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RAPID CHROMATOGRAPHIC PURIFICATION OF URINARY STEROID GLUCURONATES FOR DETERMINATION OF ALDOSTERONE SECRETION RATE

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SUMMARY

Aldosterone secretion rate (ASR) is utilized as a rapid screening procedure to detect subtle forms of hypertension. A rapid and robust chromatographic method has been developed, based on easily prepared, rigid matrices, which permit the flow of urine by suction through sequential purification columns. Both major metabolites, aldosterone glucuronate and tetrahydroaldosterone glucuronate, are isolated in *ca.* 75% yield. They are hydrolyzed and quantitated by high-performance liquid chromatography–radioimmunoassay (HPLC–RIA) or, for bulk preparation, further purified by preparative chromatofocusing. These two polar conjugates are isolated in nearly pure form by HPLC on a C_2 column with a two-step gradient. An ASR determination can be completed in one to two working days; preparative-scale work takes somewhat longer.

INTRODUCTION

Several complex physiological mechanisms mediate blood pressure homeostasis. Aldosterone is a major hormone in this process by an indirect control of ion transport. Several types of alterations of metabolism of mineralocorticoids may occur; one is altered secretion rate, so that the steady-state level of aldosterone differs from that of the normal population. When the standard screening method of saline infusion is used, about 2% of demonstrably hypertensive patients are characterized as cases of primary aldosteronism[1]. However, it has been suggested that alternate protocols may show that the incidence of this subset of hypertension may be high[2,3]. Currently, indirect methods, based on plasma aldosterone levels[1] or urinary metabolites[3,4], are standard. A direct measurement of secretion rate is likely to uncover subtle forms of mineralocorticoid abnormalities.

A convenient method for determination of the secretion rate utilizes a pulse-chase technique, in which known amounts of labeled aldosterone are presented at the start of the protocol, and pooled urine is examined for labeled material to find the rate of excretion. This value, compared with total aldosterone, permits computation of the secretion rate. Previous methods for the purification of aldosterone or its major metabolite, tetrahydroaldosterone (THA), to constant specific activity were

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slow and tedious[6–8]. The development of an improved method would facilitate rapid screening of hypertensives and would permit other applications of aldosterone secretion rate (ASR) determination.

The procedure described here is based on physical properties of the two major excretion products of aldosterone, aldosterone glucuronide (AG) and tetrahydroaldosterone glucuronide (THAG)[3,5], comprising about 1/4 and 3/4 respectively, of the total metabolites and allows rapid purification of these components for ASR or other studies. The glucuronates are removed from the majority of interfering materials in urine by using three bonded silica matrices in sequence. This method utilizes obverse chromatography, in which interfering components are removed by binding to differing functionalities, while the compounds of interest are unretained. For ASR, the glucuronate of interest is hydrolyzed[9], the released aldosterone is separated from other polar components, isolated by high-performance liquid chromatography (HPLC) and quantitated by radioimmunoassay (RIA). Alternatively, for bulk purification of glucuronates, the partially purified urine is fractionated further by preparative chromatofocusing (CF), followd by HPLC of the intact glucuronates.

MATERIALS AND METHODS

Materials

Bonded-phase silica derivatives (Sepralytes): methyl (C_1), ethyl (C_2), octadecyl (C_{18}), strong anion exchanger (SAX), phenyl (PH), and strong cation exchanger (SCX) were purchased from Analytichem (Harbor City, CA, U.SA.). PB 94 and buffer PBE were obtained from Pharmacia (Piscataway, NJ, U.S.A.) and imidazole from Sigma (St. Louis, MO, U.S.A.). Econocolumns (Bio-Rad Labs., Richmond, CA, U.S.A.) 30 × 1.0 cm I.D., were used for narrow-range CF with a Gilson Rabbit peristalic pump and an Amicon (Danvers, MA, U.S.A.) 60 × 1.5 cm I.D. column for wide-range CF. An ISCO (Lincoln, NE, U.S.A.) Retriever fraction collector with graduated test tubes or scintillation vials was used for collecting the effluent. Methanol, ethanol, 2-propanol, isooctane, hexane, dichloromethane, and ethyl acetate HPLC-grade solvents were from Fisher Scientific (Pittsburgh, PA, U.S.A.) or Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) Water was deionized and distilled. All solvents were filtered through a Rheodyne (Cotati, CA, U.S.A.) 0.45-μm nylon disk prior to use. Samples for HPLC were filtered through a Millipore (Bedford, MA, U.S.A.) HV 0.45-μm filter or Supelco (Bellefont, PA, U.S.A.) pipette tip 2-μm filter. Syringes used as mini-columns were purchased from Monoject (St. Louis, MO, U.S.A.) and used with 20-μm frits, purchased from Analytichem. A Glenco (Houston, TX, U.S.A.) HPLC apparatus with a Rheodyne 7125 injector and 200-μl sample loop was utilized with an Analytichem Diol column (5-μm packing, 30 × 0.46 cm I.D.) with a Brownlee (Santa Clara, CA, U.S.A.) 10-μm Diol guard column or Analytichem C_2 column with C_2 guard column. [1,2-^{3}H]Aldosterone and [4-^{14}C]-aldosterone were obtained from New England Nuclear (N. Billerica, MA, U.S.A.) and repurified by HPLC prior to use. A Packard (Downers Grove, IL, U.S.A.) 460 C scintillation counter was used for ^{3}H determination. Aquasol II (New England Nuclear) was used as the counting cocktail, except for the RIA of aldosterone, where a toluene–methanol (98:2, v/v) cocktail, containing 0.4% 2,5-diphenyloxazole, 0.008% *p*-bis(O-methylstyryl benzene) was used.

Preparation of [1,2-^{3}H]aldosterone for the secretory rate determination

[1,2-^{3}H]Aldosterone was purified by HPLC on a Diol column with isooctane–dichloromethane–ethyl acetate–ethanol (65:15:15:5) as the eluent. The peak was collected, evaporated to dryness, reconstituted in 100% ethanol and filtered through a Millipore HV (0.45-μm) filter. An aliquot equivalent to 1.0 μCi was taken and made up in 150 ml of 5% glucose solution.

Clinical procedure

Normal and hypertensive patients were placed on a restricted, salt-controlled diet for 4 days[2], at which time the 1.0 μCi prepurified [1,2-^{3}H]aldosterone solution was infused. Refrigerated urine was pooled for the first 24-h period and stored at −20°C until used. The study protocol (approved by the Human Research Committee of UTMB) was explained to all subjects.

Urochrome removal

Initial studies showed that if large volumes of urine are to be processed, the removal of interfering components is mandatory, since they form an intractable, tarry mass during sample concentration. It was ascertained that the visual criterion of removal of colored material from the sample is sufficient to indicate purification adequate for further processing. The first series of steps removes the bulk of these urochromes, while permitting AG and THAG to be collected. Urine volumes are processed in multiples of 1/25 daily output. Sepralytes are packed dry into suitably-sized Monoject syringes with 20-μm frits, primed with 5 bed volumes of methanol and 5 bed volumes of water just prior to use, and urine is passed through these columns by suction into sidearm flasks at 3–5 ml/min (see Fig. 1).

(1) A volume of untreated urine, corresponding to 1/25 of the 24-h pool is first processed on a 5 g (10 ml) C_2column; this eluate (containing most of the inorganic ions) is discarded. A 10-g (20-ml) SCX column is positioned below the C_2 column, and the steroid glucuronates are eluted with 40 bed volumes (1.2 l) of distilled water. Hydrophobic urochromes and the small amount of free steroids present in the urine are retained on the C_2 matrix, and cationic urochromes are trapped by the SCX column.

The eluent is brought to pH 7.5–8.0 with 2.0 *M* imidazole–hydrochloric acid (pH 8.0) and passed through 5 g (10 ml) SAX Sepralyte, primed with 5 bed volumes of each: methanol, water and 50 m*M* imidazole–hydrochloric acid (pH 8.0). The

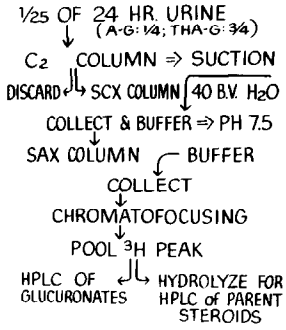

Fig. 1. Flow chart for steroid glucuronate purification from crude urine.

material eluted from C_2-SCX is passed through and collected with 3 bed volumes of 50 m*M* imidazole buffer; anionic urochromes are retained by the SAX column.

(2) For ASR studies, this eluate is brought to pH 1.0 ± 0.1 with *ca.* 0.05 volumes of 2.5 *M* hydrochloric acid–potassium chloride buffer, heated to 100°C for 5 min for the specific hydrolysis of aldosterone glucuronate, then cooled and separated from the intact THAG. This method depends on the weak adsorption of highly polar glucuronates (THAG) *vs.* the strong adsorption of liberated aldosterone on the hydrophobic column. The hydrolyzed material is brought to pH 6.5–7.5 with about 0.05 volumes 2.5 *M* Tris, and passed through a 2.5-g (5-ml) C_1 column. The THAG is eluted selectively with 10 bed volumes of 10% ethanol, and the liberated aldosterone is eluted through a Millipore or Supelco filter in 3 bed volumes of 100% ethanol. This highly purified aldosterone is dried and used for HPLC. Fractions of 1 ml are collected, analyzed for ^3H and aldosterone (off-line RIA), and the secretion rate is calculated[10]. This procedure can be completed in 1.5 working days.

Bulk glucuronate preparation

For bulk purification of AG and THAG, the procedure is scaled up by using 50% or more of the 24-h urine sample, with proportionate increases in the Sepralyte amounts. The urine from the SAX clean-up step is left at pH 7.5 and applied directly to the CF column, or acidifed as described above and passed through a Sepralyte C_{18} column, (2.5 g, per 1/25 daily urine collection). The column is air-dried, and the glucuronate is eluted with 3 bed volumes acetone. The eluate is concentrated under a nitrogen stream in a warm water bath, reconstituted in a suitable buffer, and applied to the CF columns.

Chromatofocusing

A 30 × 1.0 cm I.D. Econocolumn was packed with PB 94 to a 10.6-ml volume with 25% ethanol at 100 ml/h. The column was washed with 5 bed volumes water, and equilibrated with 15 bed volumes of 25 m*M* piperazine–hydrochloric acid (pH 5.5) for the narrow-range gradient. The eluate from the SAX column was brought to this pH with 2.5 *M* piperazine–hydrochloric acid and passed through the column at 60 ml/h. PBE 74–HCl pH 3.8, diluted 1:10 was used to elute the glucuronates at 60 ml/h. Fractions of 5 ml were collected, the pH of each was measured, and small

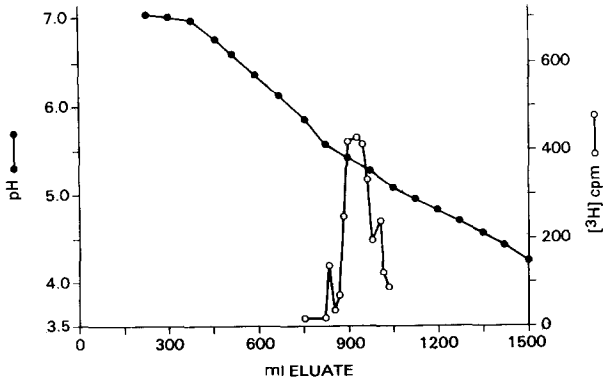

Fig. 2. Purification of steroid glucuronates on wide range chromatofocusing.

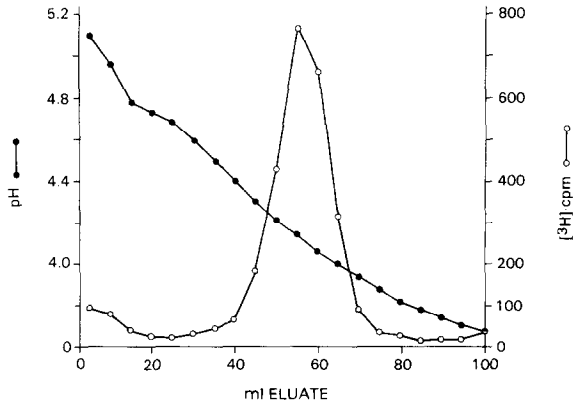

Fig. 3. Purification of steroid glucuronates on narrow range chromatofocusing.

aliquots were taken for ^{3}H-determination (see Fig. 2).

Alternatively, for wide-range pH gradient, a 40 × 1.5 cm I.D. column was packed to a 150-ml bed volume (19-cm leight). The column was primed with 15 bed volumes of imidazole–hydrochloric acid pH 7.4, and urine extract equivalent to ½ of a 24-h period at pH 7.5 was applied to the column; PBE 74–hydrochloric acid (pH 3.7) was used to elute the glucuronates. Volumes of 15 ml were collected, the pH was measured, and aliquots were taken for ^{3}H determination (see Fig. 3).

Both columns were purged for reuse with 1 *M* sodium chloride and 25% ethanol (5 bed volumes each). This also demonstrated that residual radioactivity was not detected after the PBE elution.

The radioactive peak from the CF purification was pooled and concentrated on a phenyl Sepralyte column. Of this matrix 5 g (10 ml) per 1/25 total daily urine collection was packed in a suitable Monoject syringe, primed with 5 bed volumes of each, methanol and water, and the CF pooled glucuronate peak was applied. The PBE buffer was washed out with 5 bed volumes water and the glucuronates were eluted with 3 bed volumes 100% ethanol. This eluate was filtered and evaporated to dryness, and the two polar conjugates were separated by HPLC on a C_2 column.

Glucuronate resolution

The CF-purified material contains both AG and THAG. They are resolved by a step gradient on a C_2 column.

A buffer of 25 m*M* ammonium acetate–acetic acid (pH 4.3) with either 7.5% or 12% acetonitrile was prepared. The C_2 column was equilibrated in the 7.5% acetonitrile buffer and the glucuronate mixture was applied to the column. The flow-rate was maintained at 0.5 ml/min; after 4 ml was collected, the solvent was switched to 12% acetonitrile buffer, an additional 26 ml collected, and the mobile phase was changed to 100% methanol.

Each ^{3}H peak (8–12 ml, 20–25 ml and 40–43 ml) was pooled and analysed individually. Hydrolysis and further separation of aldosterone was used as proof of identity. Acid hydrolysis releases aldosterone only; enzymatic hydrolysis releases THA but not aldosterone from the respective glucuronates. These procedures (data not shown) give evidence that the first peak was AG, the second THAG, and the

methanol fraction consisted of low-polarity degradation products. Large amounts of UV-absorbing material were eluted with the AG and methanol degradation products; the THAG peak showed very low absorbance at 260 nm and was presumably quite pure.

RESULTS

Urine contains large amounts of highly heterogenous urochromes; the majority of the visible material is between 500 and 5000 daltons, as shown by diafiltration (Amicon stirred cell with suitable cut-off membranes) in a series of preliminary experiments (data not shown). Initial studies showed that it is essential to remove these materials, which become intractable if retained. Therefore, a series of sequential chromatographic procedures was developed to remove the three major classes of these components while allowing the steroid glucuronates to be eluted in high yield.

Visual monitoring showed that different classes of urochromes were removed at each of the three steps of Sepralyte separation. Radioactivity was monitored at each step to determine recovery (Table I). C_2 Sepralyte has the convenient property of fractionating urine into three components: (1) unretained material, consisting of very polar molecules, such as inorganic ions and small very hydrophilic molecules and most of the proteins; (2) steroid glucuronates and other moderately polar material eluted at very low ionic strengh (40 bed volumes) or with 10% ethanol (20 bed volumes); and (3) residual free steroids, hydrophobic urochromes, and other components eluted by 100% ethanol. For this reason, this matrix obviates the lengthy and difficult usual first step of liquid–liquid extraction in the removal of trace amounts of free aldosterone.

The remaining cationic urochromes were removed on the SCX column, and most anionic urochromes on the SAX column. To preclude retention of steroid glucuronates on the SAX column, they are in the uncharged state (pH 7.5) where they have little affinity for this column.

CF allows further purification of the glucuronates, since only material of a similar pK_a is pooled. This is an obligatory step for bulk preparation of the glucuronates, since, if it is bypassed, the C_2 HPLC column is readily overloaded.

The identity of the two major peaks on the C_2 column was established by differential hydrolysis, allowing our procedure to utilize the specificity of acid hydrolysis (aldosterone glucuronate only) and enzymatic hydrolysis (THAG and other steroids not germane to this study). It must be noted that long-term storage and/or improper handling of samples leads to a large ^3H degradation peak on the C_2 column.

TABLE I

RECOVERY (%) OF ^3H LABEL DURING PURIFICATION

Step	*Recovery ± S.D. (n = 14)*
Crude urine	100
C_2-SCX	72.8 ± 6.6
SAX	70.2 ± 6.0
Chromatofocusing	63.2% (n = 3)

DISCUSSION

Our goal was to develop a rapid, easy, and robust method for treating urine for both ASR and, when scaled up, for the bulk preparation of steroid glucuronates. The former method is used for rapid screening in hypertensive patients. The latter is needed for purification of the major excretory product of aldosterone, THAG. This metabolite is being prepared in milligram quantities for the preparation of monoclonal antibodies as an alternative method of aldosterone determination, since hydrolysis would be obviated.

Many early studies of ASR involved a lengthy procedure, including a radiolabel derivatization step[11–14]. This method is arduous and therefore not suitable for a screening test.

Previous methods for ASR determinations have involved acid hydrolysis with subsequent purification of liberated aldosterone[7–9] or glucuronidase hydrolysis with subsequent purification of liberated THA[6,9]. We prefer the acid hydrolysis method, since it allows separation of aldosterone from its metabolites and other steroids. However, aldosterone is not the only steroid liberated by acid hydrolysis[15]. Furthermore, a disadvantage of this method is that the major metabolite of aldosterone is not isolated, and this necessitates an increase in the amount of urine that must be processed. Although this method has been developed to purify THA, the major metabolite of aldosterone, as well, we prefer the specificity of acid hydrolysis (for AG), since glucuronidase (for THAG) liberates large amounts of tetrahydro metabolites of cortisol and other steroids[16] which must be separated from THAG due to RIA cross-reactivity[10].

In earlier studies, conversion of the partially purified aldosterone to a ^{14}C-acetylated derivative was carried out to allow quantitation. Later comparisons of the double isotope method validated aldosterone quantitation by simpler fluorometric methods[13,16,17] or by RIA of a 21-monoacetylated derivative [12]. Aldosterone quantitation has also been reported by gas chromatography (GC)[18] and GC–mass spectrometry[19]. RIA has advantages over fluorometric and GC methods, since it requires less costly instrumentation and is more sensitive. The method based on formation of the 21-monoacetate derivative of aldosterone has utility, but it is flawed by possible incomplete conversion to the derivative and instability of the 21-monoacetate in either the patient sample or standards.

Purification of aldosterone after acid hydrolysis has been accomplished by several methods. Paper chromatography was commonly used in earlier methods, either exclusively or in combination with thin-layer or column chromatography[20,21]. Though these methods are relatively inexpensive, they are quite tedious and time-consuming. All except column chromatography are poorly suited to the removal of various interfering materials. Furthermore, their ability to separate aldosterone from other cross-reacting steroids and/or metabolites of aldosterone is limited. HPLC offers advantages over other types of chromatography, because it has high separation efficiency, is rapid, and is suitable for bulk preparation and collection.

Other bonded-phase materials, including C_{18} Sep-Pak, have been utilized for the isolation of steroids and their conjugates[19,22–24]. However, we were unable to elute aldosterone conjugates with 10% ethanol, and sufficiently high concentrations of ethanol for elution also caused elution of free aldosterone and large amounts of

urochromes. As we found that aldosterone could be eluted more easily from C_1 than C_2 columns with small volumes of ethanol, C_1 was utilized after hydrolysis. It is important to note that bonded-phase materials supplied by different manufacturers may not behave alike, primarily due to variations in the extent of end capping of the silanol residues. These differences may cause considerable variations in their binding capacity and must be considered when optimizing the technique for specific applications.

Minicartridges of diatomaceous earth (Extralut, E. Merck, Darmstadt, F.R.G.) have been reported to perform similar functions of binding steroids from biological fluids[25]. However, lengthy equilibration is required, and their binding capacity appears to be only about 1/10th that of C_2. A similar low capacity has been observed with Du Pont (Wilmington, DE, U.S.A.) Type W cartridge[26]. Previously, Amberlite XAD resin (Rohm & Haas, Philadelphia, PA, U.S.A.) was used to bind steroids and steroid conjugates[27], though it has not been found to work as well as Sep-Packs[23].

Several classes of urochromes are known to exist, though the majority are known to be glycoproteins[30] and bilirubin conjugates[28,29]. Unreported studies in our laboratory have shown that they are between 500 and 5000 daltons (based on diafiltration studies). The utilization of different functional groups on the bonded phases permitted high capacity and rapid removal of these heterogenous urochromes. Therefore, we feel that this matrix has advantages over the previously described matrices.

Normal-phase HPLC (with a Diol column)[31] has advantages over reversed-phase HPLC, since with volatile solvents the time for evaporation before RIA is reduced. We noted that optimization of the system allowed satisfactory purification of the liberated steroids, and insignificant contamination with cross-reacting materials.

Several technical details should be specified for others who wish to use this procedure: (1) The C_2 Sepralyte column was found to bind glucuronides of aldosterone, which are selectively eluted by either 10% ethanol or larger volumes of water, while free steroids and significant amounts of urochromes are retained. We found that application of material should be at the rate of only 3–5 ml/min, since higher loading rates cause overloading of the column. (2) We found it necessary to equilibrate the C_2 column with methanol, as recommended, to maintain full binding capacity but used ethanol for elution to avoid formation of methoxy derivaties[32] of aldosterone at later stages. (3) It is interesting to note that the pH at which the glucuronates of interest are eluted in CF depends on the starting buffer equilibration. Minor changes of pH affect both binding and competitive elution in this technique[33], and are presumably related to both charge and other types of binding to the medium. Work by Hutchens has shown that steroids bind to various polyamine constituents of the polybuffer (PBE 94) depending on pH and other parameters[34]. Therefore, the mechanism responsible for the CF separation probably involves the steroid glucuronate–polyamine complex. The specificity of the particular class of polyamines complexing with the steroid is determined by the initial pH, and thus, characteristics of the separation are influenced by column preparation.

Even though we have evaluated this procedure only for the determination of ASR and AG and THAG purification, this method, with minor modifications, is

undoubtedly applicable to a wide variety of steroid glucuronides. For example, many procedures for determining steroid secretion rates involve glucuronidase hydrolysis to obtain a metabolite of the steroid which is subsequently purified to constant specific activity. Initial isolation of the steroid glucuronides allows smaller amounts of glucuronidase to be used, and facilitates the removal of extraneous interfering materials.

An additional advantage of isolation by using suction for chromatography on rigid-bonded phases is that large amounts of urine can be processed readily. This facilitates the recovery of the desired class of compounds and allows the administration of less tritiated tracer to the patient. We found that when 1 μCi [1,2-^{3}H]-aldosterone was administered, 1/25th of the 24-h urine pool provided sufficient radioactive marker for monitoring the procedure. Additional development of this procedure will permit further reduction of tracer administered. Thus, this technique has simplified the determination of ASR and should facilitate such studies[35] and may have wider applicability to the purification of steroid conjugates.

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