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IMPORTANCE OF THE LACTONIC RING IN THE ACTIVITY OF STEROIDAL ANTIALDOSTERONES

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Abstract—The *in vivo* pharmacological activity of several spirolactone compounds was tested in rats and compared to their ability to compete for [^{3}H] aldosterone renal binding sites. Spironolactone,* canrenone, dihydrocanrenone and K-canrenoate were all active *in vivo*, but 17-*O*-methyl 5,6-dihydrocanrenoic acid, a derivative which cannot be lactonized, was inactive at doses up to 20 mg/kg. Competition experiments were performed on cytosolic renal aldosterone sites labelled with 5×10^{-9} M [^{3}H] aldosterone. Spironolactone, canrenone and dihydrocanrenone were almost equally potent, whereas K-canrenoate and its derivatives exhibited practically no affinity for aldosterone sites. These results strongly suggest that K-canrenoate is only active *in vivo* when converted into canrenone, a steroid possessing a γ-lactone ring.

Spironolactone, canrenone and potassium canrenoate (Fig. 1) are competitive inhibitors of mineralocorticoids *in vivo* in experimental animals [1, 2] and their pharmacological activity in man has been demonstrated [3–5]. However, recent studies in animals [6, 7] and in men [8–10] have shown that these drugs have a complex metabolism. This raises the problem of the pharmacological activity of these drugs and their metabolites. For instance, spironolactone *in vivo* is rapidly dethioacethylated into canrenone, which then reaches equilibrium with potassium canrenoate. The major difference between all these molecules is the presence of a lipophilic lactone ring in spironolactone and canrenone, and a polar hydroxycarboxylic acid group in K-canrenoate. Spironolactone and K-canrenoate are equipotent in rats [2] and equivalent doses of these drugs produce similar levels of canrenone in plasma [8].

This study concerns the comparison between the *in vivo* pharmacological activity and the *in vitro* potency of these drugs in competing for aldosterone binding sites. Antialdosterone compounds have been shown to act by competing with aldosterone at receptor level [11–13]; thus they very probably inhibit the active sodium transport induced by

* Spironolactone: 3-(-3-oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl) proprionic acid γ-lactone; Canrenone: 3-(17β-hydroxy-6,7-dehydro-3-oxo-4-androsten-17α-yl) proprionic acid γ-lactone; Dihydrocanrenone: 3 (17β-hydroxy-6β, 7β dihydro-3-oxo-4-androsen-17-α-yl) proprionic acid γ-lactone; K-canrenoate: potassium 3-(17β-hydroxy - 6,7 - dehydro - 3 - oxo - 4 - androsten - 17α - yl) proprionate.

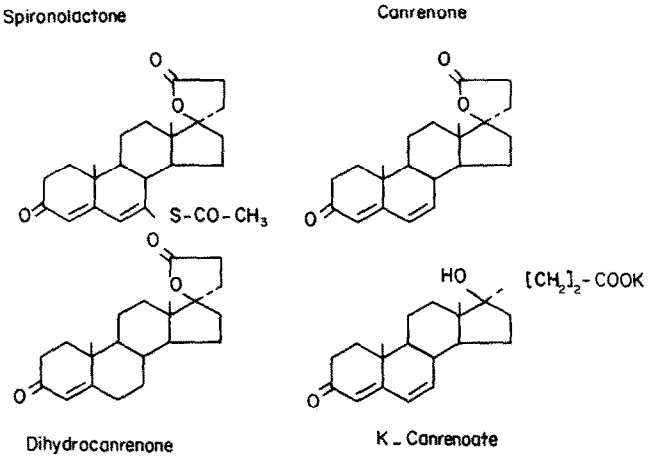

Fig. 1. Formulas for spironolactone and its derivatives.

mineralocorticoids. However, it has never been definitively established whether or not the lactonic ring is essential to such activity. This is why, in addition, a canrenoic acid derivative which cannot be lactonized was synthetized and tested in the present work for *in vivo* and *in vitro* activity.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley and Wistar rats (180/200 g) were used, respectively, for *in vivo* and *in vitro* assays. Animals were adrenalectomized 48 hr before the experiments and maintained on normal saline *ad libitum*.

Chemicals and radio-chemicals. Spironolactone, canrenone and K-canrenoate came from Searle, aldosterone -21- acetate from Ikapharm, and aldosterone from Sigma; 5,6 dihydrocanrenone (Fig. 1) was from Roussel–Uclaf; [1,2-^{3}H] aldosterone (52 Ci/mmole) was supplied by Amersham. All other chemicals and solvents were of analytical grade from Merck.

Chemical synthesis of 17-O-*methyl* 5,6 *dihydro canrenoic acid* (*Fig.* 2). This compound (D) was obtained in 4 steps from the oxirane (A) [14] A → B → C → D.

B was prepared by condensing the acetonitril carbanion group on the epoxy group of A according to Greger [15], followed by acidic hydrolysis in 5N HCl. The crude product was purified by silica gel chromatography (benzene/ethylacetate, 7/3) and recrystallized from methylen chloride isopropylether (yield: 81%, m.p.: 154°).

The B keto group was then protected by ketal formation and the 17-OH group methylated: *p*-toluene sulfonic acid monohydrate (80 mg) was added to a stirred suspension of B (12 mM) in a mixture of ethylen glycol (12 ml) and ethylorthoformate (8 ml) heated to 50°. After 1–2 min, dissolution of B was followed by crystallization of the ketal (C1). The resulting suspension was cooled to 0° and after 20 min, diluted with water containing pyridine (8 ml/0.25 ml). The crude ketal was collected, washed with water and dried under vacuum (yield: 86%). Analytical sample was prepared by recrystallization from ethanol (m.p.: 200°). Methylation of the 17-OH group was performed using methyliodide in the presence of potassium *t*-butoxide. The crude 17-OCH_3 derivative (C2) was recrystallized from chloroform–isopropylether (yield: 84%, m.p.: 166°). For synthesis of the final compound D, a mixture of C2 (2 mM), KOH (0.56 g) and ethylene glycol (8 ml) was heated to reflux until the evolution of ammonia stopped (≃1.5 hr). The cooled mixture was diluted with water, acidified with 2.5 ml of concentrated HCl and extracted with ethylacetate. After removal of the solvent, the residue was kept for 2 hr at 20° in 20 ml of acetone containing 5 ml of 5 N HCl; after dilution with water and chloroform extraction, the organic layer was evaporated to dryness leaving the crude D which was further purified by silica gel chromatography (benzene/ethylacetate/acetic acid, 80/20/1) and recrystallized from ethyl ether (yield: 80%, m.p.: 199°).

$\alpha_D^{20} = +54°$ (1% solution in ethanol on Roussel–Jouan electronic polarimeter).

i.r. (cm^{-1}): 3500 and 1704 (COOH), 1660 and 1619 (conjugated ketone).

u.v. (240 nm): $\varepsilon = 16{,}350$.

n.m.r. (ppm): 0.98 (s, 3H, $CH_3$18), 1.2 (s, 3H, $CH_3$19), 3.23 (s, 3H, OCH_3), 5.78 (s, 1H, H4), 6.0 (broad, CO_2H).

Melting points were determined on a Kofler–Heizbank apparatus. Infrared spectra were obtained in chloroform on a Spectromaster spectrometer. Ultraviolet spectra were measured in 95% ethanol using a Cary Model 14 instrument. Proton nuclear magnetic resonance spectra were taken in deuteriochloroform on a Varian Model A-60 spectrometer with TMS as an internal standard.

In vivo *experiments. In vivo* determination of the antimineralocorticoid activity of the various spirolactones and derivatives was performed according to a method derived from Kagawa [1]. Adrenalec-

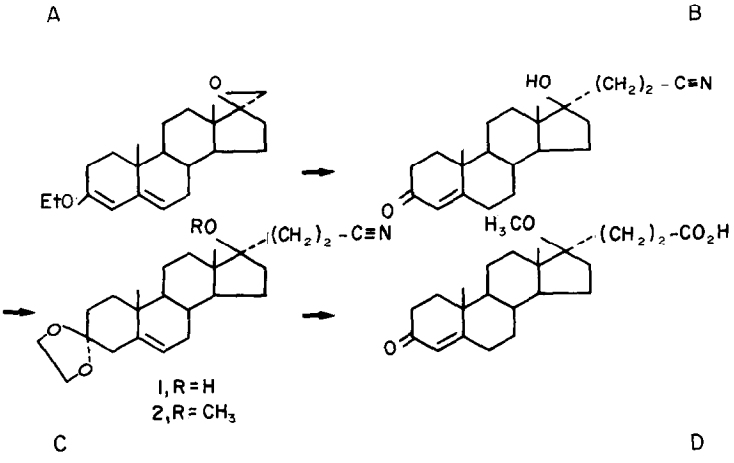

Fig. 2. Structural formulas of the intermediates A [(3-ethoxy 17,20 epoxy) 17α-methyl androst 3,5-diene], B [3-oxo 17-hydroxy (17α) pregen 4-ene 21-carbonitrile], C [3,3-ethylenedioxy 17-hydroxy (17α) pregn 5-ene 21-carbonitrile], for the synthesis of the 17-*O*-methyl 5,6 dihydro canrenoic acid D (17β-methoxy 3-oxo androst 4-ene 17α-yl) propionic acid.

tomized rats were fasted for 17 hr before experimentation. The drugs to be tested were administered subcutaneously 1 hr before the animals were placed in metabolism cages. Just before caging they were given intraperitoneally an hydrosaline solution (5 ml NaCl 9‰/100 g body wt) together with a subcutaneous injection of aldosterone acetate (1 μg/kg in 2.5% ethanol/water). After a 4 hr period, urine was collected, the bladder of each animal was emptied by slight pressure and the pooled fractions were diluted to 50 ml with distilled water. Na^+ and K^+ were determined in the solution using a flame photometer (Electro-synthese). Results are expressed as mEq/rat for the 4 hr experimentation period. Na^+/K^+ ratios were calculated and antimineralocorticoid activity was established as a percentage of the Na^+/K^+ ratio increase compared with the Na^+/K^+ ratio for the controls (animals injected with aldosterone only).

In vitro *experiments. In vitro* competition experiments for [^{3}H] aldosterone binding to kidney, receptor sites were performed as described in an earlier paper [16]. Adrenalectomized rat kidneys were homogenized in Tris–HCl buffer (10 mM Tris HCl, 1 mM EDTA, 1 mM dithiothreitol, glycerol 10% v/v, pH 7.4) and the homogenate was centrifuged at 1000 *g*. 5×10^{-9}M[^{3}H] aldosterone, with or without increasing concentrations of the various steroids to be tested, was added to the 1000 *g* supernatant and the solution centrifuged at 30,000 *g* for 30 min; the supernatant was collected and kept until the end of a total incubation period of 2 hr 30 (steady state); bound and free hormone were then separated by the charcoal dextran method [16] and samples were counted for radioactivity; results were expressed as the percentage of the maximal binding obtained by incubation with [^{3}H] aldosterone alone.

RESULTS

Subcutaneously administered spironolactone, canrenone and K-canrenoate inhibited the mineralocorticoid effects of aldosterone acetate (Fig. 3). K-canrenoate was less efficient than spirolactone and canrenone. 17-*O*-methyl 5,6-dihydro canrenoic acid was completely ineffective in blocking the antinatriuretic and kaliuretic effects of aldosterone acetate, even at doses as high as 20 mg.

In a similar separate experiment (not shown), dihydrocanrenone exhibited nearly the same antimineralocorticoid activity than spironolactone (60% increase of the Na^+/K^+ ratio compared to the aldosterone treated group, at a dose of 2 mg/kg).

In vitro inhibition of aldosterone binding by the

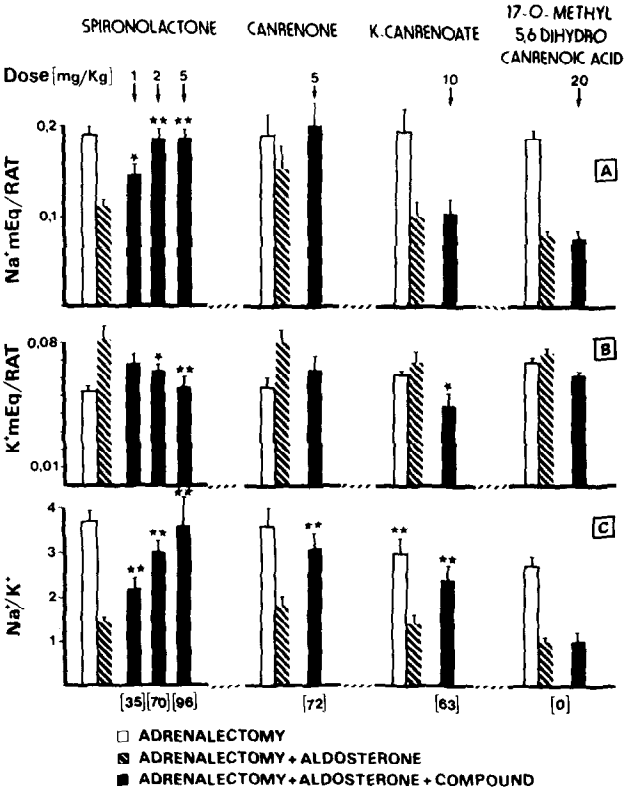

Fig. 3. Antimineralocorticoid activity of spironolactone and its derivatives *in vivo*. Adrenalectomized rats were either not treated, or treated as indicated under Materials and Methods, i.e. with aldosterone alone or with aldosterone and the drugs to be tested. Urinary Na^+ (A) K^+ (B) and Na^+/K^+ ratio (C) are given for the 4-hr period of the experiments. S.D. is mentioned (8–15 animals per group) and the $P < 0.05$ (*) or $P < 0.01$ (**) significance of the Dunett test [25] is indicated. Numbers in brackets under panel C indicate the percentage of increase in the Na^+/K^+ ratio compared to the aldosterone-treated reference group.

various compounds tested is shown in Fig. 4. Spironolactone, dihydrocanrenone and canrenone were, respectively, about 6, 10 and 40 times less efficient than aldosterone as regards 50% inhibition of [^{3}H] aldosterone binding to its receptor sites. K-Canrenoate efficiency was much lower (≈200 times) and there was practically no competition of 17-*O*-methyl 5,6-dihydrocanrenoic acid for aldosterone binding sites. Table 1 summarizes and compares the *in vivo* and *in vitro* results.

Table 1. Comparison of *in vivo* and *in vitro* activity of the various steroidal antialdosterones tested

Steroids	Activity *In vivo**	*In vitro*†
Spironolactone	5 mg/kg 96%	24%
Canrenone	5 mg/kg 72%	2.5%
Dihydrocanrenone	2 mg/kg 60%	20%
K-Canrenoate	10 mg/kg 63%	0.06%
17-*O*-methyl 5,6-dihydro canrenoic acid	20 mg/kg 0%	0%

* Data obtained from Fig. 2. Dose and corresponding activity (Na^+/K^+ ratio increase) are indicated.

† Activity is expressed as per cent of the competitive power of aldosterone taken as a reference. 100% corresponds to the concentration ratio of unlabelled to tritiated aldosterone needed to obtain 50% inhibition of binding (see Fig. 3).

DISCUSSION

Spironolactone, the most common antialdosterone agent used in human therapy, is rapidly metabolized since it cannot be detected in plasma after oral administration [17]. Canrenone and K-canrenoate account for about 80% of this metabolism and sulfhydryl compounds for 20% [9, 10]. Conversely, potassium canrenoate is metabolized into canrenone after injection and both compounds have relatively comparable plasma concentration in man [7, 17]. Similar metabolism has been found for spironolactone in rats [4] and in dogs [2]. All three compounds exhibit biological activity, although at the dose used (10 mg), the potency of K-canrenoate found in this study was somewhat lower than that of canrenone and spironolactone.

Comparison between *in vivo* and *in vitro* results concerning competition for aldosterone receptor sites makes it possible to determine the pharmacological activity of each compound. *In vitro* studies have shown that spirolactones antagonize binding of aldosterone to its receptor sites [13, 18, 19], which is believed to be the first step in the aldosterone mechanism of action [20]. Recent studies [19, 21, 22] showed that various spirolactones could compete for aldosterone binding sites in the kidney and that there was in general a good correlation between *in vitro* competition and *in vivo* antimineralocorticoid activity. Since K-canrenoate [19] and K-prorenoate [21, 23], a new aldosterone antagonist with a similar structure, were poor competitors for [^{3}H] aldosterone binding sites, these authors suggested that a lactone ring was necessary to obtain biological activity. The use in this work of 17-*O*-methyl 5,6-dihydro canrenoic acid, a compound which cannot be lactonized, supports this hypothesis and implies that in all probability K-canrenoate is only active when converted into the lactonic form. No

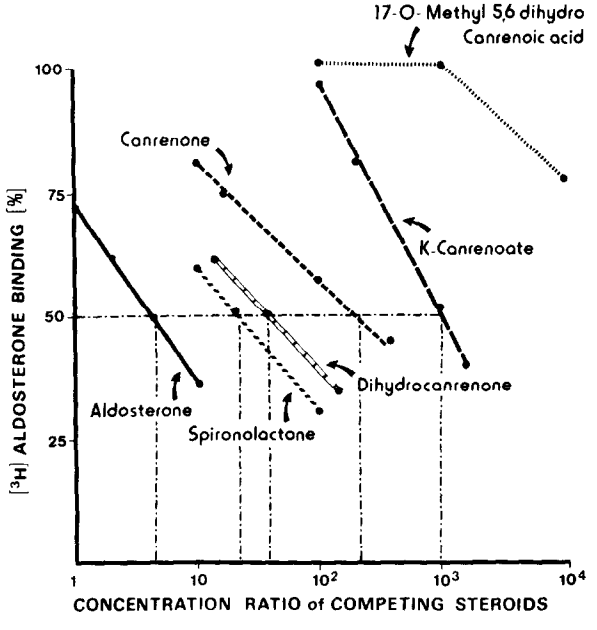

Fig. 4. Competition experiments between unlabelled steroids and 5×10^{-9} M [^{3}H] aldosterone. Results are expressed as per cent of total binding obtained with [^{3}H] aldosterone alone. The figure represents the mean of 3 separate experiments.

antimineralocorticoid effect was detected for the 17-*O*-methyl derivative, nor did it compete for [^{3}H] aldosterone binding sites. This observation is further strengthened by the fact that dihydrocanrenone which does not possess a double-bond in the 5,6 position as 17-*O*-methyl 5,6 dihydrocanrenoic acid exerts a potent antimineralocorticoid activity *in vivo* and binds to the mineralocorticoid receptor.

The restricted *in vitro* competitive power of K-canrenoate found in the competition experiments could be due either to its minimal conversion into an active metabolite or to slight displacement of [^{3}H] aldosterone from glucocorticoid binding sites [24]. From our experiments, it appears likely that part of the pharmacological activity of antialdosterone compounds with a lactonic ring is lost *in vivo* because of their conversion into hydroxycarboxylic form.

REFERENCES

1. C. M. Kagawa, *Endocrinology* **67**, 125 (1960).
2. C. M. Kagawa, D. J. Bouska, M. L. Anderson and W. F. Kroz, *Archs int. Pharmacodyn. Thér.* **149**, 8 (1964).
3. G. W. Liddle, *Metabolism* **10**, 1021 (1961).
4. W. J. Mroczek, M. E. Davidov, A. Horoschak and F. A. Finnerty, *Clin. Pharmac. Ther.* **16**, 336 (1974).
5. L. E. Ramsay, J. R. Shelton, D. Wilkinson and M. J. Tidd, *Br. J. clin. Pharmac.* **3**, 607 (1976).
6. C. M. Kagawa, D. J. Bouska and M. L. Anderson, *Proc. Soc. exp. Biol. Med.* **115**, 837 (1964).
7. W. Sadee, S. Riegelman and S. C. Jones, *J. pharm. Sci.* **61**, 1129 (1972).
8. W. Sadee, M. Dagcioglu and R. Schroder, *J. Pharmac. exp. Ther.* **185**, 686 (1973).
9. A. Karim, J. Zagarella, J. Hribar and M. Dooley, *Clin. Pharmac. Ther.* **19**, 158 (1976).
10. A. Karim, J. Hribar, W. Aksamit, M. Domerty and J. L. Chinn, *Drug Metab. Disposition* **3**, 467 (1976).
11. T. S. Herman, G. M. Fimognari and I. S. Edelman, *J. biol. Chem.* **243**, 3849 (1968).
12. W. Sadee, M. Dagcioglu and R. Schroder, *J. Pharmac. exp. Ther.* **185**, 686 (1973).
13. D. Marver, J. Steward, J. Funder, D. Feldman and I. S. Edelman, *Proc. natn. Acad. Sci. U.S.A.* **71**, 1431 (1972).
14. Roussel-Uclaf Patent, *Ger. Offen.* **2**, 404, 947 (8 August 1974). *Chem. Abstr.* **81**, 13638k (1974).
15. P. L. Greger, *J. org. Chem.* **37**, 1907 (1972).
16. M. E. Rafestin-Oblin, A. Michaud, M. Claire and P. Corvol, *J. Steroid Biochem.* **8**, 19 (1977).
17. A. Karim, R. E. Ranney and H. I. Maibach, *J. Pharm. Sci.* **50**, 708 (1971).
18. T. S. Herman, G. M. Ifmognari and I. S. Edelman, *J. biol. Chem.* **243**, 3849 (1968).
19. J. W. Funder, D. Feldman, E. Highland and I. S. Edelman, *Biochem. Pharmac.* **23**, 1493 (1974).
20. I. S. Edelman and G. M. Fimognari, *Recent Prog. Horm. Res.* **24**, 1 (1968).
21. J. W. Funder, J. Mercer and J. Mood, *Clin. Sci. molec. Med.* **51**, 333 (1976).
22. M. Claire, M. E. Rafestin-Oblin, A. Michaud, C. Roth-Meyer and P. Corvol, *Endocrinology*, in press.
23. C. M. Kagawa, in *Methods in Hormones Research* (Ed. Dorfmann) Vol. III, Chap. 7, p. 355 (1964).
24. J. W. Funder, D. Feldman and I. S. Edelman, *Endocrinology* **92**, 1005 (1973).
25. C. W. Dunett, *Am. Stat. Ass. J.* **50**, 1098 (1955).