

An Abbreviated History of Aldosterone Metabolism, Current and Future Challenges

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ABSTRACT

The initial isolation of adrenal steroids from large quantities of animal adrenals resulted in an amorphous fraction resistant to crystallization and identification and had potent effects on electrolyte transport. Aldosterone was eventually isolated and identified in the fraction and was soon shown to cause hypertension when in excess. The autonomous and excessive production of aldosterone, primary aldosteronism, is the most common cause of secondary hypertension. Aldosterone is metabolized in the liver and kidney, and its metabolites are conjugated with glucuronic acid for excretion. The most common liver metabolite is 3 α ,5 β -tetrahydroaldosterone-3-glucuronide, while that of the kidney is aldosterone-18-oxo-glucuronide. In terms of their value, especially the aldosterone-18-oxo-glucuronide, is commonly used for the diagnosis of primary aldosteronism because they provide an integrated value of the total daily production of aldosterone. Conversion of aldosterone to 18-oxo-glucuronide is impeded by drugs, like some common non-steroidal anti-inflammatory drugs that compete for UDP-glucuronosyltransferase-2B7, the most important glucuronosyltransferase for aldosterone metabolism. Tetrahydroaldosterone is the most abundant metabolite and the most reliable for the diagnosis of primary aldosteronism, but it is not commonly measured.

Introduction

History of adrenal discovery and function

A comprehensive history of the adrenal gland was recently published [1]. The anatomy of the human adrenal gland was first described by Bartolomeo Eustachi (1520–1574) in his book *Opuscula Anatomica*, published in 1564, with the first 8 plates of 47 engraved copper plates comprising anatomical illustrations by the Roman artist Pier Matteo Pini. A detailed drawing of the anatomy of the kidney clearly depicts the suprarenal (adrenal) glands. The plates remained in the Vatican library for 150 years until Pope Clement XI gave them to his personal physician, Giovanni M. Lancisi, who published them in 1714 as *Tabulae Anatomicae Clarissimi viri Bar-*

tholomaei Eustacci. The physiological importance of the adrenal gland was first reported in 1855 by Thomas Addison, who described hyperpigmentation, anemia, general languor, debility, feebleness of the heart's action, and gastric irritability associated with diseased suprarenal capsules [2]. Soon thereafter, Charles-Edouard Brown-Sequard reported that adrenalectomy in dogs led to death, indicating that adrenal glands were essential for life [1]. The search for an adrenal secretion led to the isolation and crystallization of epinephrine by Takamine [3]; however, functions of the adrenal medulla and cortex were quickly recognized to be distinct, and that lipoidal extracts of the adrenal were required to sustain the life of adrenalectomized animals [4, 5].

Isolation and chemical characterization of adrenal cortical steroids

The isolation of steroid hormones and chemical characterization of their structures were performed by many groups during the 1930–1940s [1]. Reichstein isolated and determined the structure of 28 adrenal corticoids, of which only 5 had biological activity [6]. Among these was cortisone, also isolated at Mayo Clinic by Kendall, who first demonstrated its biological activity [7]. Desoxycorticosterone (deoxycorticosterone or DOC) was first isolated and synthesized by Reichstein [8] and was shown to be useful in the treatment of Addison's disease [9]. Under low-stress conditions, mineralocorticoids like DOC were more potent in keeping adrenalectomized animals alive; however, under stress conditions, glucocorticoids like cortisol were more effective. However, DOC production was found to be too low [1] to explain sodium retention and edema in patients with nephrosis and congestive heart failure, so the search for another mineralocorticoid continued.

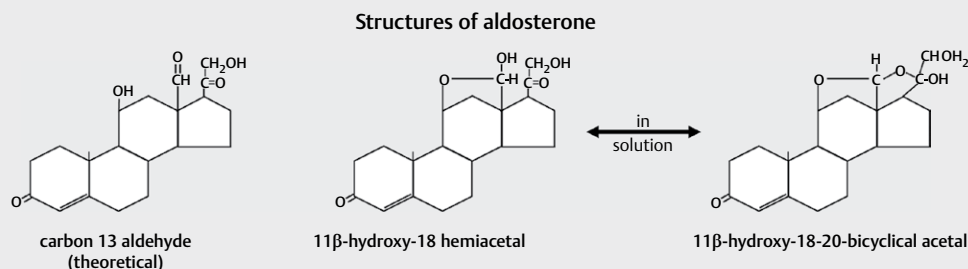
Steroids were initially isolated from large quantities of beef or hog adrenals by extraction with organic solvents, followed by various chemical and physical separatory processes, and finally by crystallization. During isolation, a significant amount of salt-retaining activity remained in a residue called the amorphous fraction which could not be crystallized [10]. The use of large doses of cortisone in clinical applications in the late 1940s and early 1950s demonstrated that it had both glucocorticoid and mineralocorticoid properties, leading several prominent steroid researchers to propose the unitarian hypothesis that cortisol was the only adrenal hormone responsible for both glucocorticoid and mineralocorticoid activity, and subsequently, also for the adrenal steroid-mediated hypertension [11, 12]. Demonstration that cortisone was efficiently converted to cortisol in extra-adrenal tissues supported this premise [13]. Others believed that a yet undiscovered steroid or mixture of steroids was responsible for the mineralocorticoid properties of the amorphous fraction and that discrepancies between results from different studies were due to different bioassays employed to determine biological effects. Kendall used the Ingle work test, which is particularly suited for detecting glucocorticoids but not mineralocorticoids, while Reichstein used the Everse and de Fremery assay, which was more specific for mineralocorticoid activity [14]. The isolation of aldosterone by the Reichstein laboratory was probably delayed by the need to acetylate the steroids prior to purification using alumina columns and because, though most 21-monoacetylate steroids are very active, the diacetate of aldosterone is completely inactive.

The development of bioassays based on the direct action of mineralocorticoids on electrolyte metabolism in the late 1940s simplified the determination of mineralocorticoid activity [15]. The use of radioactive sodium (Na^{24}) and potassium (K^{42}) injections before the collection of urine to determine the mineralocorticoid activity of the test substances increased the sensitivity of early flame photometric measurements of these analytes [16], but the use of radioisotopes became unnecessary with the development of more sensitive flame photometers [17]. A crucial development for the purification of the amorphous fraction was the development of paper partition chromatography using the Zaffaroni system of a stationary phase of propylene glycol saturated with toluene and a mobile phase of toluene saturated with the stationary phase. The

biologically active mineralocorticoid migrated at the same speed as cortisone when the system was run for 3 days, but was only partially separated after 7 days [18, 19]. The Bush system, in which the stationary phase was methanol and water saturated with benzene and the mobile phase was benzene saturated with the stationary phase (Bush B5 system), clearly separated cortisone from the mineralocorticoid active fraction [18, 20]. Material extracted from adrenal blood migrated at the same speed as the fraction isolated from beef adrenals and had been called electrocortin, later renamed aldosterone [18]. The purified fraction retained its biological function upon partial acetylation but was inactivated by extensive acetylation; its function was restored by gentle hydrolysis [21].

At the same time, John Luetscher at Stanford University was working on the identification of a salt-retaining hormone in the urine of patients with nephrotic syndrome. He initially believed it was a vasopressin-like substance, but discovered that it was very similar and more potent than DOC [22]. His attempts to purify and characterize the substance were initially unsuccessful. After the structure of aldosterone was published, the Luetscher group was able to isolate and crystallize aldosterone from human urine and demonstrate that it was identical to that isolated from beef adrenals [23]. His group's method of isolating the biologically active aldosterone from urine acidified to pH 1 to hydrolyze it became standard for measuring urinary aldosterone as it substantially increased the amount isolated [23–25].

The original identification of the structure of aldosterone used microchemical methods, including the Bush soda fluorescent method specific for the 4-en-3-one group, rapid reaction with tetrazolium, the release of formaldehyde after oxidation with bismuthate to detect the 20,21-ketols, and acetylation with [^{14}C]-acetic anhydride showing that electrocortin had two acetylable hydroxy groups, one at position 21 and the other that was initially unclear [21]. There were several possibilities for the position of the second hydroxy group. Reichstein, who had isolated 50 mg of the steroid, demonstrated that oxidation of the steroid yielded a γ -lactone [21, 26–28], suggesting the presence of an aldehyde at position 18, which, due to the proximity of the 11β -hydroxy group, forms a hemiacetal and a bicyclic acetal [21, 28, 29]. Once X-ray crystallography and NMR became available, it was shown that aldosterone in crystal form is the bicyclic acetal [30] (► **Fig 1**), while in solution, it is a mixture of the readily interconverted hemiacetal and bicyclic acetal forms [29, 31]. The parent structure of an aldehyde in position 13 drawn in many publications has never been confirmed in solution or solid state due to the very high reactivity of the -CHO group at C-13 with the 11β -hydroxy (forming a hemiacetal) and the 11β -hydroxy, the -CHO group at C-13, and 20-keto which form the more stable bicyclic acetal. The synthesis of aldosterone made it available for studies and the design of multiple methods for its measurement [32–34]. A recent quantitative NMR study of aldosterone samples from two commercial sources that were at least 95% pure by liquid chromatography with tandem mass spectrometric (LC-MS-MS) demonstrated by quantitative ^1H NMR that in the purest sample, the diastereomers are in a tautomeric equilibrium of the bicyclic acetal $18R,20S$ and $18S,20$ -oxo in a proportion of 3:4 [29]. ^1H NMR showed that this sample was 93.8% pure, while the other was only 81.85% pure. The contaminants present included dimers, aldosterone acetal, and aldosterone- γ -



► **Fig. 1** Structures of aldosterone as the theoretical carbon 13 aldehyde of aldosterone and the two acetals found in solution, 11β-hydroxy-18 hemiacetal and 11β-hydroxy-18-20-bicyclic acetal of aldosterone.

lactone. The use of different sources of aldosterone as standards for its quantification has significant repercussions in precision and reproducibility between laboratories [29].

Metabolism of aldosterone

Aldosterone is synthesized in the zona glomerulosa of the adrenal [35] and released upon stimulation by angiotensin II, potassium, and acutely by ACTH and other peptides [36, 37]. Aldosterone secretion rates in the human were initially measured by the isotope dilution method using [³H]-aldosterone produced by incubating [³H]-progesterone with adrenal glands, then purified [38]. A known amount and specific activity of [³H]-aldosterone was administered intravenously in humans, then an aldosterone metabolite (tetrahydroaldosterone or aldosterone-18-oxo-glucuronide) was isolated from the urine and its specific activity measured. The secretion rate was calculated by comparing the specific activity of the metabolite compared to the specific activity of the infused aldosterone [39, 40]. The distribution volume and turnover rate of aldosterone are greater than those of cortisol [41, 42] because cortisol is extensively and tightly bound to corticosteroid-binding protein, whereas aldosterone is weakly bound.

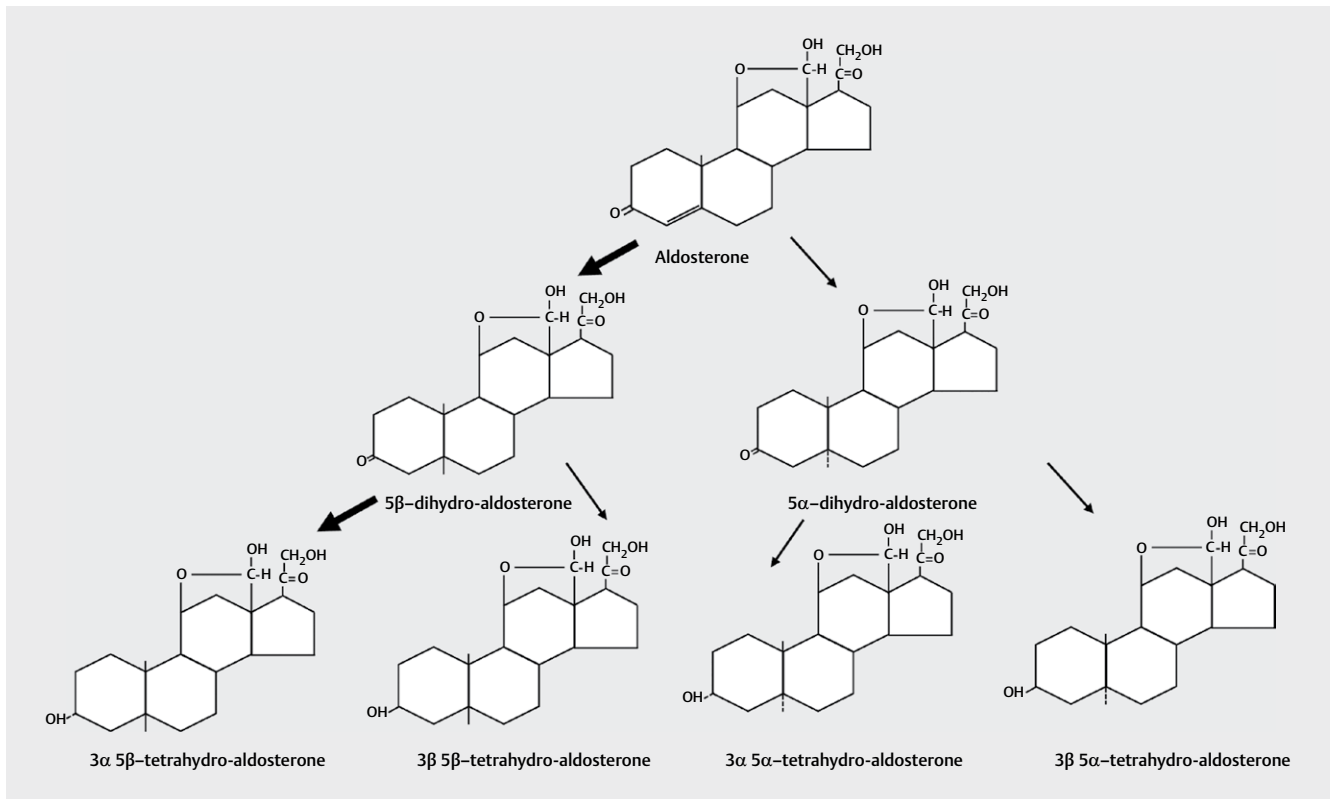
Studies of the metabolic clearance rate measured after the continuous infusion of [³H]-aldosterone demonstrated that most aldosterone was cleared by the liver, with a small portion cleared by the kidney and even less by other tissues [43]. The primary catabolic process is the reduction of the 4–5-double bond, producing both the 5β- and 5α-reduced dihydro steroid and further reduction of the 3-ketone to a 3α- or 3β-hydroxy group (► **Fig 2**). The most abundant metabolite is 3α,5β-tetrahydroaldosterone [44], corresponding to about 30–50% of the secretory rate of aldosterone [45, 46]. Smaller amounts of 3α,5α-tetrahydroaldosterone and 3β,5β-tetrahydroaldosterone are also excreted in the urine. 3α,5β-tetrahydroaldosterone is also the main metabolite when aldosterone is incubated with a liver homogenate. The tetrahydroaldosterones are conjugated with glucuronic acid for excretion, as described below.

At the time aldosterone was isolated, the techniques available for the identification of steroid metabolites required large amounts of steroid. Aldosterone metabolites were often isolated from the urine of individuals who consumed a large amount of aldosterone, 100–200 μg (considering that only about 100 μg are normally secreted by the ad-

renal), that included a known amount of tritiated aldosterone to follow the purification [47–49]. Among the several other metabolites isolated was one described as 11β:18(S),18:20α-diepoxy-5β-pregnan-3α-ol [48] and called the Kelly M1 metabolite. It was found in normal individuals and in higher concentrations in patients with 21-hydroxylase deficiency [50]. Aldosterone in the gut is deoxygenated to 21-deoxyaldosterone and other metabolites by gut bacteria [51] and is probably the origin of the Kelly M-1 metabolite. Acid hydrolysis of urine for the measurement of aldosterone can lead to significant modification of the aldosterone structure with the formation of 18,21-anhydroaldosterone, aldosterone-γ-etiolactone, and dimers of aldosterone [52, 53]. In addition to 3β,5β-tetrahydroaldosterone, incubation of aldosterone with liver homogenates produces other metabolic products, including 2α-hydroxyaldosterone [54], 6β-hydroxyaldosterone [55], and others [56]. Some metabolites of aldosterone have similar, albeit weaker, electrolyte-modulating activities than aldosterone [57, 58]. Interestingly, the reduction at the 3 and 5 positions can be modified by changes in sodium intake; a low sodium intake increases the proportion of 5α-dihydroaldosterone, and a high sodium diet increases the proportion of 5β-dihydroaldosterone [59]. The 5α-reduced aldosterone is a more potent promoter of sodium retention than the 5β-reduced aldosterone [60].

Other metabolites of aldosterone

A small amount of aldosterone-21-sulfate, corresponding to approximately 2.5% of the adrenal production of aldosterone, was found in the urine [61]. Methods for the synthesis of aldosterone-21-sulfate and its separation from the aldosterone-18-oxo-glucuronide in the urine were designed, allowing the measurement of its excretion by hydrolyzing aldosterone-21-sulfate with sulfatase, then determining the free steroid by conventional double isotope technique [61]. Excretion of aldosterone-21-sulfate in five normal subjects on a normal sodium diet was 2.09 ± 0.64 μg/24 h; one patient with primary aldosteronism had six times greater excretion [61]. Further studies demonstrated that aldosterone-21-sulfate concentration in adrenal vein blood is higher than that in peripheral blood and about one-tenth that of aldosterone [62]. Its biological activity using the adrenalectomized rat bioassay is approximately 1% that of aldosterone [62]. Aldosterone incubated with aldosterone-producing adenomas was converted into a water-soluble compound shown to be aldosterone-21-sulfate [62].



► **Fig. 2** Metabolic products of aldosterone metabolism.

The urinary measurement of aldosterone-3-oxo-glucuronide (urinary aldosterone), 3 α ,5 β -tetrahydroaldosterone, and urinary free aldosterone have been used for diagnostic purposes for aldosterone disorders of overproduction and less often for diagnosing underproduction. Multiple other metabolites of aldosterone have been described, but most correspond to a very low proportion of the metabolized aldosterone and have not been found to have physiological or diagnostic consequences. The far less abundant metabolites were usually found when large amounts of aldosterone (100–200 mg or about 1,000 times the average production rate of aldosterone) were administered orally and the urine collected for isolation and determination of the metabolites [40, 44, 47–49].

Aldosterone-18-oxo-glucuronide (urinary aldosterone or acid-labeled metabolite)

Luetscher's group was the first to report that acidification of the urine significantly increased the mineralocorticoid activity of its extract, later identified as aldosterone [22, 24]. Urine from normal subjects extracted at pH 6.5 yielded only trace amounts of aldosterone, but upon keeping the urine for a day at pH 1, a far larger amount [22, 24, 63, 64] was isolated. Further determination of the nature of the conjugate of urinary aldosterone showed that glucuronidase-treated urine also yielded minimal amounts of aldosterone. The reaction of the isolated urinary aldosterone with naphthoresorcinol confirmed that the compound was a conjugate of glucuronic acid [64]. It was originally referred to as a 3-oxo conjugate or

acid-labile conjugate. Studies by Pasqualini et al. [65] and Underwood and Tait [66] suggested that it was an 18-oxo-glucuronide. Studies indicated that about 7.8% of the secretion of aldosterone is converted to this glucuronide in normal subjects, with a range of 5 to 15% [66, 67]. Factors involved in the measurement of urinary aldosterone depend on the adequacy of the urine collection, the standardization of the conditions for acid, including pH, temperature, and duration of the exposure to the acid, due to the competition between the hydrolysis of the conjugate and acid-induced degradation of aldosterone [64, 68, 69], and the assay for the measurement of aldosterone [69–71]. The proportion of aldosterone excretion as the aldosterone-18-oxoglucuronide in women with a standard sodium intake is greater in pregnancy [72]. Measurement of urinary aldosterone, which is mostly 18-oxo-glucuronide, on a high sodium diet is one of the recommended confirmatory tests for primary aldosteronism [73] and has also been used as a screening and confirmatory test for primary aldosteronism in patients with low renin hypertension [74]. Repeated measurements of urinary aldosterone excretion of individuals on a high sodium diet show marked intraindividual variability with differences between 1–88% (median of 31%) when two measurements were done 2 weeks apart [75]. This is a probable reason why urinary aldosterone excretion appeared to be less reliable in the diagnosis of primary aldosteronism, especially in normokalemic patients [70]. The basis for the current criteria of > 12 $\mu\text{g}/24\text{ h}$ urinary aldosterone excretion while consuming a high salt diet for the diagnosis of primary aldosteronism [74, 76] is arbitrary and not well documented.

Others have used 14 µg/24 h [77]; early studies used 20 µg/24 h [78]. Moreover, around 14% of normotensive individuals have values above this number, which might have prognostic significance or might simply represent the high end of the normal conversion rate of aldosterone to aldosterone-18-oxo-glucuronide and, therefore, might not be representative of the production rate of aldosterone [74].

The glucuronidation of mineralocorticoids and glucocorticoids is exerted by several of the UDP-glucuronosyltransferases [79–81]. H293 cells transfected with different human [79] and monkey [80, 81] UDP-glucuronosyltransferase cDNAs demonstrated that of the 18 catalytically active human UD-glucuronosyltransferases found in the liver and kidney, only UGT1A10 and UGT2B7 were capable of converting aldosterone to the 18-oxo-glucuronide; only the UGT2B7 is expressed in the kidney where the bulk of aldosterone is conjugated with the glucuronide [79]. These enzymes also glucuronidate the tetrahydroaldosterones, mainly in the liver. Non-steroidal anti-inflammatory drugs are glucuronidated by the same enzymes, thus, are competitive antagonists of aldosterone glucuronidation, potentially increasing the concentration and action of aldosterone within the kidney, as well as decreasing the proportion of aldosterone excreted as the 18-oxo-glucuronide [79, 82]. In humans, immunohistochemistry using an antibody against the UGT2B7 demonstrated enzyme expression throughout the proximal, distal convoluted tubules, the loop of Henle, and the collecting ducts [83]. In monkeys, it appears to be primarily in the proximal duct [81]. A further source of variation for the conjugation of aldosterone to glucuronic acid in the human is the existence of two allelic variants of the UGT2B7 [81]. The UGT2B7H⁽²⁶⁸⁾ possesses 11-fold higher aldosterone glucuronidation efficiency than the UGT2B7Y⁽²⁶⁸⁾. The multiple drug-resistant *MDR1* gene product P-glycoprotein belongs to the family of ATP-binding cassette transporters that function as transmembrane efflux pumps to translocate substrates from the intracellular to the extracellular space. Aldosterone is transported by P-glycoprotein in the adrenal glomerulosa cell and participates in the efflux of aldosterone from the cells of the zona glomerulosa [84] and other cells, including the kidney where P-glycoprotein is located in the luminal membrane of collecting duct cells [85]. Polymorphisms of the P-glycoprotein do not appear to have a major effect under basal conditions; however, their inhibition by P-glycoprotein inhibitor drugs affects the urinary excretion of aldosterone differently depending on the polymorphism [86].

Tetrahydroaldosterone

The main metabolite of aldosterone is the 3α,5β-tetrahydroaldosterone glucuronide. It is primarily a liver metabolite, corresponding to around half of the aldosterone metabolites [67, 87]. Measuring tetrahydroaldosterone in the urine involves hydrolysis of the glucuronide conjugate with a beta-glucuronidase and measurement by several methods, including radioimmunoassay, high-performance liquid chromatography (HPLC)-MS-MS [88–93]. Since most aldosterone is metabolized to 3α,5β-tetrahydroaldosterone, it has been proposed that it is a better indicator of aldosterone secretion; however, the metabolism of aldosterone to the 3α,5β-tetrahydroaldosterone is affected by different disease states; in particular, it is decreased in congestive heart failure and cirrhosis of

the liver [94, 95]. Antihypertensive drug therapy and sodium diet can also affect the proportion of aldosterone metabolized to the 3α,5β-tetrahydroaldosterone; thiazide diuretics decrease and converting enzyme inhibitors increase the proportion of aldosterone metabolized to 3α,5β-tetrahydroaldosterone [87]. Patients with low renin hypertension also preferentially metabolize aldosterone to 3α,5β-tetrahydroaldosterone [96]. A radioimmunoassay was designed using an antibody against 3α,5β-tetrahydroaldosterone-glucuronide isolated from the urine, allowing its direct measurement in urine [97, 98]. This simplified the assay, but unfortunately, the antibody and standards are no longer available. Although there is a good correlation between aldosterone-18-oxo-glucuronide and 3α,5β-tetrahydroaldosterone-glucuronide excretion in normal and hypertensive individuals, there are significant differences in the excretion of these metabolites and urinary 3α,5β-tetrahydroaldosterone appears to be the most reliable in the diagnosis of primary aldosteronism [88, 90].

Urinary free aldosterone

Urinary free aldosterone represents a very small proportion, about 1%, of the total aldosterone secretion [88]. Measurement of free aldosterone excretion was reported to be equally as good as that of aldosterone-18-oxo-glucuronide for the diagnosis of primary aldosteronism [99]; however, others have reported that urinary free aldosterone is frequently normal in patients with primary aldosteronism, while the excretion of 3α,5β-tetrahydroaldosterone and aldosterone-18-oxo-glucuronide are consistently elevated [88]. As it does not have advantages over the measurements of 3α,5β-tetrahydroaldosterone and aldosterone-18-oxo-glucuronide, urinary free aldosterone is seldom used for diagnostic purposes. Interestingly, a recent publication with a CYP11B2 inhibitor, baxdrostat, used urinary free aldosterone measurements to document the in vivo inhibition of the enzyme [100].

Conclusion

Aldosterone is metabolized primarily in the liver to form several 5α and 5β dihydroaldosterone and tetrahydroaldosterone metabolites, the most abundant of which is 3α,5β-tetrahydroaldosterone, which is glucuronidated for excretion in the kidney. A smaller proportion of aldosterone is glucuronidated in the kidney at carbon 18 to form aldosterone-18-oxo-glucuronide, commonly called urinary aldosterone. Aldosterone metabolism is affected by drugs that alter metabolism and glucuronidation. Urinary excretion of aldosterone-18-oxo-glucuronide is one of the most frequently used measured metabolites for the confirmation of the diagnosis of primary aldosteronism. Although not frequently used, urinary 3α,5β-tetrahydroaldosterone is probably the most reliable metabolite for the estimation of aldosterone secretion in primary aldosteronism.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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