

A capillary gas chromatography/mass spectrometric method for the quantification of hydroxysteroids in human plasma[☆]

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Abstract

A specific and sensitive methodology for the quantitative determination of hydroxysteroids dehydroepiandrosterone and pregnenolone and their main metabolites in human plasma is described. Hydroxysteroids were extracted using methanol and steroids were further separated by reverse-phase high-performance liquid chromatography, allowing for minimization of the possible chromatographic interferences. Eluted fractions were collected, pooled, and analyzed by gas chromatography–mass spectrometry as trimethylsilyl ether derivatives. The quantification was performed with single-ion monitoring of the highly abundant m/z 129 or m/z 358 fragments. The combination of the chromatographic characteristics to the specific fragments ensured the selectivity and specificity of the method. Under these conditions the method was linear (typical R^2 is superior to 0.98 for all hydroxysteroids studied) over the concentration range of 2×10^{-9} to 10^{-6} M with good precision and accuracy.

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Hydroxysteroid neurosteroids were initially described as steroids synthesized in the central nervous system [1–4] in a manner independent of peripheral steroidogenesis [5–7]. Among these steroids, pregnenolone, dehydroepiandrosterone (DHEA)¹, and their sulfate esters are the main biologically active neurosteroids, reported to alleviate amnesia and to enhance learning and long-term memory in mice [8–10]. Numerous studies were conducted to correlate DHEA and DHEA–sulfate serum levels to the onset and/or progression of Alzheimer’s

disease and to identify biomarkers via their corresponding hydroxylated metabolites [11]. Moreover, numerous gas chromatographic methods coupled to mass spectrometry (GCMS) for the determination of steroids and their hydroxylated metabolites have been described in the literature [11–16].

To develop a profile of the hydroxysteroid neurosteroids in biological fluids, we describe herein a rugged and fully validated gas chromatography/mass spectrometric methodology using electron impact ionization (from isolation by extraction to GCMS analysis via a RP-HPLC preparative purification) for the quantitative determination of hydroxysteroids (dehydroepiandrosterone, pregnenolone, and their main hydroxyl metabolites) using a selected single-ion monitoring (SIM) (m/z 129 or m/z 358) procedure in human plasma samples. The identification was assessed by combination of the absolute retention times to the mass spectrometric characteristics (i.e., specific MS fragments) of the studied neurosteroids and comparison to those stored in our own library built under the same gas

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¹ Abbreviations used: SIM, single-ion monitoring; MIM, multiplex monitoring; DHEA, dehydroepiandrosterone; RP-HPLC, reversed-phase high-performance liquid chromatography; m/z , mass to charge; GCMS, gas chromatography–mass spectrometry; RT, retention time; CV, coefficient of variation; S/N , signal to noise; LOD, limit of detection; LOQ, limit of quantification; RIA, radioimmunoassay; tms, trimethylsilyl.

chromatographic conditions and by specific multiple-ion monitoring.

Under these conditions, quantitative determination of DHEA, pregnenolone, and their main hydroxyl metabolites at the low-level part per billion (ppb) range become easily possible in human plasma samples. This method has been fully automated and adapted for the future determination of neurosteroids and their hydroxy metabolites in biological samples (i.e., sera or cerebrospinal fluid) obtained from patients suffering from Alzheimer's disease, in which lower levels of DHEA have been reported [17–20].

Materials and methods

Reagents and chemicals

DHEA, pregnenolone, dextran, charcoal, and gelatin were purchased from Sigma (St. Louis, MO, USA). 5-Androsten-3 β -ol-7, 17-dione (7-oxo-DHEA), 5-androsten-3 β , 7 α -diol, 17-one (7- α -hydroxy-DHEA), and 5-androsten-3 β , 7 β -diol, 17-one (7- β -hydroxy-DHEA) were purchased from Steraloids (Newport, RI, USA). Methanol, ethyl acetate, chloroform, acetonitrile, and water (HPLC-grade purity) were purchased from Merck (Darmstadt, Germany). Phosphate-buffered saline (1 \times) was purchased from Biosource International (CA, USA). The antibody anti-DHEA was supplied from ICN Pharmaceuticals (NY, USA). The derivatization reagent consisted of N,O-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane purchased from Supelco (Bellefonte, PA, USA). The radiolabeled compounds [3 H]dehydroepiandrosterone (specific activity, 60.00 Ci/mmol) and [3 H]pregnenolone (specific activity, 17.50 Ci/mmol) were purchased from Perkin-Elmer (Boston, MA, USA).

Samples

Plasma samples from 12 control (nondemented) subjects, six men and six women, mean age 71.3 ± 3.8 years, were from the Alzheimer's Disease Research Center (Department of Psychiatry, Mount Sinai School of Medicine, New York, NY, USA). Protocols for the use of human samples were approved by the Mount Sinai and Georgetown University Institutional Review Boards.

Steroid extraction

The extraction was performed using a liquid/liquid extraction with methanol allowing both protein precipitation (defecation) and concomitant solubilization of the hydroxysteroids present in the aqueous layer. The extraction yield was assessed by adding known amounts

of radiolabeled DHEA and pregnenolone in sera, and radioactivity was measured after extraction on the methanolic extract.

RP-HPLC preparative purification

The HPLC system used was a System Gold equipped with a gradient solvent delivery system, an autosampler AS 508, a 166 NM detector (Beckman, CA, USA), and a Rheodyne loop injector, 100 μ L (Cotati, USA). The chromatographic separations were carried out on an Ultra sphere column, 5 μ m, 4.6 mm ID \times 250 mm of length (Beckman). The mobile phase (acetonitrile + water; 55 + 45; v/v) was ultrasonicated for 30 min and isocratically set at 1.0 mL/min. The detection was set at 212 nm and the absolute retention times (RT min) were targeted for the collection of the studied steroids. Only HPLC fraction corresponding to "collect times" were pooled and used for further analysis.

Radioimmunoassay (RIA)

The RIA method was performed using a commercially available antibody raised against the DHEA (ICN Diagnostics, CA, USA).

GCMS analysis

The GCMS system (Shimadzu, Kyoto, Japan) consisted of a GC 17A gas chromatograph equipped with an automatic flow control, an AOC-20 autosampler, and a mass spectrometer QP 5050A quadrupole electron impact detector with a fixed electron voltage of 70 eV. The chromatographic separation was performed on a fused silica capillary column (30 m \times 0.25 mm ID) coated with a 0.25- μ m layer of methylsilicone plus 5% phenyl-methylsilicone, and the injection port was equipped with double-gooseneck splitless liner with siltek deactivation coating (Restek Co., Bellefonte, PA, USA). The temperatures were set at 280 and 310 $^{\circ}$ C for the injection port and interface, respectively. The column temperature program was ramped as follows: the initial temperature of the column was held at 120 $^{\circ}$ C for 3 min and then increased at 40 $^{\circ}$ C/min to 310 $^{\circ}$ C followed by a decrease at 10 $^{\circ}$ C/min to 120 $^{\circ}$ C. The total run time analysis was 34 min. A splitless injection mode was used. The flow of the carrier gas (helium) was 1.2 mL/min. The detector voltage was set at 1.2 kV and the sampling was 0.25 s. For full-scan acquisition (GCMS), the mass spectrometer was operated in an impact electron ionization mode at 70 eV and scanned from 20 to 920 U. Electron impact with a single-ion monitoring mode was used for this procedure. The most abundant ion fragments, m/z 129 or m/z 358, in the steroids studied were monitored for the quantification. The specificity of the identification was increased by

Table 1
Molecular mass and mass spectral characteristics of the studied hydroxysteroids

Steroid to be monitored	MW	Target SIM m/z fragment	Target m/z fragments for MIM
DHEA	288.4	129	360(M); 304(M-56); 270(M-90); 231(M-129)
7 α -Hydroxy-DHEA	304.4	358	448(M); 129(M-319); 343(M-105)
7 β -Hydroxy-DHEA	304.4	358	448(M); 129(M-319); 343(M-105)
7-Keto-DHEA	302.4	129	374(M); 318(M-56); 284(M-90); 269(M-90-15)
Pregnenolone	316.5	129	388(M); 373(M-15); 253(M-90-15)

monitoring multiple fragments (MIM mode) corresponding to further fragmentation (Table 1).

Operating procedure

HPLC fractions were pooled and evaporated to dryness by centrifugation in vacuum using the Speed-Vap instrument (ATR Laurel, MD, USA). One to two milligrams of anhydrous granular sodium sulfate was added, and a volume of 400 μ L of ethyl acetate was added to the dried residue. The mixture was shaken by vortex for few seconds and the organic phase was gently collected and dried by centrifugation in vacuum. Finally a volume of 100 μ L of the silylated reagent was added and the solution kept incubated at 80 °C for 45 min. The excess of the silylated reagent was removed under a gentle stream of nitrogen to dryness, a volume of 400 μ L of chloroform was added, and a 6- μ L sample was injected for GCMS analysis.

Results and discussion

It has been reported that biotransformation of DHEA and pregnenolone leads to their corresponding 7-hydroxylated metabolites [21–23]. Moreover, Rose et al. [24] reported the importance of a novel brain cytochrome P450 catalyzing the formation of 7-hydroxylated metabolites (particularly DHEA and pregnenolone). Cascio et al. [5] reported an alternative pathway for the formation of DHEA in brain, and, recently, Brown et al. [6] corroborated this phenomenon in human sera using radioimmunoassay on HPLC-purified fractions to measure the DHEA levels. The main objective of the present study was to qualitatively and quantitatively determine trace amounts of human plasma 3-hydroxysteroids and their hydroxylated metabolites in a one-run analysis using a gas chromatography method after extraction and liquid chromatography purification to investigate changes in their profiles in various neurodegenerative diseases.

Extraction

A liquid/liquid extraction based on the property of methanol to both precipitate plasma proteins and to

simultaneously solubilize the steroids present in the aqueous phase was used. Due to the strong interactions between steroids and tissue proteins, we adopted this extraction protocol allowing minimization of operations, thus avoiding inherent loss due to supplementary steps. For the extraction, two main representative compounds of the studied class of steroids were chosen because of their chemical and physical properties: one strongly soluble in apolar solvent (i.e., chloroform) (pregnenolone) and one sparingly soluble in chloroform (DHEA). The use of the radiolabeled compound was based on the specificity of this approach with regard to the possible interference of plasma endogenous steroids when using HPLC coupled to ultraviolet detection. The extraction yield was assessed as described under Materials and methods and recoveries were determined by comparing the radioactivity of the extracts of plasma spiked with known amounts of various radiolabeled steroids versus radioactivity of direct measurements performed on the standard solutions at the same amounts. In the amount range of 10^{-11} to 10^{-8} mole, the interassay recoveries of the extraction procedure from plasma were 0.63 ± 0.03 and $0.80 \pm 0.04\%$ ($n = 5$) for [3 H]DHEA and [3 H]pregnenolone, respectively. The efficiency of the liquid chromatographic process was assessed by injecting known amounts of radiolabeled DHEA (the efficiency ranged from 0.90 to 0.102).

Mass spectral characteristics

The hydroxysteroids under investigation exhibited an important base peak (m/z 129) in comparison to other fragments, with the exception of the 7-hydroxylated metabolites (7 α - and 7 β -hydroxy-DHEA). The 7-hydroxy-DHEA derivatives exhibited both a m/z 129 fragment with less intensity and an abundant m/z 358 fragment corresponding to a prominent loss of a trimethylsilanol. Under these silylation conditions the quantitation of the hydroxylated metabolites is achieved via the m/z 358 selected ion monitoring. The M-105 (m/z 343) and the m/z 129 were in these cases chosen as fragments for assessing specificity. Fig. 1 depicts typical mass spectral profiles of the HPLC fractions corresponding to the studied steroids. Fig. 2 shows the chemical structures of the specific MS fragments obtained by electron impact ionization of the molecular

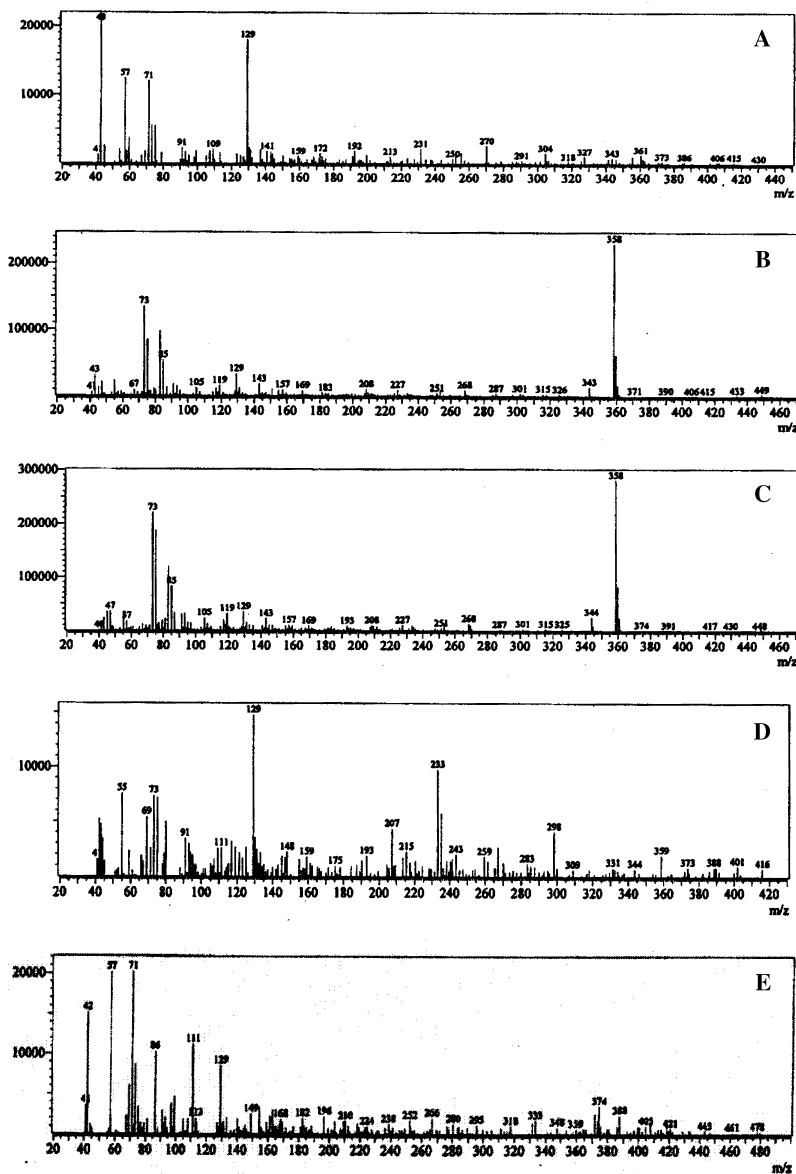


Fig. 1. Mass spectral characteristics of the studied hydroxysteroids as trimethylsilyl (tms) derivatives under electron impact ionization from HPLC fractions. (A) DHEA-tms; (B) 7- α -hydroxy-DHEA-2tms; (C) 7- β -hydroxy-DHEA-2tms; (D) pregnenolone-tms; (E) 7-oxo-DHEA-tms.

ion radical. Table 1 summarizes the process used for the characterization of the studied hydroxysteroids. For example in the case of DHEA, the m/z 129 fragment in the A ring, the m/z 304 resulting from the opening and loss of the D ring from the molecular ion radical ($M^{+\cdot}$) (M 360), the $M-90$ (m/z 270) resulting from the loss of trimethylsilanol with subsequent electronic rearrangement of the steroidal moiety, and the m/z 231 ($M-129$) are depicted in Fig. 2.

It has been reported that the m/z 129 fragment is frequently encountered in several mass spectra of several substances (trimethylsilyl ether derivatives or not) which would suggest a hypothetical lack of specificity. Shackleton et al. [25] reported the presence of Δ^7 and Δ^8 dehydroanalogues of the classical 3- β -hydroxy- Δ^5 -

steroids in plasma and urine of patients associated with the Smith Lemli Opitz syndrome. Among these, the corresponding analogues of dehydroepiandrosterone exhibited a m/z 129 fragment in their spectral pattern upon electron impact [25]. Furthermore the whole process of the methodology (HPLC purification, specific derivatization, and GC retention characteristics) should favorably contribute to increase the specificity of the procedure. Under these experimental conditions, no fragment of charge to mass ratio of 129 or 358 was monitored at the retention times corresponding to the studied hydroxysteroids in a chromatogram of a blank silylated solution. For the identification, unstable specific fragments with lower mass were monitored to ensure the specificity of the substance to be monitored.

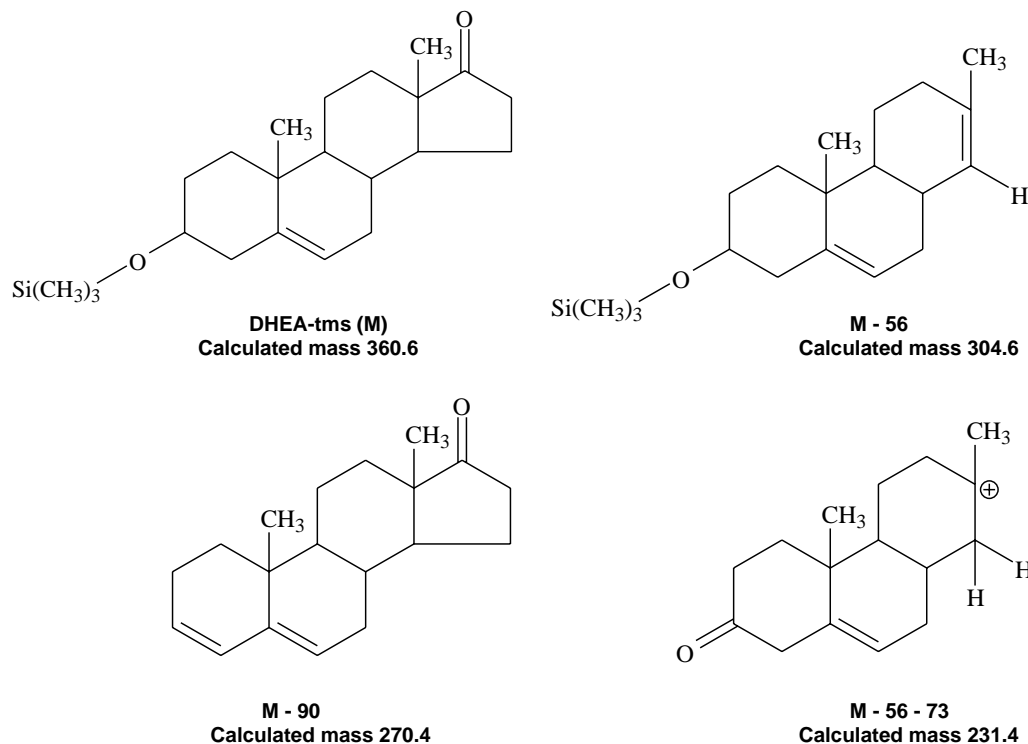


Fig. 2. Proposed chemical structures of the specific MS fragments of DHEA-tms selected for multiple ion monitoring.

HPLC analysis

HPLC purification was used to eliminate contaminants and selectively collect the steroids before the GCMS analysis. In preliminary experiments, although we noted that the commonly used solid-phase cartridge microextraction for the isolation of steroids [26,27] is a less time-consuming and more selective method, because of the higher energy involved, we opted for a protein-precipitation method with a concomitant solvent (i.e., methanol) water miscible recovery (solubilization). This choice was based on the fact that this approach is less selective and exhibits higher extraction recovery, thus eliminating the addition of supplementary steps during sample preparation, which led to increased risk of analyte loss. Because of the poor selectivity of the methanol and to lower the analytical interferences, RP-HPLC was used to clean up the solution before injection into the gas chromatograph. This procedure led to

relatively pure eluted substances and minimized the possible analytical interference during the derivatization step, GC analysis, and MS detection. The partition allowed elution of the more polar to the less polar hydroxysteroid, and this operation allowed collection of the hydroxysteroids in 15 min and lowered the background during MS analysis. In addition, this methodology allowed us to collect enough material to perform measurements using two distinct approaches, i.e., RIA and GCMS.

Table 2 shows the chromatographic data obtained from a mixture of steroids. A good resolution is obtained between DHEA and pregnenolone, and on the other hand no separation is obtained between 7 α -hydroxy-DHEA, 7 β -hydroxy-DHEA, and 7-oxo-DHEA (Table 2); meanwhile this was achieved by the GC analysis as indicated in the chromatograms displayed in Fig. 3. The double chromatographic retention characteristics (HPLC and GC) allowed us to eliminate a

Table 2
Liquid chromatography and gas chromatography characteristics of the studied hydroxysteroids (values are means \pm SD; $n = 15$ to 30)

Compound to be Monitored	RP-HPLC		GC
	RT (min)	Collect time (min)	RT (min)
DHEA	6.4 \pm 0.02	6.0 to 7.0	8.93 \pm 0.02
7 α -Hydroxy-DHEA	3.7 \pm 0.03	3.0 to 4.5	9.02 \pm 0.02
7 β -Hydroxy-DHEA	3.7 \pm 0.02	3.0 to 4.5	9.44 \pm 0.01
Pregnenolone	13.4 \pm 0.02	13.0 to 14.0	9.86 \pm 0.02
7-Keto-DHEA	3.7 \pm 0.03	3.0 to 4.5	10.2 \pm 0.02

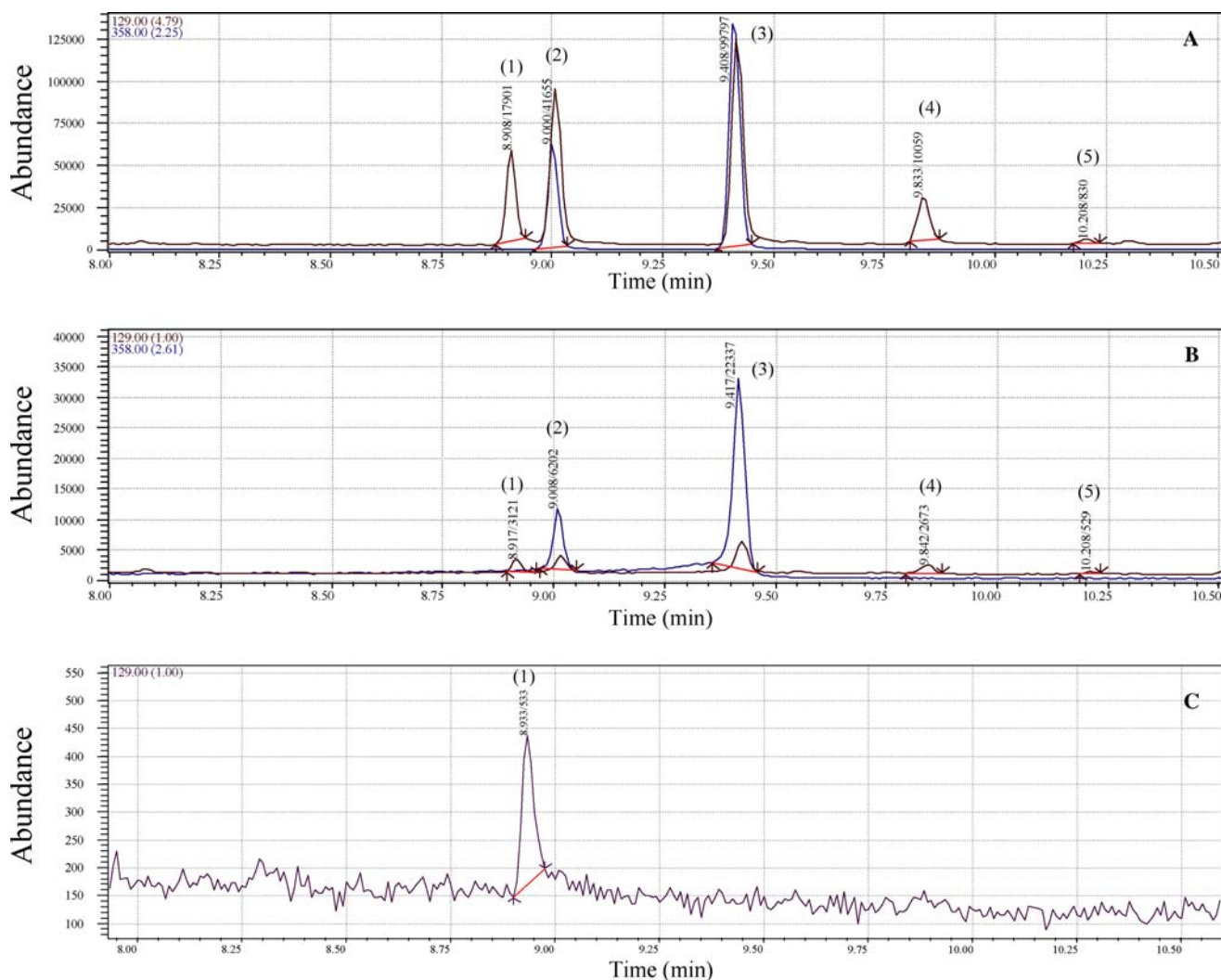


Fig. 3. Typical GCMS reconstructed ion chromatograms as trimethyl derivatives on SIM analysis (m/z 129 and m/z 358). (A) Solution containing five hydroxysteroids; (B) plasma spiked with five steroids; (C) HPLC fraction of human plasma. (1) DHEA-tms; (2) 7- α -hydroxy-DHEA-2tms; (3) 7- β -hydroxy-DHEA-2tms; (4) pregnenolone-tms; (5) 7-oxo-DHEA-tms.

number of interferences which should exhibit m/z 129 or m/z 358 mass fragments.

Analytical performance characteristics and assay in human plasma samples

The interserial repeatability of the absolute RT (min) and intensity were evaluated. The internal

interday reproducibility of the retention times as the intensity of the peak in area was studied for 3 days running identically prepared pooled plasma spiked with the steroids to be monitored, and the data are shown in Table 3. The standard curves for the measurement of the hydroxysteroids were constructed by using the m/z 129 or m/z 358 fragments which constituted under these derivatization conditions the base

Table 3
GC retention data and analytical performance characteristics of the trimethylsilyl hydroxysteroid derivatives

Compound to be monitored	LOD (nM)	LOQ (nM)	Repeatability CV%	Reproducibility CV%	Linearity (R^2) range
DHEA	0.8	2	5.8	8.6	0.995 (2 nM to 2 μ M)
7 α -Hydroxy-DHEA	2	5	6.5	9.1	0.997 (5 nM to 2 μ M)
7 β -Hydroxy-DHEA	2	5	5.3	7.6	0.999 (5 nM to 2 μ M)
Pregnenolone	3	9	7.2	9.7	0.994 (9 nM to 2 μ M)
7-Oxo-DHEA	nd	nd	10.4	12.7	0.896 (50 nM to 2 μ M)

nd, not determined.

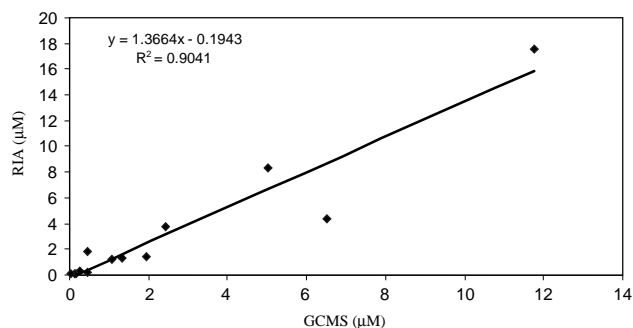


Fig. 4. DHEA quantification: correlation analysis between GCMS and RIA data.

peaks. Good linearity of the plots correlating peak areas and concentration (R^2 always greater than 0.9) were obtained. Standards were run each time that serum hydroxysteroids were quantified. It should be also noted that this assay was validated in a 1-month time period.

LOD was determined by the lowest concentration at which the single monitored fragment was seen under these chromatographic and detection conditions used with a signal/noise ratio greater than 3. LOQ was determined by the lowest concentration at which the monitored fragment was seen under the same conditions described above with a signal/noise ratio greater than 10 and at which the accuracy was maintained within $\pm 20\%$. Based on these criteria, the data for the LOD and LOQ of the method are summarized in Table 3, indicating that the proposed method is relevant to the detection of neurosteroid concentrations present in biological fluids [11,15–20]. The method was subsequently applied to the analysis of human plasma samples obtained from control (nondemented) 65- to 75-year-old individuals. Using the sample processing and chromatographic conditions described herein, no chromatographic peak interfering with that of the studied hydroxysteroids was observed and the substances were well resolved. Representative chromatograms of the reconstituted ion current analysis (m/z 129 and m/z 358) of a mixture of steroids (A), human plasma spiked with steroids (B), and control plasma (C) are shown in Fig. 3.

To determine the precision of the evaluated methodology, the results (plasma DHEA content) obtained in both RIA and GCMS were analyzed by linear regression. The Spearman correlation coefficient was calculated from a total of 12 human plasma samples and found to be at 0.90 for the typical R^2 as indicated in Fig. 4. As noted earlier, the long-term goal of this study is to determine hydroxysteroid levels in plasma obtained from patients with various pathologies in which steroid metabolism may be affected, such as Alzheimer's disease [6].

Conclusion

In the present study an optimal qualitative and quantitative GCMS analysis is presented offering (i) good resolution as shown by sharp and symmetric peaks, (ii) high repeatability and reproducibility of the absolute retention times, (iii) optimal qualitative identification using multiple-ion monitoring of the specific fragments, (iv) high precision and accuracy in quantification based on area measurements, (v) high specificity provided by the combination capacity factors in both HPLC and GC (as trimethylsilyl ether derivatives), and (vi) high sensitivity provided by monitoring the highly abundant base peaks (m/z 129 or 358 fragments) in SIM mode.

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