr (141 per cent of control), while SKF 525-A did not affect liver levels of radioactivity at any time period.

These results indicate that spironolactone pretreatment markedly increases the metabolism and/or excretion of digitoxin, while phenobarbital pretreatment apparently does not affect these processes. The apparent inhibitory effects of SKF 525-A on digitoxin metabolism are seen only at 8 hr after the administration of digitoxin.

DIGITOXIN, a steroid cardiac glycoside, causes convulsions and death in toxic doses. Recent reports have established that pretreatment of rats and mice with spironolactone antagonizes these toxic effects.[1,2] Indirect evidence has been presented which suggests that this antagonism may be the result of increased metabolism of digitoxin.[2,3] Although several reports have appeared which indicate that spironolactone accelerates the disappearance of some drugs from the blood,[4–6] little work has been

* Supported by U.S. Public Health Service Research Grant HE 12069 and by a grant from the Kansas Heart Association.

done to determine the effect of spironolactone on blood and tissue levels of digitoxin. Solymoss *et al.*[3] have recently reported that pretreatment of rats with spironolactone lowers the blood levels of "digitoxin" 6 hr after an oral administration. The altered blood levels were postulated to be the result of stimulation of the drug-metabolizing enzymes of the liver. Phenobarbital, which stimulates a wide variety of hepatic drug-metabolizing enzymes, accelerates the disappearance of many drugs from the blood,[7] but the effect of this enzyme inducer on blood and tissue levels of digitoxin has not been investigated. Likewise, it is not known if these levels are affected by pretreatment with β-diethylaminoethyl diphenylpropylacetate (SKF 525-A), the well known inhibitor of a variety of drug-metabolizing enzymes.

We have, therefore, used sensitive radiotracer techniques to investigate the effect of pretreatment with spironolactone on the levels of tritiated compounds in the blood, heart and liver at various times after the administration of [^{3}H]digitoxin. We have also compared these results with data obtained from animals pretreated with phenobarbital or with SKF 525-A.

MATERIALS AND METHODS

Chemicals. The randomly-labeled tritiated digitoxin used in these studies was obtained from New England Nuclear (Boston, Mass.) and had an initial specific activity of 5·88 mc/mg. Non-radioactive digitoxin (Sigma Chemical Company, St. Louis, Mo.) was added to the radioactive digitoxin to give a final specific activity of 0·300 mc/mg. Phenobarbital sodium was purchased from Merck & Co., Inc., Rahway, N.J. and spironolactone from Sigma Chemical Company, St. Louis, Mo. SKF 525-A was kindly supplied by Smith Kline & French Labs., Philadelphia, Pa. Phenobarbital sodium and SKF 525-A were prepared as aqueous solutions. Spironolactone, fine ground with a mortar and pestle, was given as a suspension in 0·5 per cent methyl cellulose.

Treatment of animals. Male Holtzmann rats (Madison, Wis.), 150–200 g, were allowed Purina lab chow and water *ad lib.* throughout the experiment. Phenobarbital sodium (75 mg/kg), saline (0·9%), and spironolactone (125 mg/kg) were injected i.p in the appropriate animals once daily for 3 days, the last injection given 24 hr before the administration of digitoxin. SKF 525-A animals were given normal saline for 3 days and on the fourth day received SKF 525-A (15 mg/kg) 45 min before the administration of digitoxin. The volume of all injections was 2 ml/kg. [^{3}H]digitoxin (1 mg/kg; 0·300 mc/kg) was given i.p. in 47·5% ethanol (1 ml/kg). At the appropriate time after injection of digitoxin, the animals were anesthetized with ether, and approximately 2·5 ml of blood was withdrawn by cardiac puncture. Sodium oxalate (75 mg) was used as an anticoagulant. After exsanguination, the heart and liver were quickly removed, blotted and weighed. The tissues were frozen on dry ice and remained frozen at −70° until assayed.

Analytical methods. Blood tritium levels were determined by extracting 1·0 ml of oxalated, whole blood three times with 20 ml of hot methanol. The extractions were combined and evaporated to dryness at 50°. The methanol residue was dissolved in 5 ml of absolute ethanol with the aid of a sonic cleaner. The solution was transferred to a counting vial, and 15 ml of toluene-based scintillation mixture[8] was added. The final solution was decolorized by adding 0·5 ml of 30% hydrogen peroxide. The adequacy

of this procedure was checked by combusting dried blood samples to carbon dioxide and water and counting the tritiated water.[9] The values from the extraction procedure were not significantly different from those obtained by the combustion method (98·3 ± 1·2 per cent).

Whole hearts or approximately 1 g of liver were weighed and homogenized in 5 ml of normal saline. Each homogenate was diluted to 10 ml with saline and a 0·5-ml aliquot was placed in a counting vial along with 2 ml of NCS tissue solubilizer (Amersham/Searle, Arlington Heights, Ill.). The vials were capped and the tissues were digested at 50° for 48 hr. The vials were allowed to cool to room temperature, and 2·5 ml of toluene was added along with 15 ml of toluene-based scintillation mixture. The adequacy of this procedure was checked by combusting a known amount of dried tissue and counting as described above. The values obtained from the digested tissues were not significantly different from those obtained by the combustion procedure (98·7 ± 0·9 per cent).

Counting techniques. All samples were kept in the dark for 48 hr prior to counting to eliminate chemiluminescence. The samples were counted in a Beckman liquid scintillation spectrometer. The final counting solution contained 50 mg/l. of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (POPOP) and 4 g/l. of 2,5-diphenyloxazole (PPO). All samples were counted for 30 min and were corrected for quenching by the addition of a known amount of tritiated toluene as an internal standard.

Statistical analysis. Disintegration rates were converted to nanocuries per milliliter for whole blood and to nanocuries per gram for heart and liver tissues. A randomized complete block analysis of variance[10] was carried out for each tissue. In all groups where the analysis of variance was significant at $P < 0.05$, the Student–Newman–Keuls test[10] was performed on all possible combinations at each time period. Significant differences ($P < 0.05$) between the means are noted in the Results section.

RESULTS

Each of the bars in Figs. 1–3 represents the mean plus or minus standard error of four animals. It should be emphasized that we measured only levels of radioactivity in each tissue and did not attempt to separate and quantitate metabolites and unchanged digitoxin. Since the [^{3}H]digitoxin was randomly labeled, our data probably represent not only unchanged digitoxin, but also metabolites of digitoxin, some of which are active.[11] Figures 1–3 illustrate two concepts: (1) the effect of pretreatments with various drugs upon the tritium levels in each of the tissues, and (2) the change of these levels with time.

As illustrated in Fig. 1, tritium levels in the blood of spironolactone-pretreated rats were less than one half of the levels in control animals 1 hr after an i.p. administration of [^{3}H]digitoxin. The spironolactone-pretreated groups also had significantly lower levels than control groups at all other time periods tested. Tritium levels in phenobarbital-pretreated animals were not significantly different from control animals at any time period. SKF 525-A-pretreated animals had significantly lower levels of tritium than control animals at 1 hr and significantly greater levels at 8 hr, but the levels were not different from control animals at 2 or 4 hr.

In Fig. 2, tritium levels in the heart were maximal at 1 hr and declined steadily

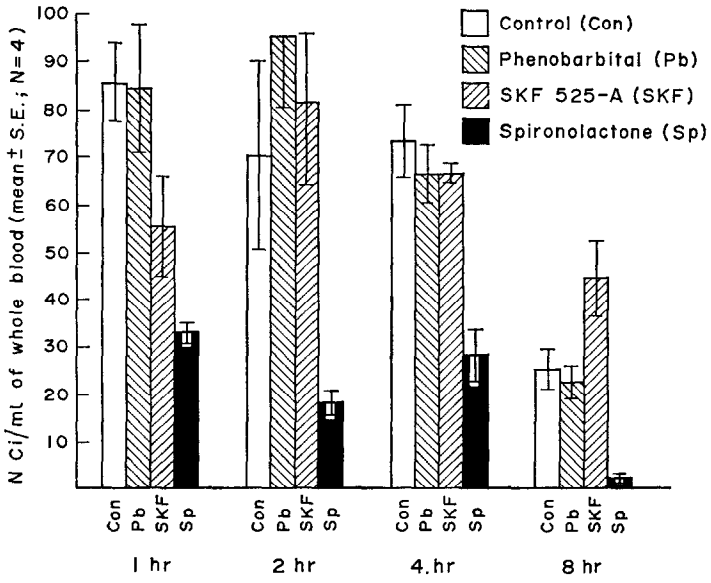

FIG. 1. Effect of pretreatment with spironolactone, phenobarbital or SKF 525-A on tritium levels in blood at various times after administration of [^{3}H]digitoxin.

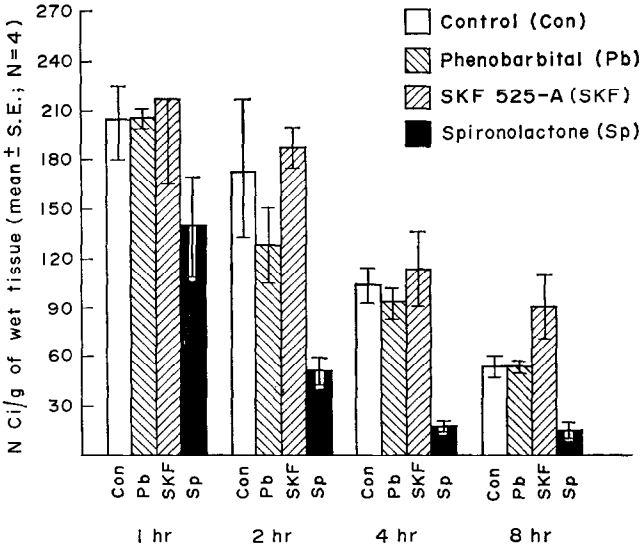

FIG. 2. Effect of pretreatment with spironolactone, phenobarbital or SKF 525-A on tritium levels in heart at various times after administration of [^{3}H]digitoxin.

with time for all groups. Although spironolactone-pretreated animals had lower levels in the heart at 1 hr than did control animals, these differences were not significant, probably as a result of the large variations within the groups. Spironolactone pretreatment did result in significantly lower levels of tritium in the heart than control animals at 2, 4 and 8 hr. SKF 525-A-pretreated animals were significantly different

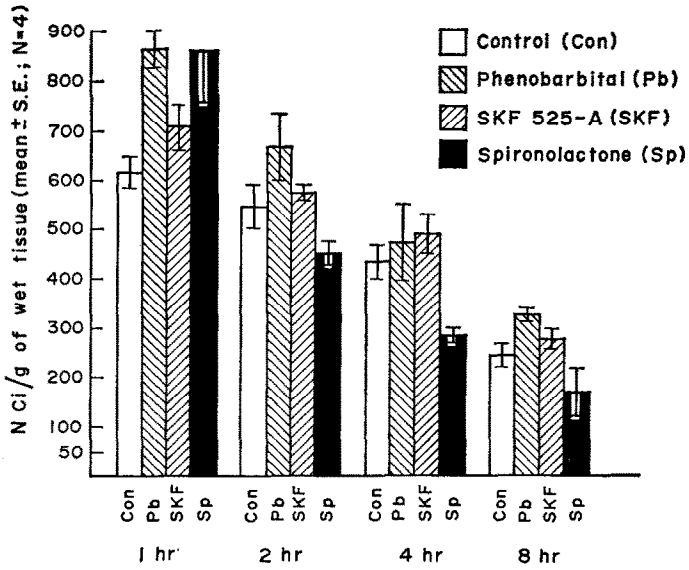

FIG. 3. Effect of pretreatment with spironolactone, phenobarbital or SKF 525-A on tritium levels in liver at various times after administration of [^{3}H]digitoxin.

from control animals only at the 8-hr time period when the level was almost twice that of control animals. Pretreatment with phenobarbital did not significantly alter the tritium levels in the heart at any time period.

As shown in Fig. 3, spironolactone-pretreated animals had more radioactivity in the liver at 1 hr and less at 4 hr than did control animals. However, there were no differences at either 2 or 8 hr. Phenobarbital pretreatment caused elevated levels of tritium in the liver at 1 hr, but did not affect these levels at any other time period. Pretreatment with SKF 525-A did not alter the amount of radioactivity in the liver at any of the time periods studied.

Comparisons among the pretreatment groups yield many significant differences. The SKF 525-A-pretreated animals had significantly higher levels of tritium in the blood and heart than did the phenobarbital-pretreated animals at the 8-hr time period. Spironolactone-pretreated animals had significantly lower tritium levels than the SKF 525-A-pretreated animals in blood and heart at 2, 4 and 8 hr, and in the liver at 4 and 8 hr. Spironolactone-pretreated animals also had significantly lower levels than the phenobarbital-pretreated animals in the blood at all time periods, in the heart at 2, 4 and 8 hr, and in the liver at 4 and 8 hr. All other comparisons are not significant.

DISCUSSION

When Selye *et al.*[1] first demonstrated antagonism of digitoxin toxicity by the potassium-sparing diuretic, spironolactone, they were able to rule out increased potassium levels as the mechanism of this protective effect. Spironolactone and digitoxin are structurally similar in that both are steroids containing a lactone ring. It was, therefore, suggested that spironolactone might be a competitive antagonist of digitoxin.[1] It soon became apparent, however, that spironolactone also inhibits the

actions of a variety of structurally unrelated drugs including pentobarbital,[4] hexobarbital[12] and ethanol[13] as well as several steroids.[13] Other studies demonstrated increased metabolism of some drugs by animals pretreated with spironolactone.[4–6] These studies pointed to enzyme induction as the mechanism by which spironolactone alters the activity of many drugs. Furthermore, simultaneous administration of ethionine, which blocks enzyme induction, abolished the protective effect of spironolactone to digitoxin toxicity.[2] These studies have led to the postulation of enzyme induction as the mechanism by which spironolactone antagonizes the toxic effects of digitoxin.[2,3] Our data are consistent with this mechanism. As early as 1 hr after an i.p. injection of [^{3}H]digitoxin, blood tritium levels in spironolactone-pretreated rats were less than one half those of control animals. This apparent accelerated disappearance of digitoxin from the blood may be the result of increased metabolism. It should be pointed out, however, that increased excretion of unchanged digitoxin and/or its metabolites could also explain both the protective effect and the accelerated disappearance of tritiated compounds from the blood after pretreatment with spironolactone. This mechanism cannot be ruled out at this time.

Phenobarbital is one of the most powerful and universal inducers of microsomal drug-metabolizing enzymes.[7] Nevertheless, rats pretreated with phenobarbital had the same blood levels of tritium as control animals even at 8 hr after the administration of [^{3}H]digitoxin. This lack of effect of phenobarbital could suggest that phenobarbital does not stimulate the metabolism of digitoxin in rats. It could also mean that if the metabolism of digitoxin is stimulated by phenobarbital, then the metabolites have approximately the same half-life as unchanged digitoxin. Phenobarbital has been shown to stimulate the C-12 hydroxylation of digitoxin to digoxin,[14] and to stimulate the formation of polar metabolites from the aglycone, digitoxigenin.[15] However, it has also been demonstrated in monkeys that neither 1,1,l-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) nor chlordane pretreatment stimulated the metabolism *in vitro* of digoxin, which is metabolized through many of the same pathways as digitoxin.[16] Other studies with livers from these same monkeys indicated that these insecticides do increase the metabolism of other drugs.[17,18] If spironolactone does indeed alter the metabolism of digitoxin, then it is apparently stimulating enzymes which are unaffected by pretreatment with phenobarbital, DDT or chlordane. It is conceivable that these may not be microsomal enzymes, but may be located in some other part of the cell (e.g. the cytoplasm or the cell membrane).

One hour after the administration of [^{3}H]digitoxin, blood of animals pretreated with SKF 525-A contained less radioactivity than blood from control animals. This is somewhat unexpected since SKF 525-A is known to inhibit the metabolism of many drugs.[19] The increased blood levels of tritiated compounds in SKF 525-A-pretreated animals at 8 hr may indicate that digitoxin metabolism is inhibited only after a considerable delay.

The failure of all three pretreatments (i.e. spironolactone, phenobarbital or SKF 525-A) to alter the tritium levels in the heart at 1 hr may indicate that none of these drugs affect the uptake or binding of digitoxin by the heart. The decreased levels of radioactivity in the heart of spironolactone-pretreated animals at 2, 4 and 8 hr and the elevated levels in SKF 525-A-pretreated animals at 8 hr are probably not direct effects upon uptake or binding by the heart since these changes parallel changes in the blood at these time periods.

Both spironolactone and phenobarbital apparently increase the uptake and/or binding of digitoxin by the liver since animals pretreated with these drugs showed higher hepatic concentrations of radioactivity at 1 hr than did control animals. This probably is an effect upon uptake or binding since blood levels of tritium in phenobarbital-pretreated animals at this time period were not different from control animals and spironolactone-pretreated animals showed blood levels much less than control animals. Although the hepatic tritium levels were identical at the 1-hr time period for these two pretreatments, the rate of disappearance of radioactivity from the liver was much more rapid in the spironolactone-pretreated animals than in the phenobarbital-pretreated animals. The exact meaning of these results is difficult to interpret at this time, but it appears that spironolactone has a greater effect than phenobarbital on the metabolism and/or excretion of digitoxin.

It is interesting that all of the studies involving the antagonism of digitoxin toxicity in rats have been done with females. Stripp *et al.*[20] have recently reported that spironolactone-pretreatment in female rats decreases hexobarbital sleeping time and increases the metabolism *in vitro* of hexobarbital. In male rats, however, they found no change in the hexobarbital sleeping time from controls and a decrease in the metabolism *in vitro* of hexobarbital. In addition, spironolactone-pretreatment decreased the cytochrome P-450 levels in females, but did not significantly alter these levels in male rats. These observations together with the data of Lage *et al.*[21] with DDT and chlordane and our results with spironolactone and phenobarbital suggest that spironolactone may be stimulating the metabolism of digitoxin by a different mechanism of enzyme induction than that which occurs with the classical enzyme inducers.

Our data are in agreement with the theory that enzyme induction may be the mechanism by which spironolactone protects against digitoxin toxicity. However, further studies to separate and quantitate the metabolites of digitoxin in the blood, tissues and excretory products are needed to confirm this mechanism. These studies are currently in progress in our laboratory.

REFERENCES

1. H. Selye, M. Krajny and L. Savoie, *Science, N.Y.* **164,** 842 (1969).
2. S. H. Buck and G. L. Lage, *Archs. int. Pharmacodyn. Ther.* **189,** 192 (1971).
3. B. Solymoss, S. Toth, S. Varga and H. Selye, *Toxic. appl. Pharmac.* **18,** 586 (1971).
4. B. Solymoss, H. G. Classen and S. Varga, *Proc. Soc. exp. Biol. Med.* **132,** 940 (1969).
5. B. Solymoss, S. Toth, S. Varga and M. Krajny, *Steroids* **16,** 263 (1970).
6. B. Solymoss, S. Varga and M. Krajny, *Arzneimittel-Forsch.* **21,** 384 (1971).
7. A. H. Conney, *Pharmac. Rev.* **19,** 317 (1967).
8. K. C. Wong and J. L. Spratt, *Biochem. Pharmac.* **12,** 577 (1963).
9. V. T. Oliverio, C. Denham and J. D. Davidson, *Analyt. Biochem.* **4,** 188 (1962).
10. R. R. Sokal and F. J. Rohlf, in *Biometry*, p. 239. W. H. Freeman, San Francisco (1969).
11. G. L. Lage and J. L. Spratt, *J. Pharmac. exp. Ther.* **152,** 501 (1966).
12. M. C. Gerald and D. R. Feller, *Archs. int. Pharmacodyn. Ther.* **187,** 120 (1970).
13. H. Selye, I. Mecs and L. Savoie, *Anesthesiolgy* **31,** 261 (1969).
14. R. W. Jelliffe and D. H. Blankenhorn, *Clin. Res. Proc.* **14,** 160 (1966).
15. S. J. Stohs, L. A. Reinke, and M. M. El-Olemy, *Biochem. Pharmac.* **20,** 437 (1971).
16. K. Repke, in *Proc. First Int. Pharmac. Meetings*, Stockholm, 1961, Vol. 3, p. 47. Pergamon Press, Oxford (1963).
17. R. L. Cram, M. R. Juchau and J. R. Fouts, *J. Lab. clin. Med.* **66,** 906 (1965).
18. M. R. Juchau, T. Gram and J. R. Fouts, *Gastroenterology* **51,** 213 (1966).
19. J. R. Gillette, *Adv. Pharmac.* **4,** 219 (1966).
20. B. Stripp, M. E. Hamrick, N. G. Zampaglione and J. R. Gillette, *J. Pharmac. exp. Ther.* **176,** 766 (1971).
21. G. L. Lage, D. Oeth and J. L. Spratt, *Archs int. Pharmacodyn. Ther.* **169,** 255 (1967).