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SYNTHESIS OF A DEUTERIUM-LABELED CORTISOL FOR THE STUDY OF ITS RATE OF 11β-HYDROXY DEHYDROGENATION IN MAN

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Summary—11β-Hydroxy dehydrogenation of cortisol to cortisone is specifically impaired in the syndrome of apparent mineralocorticoid excess. This defect bears on the pathogenesis of the disorder by unmasking the potential mineralocorticoid agonism of unmetabolized cortisol at or near mineralocorticoid target tissues. A specific index of this defect is provided by measurement of the formation of tritiated water following the administration of [^{3}H]11α-cortisol. We have explored the use of a non-radioactive tracer to follow this unidirectional dehydrogenation reaction but because of the relatively lower sensitivity of measurement of ^2H_2O compared to ^3H_2O in body fluids, use of the corresponding [^{2}H]11α-cortisol was not feasible. We have devised instead a method incorporating additional deuterium atoms into cortisol to measure unidirectional 11β-hydroxy dehydrogenation not by the formation of labeled water but by the determination of the dehydrogenated cortisol product from its residual deuterium content. Cortisol-d_4 metabolized to cortisone-d_3 is conveniently measured by the techniques of organic mass spectrometry. The synthesis of cortisol-9α,11α,12α12β-d_4 and the validation of its isotopic distribution by mass spectrometry and nuclear magnetic resonance is described.

INTRODUCTION

The syndrome of apparent mineralocorticoid excess is associated with defective peripheral metabolism of cortisol to cortisone [1–4]. The mechanism of the mineralocorticoid excess is considered to be the persistence of unmetabolized cortisol as a mineralocorticoid agonist at or near target tissues containing mineralocorticoid receptors [5, 6]. Several methods have been used to demonstrate and quantitate this abnormality, but only the rate of removal of the 11α-hydrogen isotope from [^{3}H]11α-cortisol reflects specifically the unidirectional 11β-hydroxy dehydrogenation reaction [1, 3, 7]. Other indices such as the urinary cortisol:cortisone metabolite ratio measure the 24 h oxido-reduction equilibrium position and the turnover rate or half time of disappearance of labeled cortisol from blood or urine reflects its rate of removal by all metabolic routes.

Measurement of the rate of unidirectional dehydrogenation may be a more sensitive index than the cortisol:cortisone metabolite ratio as suggested by the description of the type 2 variant of the syndrome of apparent mineralocorticoid excess in which overall cortisol turnover is decreased while the cortisol:cortisone metabolite ratio remains normal [2, 8]. In considering the substitution of the non-radioactive deuterium label for tritium to measure the rate of cortisol dehydrogenation *in vivo*, the advantage of a nonradioactive tracer is offset by the disadvantage of the very much lower sensitivity of measurement of ^2H_2O compared to ^3H_2O so that the rate of appearance of the former in body water is not readily measurable. In this report we have overcome this limitation of deuterium labeling by synthesizing a molecule containing additional deuterium atoms which could still be recognized by organic mass spectrometry following specific removal of [^{2}H]11α. This was accomplished by base-catalyzed deuterium exchange of the 3 enolizable positions in the 11-ketone form followed by borodeuteride reduction to yield as final product, cortisol-9α,11α,12α,12β-d_4 for which the synthesis and validation of isotopic distribution is herein described.

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EXPERIMENTAL AND RESULTS

Syntheses

General methods. When possible the course of reaction was followed by HPLC analysis using methanol–H_2O mixtures as mobile phase and a C-18 reversed phase column. 3-Keto-4-pregnene compounds were monitored at 240 nm and those containing at least 1 double bond or carbonyl group were monitored by end absorption at 200 nm. For mass spectrometry, underivatized steroids were analyzed using the solids probe under electron impact conditions of a V.G. TS250 mass spectrometer. GC/MS analyses were carried out on methoxime trimethylsilyl ether derivatives prepared as described [9] using a Hewlett–Packard 5970 mass spectrometer. All deuterated reagents contained at least 99 atom percent deuterium and were obtained from the Aldrich Chemical Company. Whenever possible reactions were carried out in 10 ml conical minivials whose cap threads could be adapted directly to a rotary evaporator for removal of solvent.

NMR spectra were recorded at room temperature in acetone-d_6 using a Varian Unity 400 MHz spectrometer. Chemical shifts are in ppm relative to the internal acetone resonance set at 2.05 ppm.

Cortisone-3,20-diethylene ketal (I). The reaction vessel was fitted with a magnetically driven stirring bar and connected to a water aspirator pump as vacuum source. A mixture of 200 mg cortiscone, 40 ml diethylene glycol and 20 mg *p*-toluenesulfonic acid was heated under partial reflux at a temperature of approx. 110°C so that half of the solvent distilled over 4 h. The product was extracted with methylene chloride and the extract washed with 1% $NaHCO_3$ and water, dried and evaporated. HPLC analysis showed 95% conversion to the bisketal derivative (I) confirmed by solid probe

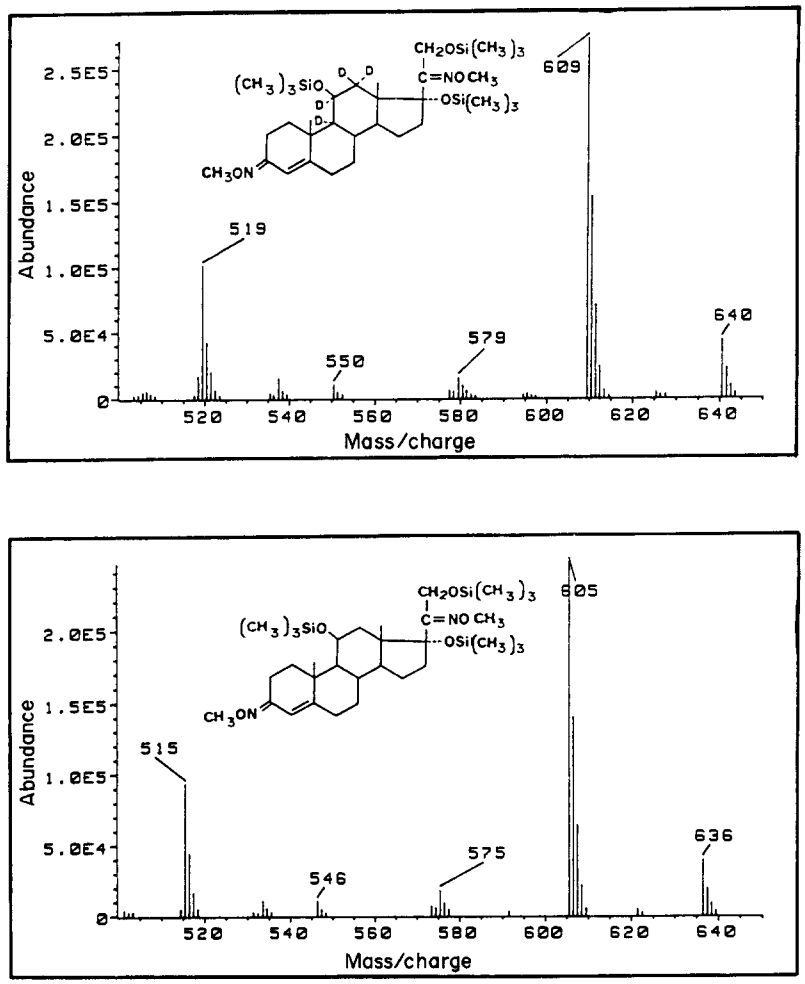

Fig. 1. Mass spectra of methoxime trimethylsilyl ether derivatives of cortisol-d_4 (upper curve) and of cortisol.

mass spectrometry showing a molecular ion $m/z = 448$.

Cortisone 3,20-diethylene ketal-9α,12α,12β-d_3 (II). Bisketal I, (100 mg) in 5 ml CH_3OD was treated with 1.5 ml D_2O and 0.5 ml NaOD (40% in D_2O) in a tightly capped 10 ml minivial. In preliminary experiments, the course of the exchange reaction and optimum conditions were monitored by solids probe mass spectrometry of the bisketal form and by GC/MS analysis after acid hydrolysis and derivatization to the methoxime-trimethylsilyl ether. Exchange at C-12 appeared to take place more readily then at C-9. A temperature of 60°C for 16 h resulted in exchange of all three enolizable hydrogen atoms as shown below.

Cortisone 9α,12α,12β-d_3 (III). After base catalyzed exchange as described above, the bisketal derivative (II) was dissolved in 4 ml 40% formic acid maintained at 60°C for 1 h. The solvent was removed and the product washed with cold acetonitrile and recrystallized from 95% ethanol. The hydrolyzed product had the mobility of cortisone by HPLC. Molecular ions by mass spectrometry were $m/z = 363$ for the underivatized form and $m/z = 565$ for the methoxime trimethylsilyl ether form indicating the incorporation of 3 deuterium atoms. Both spectra however showed an M + 2 ion approximately 30% as intense as M + 3, indicating that some exchange of hydrogen for deuterium had taken place. Exchange could be avoided if the 11-ketone form was not isolated but reduced directly as described below.

Cortisol-3,20-diethyleneketal-9α,11α,12α,12β-d_4 (IV) and cortisol-9α,11α,12α,12β-d_4 (V). The best incorporation of isotope was obtained when the 11-ketone of cortisone was reduced with borodeuteride directly without the isolation and hydrolysis of the exchanged cortisone product. The crude cortisone bisketal-d_3 product II was treated directly with 80 mg

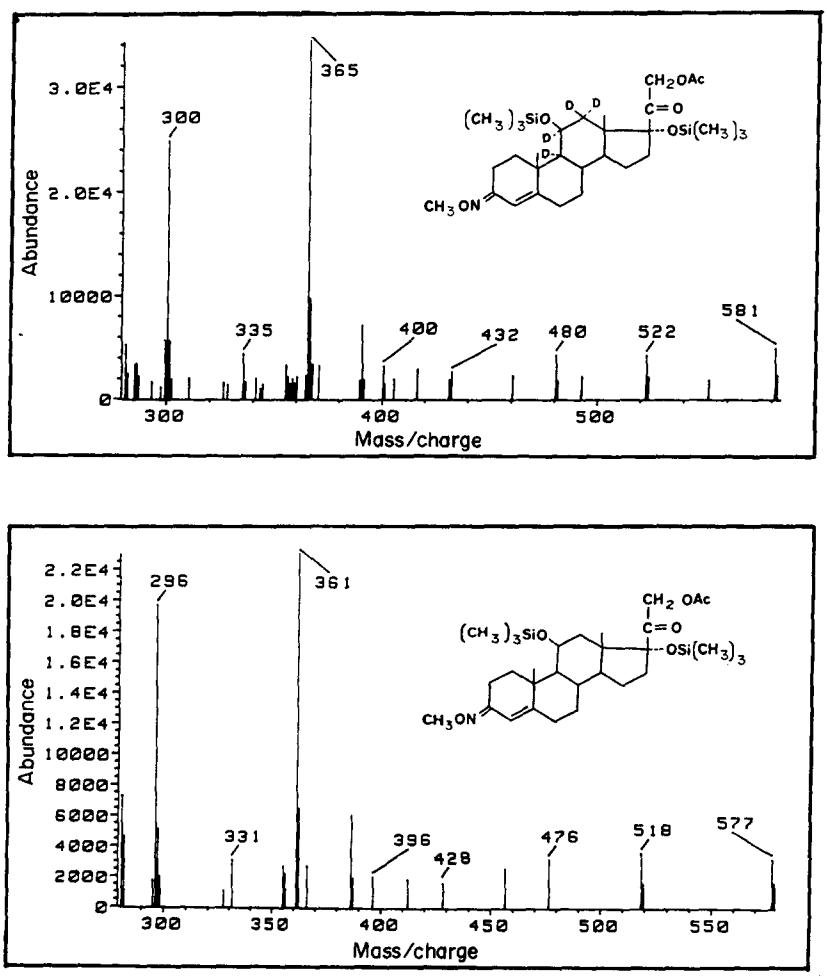

Fig. 2. Mass spectra of methoxime trimethylsilyl ether derivatives of cortisol-21-acetate-d_4 (upper curve) and of cortisol-21-acetate.

$NaBD_4$ at 60°C for 16 h. The solvent was removed and the residue extracted with methylene chloride. The filtered extract was evaporated and the product dissolved in 4 ml 40% formic acid. After 1 h at 60°C the solvent was evaporated and the residue washed with cold acetonitrile and recrystallized from 95% ethanol, m.p. 212–215°C. HPLC analysis showed a single component with the retention time of cortisol. GC/MS analysis of its methoxime trimethylsilyl ether derivative (Fig. 1) showed molecular and fragment ions 4 atomic mass units greater than that of the unlabled derivative indicating that 4 deuterium atoms had been incorporated.

Validation of isotopic distribution

Cortisol-21-acetate-d_4. Cortisol-d_4 (200 μg) was treated with 50 μl acetic anhydride and 100 μl pyridine, heated at 100°C for 1 h and the reagents removed on a rotary evaporator. HPLC analysis showed the product to be cortisol-21-monoacetate. The methoxime trimethylsilyl ether derivatives was formed and analyzed by GC/MS. The corresponding derivative of unlabeled cortisol was similarly prepared and analyzed. Under these conditions the derivative was the C-3-monomethoxime instead of the C-3,20-bismethoxime, shown in Fig. 2. The molecular mass of the deuterated product was 4 atomic mass units higher than that of the unlabeled moiety in keeping with the presence of 4 deuterium atoms. There was no evidence of back exchange of hydrogen for deuterium in the form of M + 3 ions. The deuterated cortisol was stable in ethanol solution over many months.

Cortisone acetate-d_3. Cortisol-21-acetate-d_4 (100 μg) was treated with 5 mg pyridinium dichromate in 1 ml methylene chloride for 15 min at room temperature. The reaction mixture was washed with 0.1 N HCl, 0.2 M acetate buffer pH 5 and water. The solvent was dried and evaporated and an aliquot converted to a

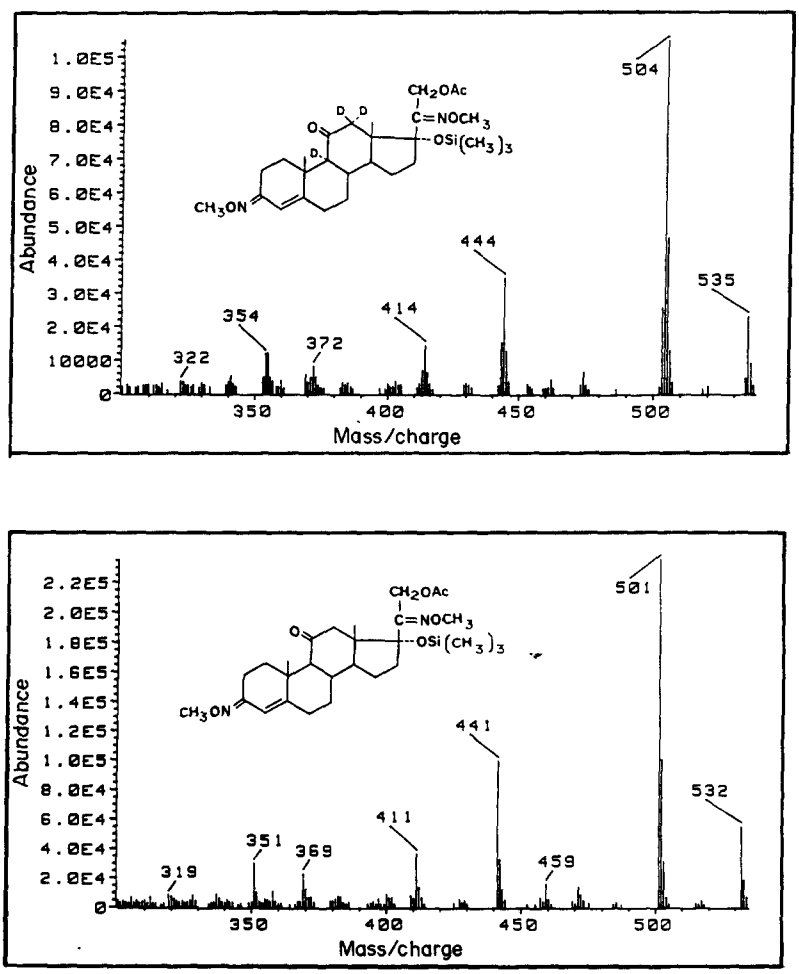

Fig. 3. Mass spectra of methoxime trimethylsilyl ether derivatives of cortisone-21-acetate-d_3 (upper curve) and cortisone-21-acetate.

methoxime trimethylsilyl ether derivative and analyzed by GC/MS. Its spectrum compared in Fig. 3 to that of derivatized unlabeled cortisone-21-acetate shows the molecular ion and relevant fragment ions 3 atomic mass units higher in the deuterated form corresponding to the presence of 3 deuterium atoms and demonstrates the selective removal of the 11α-hydrogen isotope in the course of the conversion of cortisol to cortisone. There was however an ion at $m/z = 534$ that was 20% of the m/z 535 molecular ion and also one at $m/z = 503$ that was 20% of the m/z 504 M-31 fragment. The presence of these ions suggested a 20% loss of deuterium during the chemical conversion of cortisol-d_4 to cortisone-d_3. Different oxidation conditions using chromium oxide in pyridine gave a similar product with a similar isotopic distribution.

NMR evidence. The spectrum of cortisol-d_4 is shown in Fig. 4 and unlabeled cortisol in Fig. 5. The level of deuteration at H-9, H-11, and 12α-H could be determined quite accurately since these protons appear as completely resolved signals. The residual H in all three resonances are comparable and are estimated at $2 \pm 1\%$ of stochiomistry. Even though the residual 12β-H is obscured by H-8, it is evident that its H content is not appreciably higher than at the other sites since it would otherwise distort the normal looking double triplet pattern of H-8. A further indication of massive deuteration at the 12β site is the loss of long-range coupling between the 12β-H and the 18-methyl. This weak coupling is generally unresolved but is manifested by a slight broadening of the 18-methyl signal which results in its peak height being lower than that of the 19-methyl. In the present case the coupling is resolved with the 18-methyl appearing as a doublet ($J \simeq 0.5$ Hz). Replacement of the 12β-H with deuterium effectively removes this coupling and as a result the peak height of the 18-methyl signal in the labeled preparation is higher than that of the 19-methyl.

The $2 \pm 1\%$ conservative estimate was arrived at by comparing the relative area of the residual H-9 signal at 1.01 ppm with the nearby axial H-7 at 1.09 ppm. The axial H-7 signal is composed of 16 lines of approximately equal intensity and it is apparent that the area of the

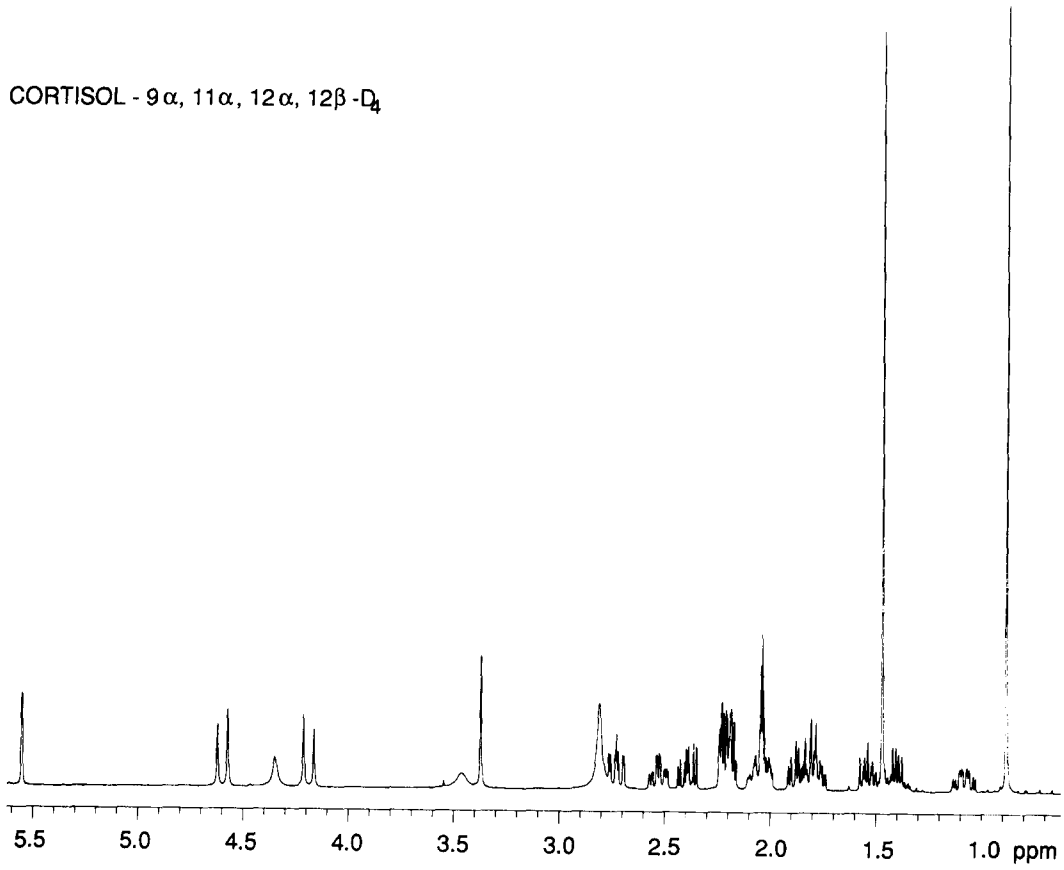

Fig. 4. NMR spectrum of cortisol-9α,11β,12α,12β-D_4 in acetone D_6.

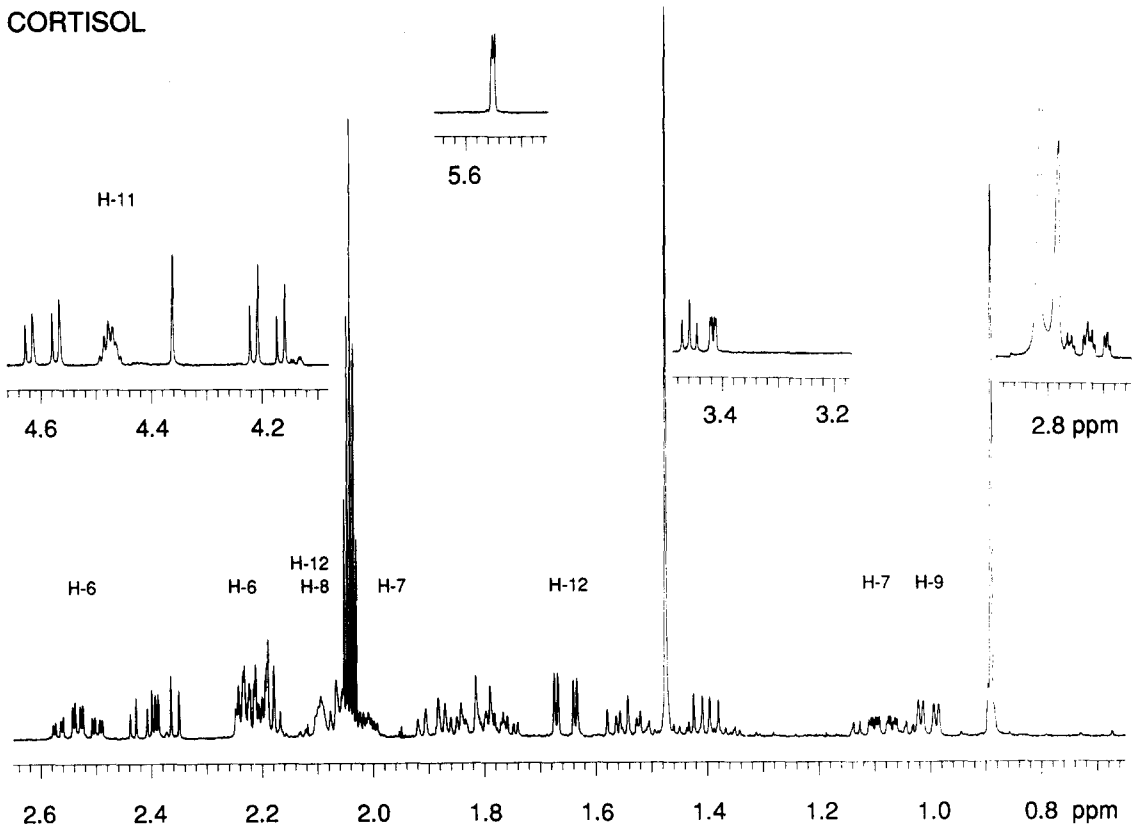

Fig. 5. NMR spectrum of cortisol in acetone D_6.

four weak lines of H-9 add up to somewhat less than one-half of one of the 16 lines, i.e. less than 3%.

DISCUSSION

We describe the synthesis of a deuterium-labeled tracer as an alternative to the 11α-tritium-labeled cortisol used by Hellman *et al.* [7] for the study of the dynamics of the cortisol–cortisone interchange in man. The use of a nonradioactive tracer offers obvious advantages but because of the much greater sensitivity of measurement of tritium compared to deuterium, a different analytic approach was necessary. Measurement of the dehydrogenation product directly had the advantage that it could be accomplished by the techniques of the mass spectrometry of organic molecules and not requiring a specialized instrument for the analysis of gas phase isotopes.

Concerning the proof of localization of the 4 deuterium atoms in the product, labeling of the 11α position was unequivocally established by its removal in the conversion of cortisol to cortisone. Substitution of the positions 9α,12α,12β during base-catalyzed exchange of the 11-ketone form is strongly suggested by the chemistry of these 3 enolizable positions in the molecule. Three residual deuterium atoms remained after oxidation of cortisol to cortisone, serving to identify the oxidation product as a d_3-moiety. The finding of some exchange of deuterium during chemical oxidation of cortisol to cortisone, does not necessarily indicate that such exchange would occur in biologic experiments. Nevertheless, the possibility of exchange of the labeled cortisone product should be examined and if necessary, corrected for in each biological system. Another preparation of a 9,12,12-deuterium-labeled cortisol has been shown to be stable in another type of study in man [10]. This distribution of deuterium as 9α,11α,12α,12β was unambiguously demonstrated by nuclear magnetic resonance. Both NMR and mass spectrometry indicated at least 98% incorporation of deuterium into the product. Cortisol-9α,11α,12α,12β-d_4 was thus demonstrated to be a valid tracer for the study of unidirectional 11β-hydroxy dehydrogenation *in vivo*.

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