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Note

Separation of spironolactone and its biologically active sulfur-containing metabolites by high-performance liquid chromatography

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Spironolactone (SL) is a mineralocorticoid antagonist that is widely used in the treatment of essential hypertension, primary aldosteronism and various edematous states [1, 2]. The drug competitively blocks aldosterone binding to renal receptors, resulting in sodium loss and potassium retention. Other effects of SL have also been described including hepatic enzyme induction and inhibition of steroidogenesis in the adrenal cortex and testes [3—10].

SL is rapidly metabolized in all species including humans and very little of the parent compound is detectable in biological fluids [11, 12]. It is generally believed that SL is rapidly converted to the metabolite, canrenone (Fig. 1), and that the latter compound is the major circulating form of the drug. In addition, since canrenone has antimineralocorticoid activity, most of the biological activities of SL have been attributed to canrenone. However, in most prior

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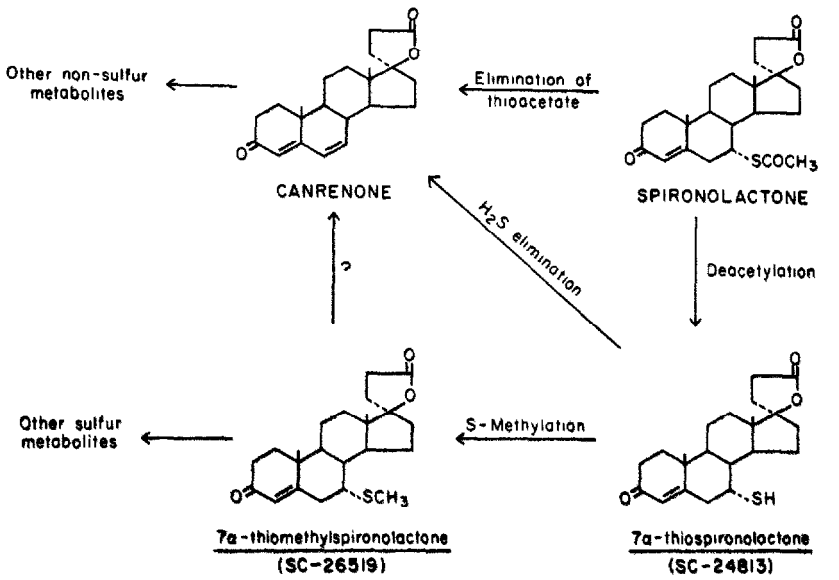

Fig. 1. Proposed pathways for the conversion of spironolactone to 7α-thiospironolactone; 7α-thiomethylspironolactone and canrenone.

investigations, plasma levels of canrenone were determined by a relatively non-selective fluorimetric method [13], and other compounds may have been inadvertently masured.

In recent years, highly selective high-performance liquid chromatographic (HPLC) analyses of plasma canrenone concentrations after SL administration have clearly indicated that the fluorimetric method grossly overestimates circulating canrenone levels [14—20]. In addition, pharmacokinetic studies indicate that canrenone can account for only a small part of the antimineralocorticoid activity of SL [14—20] and other investigations have established that the effects of SL in steroidogenic tissues are also not mediated by canrenone [10]. As a result of these findings, several investigators have proposed that metabolites of SL other than canrenone are important in mediating the biological effects of the parent drug [14- 20]. Of particular interest are the sulfur-containing metabolites 7α-thio-SL and 7α-thiomethyl-SL (Fig. 1), both of which have a very high affinity for the renal aldosterone receptor [21, 22]. Although neither compound has been definitively identified in body fluids, both represent logical precursors to various other known metabolites (see refs. 11 and 12). In addition, 7α-thiomethyl-SL is a highly fluorescent compound, which might contribute to the overestimates of plasma canrenone levels when measured fluorimetrically. Studies on the involvement of 7α-thio-SL and/or 7α-thiomethyl-SL in the actions of SL have been limited in part by the absence of a relatively simple but selective assay for each compound. In this paper an HPLC method for the separation and quantitation of SL, 7α-thio-SL, 7α-thiomethyl-SL and canrenone is described which is suitable for in vitro and in vivo analyses.

EXPERIMENTAL

Materials

Spironolactone (7α-acetylthio-17β-hydroxy-3-oxo-17-pregn-4-ene-21-carboxylic acid-γ-lactone), 7α-thiospironolactone (SC-24813), 7α-thiomethyl-spironolactone (SC-26519) and canrenone were provided by Searle (Chicago, IL, U.S.A.). Progesterone was obtained from Sigma (St. Louis, MO, U.S.A.). The purity of all steroids was confirmed by HPLC. All solvents used were HPLC grade and were obtained from Fisher (Pittsburgh, PA, U.S.A.).

Animals

Adult (600—800 g) male English Short Hair guinea pigs were obtained from Camm Research Institute (Wayne, NJ, U.S.A.). Animals were killed by decapitation and blood was collected in heparinized beakers. Plasma was obtained by centrifugation and stored frozen until needed. In pretreatment experiments guinea pigs received spironolactone (25 mg/kg body weight) as intraperitoneal injections (in saline containing three drops Tween 80 per ml) 24 and 4 h before sacrifice. Controls received the vehicle alone.

Extraction procedure

Each milliliter of plasma was diluted with 2 ml of distilled water and extracted with 5 ml of ethyl acetate. After removal of the organic phase, the aqueous phase was again extracted with 5 ml of ethyl acetate. The organic extracts were combined, taken to dryness under a stream of nitrogen, and reconstituted in 400 μl of HPLC-grade acetonitrile. Samples could not be reconstituted in methanol because SL, when kept in methanol at room temperature, is slowly converted to 7α-thio-SL. However, in acetonitrile, SL, 7α-thio-SL, 7α-thiomethyl-SL and canrenone are all stable. Extraction efficiencies were determined by spiking guinea pig plasma with various amounts of the standards and carrying them through the procedure described above.

HPLC equipment

A Waters HPLC system (Waters Assoc., Milford, MA, U.S.A.), 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 RCM-100 radial compression separation system, was used. The HPLC analyses were carried out with a 5-μm C_{18} Radial Pak cartridge.

Chromatographic conditions

Compounds were separated using a 30-min concave gradient (curve 9) of 65--100% methanol–water. The gradient is defined by the following equation: percentage methanol = percentage $\text{methanol}_{\text{start}}$ + (percentage $\text{methanol}_{\text{end}}$ — percentage $\text{methanol}_{\text{start}}$) × $(t - t_0/t_1 - t_0)^4$ where, percentage $\text{methanol}_{\text{start}}$ = percentage methanol at start of gradient; percentage $\text{methanol}_{\text{end}}$ = percentage methanol at end of gradient; t = elapsed time from sample injection; t_0 = time lag between sample injection and start of gradient; t_1 = duration of gradient.

The solvent flow-rate was 1.0 ml/min and the chromatographic system was

operated at ambient temperature. The eluent was monitored by absorbance at 254 nm and peak areas were automatically integrated by the data module. The UV detector was operated at 0.05 or 0.01 a.u.f.s. Standard curves were constructed by adding various amounts of the steroids to 3.0 ml of guinea pig plasma, extracting as described above and plotting peak area ratios (relative to the internal standard) versus amount of each compound. The reproducibility of the HPLC assay was estimated from five successive determinations of two concentrations of each compound.

Mass spectroscopy

Mass spectral analyses of authentic 7α-thiomethyl-SL and the plasma metabolite obtained from SL-treated rats were carried out with a Finnigan Model 4021 automated gas chromatograph–mass spectrometer and INCOS automatic data system. Samples were run at 20 eV by direct probe in the electron-impact (EI) mode.

RESULTS AND DISCUSSION

The separation of SL, 7α-thio-SL, 7α-thiomethyl-SL and canrenone standards by the HPLC method described is illustrated in Fig. 2. We have routinely used progesterone as an internal standard because it is well separated, not only from the SL metabolites shown, but from other tissue metabolites of SL which are also of interest to us. However, if only SL, 7α-thio-SL, 7α-thiomethyl-SL and canrenone are to be separated, any of a large number of UV-absorbing (Δ^4-3-keto) steroids may be used as an internal standard. The total run time for the assay can be reduced substantially by using a more polar internal standard than progesterone. The time can also be reduced by increasing the solvent flow-rate to as high as 1.5 ml/min without significantly compromising the separation.

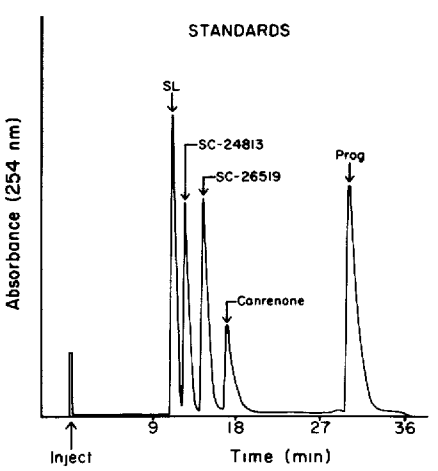

Fig. 2. Separation of spironolactone (SL), 7α-thio-SL (SC-24813), 7α-thiomethyl-SL (SC-26519), canrenone and the internal standard, progesterone (Prog), by HPLC. Of each compound 100 ng were injected and the UV absorbance at 254 nm was monitored with the detector set at 0.05 a.u.f.s. The HPLC conditions were exactly as described in Experimental.

As can be seen in Fig. 2, the extinction coefficient for canrenone at 254 nm is considerably lower than those for the other compounds. The peak absorbances for SL, 7α-thio-SL and 7α-thiomethyl-SL are at 240—250 nm, but the absorbance of canrenone is far greater at 280—290 nm. Accordingly, prior HPLC assays for canrenone have monitored absorbance in the latter range

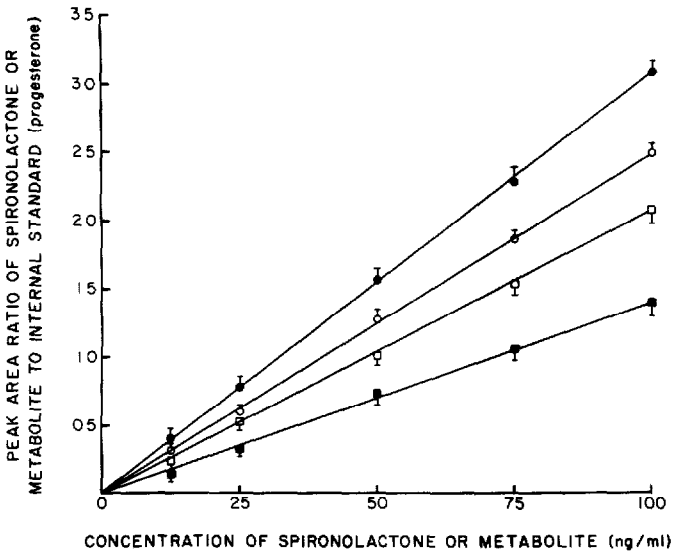

Fig. 3. Standard curves for the HPLC analyses of spironolactone (●; r = 0.996), 7α-thio-SL (○; r = 0.997), 7α-thiomethyl-SL (□, r = 0.996) and canrenone (■; r = 0.995) in guinea pig plasma. Extractions and HPLC conditions were as described in Experimental. Each point represents the mean value of five determinations (± S.E.).

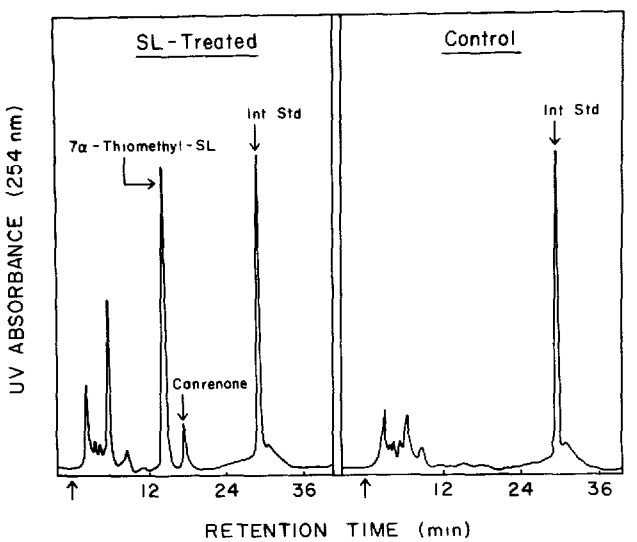

Fig. 4. HPLC analysis of the plasma extracts from control and spironolactone (SL)-treated guinea pigs. Animals were pretreated and plasma samples analyzed as described in the methods. The arrows indicate when the sample was injected. Progesterone was added to each plasma sample as the internal standard (Int. Std.).

[17–20, 23–25]. If the HPLC method described in this paper is to be used for canrenone as well as the sulfur-containing metabolites, 7α-thio-SL and 7α-thiomethyl-SL, monitoring of UV absorbances at both the lower and higher ranges will give maximal sensitivities for all compounds.

Standard curves for SL, 7α-thio-SL, 7α-thiomethyl-SL and canrenone following addition of standards to guinea pig plasma are shown in Fig. 3. Similar curves were obtained for extraction of the compounds from liver or kidney microsomal suspensions. The curves remained linear up to concentrations of 500 ng/ml. The mean recoveries (± S.D.) for the 25 ng/ml concentrations of SL, 7α-thio-SL, 7α-thiomethyl-SL and canrenone were 87 ± 3%, 84 ± 3%, 86 ± 4% and 87 ± 3%, respectively. At the 100 ng/ml concentrations, the cor-

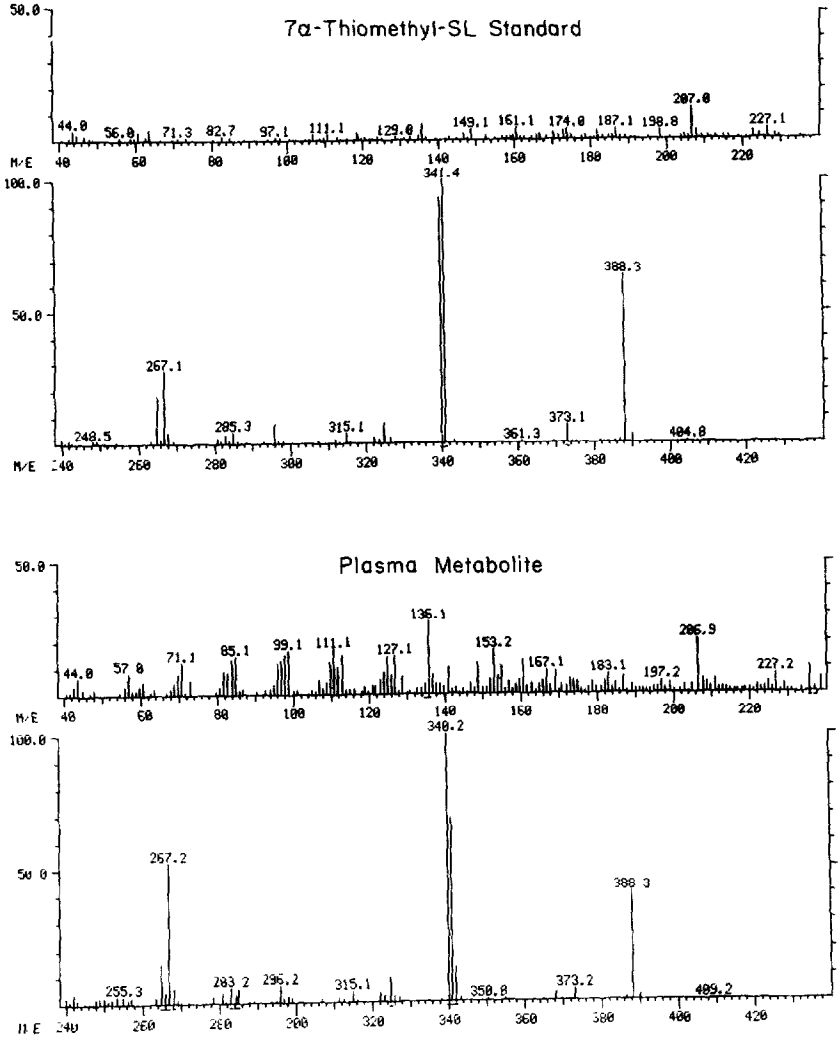

Fig. 5. Mass spectra of authentic 7α-thiomethylspironolactone (7α-thiomethyl-SL) and of the plasma metabolite isolated from spironolactone (SL)-treated guinea pigs. The plasma metabolite co-chromatographing with the 7α-thiomethyl-SL standard was isolated by HPLC and mass spectral analysis was carried out as described in Experimental.

responding recoveries were 85 ± 3%, 81 ± 3%, 86 ± 3% and 86 ± 4%, respectively.

The method described is a relatively simple and rapid procedure for separating SL and those metabolites believed to be important in mediating the actions of the drug. Selective HPLC assays for canrenone have previously been described [17—20, 23—25] and use of those assays has clearly established that canrenone is only a minor contributor to the biological activity of SL. The sulfur-containing SL derivatives, 7α-thio-SL and 7α-thiomethyl-SL, have high affinities for the renal aldosterone receptor and may therefore contribute to the antimineralocorticoid effects of SL. In addition, the results of prior studies [6—8] suggested that 7α-thio-SL might contribute to the side-effects of SL in the testes and adrenal cortex. In preliminary studies, using this HPLC assay, we have examined plasma metabolite levels following high-dose SL administration to guinea pigs (Fig. 4). The results indicate that 7α-thiomethyl-SL is a major plasma metabolite of SL. Plasma levels of 7α-thiomethyl-SL were 425 ± 39 nm/ml (mean ± S.E., n = 4) compared to canrenone levels of 285 ± 31 ng/ml. Identity of the plasma metabolite as 7α-thiomethyl-SL has been confirmed by mass spectroscopy (Fig. 5). Thus, 7α-thiomethyl-SL may have a role in mediating the renal actions of SL. Additional studies are now underway to test this hypothesis. The HPLC assay described in this paper should expedite such investigations as well as other studies on the biological importance of the sulfur-containing metabolites of SL.

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