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MEASUREMENT OF 4 URINARY C-18 OXYGENATED CORTICOSTEROIDS BY STABLE ISOTOPE DILUTION MASS FRAGMENTOGRAPHY

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Abstract—The cortisol C-18 oxidation pathway leading to the production of 18-hydroxy- and 18-oxocortisol is expressed in adenomatous primary aldosteronism and glucocorticoid remediable aldosteronism. In order to better define the significance of the pathway and its usefulness in differential diagnosis, we have developed a stable isotope dilution mass fragmentographic method for the determination of the tetrahydro metabolites of aldosterone, 18-hydroxycorticosterone and 18-oxocortisol and of unmetabolized 18-hydroxycortisol in urine. Stereochemically correct tetrahydro steroids containing 3 deuterium atoms were synthesized from the available 3-keto-4-pregnenes in 2 steps and 1,2-deuterium-labeled 18-hydroxycortisol was prepared by selective deuteration of the 1,2-double bond of a dienone precursor. Simultaneous measurement of the 4 steroids permitted a comparison of the abnormal products of the C-18 oxidation of cortisol with the normal C-18 oxidation products of corticosterone, 18-hydroxycorticosterone and aldosterone. Application of the method to the definition of the normal range is described.

INTRODUCTION

The cortisol C-18 oxidation pathway was first demonstrated in a patient with an aldosterone producing adenoma [1] and subsequently in glucocorticoid remediable aldosteronism, another disorder of mineralocorticoid excess [2]. In the latter disorder, marked overproduction of 18-hydroxy and 18-oxocortisol was thought to reflect the acquisition of cytochrome *P*450CMO activity at the locus of ACTH-dependent cortisol synthesis in the fasciculata zone [3]. In studies of the significance of this pathway, its mechanism and its usefulness in differential diagnosis, reliable quantitative data are crucial. We describe a stable isotope dilution mass fragmentographic method for the measurement of the 4 major C-18 oxygenated corticosteroids in human urine to permit comparison of the abnormal C-18 oxygenated products of cortisol with the corresponding normal C-18 oxygenated products of corticosterone, aldosterone and 18-hydroxycorticosterone. The stereospecific synthesis of deuterium-labeled internal standards for each of the 4 steroids required for this technique is also described as well as the application of the method to the determination of the normal range.

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EXPERIMENTAL

Gas chromatography/mass spectrometry

A Hewlett–Packard model 5970 was fitted with a fused silica capillary column operated in the splitless mode at an initial temperature of 100°C and a purge time of 1 min. The column was 30 m × 0.25 mm i.d. with a 1 μ film of OV101 stationary phase. The injection volume was 1 μl. The temperature program for the tetrahydro steroids consisted of a rate of 10°C/min to 280°C continued isothermally for a total run time of 50 min. For the derivatives of cortisol and 18-hydroxycortisol, the rate was 20°C/min to 280°C continued isothermally for 80 min.

Steroids

Aldosterone and 18-hydroxy-11-dehydrocorticosterone were obtained from the Makor Chemical Company. 18-Oxocortisol, 18-hydroxycortisol, and 18-hydroxyprednisolone were kindly provided by Dr Gomez-Sanchez. Synthesis of the unlabeled tetrahydro derivative forms of aldosterone [4] 18-hydroxy-11-dehydrocorticosterone [4] and 18-oxocortisol [5, 6] have been reported previously. Cortisol 9α,11α,12α,12β-d_4 was synthesized as described in Ref. [7].

Correction for natural isotope abundances

The observed ion intensities of internal standard molecules containing 2 and 3 atoms of deuterium also contained contributions from naturally occurring isotopes. In addition to ^{13}C, there was a significant contribution from ^{29}Si and ^{30}Si in those fragment ions containing trimethylsilyl groups, especially the 18-oxo-tetrahydrocortisol ion fragment at $m/z = 420$ containing 2 silicon atoms and the fragment of tetrahydroaldosterone etiolactone at $m/z = 389$ containing 1 silicon atom. Abundances of the $M+2$ and $M+3$ fragment ions were determined for all endogenous metabolites and expressed as a percentage of the M_0 fragment ion and the appropriate correction subtracted from all observed internal standard ion intensities. In the analysis of cortisol no correction was necessary because of the large, 4 Da, separation between endogenous and labeled steroid and negligible isotope abundance overlap.

Calibration curves

Fixed aliquots of stock solutions of the deuterated steroids were mixed with a range of concentrations of the corresponding unlabeled steroids and the mixture analyzed by mass fragmentography. Plots of ion ratio versus the amount of unlabeled steroid as shown in Figs 1 and 2 were used to calculate the slope of each straight line. To calculate the amount of endogenous steroid in the analyzed urinary aliquot by isotope dilution the observed ion ratio was divided by the slope of the calibration curve. Tetrahydroaldosterone and 18-hydroxycortisol were standardized by weight and the tetrahydro metabolite of 18-hydroxycorticosterone and 18-oxocortisol by the Porter Silber reaction [4] using the corresponding 3-keto-4-pregnene as reference. It was not necessary for the deuterated internal standard steroid to be pure but only that the derivative measured be well separated by the gas chromatographic/mass fragmentographic technique.

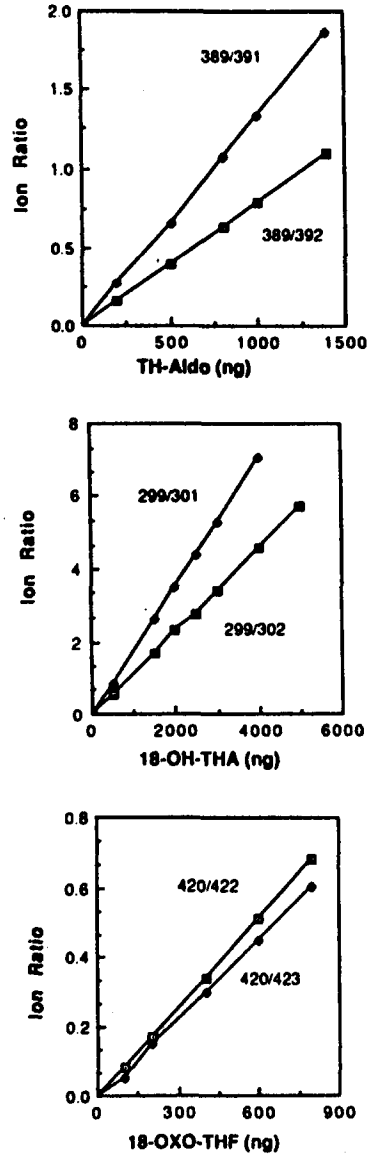

Fig. 1

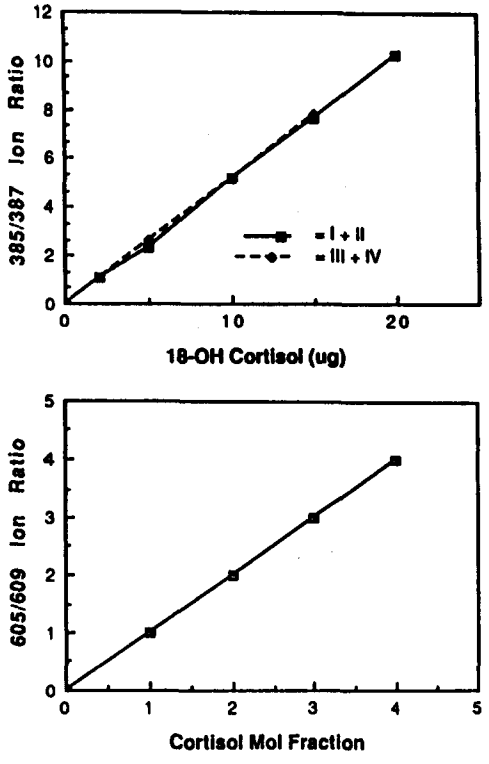

Fig. 2

Urinary steroids

Simultaneous measurement of the tetrahydro urinary metabolites of aldosterone, 18-hydroxycorticosterone and 18-oxocortisol. Aliquots of each of the deuterated tetrahydro steroids were added to 10 ml urine, 1 ml 2 M HIO_4 was added and the solution allowed to react in the dark overnight. The presence of excess periodate was verified as follows. Several drops of the mixture were added to 1 ml water, saturated with sodium bicarbonate, and a crystal of potassium iodide added. An excess of periodate was almost always observed except when there was glycosuria. If necessary, additional periodic acid was added and the oxidation cleavage step repeated. The oxidation mixture was extracted with methylene chloride and the extract washed with 1% sodium bicarbonate and water, dried over sodium sulfate, filtered and evaporated into a 10 ml conical minivial in preparation for derivatization. The trimethylsilyl ethers formed by reaction with 25 μl BSFTA (*N, O*-bis(trimethylsilyl) trifluoroacetamide) containing 10% pyridine by volume at 60°C for 1 h were analyzed by GC/mass fragmentography.

Simultaneous measurement of cortisol and 18-hydroxycortisol. To a 10 ml aliquot of urine was added known amounts of cortisol 9α,11α-12α,12β-d_4 and 18-hydroxycortisol-1,2-d_2. The mixture was adsorbed on to a C-18-Seppak resin cartridge and eluted with methanol. The eluate was evaporated and the residue converted to methoxime-trimethylsilyl ether derivatives and purified on Lipidex 5000 as described [8]. The sample was stored in 25 μl BSFTA in preparation for analysis by GC/mass fragmentography. The 385/387 ions were monitored for 18-hydroxycortisol and 605/609 for cortisol and the amounts of unlabeled steroids calculated from the calibration curves (Fig. 2).

18-Hydroxycortisol by periodic acid oxidation. In an alternative method for 18-hydroxycortisol, its labeled internal standard was added to urine along with the corresponding internal standards of the 3 tetrahydrosteroids. Under the conditions of periodic acid oxidation, the predominant product was 11β,18-dihydroxy-4-androstene-3,17-dione [1]. This 17-ketosteroid was measured by mass fragmentography using the molecular ion of its methoxime-trimethylsilyl ether derivative at $m/z = 520$ and $m/z = 522$ for its deuterated internal standard. This 17-ketosteroid was the major product under these conditions, but smaller amounts were formed of the previously described [1] C-18 glycolic acid ester and 18-hydroxy-4-androstene-3,11,17-trione. The correct molecular ion of the derivatized form of the latter is $m/z = 446$.

Syntheses

Dihydrosteroids-4,5-d_2. Typically, the 3-keto-4-pregnene form of a corticosteroid in amounts of 10–50 mg dissolved in 5 ml absolute ethanol with 10 mg palladium on activated carbon catalyst (Ventron Chemicals) was placed in an airless 3-port reaction vessel (Aldrich Chemical Z17330-4) fitted with a magnetically-driven stirring bar. The 3 ports were connected respectively to a water-aspirator vacuum pump, deuterium gas cylinder and a balloon to permit filling the system at pressures slightly greater than atmospheric. After 3 flushes consisting of evacuation and refilling of the system and balloon with deuterium gas, the vessel was closed and the reaction continued at room temperature for 30 min. Work-up consisted of filtration through sintered glass. Analysis of the filtrate by HPLC and by GC/MS indicated disappearance of the starting material and formation of the two 4,5-dihydro isomers in approximately equal amounts. Mass spectrometry confirmed the introduction of 2 deuterium atoms. The desired 5β- was separated from the 5α-dihydro isomer by preparative HPLC before the next step, 3-keto reduction. It was also possible to carry out 3-keto reduction on the unpurified dihydro mixture since other tetrahydro isomeric products were readily separated from the desired 3α-hydroxy-5β-pregnane form by GC/MS.

3α-Hydroxy-5β-pregnane tetrahydro derivatives-3β,4β,5β-d_3. The 4,5-dihydro product was reacted with 1 ml of sodium borodeuteride (Aldrich Chemical Company, 99 atom %) in pyridine (4 mg/ml) in an ice bath for 5 min. This reaction mixture was extracted with ethyl acetate and the extract washed with dilute HCl, 1% $NaHCO_3$ and water, and dried and evaporated. Deuterated tetrahydro products were converted to methoxime trimethylsilyl ethers and analyzed by GC/MS, confirming the incorporation of 3 deuterium atoms with loss of hydroxylic hydrogen isotope by exchange with the alcohol solvent. Structures were further confirmed by periodic acid oxidation to etiolactones, derivatization with BSFTA to form trimethylsilyl ethers and GC/mass fragmentography. As noted below, some exchange occurred during

these synthesis to yield a mixture of tetrahydro products containing 2 and 3 deuterium atoms. A similar mixture of deuterated tetrahydro products was obtained when ethanol, the solvent for catalytic deuteration was replaced by methanol-d_4.

18-Hydroxycortisol-1,2-d_2. The 1,2-unsaturated precursor, 18-hydroxyprednisolone (5 mg) was selectively deuterated in a reaction vessel as described above in 2 ml absolute ethanol, 1 ml benzene and 10 mg Wilkinson's rhodium catalyst (Aldrich Chemical Company) in an atmosphere of deuterium gas. Overnight reaction at room temperature indicated almost complete conversion of the dieneone to 18-hydroxy-cortisol-d_2 as shown by HPLC and GC/MS analysis. The catalyst was removed on a short column of Sephadex LH 20 developed with ethanol: cyclohexane (1:4) and the 1,2-d_2-labeled product recovered for use as an internal standard.

RESULTS

Mass spectra

Figure 3 shows the mass spectra of the etiolactone trimethylsilyl ether derivatives of tetrahydroaldosterone and of tetrahydro 18-hydroxy-11-dehydrocorticosterone and basis for the selection of diagnostic ions for quantitation: $m/z = 389$ for the metabolite of aldosterone and $m/z = 299$ for the metabolite of 18-hydroxy-corticosterone. These mass spectra show that other ions of sufficient intensity were available but their use was generally not necessary. The mass spectra of 18-hydroxycortisol methoxime trimethylsilyl ether [1] and of 18-oxo tetrahydrocortisol etiolactone trimethylsilyl ether [6] have been published previously. In the latter spectrum, the mechanism of the base peak at $m/z = 420$ was shown by accurate mass measurement to be due to loss of C_2O_3 [6].

Deuterium incorporation

In the synthesis of each of the 3 tetrahydro steroids the expected incorporation was 3 atoms of deuterium at C_3, C_4 and C_5 with exchange of hydroxylic hydrogen isotope at C-3. In practice these tetrahydro products contained stable mixtures of molecules containing 2 and 3 deuterium atoms possibly because of partial exchange at C-4 while the 3-keto group was still present. The presence of both labeled moieties was not necessarily a disadvantage. A standard curve was generated for each internal standard as shown in Fig. 1 to provide duplicate and confirming measurements in each assay. Calculated amounts of endogenous steroid using each internal standard agreed within 5%, justifying the use of the mean value.

Ion chromatograms

Figure 4 shows a representative analysis of a urinary extract in which the 3 tetrahydro

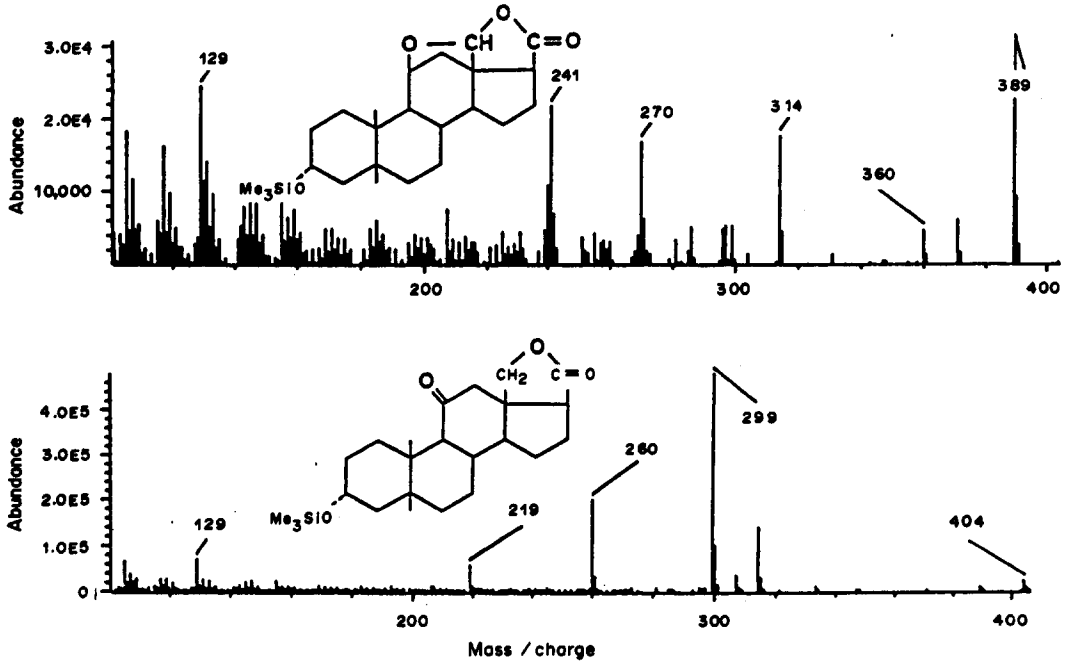

Fig. 3

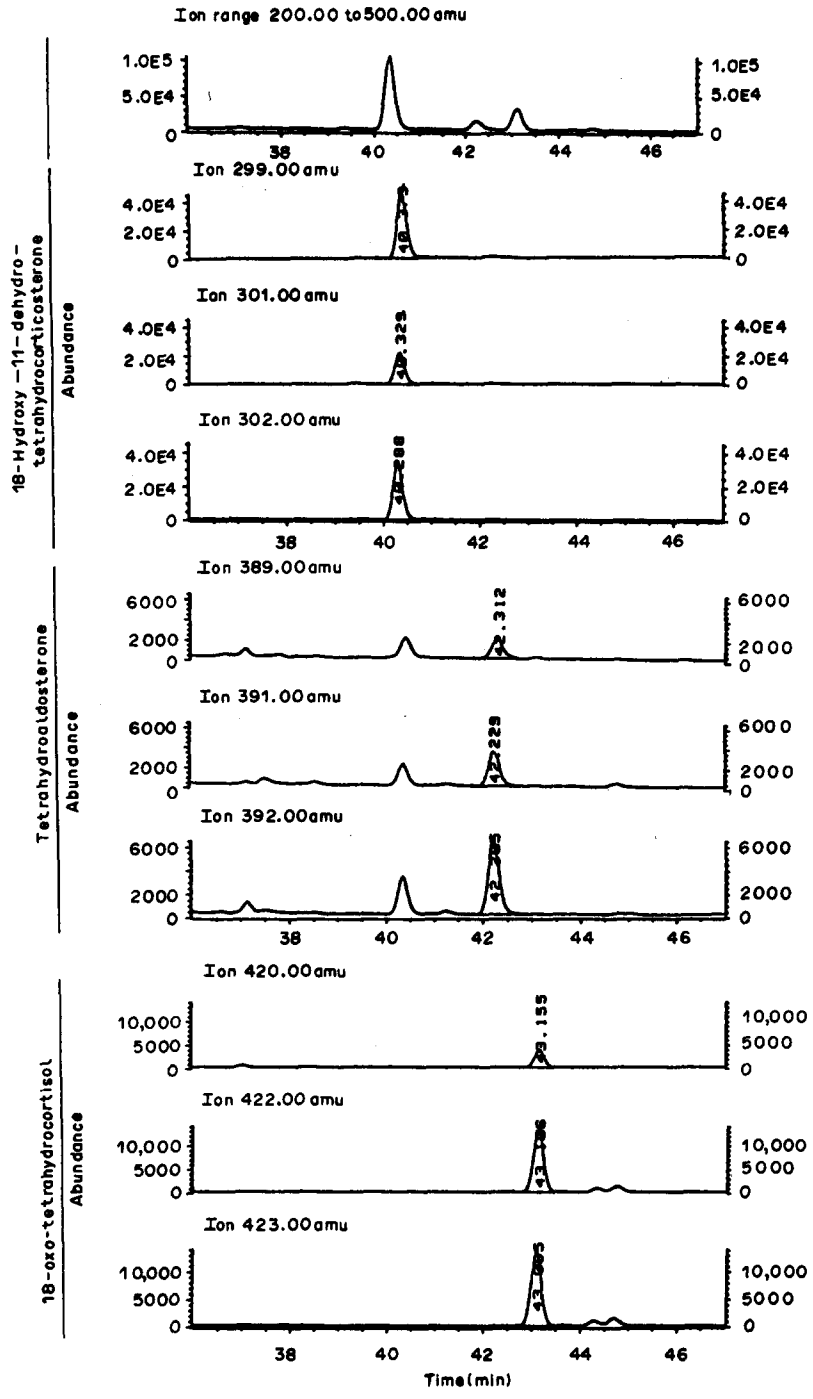

Fig. 4

metabolites were well separated. The presence of a stable mixture of internal standards containing 2 and 3 deuterium atoms is also shown. Representative chromatograms for the 2 unmetabolized urinary steroids are shown in Fig. 5. For cortisol, areas under the ion chromatogram for the pair of isomeric methoxime forms were summed. In the case of 18-hydroxycortisol there were 4 methoxime isomers of which only the last 2 were resolvable. One standard curve utilized the unresolved 64 min peak containing components I and II and the other combined the 75–76 min peaks representing components III and IV. The ion chromatograms of all unlabeled molecules and their corresponding deuterated derivatives regularly showed a chromatographic isotope effect. Retention times were always shorter for the

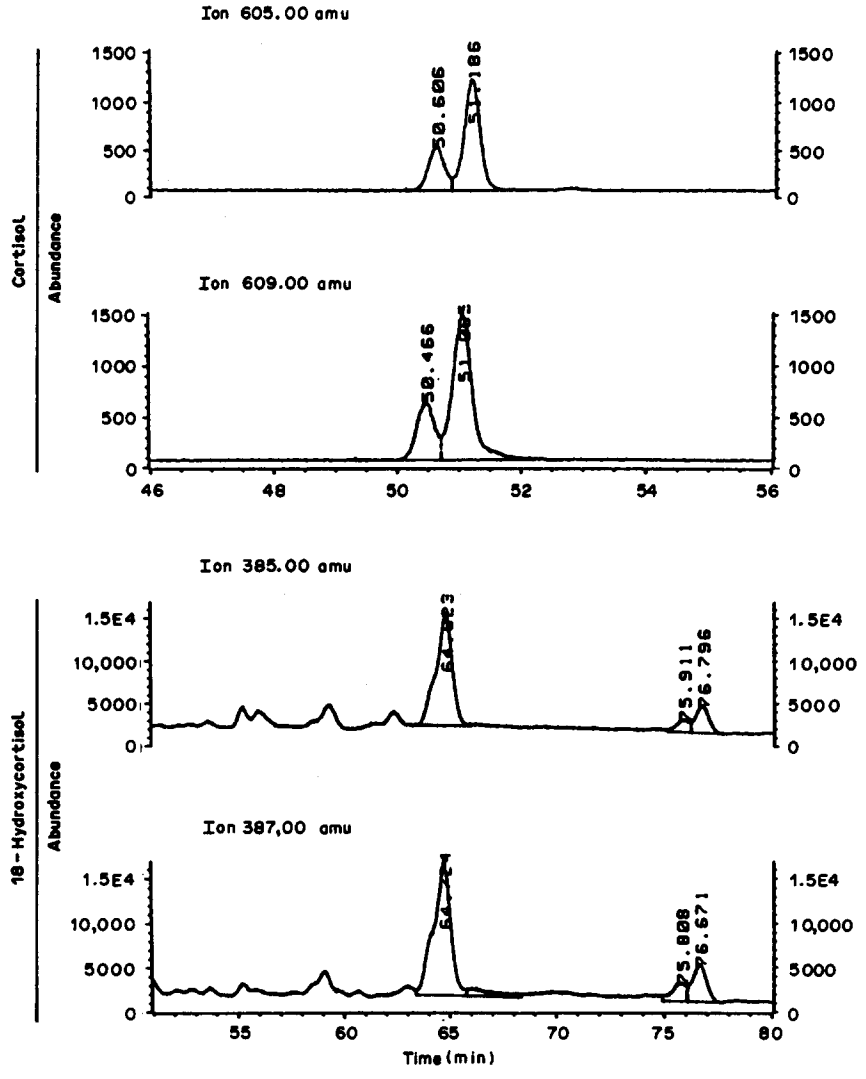

Fig. 5

deuterated species and were shortest for the form containing the most deuterium.

Normal values

A series of routine 24 h urine specimens were analyzed in individuals who were either normal or without relevant disturbances of the adrenal cortex or of aldosterone secretion. Diet was not controlled but the aldosterone excretory rate determined simultaneously served to define the state of that system as well as the cortisol C-18 oxidation pathway in relation to it. The mean values are shown in Table 1. The products of the normal cortisol C-18 oxidation pathway, the metabolites of 18-hydroxycorticosterone and aldosterone, were somewhat lower than previously determined value by a double radioisotope dilution derivative technique [9, 10], probably reflecting the improved specificity of the present method. However, the ratio of excretion of the 18-hydroxy metabolite to tetrahydroaldosterone of 2.5 in the present study agreed with previous estimates.

Table 1. Normal urinary excretory rates of steroids determined by stable isotope dilution mass fragmentography

Steroid	Urinary excretion $\bar{X}$	SD (μg/day)	n
Cortisol	26.5	7.4	(13)
18-Hydroxycortisol	42	20	(13)
Tetrahydroaldosterone	31.3	13.4	(15)
18-Hydroxy-11-dehydro-tetrahydrocorticosterone	78.9	21.8	(15)
18-Oxo-tetrahydrocortisol	2.65	2.08	(15)

Systematic names: 18-hydroxycortisol = 11β,17α,18,21-tetrahydroxy-4-pregnene-3,20-dione; tetrahydroaldosterone = 3α,18,21-trihydroxy 11β,18-oxido-5β-pregnane-20-one; 18-hydroxy-11-dehydrotetrahydrocorticosterone = 3α,18,21-trihydroxy-5β-pregnane-11,20-dione; 18-oxo-tetrahydrocortisol = 3α,17α,18,21-tetrahydroxy 11β,18-oxido 5β-pregnane-20-one.

Of the corticosterone C-18 oxidation products, the normal mean excretion of 18-oxotetrahydrocortisol was less than 3 μg/day, a value very near the limit of detection under the conditions of measurement. Values in pathological conditions greater than 10 μg/day are considered to be elevated [11]. In contrast, the normal mean excretion of 18-hydroxycortisol was 42 μg/day, the most abundant C-18 oxygenated corticosteroid in normal urine. Urinary free cortisol was 26.5 ± 7.4 μg/day.

DISCUSSION

Stable isotope dilution mass fragmentography is an analytic method with a high degree of chemical specificity. Radioimmunoassays for 18-hydroxy [12] or 18-oxocortisol [13] have also been described. In each determination with the present method the selected ion chromatogram verifies the chemical nature and purity of the product. A high degree of specificity is achieved by the combination of monitored unique diagnostic ions of the mass spectrum and a capillary gas chromatogram with a column containing at least 100,000 theoretical plates. In addition, for each peak representing endogenous metabolite there is an isotopic internal standard (in this case 2 isotope internal standards) to mark the elution time. The deuterated internal standard also provides a tracer of identical chemistry for quantitation and for correction for losses beyond its point of addition in the procedure. The only step not corrected for is the chemical oxidative cleavage of urinary glucuronides which is virtually quantitative in contrast to enzymatic hydrolysis. In addition, oxidative cleavage in yielding an etiolactone product, affords instantaneous stabilization of C-18 oxygenated corticosteroids whose reactivity toward alkali [14] or acid [15] might be a source of variability or analytic error.

The procedure itself is simple, involving only direct addition of periodic acid to urine followed by overnight reaction, extraction, and treatment of the residue with a silylating reagent and injection of the derivatized mixture directly into the gas chromatograph/mass spectrometer. The crucial components of the assay are the deuterated internal standards. These stereospecifically-labeled deuterated tetrahydro steroids were readily synthesized from available 3-keto-4-pregnene forms.

The measurement of the excretion of tetrahydro 18-oxocortisol may be useful in the differential diagnosis of primary aldosteronism. The cortisol C-18 oxidation pathway is expressed in aldosteronism associated with unilateral adenoma but not with bilateral hyperplasia [11]. In glucocorticoid remediable aldosteronism, excretion of the tetrahydro metabolite of 18-oxocortisol tends to be greater than in primary aldosteronism [3]. 18-Hydroxycortisol, reflecting decreased metabolism, is normally more abundant than the tetrahydro metabolite of 18-oxocortisol.

Urinary free cortisol tends to be overestimated by radioimmunoassay or competitive binding methods, with normal values reported in the range of 50–100 μg/day and falling to a mean of 20.5 ± 5.7 μg/day after purification [16]. The mean of 26.5 ± 7.4 μg/day by stable isotope dilution agrees with this lower normal range.

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