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ISOLATION OF ALDOSTERONE URINARY METABOLITES, GLUCURONIDES AND SULFATE*

JOHN H. GROSE,† W. NOWACZYNSKI, O. KUCHEL and J. GENEST
Clinical Research Institute and the Hôtel-Dieu Hospital of Montreal, Montreal, Canada

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SUMMARY

A simple method is described for the simultaneous isolation of aldosterone-18-glucuronide and tetrahydroladosterone-glucuronide from the urine of normal subjects following intravenous administration of purified ^{3}H-aldosterone. Using the two isolated conjugates as recovery indicators, the urinary excretion rates of the two compounds determined in normal subjects were within the ranges established by previous investigations. A method is also established for the isolation and estimation of urinary aldosterone sulfate. The mean urinary excretion rate of this compound was $2{\cdot}09 \pm 0{\cdot}6\,\mu g/24$ h in normals and $2{\cdot}83 \pm 0{\cdot}45\,\mu g/24$ h in patients with benign essential hypertension. In one instance of confirmed primary aldosteronism investigated, the pre-operative urinary aldosterone sulfate was $13{\cdot}76\,\mu g/24$ h and the same compound was undetectable in the urine of the patient after sub-total adrenalectomy.

OUR CURRENT knowledge of the physiology of aldosterone in man has been largely derived from measurements of its metabolites in the urine. The two major metabolites of the hormone, aldosterone-18-glucuronide and tetrahydroaldosterone glucuronide are the metabolites of choice because of their abundance in the urine. As these conjugates are not available commercially, most investigations were based on the estimation of the free steroid alcohols of the respective conjugates after appropriate hydrolysis of the compounds and total recovery was then estimated by using the commercially available labeled free steroids. Although this approach has proved relatively reliable for most purposes, it nevertheless does not account for subtle differences which may incur as a result of incomplete hydrolysis of the conjugates. In studies which necessitate the use of the labeled conjugates of aldosterone as markers, they are usually isolated from the human [1–3] or the monkey [4] subsequent to intravenous injections of radioactive aldosterone.

Underwood and Tait [1] have described a method for the partial purification of aldosterone- 18-glucuronide, and Pasqualini and co-workers [5] and Möhring [6] have reported methods for the partial purification of both, the 18-glucuronide and

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The following abbreviations and trivial names have been used: Aldosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione-18-al; aldosterone diacetate, 11β-hydroxy-18,21-diacetoxy-4-pregnene-3,20-dione; aldosterone-18-glucuronide, Oxo-C = 11β,21-dihydroxy-4-pregnene-3,20-dione-18-al 18-monoglucuronide; aldosterone-21-sulfate, AS = 11β-hydroxy-4-pregnene-3,20-dione-18-al-21-yl sulfate; tetrahydroaldosterone, 3α,11β,21-trihydroxy-5β-pregnan-20-one-18-al; tetrahydroaldosterone triacetate, THATA = 11β-hydroxy-3α,18,21-triacetoxy-5β-pregnan-20-one; tetrahydroaldosterone diacetate, 11β-hydroxy-3α,21-diacetoxy pregnan-18-oic; tetrahydroaldosterone glucuronide, THA-G = 3α,11β,21-trihydroxy-5β-pregnan-20-one-18-al 3-monoglucuronide; prednisolone, 11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione; BEH, benign essential hypertension.

tetrahydroglucuronide of aldosterone from human urine. On the other hand, the sulfo-conjugated metabolites of aldosterone have not been extensively studied. Although it has been demonstrated through tracer studies that aldosterone and its reduced metabolites exist in small amounts in the sulfate form in both human plasma and urine [7], the actual characterization and quantification of these urinary conjugates have not been carried out.

We now describe a simple method for the partial purification of the 18-glucuronide and tetrahydroglucuronide of aldosterone from human urine, and their application as recovery markers were also assessed. A method for the isolation and quantification of urinary aldosterone-21-sulfate is also described.

MATERIALS AND METHOD

All subjects received an identical diet containing 135 mEq of sodium and 90 mEq of potassium per day and were studied on the 4th day while on the controlled diet. The control subjects were normotensive, had normal physical examinations and no evidence by history or laboratory testing for clinical diseases. Patients with benign essential hypertension were carefully selected individuals who showed no evidence of a renal or endocrine cause for their hypertension in an investigation which included plasma and urinary electrolyte measurements, creatinine clearances and rapid sequence intravenous pyelography; electrocardiogram and renal arteriography were normal. None showed any overt signs of arterio-atherosclerosis of large vessels. All patients were off drug therapy for at least four weeks prior to the study. All female subjects were studied during the follicular phase of their menstrual cycles and those who were on oral contraceptives were excluded from the study. Twenty-four hour urine samples were collected between 8:00 h and 8:00 h the next day, with the subjects carrying out their normal activities in the Clinical Investigation Unit.

REAGENTS

All organic solvents were obtained and purified as described previously [8]. Methylene Blue (Fisher), chromium trioxide (Mallinckrodt Chemical Works), dimethylformamide (analytical reagent, BDH), dicyclohexyl–carbodiimide (Eastman Kodak Co.) were used without further purification.

STEROID STANDARDS

The following steroid standards were obtained and used without further purification: d-aldosterone (Ciba), cortisol (Merck), desoxycorticosterone, desoxycorticosterone acetate (Sigma), prednisolone (Schering).

Labeled steroids: [1,2-^{3}H] d-aldosterone (51 Ci/mmol), 14-^{14}C] d-aldosterone (56·7 mCi/mmol), acetic-1-^{14}C anhydride (2 mCi/mmol) were obtained from Amersham/Searle. [1,2,^{3}H] Tetrahydroaldosterone (100 mCi/mmol) was purchased from New England Nuclear Corp.

Radioactive aldosterone and tetrahydroaldosterone were purified in System A [9] before use. By allowing the chromatography to proceed for 16–18 h, aldosterone was well separated from 17-iso-aldosterone ($R_{\text{aldosterone}} = 0{\cdot}85$), a frequent contaminant of labeled aldosterone.

CHEMICAL SYNTHESIS OF ALDOSTERONE-21-SULFATE (AS)

Chemical synthesis of AS from aldosterone was carried out by the method described by Mumma *et al.*[10] for alkyl hydroxyl groups. Sulfation was mediated by dicyclohexylcarbodiimide (DCC) in dimethylformamide (DMF) medium using the molar ratio steroid: H_2SO_4:DOC (1:1·5:5). However, to increase the final yield of the product, the concentration of the reactants in DMF was increased proportionally to 1 mmol/8 ml of DMF. The reaction mixture was diluted to 20 ml with distilled water, filtered through glass wool, and the steroids were recovered in the methanol wash of a small column (2 × 20 cm) of Amberlite XAD-2 (Rohm and Haas, Co.) according to the method of Bradlow[11]. The AS in the extract was converted to the ammonium salt by repeated evaporation of the mixture in 10% ammoniacal methanol. The oily residue was then dissolved in 20 ml of distilled water, extracted with 2 × 20 ml of methylene dichloride to remove the unreacted aldosterone from the mixture and the aqueous phase evaporated *in vacuo* at 45°C. The residue was crystallized from methanol–methylene dichloride and methanol–acetone mixtures to give 26·5 mg of crystals (from 36 mg of pure crystalline aldosterone, 55·6% yield). This product gave a color reaction with methylene blue as typically exhibited by steroid sulfates[12]. Chromatography of an aliquot of the material in system N for 18 h indicated only one U.V. positive zone which migrated 21 cm. This U.V. positive zone did not give a positive reaction to blue tetrazolium test on the paper while a spot of standard aldosterone with similar U.V. intensity on chromatographic paper gave a positive reaction with the same reagent, indicating the absence of free α-ketol side chain on the synthetic compound. Subsequent hydrolysis of an aliquot of the conjugate with sulfatase ("Helicase", l'Industrie Biologique Française) and chromatography of the free steroid and its acetylated product in system A and in system E respectively indicated a single U.V. absorbing vomponent which corresponded in mobility to free aldosterone and aldosterone diacetate. Additional properties of the conjugate included: mp 135–8; IR analysis (Nujol Mull, Fig. 1) 3500–3100 cm^{-1} (OH and

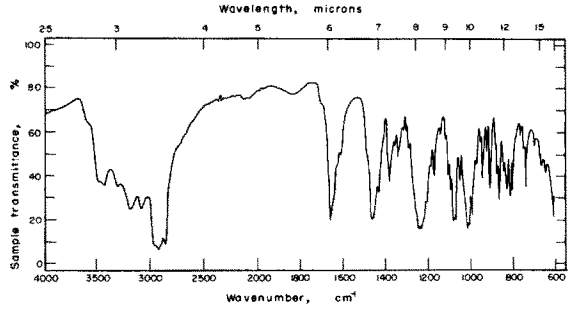

Fig. 1. Infrared spectrum of aldosterone-21-monosulfate.

C=O), 1700 cm^{-1} (C=O, 20-ketone), 1660 cm^{-1} (C=O, 4-ene-3-ketone), 1610 cm^{-1} (C=C, 4-ene-3-ketone), 1240 cm^{-1} (sulfate band). NMR (CD_3OD, Fig. 2) 4·83 ppm (hydroxyl esterification, doublet, hindered rotation), shift expected for free $-CH_2OH$ would be at about 4·4 ppm; 5·7 (C-4 proton, 4-ene-3-keto), 5·36 (C-18 proton, hemiacetal); 4·6 (C-11 proton); 1·34 (C-19 proton, angular methyl); 3·3 and 4·9 (exchange of CD_3OD). Analysis: Calc. for $C_{21}H_{32}O_8SN \cdot H_2O$ (476) C = 52·94, H = 7·14, N = 2·94%. Found C = 52·18, H = 6·9, N = 2·78.

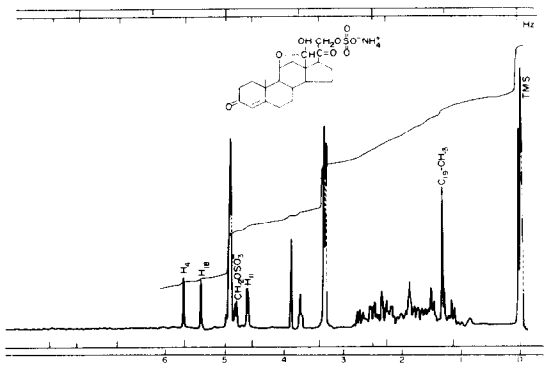

Fig. 2. NMR spectrum of aldosterone-21-monosulfate.

[1,2-^{3}H] ALDOSTERONE-21-SULFATE and [4-^{14}C] ALDOSTERONE-21-SULFATE

The general procedure for the synthesis of labeled AS was essentially the same for that of the non-labeled compound. Since the labeled aldosterones used were of high specific activity, the steroid masses were negligible and hence an equivalent amount of DOC was substituted for that of aldosterone. This step was undertaken in order to minimize the formation of undesirable labeled by-products if only labeled aldosterone alone were used. The reaction mixture was recovered as previously described and then purified by paper chromatography in system N for 18 h. An U.V. positive band with identical mobility as DOC-sulfate migrated 34·2 cm from the origin. On scanning the chromatogram, the major radioactive zone which migrated 20·2 cm had identical chromatographic mobility as AS;tracer amounts of radioactivities were also detected at 5 and 13·5 cm from the origin of sample application. The yields were 62% for ^{3}H–AS and 65% or ^{14}C–AS.

DETECTION OF RADIOACTIVITY

Radioactive compounds on paper or on thin-layer were detected by scanning 4 cm strips with a Packard Model 7201 Radiochromatogram scanner. Liquid scintillation counting was conducted by dissolving the samples in 10 ml of toluene-base scintillation fluid (Permablend II) and the samples were counted in a Packard Tri-Carb Liquid scintillation Spectrometer Model A3375S with a counting efficiency of 24% for ^{3}H and 44% for ^{14}C at double label settings (the spillage of ^{14}C into the ^{3}H channel was 7·8% and that of ^{3}H into the ^{14}C channel was $< 0{\cdot}1\%$). Samples which were insoluble in toluene were counted in the presence of 0·5 or 1 ml of methanol. Quenching was corrected by the internal standard method.

ADMINISTRATION OF [^{3}H]-ALDOSTERONE: TREATMENT OF URINE

Purified ^{3}H-aldosterone was dissolved in a solution of 5% dextrose (Abbott Laboratories Ltd.) containing 10% ethanol and passed through a millipore filter (Swinnex-12, Millipore Corp., Bedford, Mass.) into a sterilized vial. 10 ml of this solution was injected into an arm vein of a normal male. Subject Y.J. (20 yr) received $5{\cdot}26 \times 10^7$ d.p.m. ^{3}H. Urine was collected at 0–6 h and 7–48 h after the injection and was frozen (− 15°C) until analysis. Urinary steroids were extracted on Amberlite XAD-2 resin[11]. Chromatography of urinary extracts

on DEAE–Se phadex (A-25) using a linear salt gradient was carried out essentially as described by Hobkirk *et al.*[13] for estrogen conjugates.

ESTIMATION OF URINARY OXO-C AND THA-G

The procedures involved are summarized in Fig. 3. Oxo-C was estimated as free aldosterone and THA-G was estimated as free tetrahydroaldosterone after appropriate hydrolysis of the urine samples.

A one-fourth aliquot of the total 24 h collection was usually processed. About 5000 d.p.m. of each of the prepared ^{3}H-labeled markers (at a specific activity of about 40 mCi/mmol) in 0·5 ml methanol were added separately to an accurately measured urine aliquot. Identical aliquots of the markers were transferred to separate counting vials for the accurate estimation of the number of counts added as internal standards. The urine sample was extracted successively with three times half its volume of ethyl acetate and methylene dichloride to remove the free

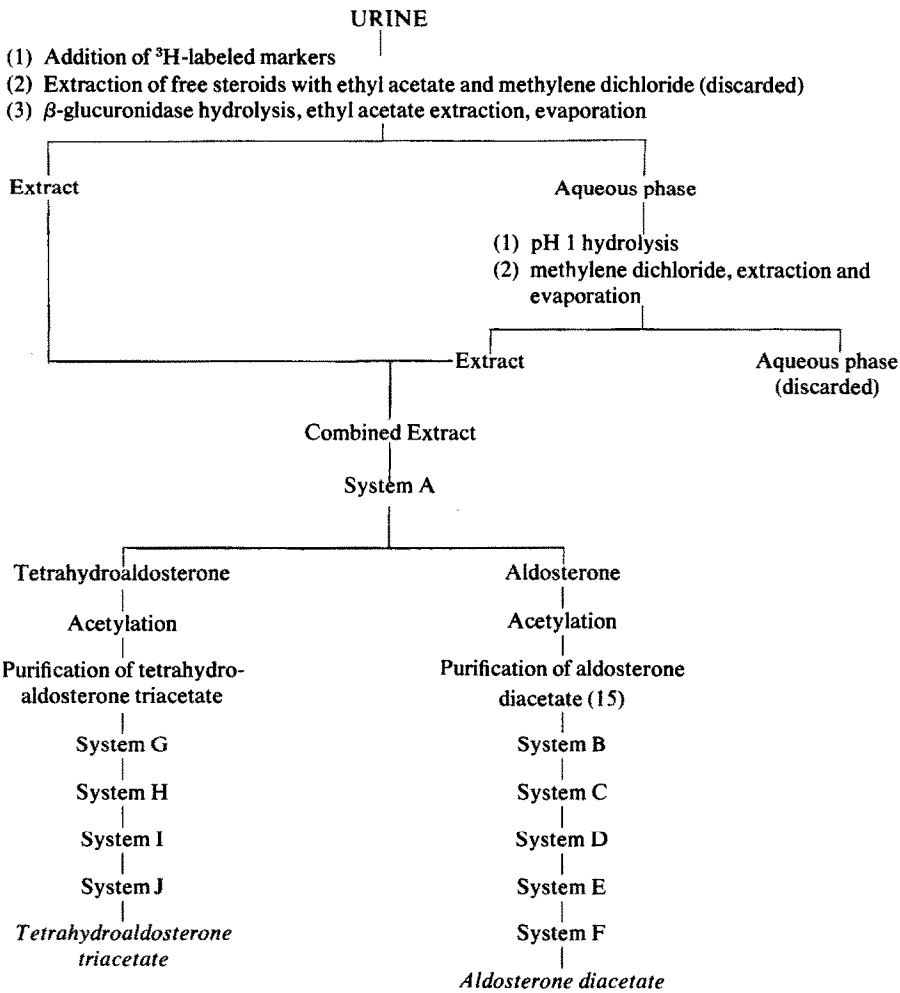

Fig. 3. Scheme for the estimation of urinary 18-glucuronide and tetrahydroglucuronide of aldosterone.

steroids in the urine and the organic extracts were discarded. The pH was then adjusted to 5 with glacial acetic acid and the sample incubated with 300 units/ml of Ketodase (Warner–Chilcott) for 48 h at 46°C in the presence of 0·1 M acetate ion (sodium acetate buffer, pH 5). The free steroids were recovered by extraction of the incubation mixture three times with equal volume of ethyl acetate. The combined ethyl acetate mixture was then washed twice with one-tenth of its volume of 0·1 N NaOH followed by three washings with one-tenth of its volume of distilled water and evaporated to dryness *in vacuo* at 40 °C. The aqueous phase was subjected to acid hydrolysis at pH 1 [1], extracted three times with equal volume of methylene dichloride and the combined extract was treated as described for the ethyl acetate extract.

Purification of free and acetates of aldosterone and tetrahydroaldosterone

The ethyl acetate and methylene dichloride extracts from the same urine sample were combined and purified by paper partition chromatography in system A for 16 h with aldosterone and prednisolone running as standards in parallel strips. Aldosterone migrated 31–33 cm and tetrahydroaldosterone, which had similar mobility as prednisolone, migrated 13–15 cm ($R_{aldosterone} = 0{\cdot}41$). The respective zones were cut out, eluted with methanol, evaporated to dryness and acetylated with ^{14}C-acetic anhydride of known specific activity.

Aldosterone diacetate was purified according to a previously described procedure [15].

Purification of tetrahydroaldosterone triacetate (THATA) was accomplished by the use of four paper chromatographic systems with DOCA running parallel as UV indicator. The sample was first chromatographed, as a 3 cm line, in the reverse phase system G for 24 h; THATA and DOCA migrated at the same rate, $R_F = 0{\cdot}4$. This area was then eluted and rechromatographed as a spot in system H for 16 h and migrated 32–35 cm, $R_{DOCA} = 1{\cdot}2$. The appropriate area was further purified in the reverse phase system I for 24 h ($R_F = 0{\cdot}4$, $R_{DOCA} = 0{\cdot}88$). In the final chromatography, the sample was allowed to run for 16 h in system J, during which time THATA migrated 32–35 cm, $R_{DOCA} = 1{\cdot}13$. The purified THATA was eluted with 5 ml of methanol directly into a counting vial for the accurate estimation of ^{3}H and ^{14}C counts. After each chromatography in systems H–J, the THATA area was located by radio-scanning. Overall recoveries of the method were 12–26%. The amount of aldosterone or tetrahydroaldosterone present in the sample was calculated as previously described [15]; the mass of the recovery marker added was then subtracted from the final calculations.

Assessment of THATA method

In order to check the purity of the purified THATA, three samples of the purified triacetate (after system J) were each divided into two portions. A one-third aliquot from each sample was counted as the triacetate while the remaining portion was oxidized to the diacetate in 0·1 ml of 0·5% chromic oxide in 90% acetic acid [16]. The diacetates which were formed in good yield were chromatographed in system J for 16 h and appeared as symmetrical peaks of radioactivity, migrating 18–21 cm ($R_{DOCA} = 0{\cdot}6$). The diacetate areas were cut out and eluted directly into counting vials. Formation of the diacetate did not appreciably alter the intrinsic ^{14}C/^{3}H ratio within the same samples (Table 2), thus indicating that the THATA obtained after system J was adequately purified. The reproduci-

Table 1. Chromatographic systems

System	Solvent composition (by vol.)
A	Cyclohexane : benzene : methanol : water (1 : 9 : 6 : 4)
B	Ethylene glycol/iso-octane : toluene (7 : 3)
C	Thin layer (silica gel), toluene : methanol (95 : 5)
D	Iso-octane : t-butanol : water (5 : 1 : 5)
E	Mesitylene : methanol : water (3 : 2 : 1)
F	Iso-octane : benzene : methanol : water (6 : 4 : 8 : 2)
G	Decalin : methanol : water (2 : 1 : 1)
H	Iso-octane : toluene : methanol : water (9 : 1 : 9 : 1)
I	Mesitylene : methanol : water (7 : 6 : 1)
J	Cyclohexane : benzene : methanol : water (9 : 1 : 9 : 1)
K	Toluene : t-butanol : n-butanol : water (12 : 15 : 50 : 50)
L	Butyl acetate : toluene : n-butanol : 12% acetic acid : methanol (5 : 3 : 3 · 5 : 5 : 5)
M	Iso-octane : t-butanol : 1 N NH_4OH (25 : 50 : 50)
N	Butyl acetate : toluene : n-butanol : 4 N NH_4OH : methanol (5 : 3 : 3 : 5 : 5)
O	Thin layer (silica gel), chloroform : methanol : NH_4OH (60 : 35 : 5)

Table 2. $^{14}C/^{3}H$ ratio of triacetate and diacetate of tetrahydroaldosterone

Sample	Triacetate	Diacetate
1	4·73	3·22
2	3·39	2·14
3	9·22	6·21

Table 3. Duplicate determinations of tetrahydroaldosterone.

Sample	Determination 1	Determination 2
	(μg/24 h)	
1	20·13	20·68
2	25·55	24·86
3	47·66	47·90

bility of the method, assessed by processing equal aliquots of three urine samples in duplicates showed close agreement within the separate determinations (Table 3).

ESTIMATION OF URINARY ALDOSTERONE SULFATE (AS)

Urinary AS was estimated as free aldosterone after enzymatic hydrolysis of the partially purified conjugate. Aldosterone diacetate was purified by a double isotope technique [15]. Since AS is present in very low concentration in the urine compared to Oxo-C, and since the sulfate is estimated as free aldosterone after enzymatic hydrolysis, it is pertinent that these two conjugates be adequately separated before final estimation of the free steroid. The procedure involved is summarized in Fig. 4.

Between 200–300 ml aliquots of 24 h urine collections were processed after addition of 10,000 d.p.m. of ^{3}H–AS in 0·5 ml methanol as internal standard. The

Fig. 4. Scheme for separation and estimation of sulfate and glucuronide of aldosterone.

urinary steroids were then extracted on an Amberlite column (2·1 × 20 cm) and the resultant residues were chromatographed on a small column (1 × 21 cm, with a reservoir capacity of 200 ml) of DEAE-Sephadex for the separation of Oxo-C from AS. After transfer of the sample, the column was eluted successively with 30 ml distilled water, 50 ml of 0·12 NaCl solution and finally with 50 ml of 0·22 M NaCl solution. The column was allowed to flow by gravity and 10 ml fractions were collected. In a series of elutions experiments whereby ^{3}H-Oxo-C and ^{14}C-AS were both added to the urine samples, it was found that fractions 5–8 (0·12 M NaCl eluate) contained Oxo-C while fractions 10–13 (0·22 M NaCl eluate) contained AS (Fig. 5). The two conjugates of aldosterone were well separated by this gradient elution technique and the elution patterns were consistently reproducible. In instances where estimation of Oxo-C was desired, the 0·12 M NaCl eluate was collected, subjected to pH 1 hydrolysis and the free aldosterone was estimated as described. The 0·22 M NaCl eluate which contained AS was evaporated and further purified by chromatography in system N for 18 h with methylene blue dye and AS running in parallel to serve as visual and U.V. indicators. The blue dye

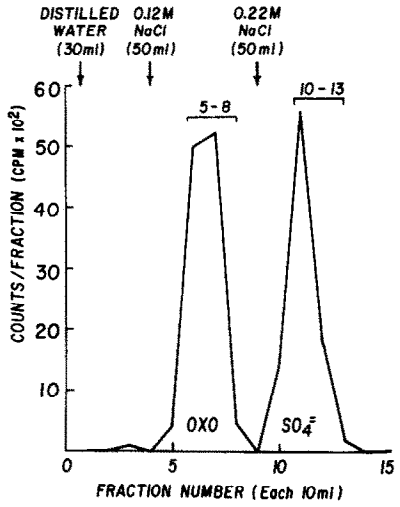

Fig. 5. Separation of urinary sulfate and glucuronide of aldosterone on DEAE-Sephadex.

migrated 10–12 cm, AS migrated 20–22 cm ($R_{dye} = 2{\cdot}08$), while Oxo-C remained close to the origin. The AS area was cut out, eluted with methanol and the eluate was evaporated and dissolved in 20 ml of 0·1 M acetate buffer (pH 5). The AS was hydrolysed by incubation of the mixture with 500 units/ml of sulfatase at 46°C for 48 h. The recovery and quantification of free aldosterone were exactly as described for Oxo-C. Under these conditions, the overall recovery of the method was 4–12 per cent.

Statistical treatments were made according to standard statistics procedures [17].

RESULTS

Analysis of labeled aldosterone urinary metabolites

The 0–6 h urinary extract was chromatographed on a column of gel dimension of 0·9 × 50 cm (0–0·4 M NaCl, 400 ml water and 400 ml 0·4 M NaCl); the 7–48 h urinary extract was chromatographed on a column with gel volume of 1·5 × 56 cm (0–6 M NaCl, 600 ml water and 600 ml 0·6 M NaCl). The elution patterns of both columns are identical, with three distinct zones of radioactivity resolved: zone A, free steroids; zone B, glucosiduronates; zone C, sulfates. A typical elution pattern is indicated in Fig. 6.

Glucosiduronates. The conjugates from zone B of the 0–6 h urine aliquot was recovered on a 4·5 × 20 cm Amberlite column and further purified by celite (Johns Manville 545, Acid washed) column partition chromatography in system K. The column (36 g celite, 1·5 cm i.d.) was dry packed according to the method of Siiteri [14]. The major peak of radioactivity (fractions 65–80; Fig. 7) was recovered and was further purified by paper chromatography (Whatman No 3MM) in system L for 16–18 h. Aldosterone-18-glucuronide (18–20 cm) and tetrahydroaldosterone glucuronide (25–27 cm) are well separated in this system (Fig. 8), with methylene blue dye (visual marker) between the two compounds at 21–23 cm from the origin. Elution of the respective peak areas yielded $1{\cdot}62 \times 10^6$ d.p.m. of aldosterone-18-

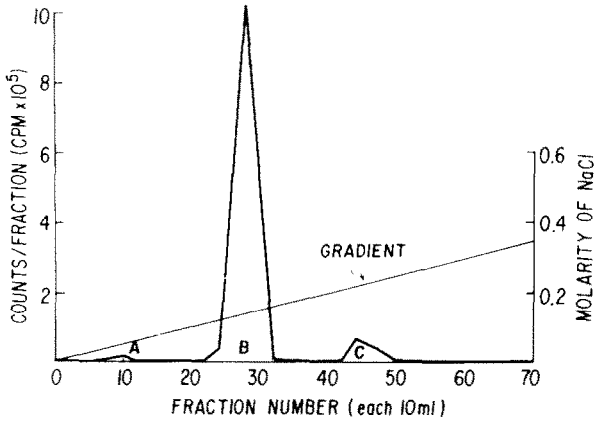

Fig. 6. DEAE-Sephadex chromatography of urinary metabolites (0–6 h) after intravenous injection of (^{3}H)-aldosterone.

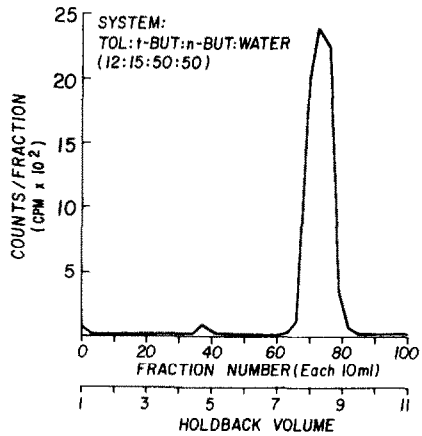

Fig. 7. Celite column chromatography (system K) of glucuronide fraction.

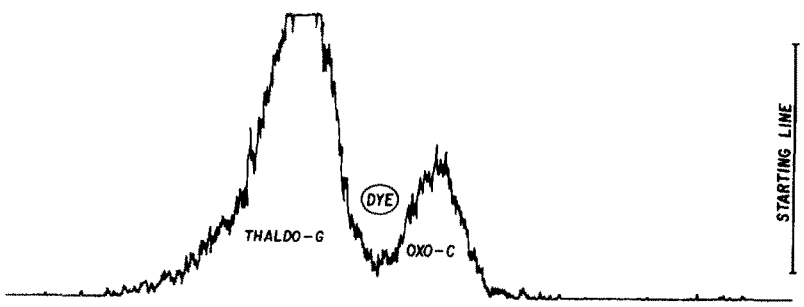

Fig. 8. Radio-scan of paper chromatographic separation of aldosterone-18-glucuronide (Oxo-C) and tetrahydroaldosterone glucuronide (THALDO-G).

glucuronide (SA $2{\cdot}52 \times 10^5$ d.p.m./μg aldosterone) and $2{\cdot}82 \times 10^6$ d.p.m. of tetrahydroaldosterone glucuronide (SA $4{\cdot}01 \times 10^5$ d.p.m./μg tetrahydroaldosterone). Subsequent hydrolysis of appropriate aliquots of each of the conjugates at pH 1 (Oxo-C) and with Ketodase (THA-G) and chromatography of the free steroids in system A for 16–18 h yielded a single peak of radioactivity with identical mobility as aldosterone or tetrahydroaldosterone. Chromatography of the acetylation products in system E (aldosterone diacetate) and in system I (tetrahydroaldosterone triacetate) also showed a single peak of radioactivity which corresponded to the respective acetates, indicating that the two isolated aldosterone metabolites are adequately purified with respect to radioactive contaminations. These two conjugates were therefore used as recovery indicators without further purification. Between determinations, the conjugates were evaporated and stored in glass flasks at −10°C. Under these conditions, minimal destruction of the conjugates was observed.

Sulfate. The conjugates from zone C (after DEAE-sephadex chromatography) of 0–6 h and 7–48 h urine aliquots were combined and recovered on an Amberlite column as described. This "sulfate pool" contained $1{\cdot}35 \times 10^6$ d.p.m. which represented 2·56% of the injected radioactivity. After addition of $8{\cdot}04 \times 10^4$ d.p.m. of [^{14}C]-aldosterone-21-monosulfate (^{3}H/^{14}C = 16·8) to this pool, the residue was further purified on a 25 g celite partition column (HBV = 60 ml) in system M. The fractions (39–52) (Fig. 9) yielded a residue containing $5{\cdot}75 \times 10^5$ d.p.m. of ^{3}H

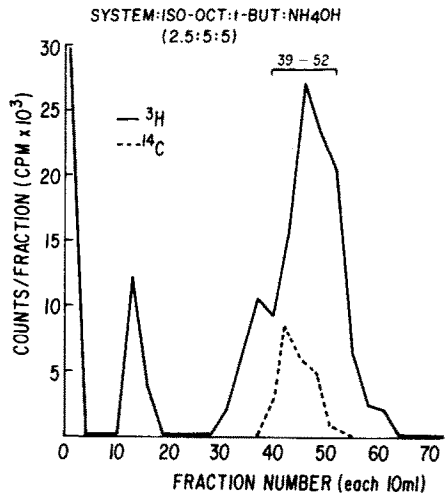

Fig. 9. Celite column chromatography of "sulfate pool."

and 6·75 d.p.m. of ^{14}C (^{3}H/^{14}C = 8·51); other radioactive peaks which did not contain ^{14}C were not investigated. Paper chromatography of this material in system N yielded a major peak of radioactivity ($2{\cdot}99 \times 10^5$ d.p.m. of ^{3}H and $6{\cdot}28 \times 10^4$ d.p.m. of ^{14}C, ^{3}H/^{14}C = 4·77) with the same mobility as AS. Further purification of this material by thin-layer chromatography in system O indicated a single radioactive peak with identical running rate as AS ($R_F = 0{\cdot}45$); this zone had $2{\cdot}74 \times 10^5$ d.p.m. of ^{3}H and $5{\cdot}79 \times 10^4$ d.p.m. of ^{14}C (^{3}H/^{14}C = 4·73). Crystallizations of this product with authentic AS in methanol–methylene dichloride and methanol–acetone mixtures resulted in no significant changes in ^{3}H/^{14}C ratio in

both the crystals and mother liquors. The mp of the crystals (AS as ammonium salt) was 135–138°C and the I.R. spectrum was identical with that of AS (Fig. 1). Using the ^{14}C counts of the crystals in the final crystallization as an indicator for the recovery of ^{3}H counts, a total of $3{\cdot}73 \times 10^{5}$ d.p.m. of ^{3}H associated with AS was estimated in the "sulfate pool"; this corresponds to approximately 0·7% of the injected radioactivity recovered in the urine within 48 h after the injection.

Urinary aldosterone glucuronides

Using the isolated radioactive Oxo-C and THA-G as recovery indicators, the mean urinary excretion of Oxo-C and THA-G estimated in five normal subjects were $10{\cdot}01 \pm 0{\cdot}89$ (SE) μg/24 h and $3{\cdot}05 \pm 5{\cdot}32$ μg/24 h respectively (Table 4).

Table 4. Urinary glucuronides of aldosterone in control subjects.

Subjects	Age	Sex	Oxo-conjugate	Tetrahydroaldosterone
			(μg/24 h)	
F.V.	23	M	8·35	20·13
Y.L.	22	M	11·24	39·33
O.D.	24	F	7·61	22·59
J.D.	22	F	10·46	25·55
N.T.	24	F	12·40	47·66
		Mean	10·01	31·05
		S.E.	0·89	5·31

Urinary aldosterone sulfate

The urinary excretion of this metabolite of aldosterone in normal subjects ranged from 0·48–4·18 μg/24 h, with a mean value of $2{\cdot}09 \pm 0{\cdot}64$ μg/24 h. In the hypertensive individuals, this range was 1·30–4·32 μg/24 h, mean $2{\cdot}83 \pm 0{\cdot}45$ μg/24 h (Table 5). Although the mean urinary excretion rate of AS was slightly higher

Table 5. Urinary aldosterone sulfate in control and hypertensive subjects

Subjects	Age	Sex	Aldosterone sulfate
Control			(μg/24 h)
D.C.	23	M	1·50
A.T.	22	M	1·49
L.L.	22	F	2·84
P.B.	24	M	0·48
J.C.	31	M	4·18
		Mean	2·09
		S.E.	0·64
Hypertensive			
B.F.	40	M	3·67
M.S.	33	M	2·29
B.	49	M	3·17
L.	33	F	1·30
G.H.	48	F	2·23
R.E.	44	M	4·32
		Mean	2·83
		S.E.	0·45

Table 6. Urinary sulfate and glucuronide of aldosterone in primary aldosteronism

Subject	Age	Sex		Sulfate	Glucuronide
				(μg/24 h)	
J.C.	28	F	(pre-op)	13·76	58·52
			(post-op)	0	0·22

in the hypertensive group, this difference was not statistically significant ($t = 0{\cdot}97$).

Urinary AS was also measured in one surgically proven case of primary aldosteronism. Prior to the operation, this patient had an elevated urinary AS of 13·8 μg/24 h. Similarly, urinary Oxo-C was also elevated at 58·5 μg/24 h. Subsequent to subtotal adrenalectomy, urinary AS was undetectable, while urinary Oxo-C decreased to a subnormal value of 0·22 μg/24 h (Table 6).

DISCUSSION

Several methods have been described for the partial purification of the major urinary metabolites of aldosterone conjugated with glucuronide acid. The method described by Underwood and Tait[1] yields the highly purified Oxo-C at the sacrifice of THA-G, since it requires pretreatment of the urine sample with mammalian β-glucuronidase to eliminate the latter glucuronide. Pasqualini and co-workers[5] and also Möhring[3] have reported methods for the partial purification of both, Oxo-C and THA-G by the use of alumina for the preliminary separation of these two conjugates. These two methods are very useful on a preparative scale, but the use of alumina as absorbant can often result in structural modification and loss by non-specific and irreversible adsorption of the steroids or their conjugates on the column.

Consequently, the method described in this communication was developed. Urine was collected for only 6 h subsequent to injection of ^{3}H-labeled aldosterone because previous studies[1, 6] have shown that within this period of time the radioactivity associated with the conjugates of choice are excreted in high yields, particularly in the case of the Oxo-C[1]. The extraction of the urinary conjugates on an Amberlite resin column gave a relatively clean extract with quantitative recovery of the radioactivity (> 98%). Group separation of the urinary conjugates into the glucuronide and sulfate fractions was then achieved by DEAE-Sephadex ion-exchange column chromatography with minimal losses. A high degree of sample purification could be achieved by the use of this type of chromatography as the bulk of the pigments were either eluted off the column within the first bed volume, or they were retained on the column. Further purification of the glucuronide fraction was then effected by celite column partition chromatography using a neutral system. Final separation of Oxo-C from THA-G was then accomplished by paper partition chromatography. The minimal use of acidic systems during the purification procedure is essential because the Oxo-C is unstable at lower pH, and frequent use of such systems could result in lower yields of this metabolite of aldosterone[1]. In the method presently described, the only acidic system used was that of the final purification step (System L). It should be pointed out that in preliminary studies, the paper chromatographic system ethyl acetate: n-butanol acetic acid: water (50:450:75:425) was used for the final separation of the two aldosterone conjugates. This system was used by Underwood and Tait

[1] for the purification of Oxo-C and subsequently used by Möhring [6] for the separation of Oxo-C and THA-G. Although this system does separate the two aldosterone conjugates, the peaks resolved (evidenced by radio-scanning and subsequent hydrolysis) were diffused and resulted in considerable overlapping of the two conjugates. As a result, system L was developed which gave excellent resolution of the conjugates as two compact peaks of radioactivity (Fig. 8). Methylene blue was chromatographed in parallel to serve as a visible reference marker to provide more precise control of the chromatography. The radioactive purity of these two isolated compounds was indicated by the recovery of one single radioactive peak which corresponded to the respective free steroids and their acetates after appropriate hydrolysis of the respective conjugates. While radioactive purity of the conjugates is pertinent as recovery markers, chemical purity is not absolutely essential.

If 5000 d.p.m. were used for each sample determination, each batch preparation according to the present procedure (injection of $5{\cdot}26 \times 10^7$ d.p.m. of [^{3}H]-aldosterone) could furnish sufficient conjugates for about 300 determinations of Oxo-C and about 500 determinations of THA-G.

The 24 h urinary excretion of the oxo-conjugate determined in five normal subjects maintained on a fixed sodium and potassium intake was 8·35–12·40 μg (Table 4). These values are within the normal ranges of urinary aldosterone reported by others using the double isotopic technique [18], by gas chromatography [19] or by radioimmunoassay measurements [20, 21]. Similarly, the twenty-four urinary excretion values for tetrahydroaldosterone (20·13–47·66 μg, Table 4) are also compatible with previous reported values for urinary tetrahydroaldosterone for normal individuals [22–24]. Thus, the values obtained by the use of the present procedures for the accurate determination of Oxo-C and of THA-G are reliable for any meaningful interpretation.

After intravenous injections of tracer amounts of tritiated aldosterone to normal subjects Pasqualini [7] demonstrated that small amounts of radioactivity corresponding to aldosterone was associated with the ester–sulfate fraction in the urines and plasmas of these individuals. To date, the actual characterization of sulfo-conjugated aldosterone has not been carried out in either human or animal species. In the present study, the chemical synthesis and characterization of AS is described. Furthermore, after intravenous injection of a tracer dose of tritiated aldosterone into a normal male subject, AS was isolated from the urine by reverse isotopic dilution technique, using a mixture of synthetic ^{14}C-labeled and unlabeled AS, thus confirming the existence of this conjugate of aldosterone in man. In order to establish the urinary excretion rate of AS, a simple method has been devised for the separation of AS from the Oxo-C; the free aldosterone liberated after sulfatase hydrolysis was then quantitated by a conventional double isotope technique. The mean urinary excretion rate of this compound estimated in five normal subjects under balanced sodium and potassium intake was $2{\cdot}09 \pm 0{\cdot}64$ μg/24 h (Table 5). This observation of low urinary excretion rate of this metabolite of aldosterone is consistent with the indirect evidence obtained by tracer studies that endogenous conversion of aldosterone to this metabolite is minimal in normal individuals [7]. In the small group of patients with BEH studied, the mean 24 h urinary excretion rate of AS was slightly higher ($2{\cdot}83 \pm 0{\cdot}45$ μg/24 h) when compared to that of the control group (Table 5). Due to the relatively small number of normals and patients studied, this apparent difference is only suggestive

and must be interpreted with caution. However, there is evidence that patients with essential hypertension excreted significantly higher amounts of sulfo-conjugated metabolites of cortisol, and that this increase was due to the presence of higher amounts of metabolites of cortisol which did not require ring A reduction [25]. Furthermore, it has also been shown that liver preparations obtained from experimentally induced hypertensive rats have significantly higher sulfokinase activity [26]. Thus the present suggestive evidence that individuals with BEH have a tendency towards excretion of higher amounts of urinary AS can possibly be related to a compensated shift of the metabolic pathway which did not require A ring reduction as previously discussed, or it may be due to an increase in the hepatic sulfokinase activity of these hypertensive individuals, or a combination of the two possibilities.

In the single case of surgically confirmed primary hyperaldosteronism studied, overproduction of aldosterone was indicated by the excessively elevated urinary oxo-conjugate excretion and a six-fold increase in urinary AS (Table 6). Subsequent to sub-total adrenalectomy, urinary oxo-conjugate fell to a sub-normal value of 0·218 μg/24 h and urinary AS was undetectable. The elevated AS observed in the pre-operative urine was probably the result of peripheral conversion of the excessive hormone produced by the adenoma, although the possibility of AS secretion by the adenoma cannot be excluded. However, adrenal secretion of AS has not been demonstrated in normal or in diseased states.

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