ACADEMIC PRESS

Bioorganic Chemistry 30 (2002) 233–248

BIOORGANIC CHEMISTRY

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Ergosteroids VII: perchloric acid-induced transformations of 7-oxygenated steroids and their bio-analytical applications—a liquid chromatographic-mass spectrometric study☆

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Received 12 December 2001

Abstract

Sulfate esters of 7-oxo-Δ^5-steroids can be selectively and quantitatively hydrolyzed to the corresponding free steroids in the presence of carboxylic acid esters by solvolysis with perchloric acid in ethyl acetate at room temperature. Sulfates as well as carboxylic acid esters, methyl ethers, and ketals can be quantitatively converted to the corresponding 3,5-diene-7-one derivatives by heating with perchloric acid in methanol at 65 °C. The dienes have a strong UV absorption with maximum centered around 284 nm. These reactions have been used for the characterization and structural elucidation of 7-oxygenated-Δ^5-steroids that are present in complex biomatrices and can also be used for the quantitative estimation of total 7-oxo-Δ^5-steroids (free as well as conjugated) in biological matrices.

☆ *Abbreviations:* APCI, Atmospheric pressure chemical ionization; DAD, diode array detector; ESI, electrospray ionization; GC, gas chromatography; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography mass spectrometry; SIM, selected ion monitoring; UV, ultraviolet. DHEA, dehydroepiandrosterone, 3β-hydroxyandrost-5-en-17-one.

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0045-2068/02/$ - see front matter
PII: S0045-2068(02)00010-X

Keywords: 7-Oxo-dehydroepiandrosterone; 7-Oxygenated-Δ^5-steroids; Sulfate conjugates; Perchloric acid; LC-MS

1. Introduction

Hydrolysis of steroid conjugates has been of major interest to steroid investigators because only a tiny fraction of steroids and their metabolites are excreted unchanged and because of difficulties associated with the analysis of intact steroid conjugates [1]. A quantum leap in the field of bio-analytical chemistry, such as the advent of bench-top mass spectrometric detectors, which can be coupled to GC and HPLC, and ready availability of HPLC columns, which can withstand the deleterious effects of extreme pH (1–11) mobile phases, has alleviated several of the difficulties associated with the analysis of intact steroid conjugates. But hydrolysis of these conjugates, prior to analysis, continues to be the most precise and direct method available. Steroids such as dehydroepiandrosterone (DHEA) are known to exist in several forms in tissues and blood (free steroid, sulfate conjugate, fatty acid esters, and glucuronides) [2,3], making it difficult to measure the total steroid concentration in the biomatrix, and leaving the scientist with no other option but to resort to hydrolysis, chemically or otherwise.

Traditionally steroid conjugates have been hydrolyzed using strong inorganic acids or enzymatically [1,4–9]. Both approaches have their advantages and limitations. The enzymatic hydrolysis is mild, but may be time-consuming or may even be incomplete in some matrices due to inhibition of enzymes [10]. Alterations in structure and artifact formation in the case of labile steroid molecules during solvolytic procedures involving the use of strong acids cannot be ruled out. Perchloric acid has been used for the hydrolysis of organic sulfates; and steroid sulfates, dissolved in organic solvents, have been solvolyzed by catalytic amount of perchloric acid. Other polar derivatives of sterols such as phosphates and glucuronides were unaffected [5]. A search of the literature revealed that the sulfate conjugate (I) of 7-oxo-DHEA (II, 3β-hydroxyandrost-5-ene-7,17-dione) was resistant to hydrolysis under various conditions (continuous ether extraction of acidified solution, enzymatic cleavage with a sulfatase-containing preparation, and acetic ester solvolysis, etc.) and a complex hydrolysis could be achieved [11] only at a high concentration of sulfuric acid, which also resulted in the partial formation of androsta-3,5-diene-7, 17-dione (VII).

During our ongoing investigation of the metabolism of DHEA and its 7-oxygenated derivatives in search of active metabolites that are responsible for the enhancement of the formation of liver mitochondrial sn-glycerol 3-phosphate dehydrogenase and cytosolic malic enzyme [12–14], we expected and encountered the problem of poor and variable recoveries of various 7-oxygenated DHEA derivatives whenever acidic conditions were employed. The hydrolysis of sulfate conjugates of 7-oxygenated DHEA derivatives was found to be highly dependent on the nature of the solvent, reaction temperature, and time. Yields were variable and often a mixture of products was obtained.

This aspect of hydrolysis of 7-oxygenated steroids has now been investigated in detail. We studied the hydrolysis and/or dehydration of 7-oxo-DHEA (II), its sulfate and carboxylic esters, and of other related 7-oxygenated compounds such as 7-hydroxy-DHEA, 3β,17β-dihydroxyandrost-5-en-7-one, androst-5-en-3β,7,17β-triol, 7-oxo-cholesterol, etc. so as to find appropriate reaction conditions, leading to the selective and exclusive formation of either hydrolyzed product or hydrolyzed and dehydrated product. The reaction conditions were developed with microgram and sub-microgram quantities so that these reactions were directly applicable to the study, in biomatrices, of 7-oxygenated derivatives of 3-hydroxy-Δ^5-steroids, in general, and 7-oxo-DHEA (II), in particular. It may be mentioned that 7-oxo-DHEA (II) is a metabolite [15–17] of DHEA, once termed "fountain of youth" [18]. 7-Oxo-DHEA (II) is more active than DHEA in two biological assays [19–21] but unlike DHEA is not converted to either androgens or estrogens [19,22]. It was isolated from human urine [15,23] and peripheral venous plasma [24] more than four decades ago but only a few methods are available for its analysis in biological fluids or tissues [25]. We have reported [26] a validated HPLC method for the determination of 7-oxo-DHEA-3β-sulfate (I) in human plasma. In the present paper, we are reporting for the first time a method, which can be used for the analysis of total 7-oxo-DHEA (II) that is present in biomatrices. The method has also been used to study the hydrolysis of fatty esters of 7-oxo-DHEA by rat brain homogenate and in the characterization of the metabolites of DHEA produced in vitro by rat liver [27].

2. Materials and methods

All reactions were carried out at nanomole (~0.1–40 nmol) scale. The reaction mixture was analyzed by HPLC and LC-MS and yields were calculated using external standard methods. The identity of the products was established by comparison with authentic samples (retention times and chromatography of spiked samples), online diode array UV spectrum, and mass spectrum analysis. For bio-analytical studies, pooled blood plasma samples from healthy human volunteers, obtained from University of Wisconsin-Madison Hospital, were used.

The chromatographic system consisted of an Agilent 1100 series LC-MSD system, which was composed of a quaternary pump (G1311A) with online degasser, thermostated column compartment (G1316A), autosampler (G1313A), diode array UV detector (DAD, G1315A), and an online mass detector (G1946A) that was coupled with atmospheric pressure electrospray (ESI) or atmospheric pressure chemical ionization device (APCI), as needed. Data were acquired and processed using HPLC/MSD Chemstation version A.08.03 software from Hewlett–Packard.

Chromatography of the chemical samples was performed on a Zorbax C_{18} analytical column (3.5 μm, 4.6 × 75 mm) that was protected with a C_{18} guard column (Zorbax SB-C_{18}, 4.6 × 12.5 mm, 5μ). The column temperature was maintained at 40 ± 0.5 °C. The flow rate was set at 1.0 mL/min and the eluent was simultaneously monitored at three wavelengths, viz., 205 nm (bandwidth 10 nm), 240 nm (bandwidth 4 nm), and 284 nm (bandwidth 4 nm), respectively. The reference wavelength was set at 360 nm for 205 and 240 nm wavelengths and 450 nm for 284 nm wavelength. The

slit width was kept at 10 nm for detection at 205 nm, 4 nm for detection at 240 nm, and 16 nm for detection at 284 nm. A methanol–water linear gradient (mobile phase A) was used to analyze the samples. The linear gradient started from 30% methanol to 96% methanol in 33 min and was brought back to an initial concentration of 30% in 3 min. A 10 min post-run was used to re-equilibrate the column and for the base line to return to the normal. For the analysis of sulfate conjugates, acetic acid (1.0% v/v) was added to methanol and to water and a slightly different gradient (15% methanol to 60% methanol in 30 min) was used (mobile phase B). 7-Oxo-DHEA-3β-stearate (III) and cholesterol (VI) were analyzed using a higher percentage of methanol (85–100% in 15 min; mobile phase C). The testosterone derivatives (X–XVI) were analyzed using acetonitrile–water gradient (20–45% acetonitrile in 25 min; mobile phase D). The products obtained by perchloric acid treatment were also analyzed using LC-MS-ESI in positive mode. Operating conditions were optimized by flow injection analysis (FIA) of 7-oxo-DHEA (II) and 7β-hydroxy-DHEA (XVII) and the final parameters used were determined as: drying gas (N_2) 12 L/min at 350 °C; nebulizer pressure 40 psi; capillary voltage 5000 V; and gain 5. The samples were run in scan mode for the mass range 200–600 and the fragmentor was set at 100 V. 7-Oxo-DHEA-3β-stearate (III) and cholesterol (VI) were analyzed in chemical ionization (APCI) mode. Operating conditions were as follows: drying gas (N_2) 5.0 L/min at 350 °C; nebulizer 60 psi; capillary voltage 4000 V; gain 5; vaporizer at 400 °C and corona at 4 μA. The samples were run in scan mode (mass range 200–600) and fragmentor was set at 70 V.

A different HPLC system was used for the analysis of plasma samples after perchloric acid treatment to convert 7-oxo-DHEA (II) to androsta-3,5-diene-7,17-dione (VII). It consisted of a Zorbax-SB column (C_8, 3.5μ, 4.6×50 mm) and methanol–water gradient containing 1% of 0.1 M phosphoric acid (50–70% methanol in 10 min) at 1.0 mL flow rate (mobile phase E). The monitoring was at 284 nm with a bandwidth of 4 nm. Plasma samples were also analyzed by LC-MS in ESI mode. Non-volatile buffers and acids are deleterious to the mass detector; hence, LC-MS was performed using methanol–water (pH 4.50 with acetic acid) gradient (65–100% methanol at 0.5 mL/min flow rate, mobile phase F). Mass detector conditions were: nebulizer 30 psi; N_2 gas flow 10 L/min at 350 °C; voltage 4500 V; fragmentor 90 V; gain 5. Detection was made in selected ion monitoring mode (SIM) at $m/z = 285$ $(M + H)^+$ and 307 $(M + Na)^+$.

2.1. Hydrolysis of sulfate esters: reaction of 7-oxo-DHEA-3β-sulfate(I) with perchloric acid in ethyl acetate

Perchloric acid (10 μL, 70%) was added to 7-oxo-DHEA-3β-SO_4 (I, 1.0 μg in 2 mL ethyl acetate) in a screw-capped tube and allowed to stand at 25 °C after cyclomixing. After 0.25 h, the ethyl acetate layer was washed with 5% aq. bicarbonate (1 mL) and water (2 × 1 mL) the organic layer was separated by snap-drying in an acetone–dry ice bath and evaporated under nitrogen. The residue was dissolved in methanol–water (1:1, 200 μL) and a 10 μL sample was analyzed by LC-MS (Table 1, entry 1). The product was identified as 7-oxo-DHEA (II) by comparison with an authentic sample: retention time (t_R) 11.71 min (mobile phase B); yield 99.5%

Table 1
The perchloric acid-induced transformations of 7-oxygenated steroids

S. No.	Compound	Solvent	Temperature (°C)	Time (h)	Product composition (%)[a]		
					a	b	c
1	I	EtOAc	25	0.25	0	>99	<1
2	I	EtOAc	40	8.0	0	1	99
3	I	Ether	25	0.75	88	12	0
4	I	CH_3OH	65	0.5	0	0	100
5	II	EtOAc	25	0.25	–	100	0
6	III	EtOAc	25	0.25	100	0	0
7	III	CH_3OH	65	0.7	0	0	100
8	IV	CH_3OH	65	0.7	0	0	100
9	V	CH_3OH	65	0.7	0	0.2	99.8
10	VI	EtOAc	25	0.5	–	99	1
11	VI	CH_3OH	65	0.7	–	0.1	99.9
12	X	EtOAc	40	1.0	1	0	99

I, 7-oxo-DHEA-3β-sulfate; II, 7-oxo-DHEA; III, 7-oxo-DHEA-3β-stearate; IV, 3β, 17β-dihydroxyandrost-5-en-7-one; V, 17β-hydroxy-3β-methoxyandrost-5-en-7-one; VI, 7-oxo-cholesterol; X, 7-hydroxytestosterone, 3-ethylene glycol ketal.

[a] a, Unreacted starting steroid; b, free steroid; c, hydrolyzed and dehydrated steroid.

(HPLC); LC-MS (m/z): 325 $(M + Na)^+$; 303 $(M + H)^+$; 285 $(M–H_2O + H)^+$; λ_{max} 242 nm (DAD).

2.2. Solvolysis followed by dehydration: reaction of 7-oxo-DHEA-3β-SO_4 (I) with perchloric acid in methanol

Perchloric acid (10 μL, 70%) was added to a methanolic solution of 7-oxo-DHEA (100 ng in 2 mL methanol) in a screw-capped pyrex tube. The tubes were closed and the contents were heated in a water bath at 65 °C. After 1 h, water (2 mL) was added and methanol was removed under nitrogen at 40 °C. The remaining aqueous solution was diluted with water (1 mL) and extracted with ether (3 mL). The ether layer was washed with water (2 × 1 mL) and the ether was separated by snap-drying in acetone–dry ice bath and evaporated under nitrogen. The residue was dissolved in methanol–water (1:1, 200 μL) and a 10 μL aliquot was injected onto the chromatogram (Table 1, entry 4). The product was identified as androsta-3,5-diene-7,17-dione (VII) by comparison with a sample synthesized in the laboratory: retention time (t_R) 19.76 min (mobile phase A); yield >99.8% (HPLC); LC-MS (m/z): 307 $(M + Na)^+$; 285 $(M + H)^+$; λ_{max} 284 nm (DAD).

2.3. Extraction procedure for plasma samples and perchloric acid treatment

Plasma sample (1 mL each) was applied to Oasis solid-phase extraction cartridge (Waters, 3 cm^3), preconditioned with methanol (1 mL) and water (1 mL). The cartridge was washed with water (1 mL) and 50% aqueous methanol containing 2% acetic acid (1 mL) and dried under water pump suction (~10–15 in. of mercury) for

1 min. It was eluted with methanol (1 mL), transferred to a screw-capped glass tube, and heated at 65 °C for 35 min after the addition of perchloric acid (70%, 5 μL). Reaction mixture was diluted with water (4 mL) and extracted with ether (3 mL). Ether layer was separated by snap-drying in an acetone–dry ice bath, washed with water (1 mL), and evaporated under nitrogen at 40 °C. The residue was dissolved in methanol–water (1:1 v/v, 300 μL) and 40 μL was injected into the HPLC system for detection with DAD-UV at 284 nm (mobile phase E) and 15 μL for detection by LC-MS (mobile phase F) in ESI-SIM mode at $m/z = 285$ $(M + H)^+$ or at 307 $(M + Na)^+$.

2.4. Hydrolysis of 7-oxo-DHEA-3β-palmitate by rat brain homogenate

7-Oxo-DHEA-3β-palmitate (10 μg) was incubated at 37 °C with rat brain homogenate (4 mL containing 1 g brain). Samples (1 mL each) were withdrawn at every hour, diluted with acetonitrile (1 mL), and centrifuged (1500*g*) for 5 min. The supernatant was concentrated (0.5 mL) under nitrogen and diluted with water (2 mL). Aqueous layer was extracted with ether (3 mL). The ether layer was concentrated under nitrogen and the residue was dissolved in methanol–water (60:40, 200 μL) and subjected to LC-MS (methanol–water gradient, 30–100% methanol in 35 min, followed by 100% methanol for 5 min). The extracted ion analysis showed the presence of 7-oxo-DHEA {t_R 11.8 min, m/z 325 $(M + Na)^+$} and 7-oxo-DHEA-3β-palmitate {t_R 37.2 min, m/z 563 $(M + Na)^+$}. The sample was analyzed again and fractions corresponding to retention times of 7-oxo-DHEA and 7-oxo-DHEA-3β-palmitate were collected separately, evaporated under nitrogen, and subjected to perchloric acid reaction in methanol as mentioned earlier. The products were identified as androsta-3,5-diene-7,17-dione (VII), as discussed above {t_R 19.76 min (mobile phase A), 307 $(M + Na)^+$; 285 $(M + H)^+$; λ_{max} 284 nm (DAD)}.

3. Results and discussion

3.1. Chemical degradations

It has been reported [4,5] that the sulfate conjugates of steroids can be solvolyzed by treatment with perchloric acid using a variety of organic solvents. However, we realized that the solvolysis of sulfate esters of 7-oxo-3-hydroxy-Δ^5-steroids was not a straightforward reaction. The solvolysis was highly dependent on the nature of the solvent, reaction temperature, time (Table 1, and Figs. 1 and 2), and mode of workup. Use of polar solvents invariably led to the formation of diene(s). Even a trace amount of residual acidity caused the dehydration during concentration, resulting in the formation of complex mixtures, the degree of complexity depending upon the nature of the 7-oxygenated-Δ^5-steroid and reaction workup conditions. We were able to achieve a highly selective hydrolysis of sulfate esters in ethyl acetate (Scheme 1) at ambient temperature in 15 min to yield the corresponding free steroids in near-quantitative yield (Table 1, entry 1). These conditions did not alter the free 7-oxo-3-hydroxy-Δ^5-steroids (Table 1, entry 5). Replacing ethyl acetate with ether (Table 1, entry 3) or methanol (Fig. 1), or a change in temperature (Fig. 2) or

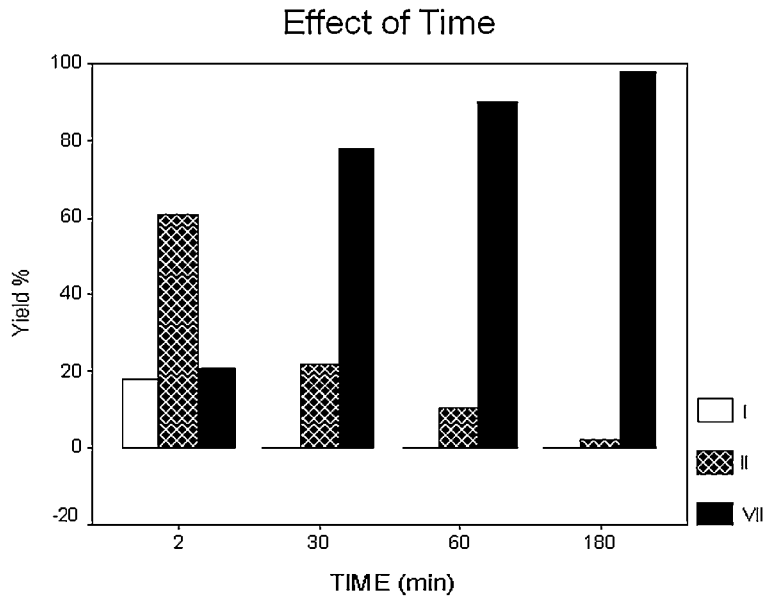


Fig. 1. The effect of reaction time on the hydrolysis of 7-oxo-DHEA-3β-sulfate using perchloric acid in methanol (0.5% v/v) at room temperature. (I) 7-Oxo-DHEA-3β-sulfate; (II) 7-oxo-DHEA; (VII) androsta-3,5-diene-7,17-dione.

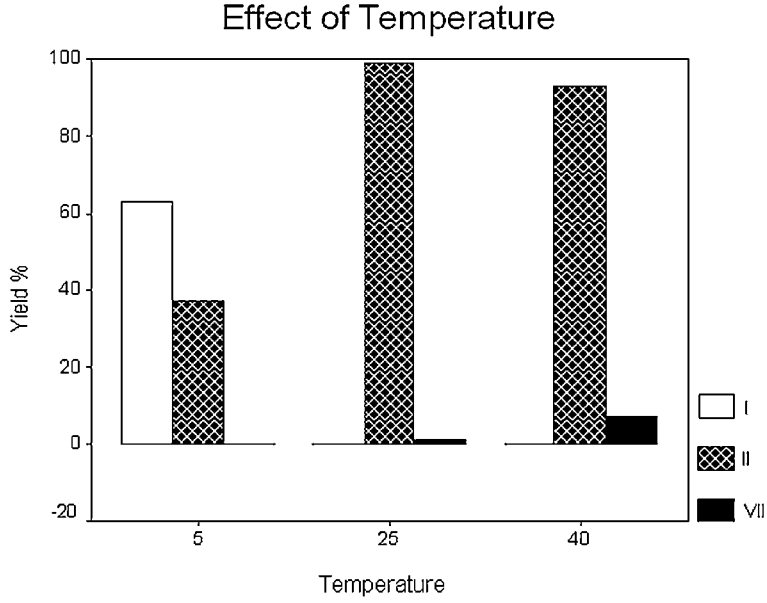
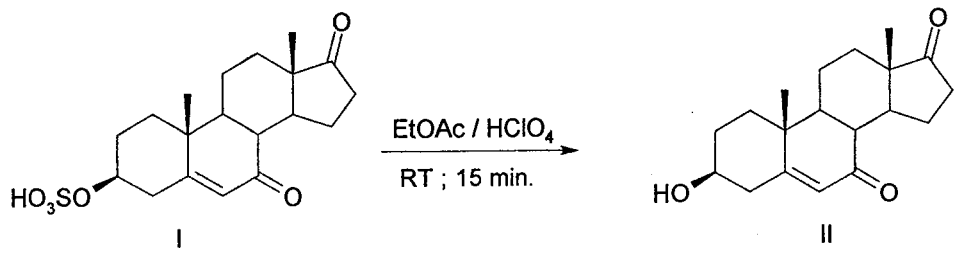
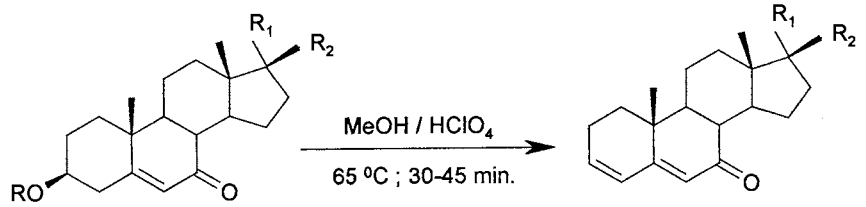


Fig. 2. The effect of temperature on the hydrolysis of 7-oxo-DHEA-3β-sulfate using perchloric acid in ethyl acetate (0.5% v/v). Reaction time 15 min. (I) 7-Oxo-DHEA-3β-sulfate; (II) 7-oxo-DHEA; (VII) androsta-3,5-diene-7,17-dione.



Scheme 1. Hydrolysis of sulfate esters with perchloric acid (0.5% v/v) in ethyl acetate at room temperature. (I) 7-Oxo-DHEA-3β-sulfate; (II) 7-oxo-DHEA.



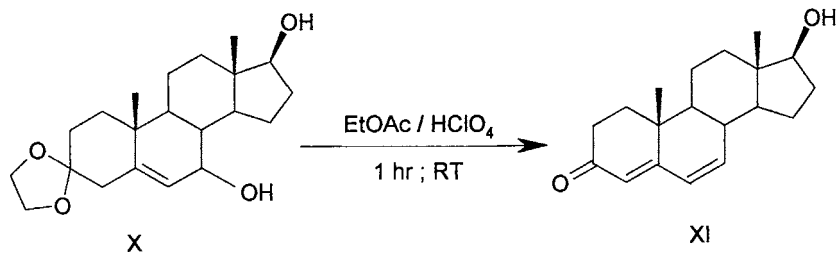
I, R=HO_3S; R_1, R_2= =O
II, R=H; R_1, R_2= =O
III, R=$CH_3(CH_2)_{16}CO$; R_1, R_2= =O
IV, R=H; R_1=H; R_2=OH
V, R=CH_3; R_1=H; R_2=OH
VI, R=H; R_1=H; R_2= -$CH(CH_3)(CH_2)_3CH(CH_3)_2$

VII, R_1,R_2= =O
VIII, R_1=H; R_2=OH
IX, R_1=H;
R_2= -$CH(CH_3)(CH_2)_3CH(CH_3)_2$

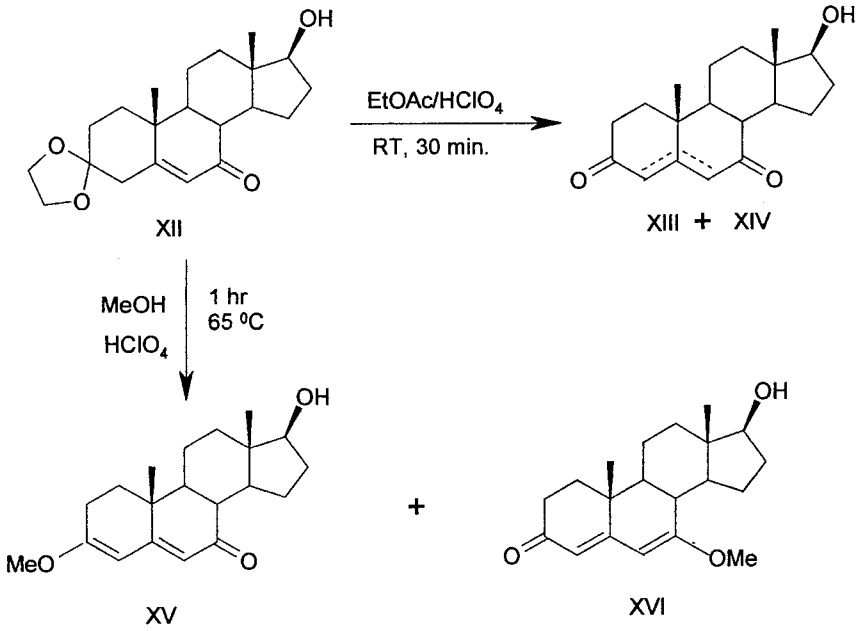
Scheme 2. Transformation of 7-oxo-Δ^5-steroids into 3,5-dien-7-one derivatives using perchloric acid in methanol at 65 °C. (I) 7-Oxo-DHEA-3β-sulfate; (II) 7-oxo-DHEA; (III) 7-oxo-DHEA-3β-stearate; (IV) 3β,17β-dihydroxyandrost-5-en-7-one; (V) 17β-hydroxy-3β-methoxyandrost-5-en-7-one; (VI) 7-oxo-cholesterol; (VII) androsta-3,5-diene-7,17-dione; (VIII) 17β-hydroxyandrosta-3,5-dien-7-one; (IX) cholesta-3,5-dien-7-one.

reaction time (Figs. 1 and Table 1, entry 2), resulted in the partial hydrolysis and/or the formation of the dienone product. Carboxylic acid esters remained unaffected (Table 1, entry 6) in ethyl acetate. However, free 7-oxo-3-hydroxy-Δ^5-steroids (Table 1, entries 8 and 11) as well as their carboxylic esters (Table 1, entry 7), sulfate conjugates (Table 1, entry 4), and methyl ether (Table 1, entry 9) were all quantitatively converted to corresponding 3,5-dien-7-one derivatives in methanol at 65 °C (Scheme 2). The unconjugated 17-hydroxyl group present in the starting steroid remained unaffected (Table 1, entries 8, 9, and 12).

This reaction was applicable with equal ease to 7-hydroxy-Δ^4-3-oxo steroids (Table 1, entry 12), the product being a 3-oxo-4,6-diene derivative (XI; Scheme 3). We next studied the behavior of 7-oxo-testosterone as its 3-ketal derivative (XII). This compound readily underwent deketalization in ethyl acetate/perchloric acid at ambient temperature (Scheme 4) to yield a mix of Δ^4 and Δ^5 products (XIII and XIV). However, upon heating in methanol/perchloric acid, XII yielded a mixture of



Scheme 3. Perchloric acid-induced transformation of 7-hydroxytestosterone, ethylene glycol ketal (X) in ethyl acetate at ambient temperature; (XI) 17-hydroxyandrosta-4,6-dien-3-one.



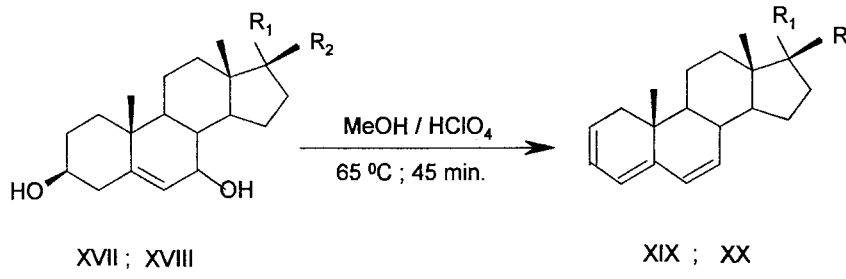
Scheme 4. Perchloric acid-induced transformation of 7-oxotestosterone, ethylene glycol ketal (XII) in methanol at 65 °C; (XIII) 7-oxo-testosterone; (XIV) 17-hydroxyandrost-5-ene-3,7-dione; (XV) 17-hydroxy-3-methoxyandrosta-3,5-dien-7-one; (XVI) 17-hydroxy-7-methoxy-androsta-4,6-dien-3-one.

17β-hydroxy-7-methoxyandrosta-4,6-dien-3-one (XV) and 17β-hydroxy-3-methoxy-androsta-4,6-dien-7-one (XVI). The formation of these products demonstrated that the keto groups (3 and 7) underwent enolization, followed by etherification. The driving force for this reaction is provided by the formation of extended conjugation (λ_{max} 322 nm). The 17-hydroxyl group remained unaffected. Therefore, perchloric acid may be used for the selective alkylation of a conjugated hydroxyl group in the presence of unconjugated alcohols. A partial formation of corresponding ethyl ethers of XV and XVI was seen, even when, reaction time was extended during the deketalization process in ethyl acetate.

7-Hydroxy-DHEA (XVII) as well as androst-5-ene-3β,7,17β-triol (XVIII) lose up to two molecules of water (resulting from loss of the 3-OH and 7-OH groups) on treatment with perchloric acid (Scheme 5), yielding a mixture of several dienes and trienes; the major products (>70%) were androsta-2,4,6-trien-17-one {XIX, m/z 269 $(M+H)^+$ and 291 $(M+Na)^+$} and androsta-2,4,6-trien-17-ol {XX, m/z 271 $(M+H)^+$ and 253 $(M+H\text{-}H_2O)^+$}. Their structures were confirmed further by ^1H NMR and ^{13}C NMR spectral analyses and λ_{max} (DAD) at 306 nm (calculated 303 nm). Table 2 lists the LC-MS data (λ_{max} as seen in diode array UV spectra, retention times, and the most abundant ions) of various 7-oxygenated steroids and the products obtained by their perchloric acid treatment.

The reactions studied in the present investigation were carried out at nanomole and sub-nanomole concentrations for LC-MS studies. Their progress and product composition were studied by HPLC and LC-MS. The products were identified, on the basis of retention times, by comparison with known standards, elution behavior in HPLC as well as LC-MS, UV spectra (obtained from an online diode array detector), co-chromatography, derivatization to known compounds, and last but not the least with the help of mass spectrometry by running the samples under different ionization modes, i.e., positive vs. negative and electrospray vs. chemical ionization, fragmentation patterns, and by comparison of mass spectra with those of known standards.

7-Oxo-DHEA (I) has a UV maximum near 240 nm; therefore, all samples were monitored at 240 nm for the presence of 7-oxo-DHEA (II) and related compounds. The chromatograms were also monitored at 205 nm for the presence of 7-hydroxy compounds. Lastly, the samples were simultaneously monitored at 284 and 310 nm for the presence of conjugated dienes, trienes, and dienones, particularly after perchloric acid treatment. The products formed by the dehydration of 7-OH-Δ^5-steroids are comparatively non-polar compounds containing two or more double bonds and the absence of polar groups makes them poor substrates for electrospray mass spectrometry. Consequently, they were also subjected to chemical ionization mass spectrometry.



Scheme 5. Perchloric acid-induced dehydration of 7-hydroxy-DHEA (XVII) and androst-5-ene-3β, 7,17β-triol (XVIII) in methanol at 65 °C; (XIX) androsta-2,4,6-trien-17-one; (XX) androsta-2,4,6-trien-17β-ol.

Table 2
LC-MS analysis of 7-oxygenated steroids and their reaction products after perchloric acid treatment

Compound	Retention time (min)	UV λ_{max} (nm)	Mass sp. (ESI)* (m/z)
I	20.2[a]	242	381 $(M–H)^-$
II	11.7[b]	242	325 $(M + Na)^+$; 303 $(M + H)^+$; 285 $(M + H–H_2O)^+$
III	14.7[c]	236	285 $(M + H–C_{17}H_{35}COOH)^+$[d]
IV	9.8[b]	242	631 $(2M + Na)^+$; 327 $(M + Na)^+$; 305 $(M + H)^+$
V	16.3[b]	242	341 $(M + Na)^+$; 319 $(M + H)^+$
VI	8.8[c]	238	401 $(M + H)^+$; 383 $(M + H–H_2O)^+$[d]
VII	19.8[b]	284	307 $(M + Na)^+$; 285 $(M + H)^+$
VIII	19.4[b]	284	595 $(2M + Na)^+$; 309 $(M + Na)^+$; 287 $(M + H)^+$
IX	13.4[c]	280	383 $(M + H)^+$[d]
X	24.6[e]	234	371 $(M + Na)^+$; 349 $(M + H)^+$; 331 $(M + H–H_2O)^+$; 287 $(M + H–H_2O–OCH_2CH_2)^+$
XI	21.9[e]	288	595 $(2M + Na)^+$; 309 $(M + Na)^+$; 287 $(M + H)^+$
XII	15.9[c]	242	369 $(M + Na)^+$; 347 $(M + H)^+$
XIII + XIV	10.9[e]	322	325 $(M + Na)^+$; 303 $(M + H)^+$
XV and XVI	21.4[e]	313	339 $(M + Na)^+$; 317 $(M + H)^+$
	22.1[e]	324	339 $(M + Na)^+$; 317 $(M + H)^+$
XVII	13.2[b]	–	327 $(M + Na)^+$; 287 $(M–H_2O)^+$; 269 $(M–2H_2O)^+$
XVIII	8.3[b]	–	329 $(M + Na)^+$; 289 $(M–H_2O)^+$; 271 $(M–2H_2O)^+$; 253 $(M–3H_2O)^+$
XIX	27.9[b]	306	559 $(2M + Na)^+$; 291 $(M + Na)^+$; 269 $(M + H)^+$; 241 $(M + H–CO)^+$
XX	28.3[b]	306	271 $(M + H)^+$; 253 $(M + H–H_2O)^+$

I, 7-Oxo-DHEA-3β-sulfate; II, 7-oxo-DHEA; III, 7-oxo-DHEA-3β-stearate; IV, 3β,17β-dihydroxyandrost-5-en-7-one; V, 17β-hydroxy-3β-methoxyandrost-5-en-7-one; VI, 7-oxo-cholesterol; VII, androsta-3,5-diene-7,17-dione; VIII, 17β-hydroxyandrosta-3,5-dien-7-one; IX, cholesta-3,5-dien-7-one; X, 7-hydroxytestosterone, 3-ethylene glycol ketal; XI, 17-hydroxyandrosta-4,6-dien-3-one; XII, 7-oxotestosterone, ethylene glycol ketal; XIII, 7-oxo-testosterone; XIV, 17-hydroxyandrost-5-ene-3,7-dione; XV, 17-hydroxy-3-methoxyandrosta-3,5-dien-7-one; XVI, 17-hydroxy-7-methoxyandrosta-4,6-dien-3-one; XVII, 7-hydroxy-DHEA; XVIII, androst-5-ene-3β,7,17β-triol; XIX, androsta-2,4,6-trien-17-one; XX, androsta-2,4,6-trien-17β-ol.

* ESI mode unless specified otherwise.

[a] Mobile phase B.

[b] Mobile phase A.

[c] Mobile phase C.

[d] APCI mode.

[e] Mobile phase D.

3.2. Bioanalytical applications

A major constraint in the analysis of steroids in biomatrices is the presence of steroids and their metabolites in nanogram quantities. The problem is complicated further for steroid derivatives, which may be present as more than one conjugate, thus,

making it difficult to determine their total concentration in the system. As discussed above, the 3-hydroxy-Δ^5-steroids oxygenated at position 7 are prone to dehydration and this tendency is particularly pronounced in the case of 7-oxo derivatives, which were selectively and quantitatively converted into 3,5-dien-7-one derivatives, which are stable and also have a better UV absorbance. The same is true for 7-hydroxy derivatives of 3-oxo-Δ^4-steroids. These features were successfully exploited in developing a strategy for the estimation of total 7-oxo-DHEA (II) and its derivatives in biological matrices (human plasma, rat liver, and brain) after their conversion to the corresponding 3,5-diene-7-one. In a typical example, human plasma was subjected to cleanup by solid-phase extraction and the total 7-oxo-DHEA was eluted with methanol. The methanol solution was treated with perchloric acid to convert the total 7-oxo-DHEA into dienone derivative (VII). After evaporation and dilution with water, the dienone was extracted with ether and, subsequently, analyzed by HPLC on a short C_8 column using methanol–water gradient and detected at 284 nm. The method is simple, fast (total run time 10 min), and sensitive (limit of detection 1 ng, for a signal-to-noise ratio of 3). Fig. 3A shows a representative chromatogram of human plasma blank that is treated with perchloric acid at 284 nm, while Fig. 3B shows a chromatogram of androsta-3,5-diene-7,17-dione (VII, 40 ng) in the same scale. The sensitivity of the method was improved further by subjecting the samples to LC-MS in electrospray mode on a C_{18} column using methanol–water gradient at pH 5 (with acetic acid, mobile phase D). The detection was made in selected ion monitoring mode at m/z 285 $(M + H)^+$ and m/z 307 $(M + Na)^+$. The formation of sodium ion adducts in electrospray LC-MS is well known and their use in quantitative analysis has been documented [28]. Fig. 4A shows an LC-MS chromatogram of perchloric acid-treated

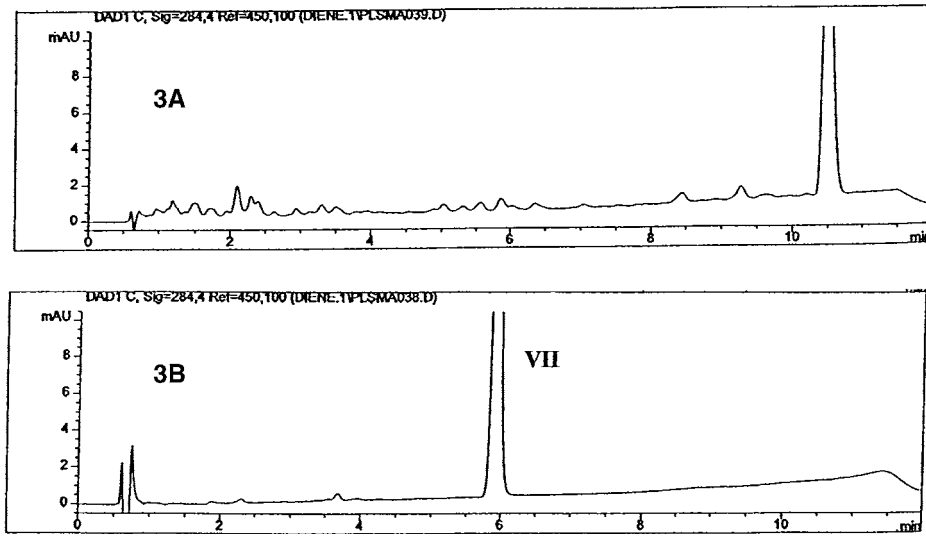


Fig. 3. (A) Chromatogram of human plasma blank (at 284 nm) after solid phase cleanup and perchloric acid treatment in methanol at 65 °C. (B) Androsta-3,5-diene-7,17-dione (40 ng). Chromatographic conditions: Zorbax-SB C_8 column (3.5 m, 4.6 × 50 mm); methanol–water (1% of 0.1 M phosphoric acid) gradient (50–70% methanol in 10 min) at 1 mL/min flow rate.

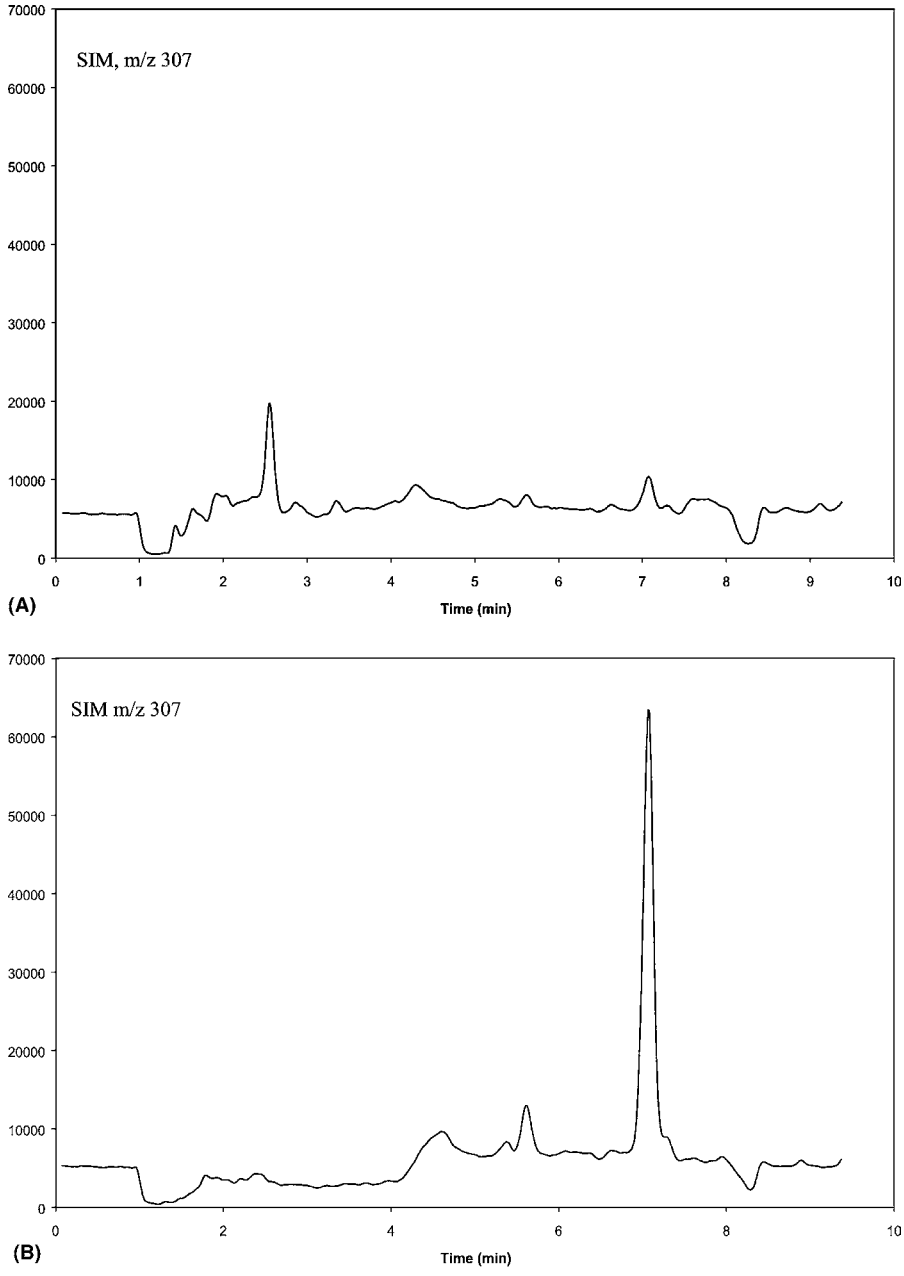


Fig. 4. (A) Chromatogram of human plasma blank (LC-MS) after solid phase cleanup and perchloric acid treatment in methanol at 65 °C. (B) Another treated plasma blank spiked with androsta-3,5-diene-7,17-dione (2 ng). Chromatographic conditions: Zorbax-SB C_{18} column (3.5 m, 4.6 × 75 mm); methanol–water (pH 4.50 with acetic acid) gradient (65–100% methanol in 10 min at 0.5 mL/min flow rate; gas flow 10 L/min at 350 °C; voltage 4500; fragmentor 90 V; nebulizer 30 psi.

human plasma extract in SIM mode (ESI), while Fig. 4B shows a chromatogram of perchloric acid treated human plasma extract spiked with 2 ng of 3,5-dien-7-one (VII).

This method is routinely used by us [27] to establish the structure of various 7-oxygenated metabolites of dehydroepiandrosterone (DHEA) that are produced by rat liver in vitro, by collecting the fractions of interest, then subjecting them to perchloric acid treatment in methanol, and comparing the dienone derivative, thus, formed, with authentic samples. In another study concerning hydrolysis of 7-oxo-DHEA fatty esters by rat brain homogenate (Fig. 5), it was difficult to prove the presence of unhydrolyzed ester solely on the basis of extracted ion chromatograms of total-ion current (TIC) spectrum, which was quite complicated for the 7-oxo-DHEA-3β-palmitate due to the presence of interfering peaks from the biomatrix (brain). Therefore, the fractions corresponding to retention times of 7-oxo-DHEA and 7-oxo-DHEA-3β-palmitate were collected and treated separately with perchloric acid and the product was identified as androsta-3,5-dien-7,17-dione (VII). This allowed us to prove beyond doubt the structures of desired compounds that are present in this complex matrix system.

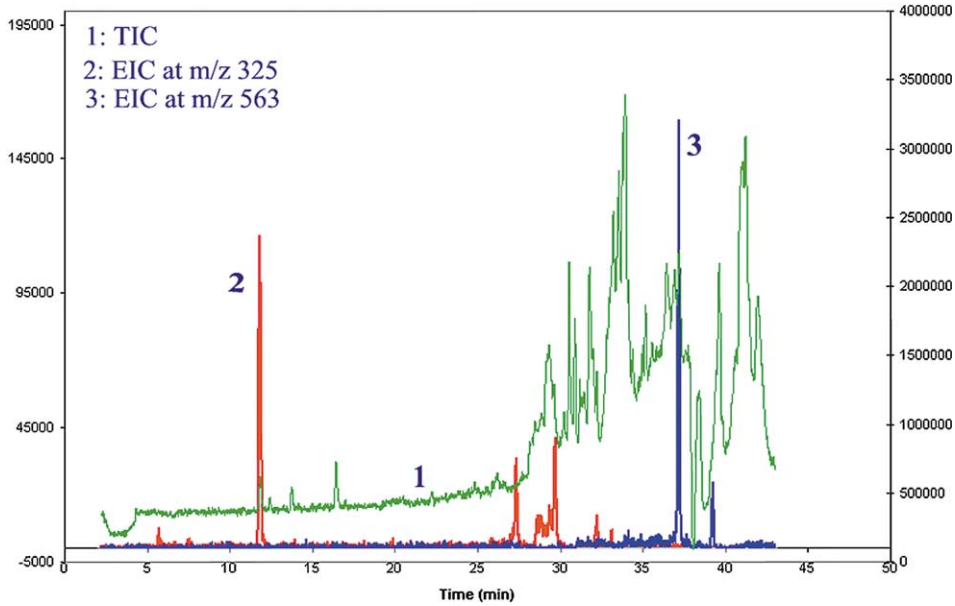


Fig. 5. Hydrolysis of 7-oxo-DHEA-3β-palmitate by rat brain homogenate. Each chromatogram in full scale. (1, blue) Total ion chromatogram of the brain homogenate extract, scale 1–3500 K. (2, red) Extracted ion chromatogram of 7-oxo-DHEA $\{m/z = 325\ (M + Na)^+\}$, scale 1–120 K. (3, green) Extracted ion chromatogram of 7-oxo-DHEA-3β-palmitate $\{m/z = 563(M + Na)^+\}$, scale 1–175 K. LC-MS conditions: methanol–water gradient (30–100% methanol in 35 min, followed by 100% for 5 min) on Zorbax-SB C_{18} column (4.6 × 75 mm) at 1 mL/min. N_2 flow 12 L/min at 350 °C; nebulizer 40 psi; voltage 5000 V; and fragmentor 75 V.

4. Conclusions

In the present study, the reaction of 7-oxygenated Δ^4- and Δ^5-steroids with perchloric acid has been studied in detail. The sulfate esters could be selectively and quantitatively solvolyzed in ethyl acetate at ambient temperature to the corresponding free steroids under mild conditions; whereas sulfate and carboxylic acid esters, ethers, ketals, etc. of these steroids were quantitatively converted to dienone derivatives in methanol at elevated temperatures. The later reaction can be utilized for the estimation of total 7-oxo-DHEA that is present in human plasma and to verify the presence of 7-oxo-Δ^5-steroids in complex biomatrices.

Acknowledgment

This work was supported by Hollis Eden Pharmaceuticals, San Diego, CA, USA.

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