Plasma estrone sulfate assay in men: Comparison of radioimmunoassay, mass spectrometry coupled to gas chromatography (GC-MS), and liquid chromatography-tandem mass spectrometry (LC-MS/MS).

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Plasma estrone sulfate assay in men: comparison of Radioimmunoassay, Mass Spectrometry coupled to Gas Chromatography (GC-MS), and Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS)

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ABSTRACT

Background: Estrogens are involved in the natural history of the prostate cancer and estrone sulfate, the quantitatively main circulating plasma estrogen in men, has been associated with an aggressive form of this cancer. A convenient and accurate plasma assay of this steroid has become important.

Methods: We simultaneously assayed estrone sulfate in the plasma of one hundred men aged 30-50 years, according to LC-MS/MS, GC-MS after solvolysis of E_1S, radioimmunoassay after a chromatographic purification step, and a direct RIA commercial kit.

Results: Estrone sulfate plasma levels obtained with the first three methods were not significantly different. However, estrone sulfate levels measured by the direct RIA were threefold higher than those obtained by the first three methods. We showed that the excessively high estrone sulfate levels obtained with the direct RIA kit had two origins: interference by high dehydroepiandrosterone sulfate plasma levels in men, and estrone sulfate inaccurate low concentrations in the standards.

Conclusion: The LC-MS/MS method can be considered as an optimum option for clinical laboratory. The GC-MS method requires solvolysis to estrone, but allows simultaneous unconjugated steroid measurement. RIA method, with chromatographic purification, is cumbersome, but less expensive. DSL-5400 kit yielded estrone sulfate plasma levels that were too high.

1. Introduction

Quantitatively, estrone sulfate (E_1S) is the major plasma estrogen in both men and in women [1]. Although this estrogen is not directly active on estrogen receptors, it becomes active in many tissues after hydrolysis [2, 3] and its reduction to active estradiol (E_2) by various 17beta-hydroxysteroid dehydrogenases enzymes [4, 5]. Several tissues, whose prostate, contain the enzyme machinery required to convert E_1S to E_2 [6]. Estrogens appear to be involved in the natural history of prostate cancer (PCa) [7-11]. We previously demonstrated an association between E_1S and aggressive PCa [12]. Therefore to determine the potency of circulating estrogens it is extremely important to determine the real concentrations of this steroid. The use of radioimmunoassay (RIA) has been well documented in the literature. For routine analysis in clinical laboratories, several authors reported RIA methods [13-18]. We described, with accuracy and precision, a specific E_1S radioimmunoassay involving a chromatographic purification C_{18} step [13]. In an attempt to replace this specific but cumbersome RIA assay method, we tried a direct convenient E_1S kit DSL-5400. In the present study, this direct E_1S RIA method was compared with previously described E_1S RIA [13] and with mass spectrometry coupled either with gas chromatography (GC-MS) or liquid chromatography (LC-MS/MS) in plasma samples from men [19].

2. Materials and methods

2.1. Subjects

One-hundred healthy, volunteer men aged 30-50 were recruited in a healthcare center (IRSA, Tours, France). The ethical committee of the institution reviewed and approved the study. Plasma was separated from blood samples collected between 8:00 AM and 10:00 AM after a 12-h overnight fast.

2.2. Methods

Four assay methods, LC-MS/MS, GC-MS, RIA after C_{18} chromatographic purification, and direct RIA using a DSL-5400 kit, were carried out simultaneously.

2.2.1. Analytical E_1S standards

The E_1S analytical standard used to quantify E_1S by LC-MS/MS, GC-MS, and RIA with chromatography was a sodium salt obtained from Research Plus Laboratory (1887-5; Barnegat, NJ, USA). As ascertained by chemical analysis, this powder contained 40.2 and 6.75 % (w/w) Tris and water, respectively. The deuterated internal standard (E_1S-d_4, or sodium estrone-2, 4, 16, 16-d_4 sulfate) was obtained from C/D/N Isotopes, Inc., (Montréal, Canada).

2.2.2. HPLC-UV analysis of the E_1S standard

To determine whether the stock solution of E_1S contained a significant proportion of the unconjugated estrone steroid (i.e., E_1), HPLC-UV analysis was carried out. For this purpose, the stock solution (255.1 mmol/L) used for the standard curve preparation was diluted five times in ethanol, and 5 µL were injected into the chromatographic system (Alliance 2695, Waters, Milford, MA, USA). E_1 and E_1S were separated with a 5 µm Luna Phenyl Hexyl 75 x 4.6 mm column (Phenomenex, Torrance, CA, USA). In all the analyses, solvent A corresponded to water 4 mM ammonium acetate, solvent B to methanol 0.1 % ammonium acetate, and solvent C to acetonitrile. E_1 and E_1S were eluted at a flow rate of 0.8 mL/min. Initial conditions were 55 % A, 40 % B, and 5 % C, followed by a linear gradient to 10 % A, 85 % B and 5 % C in 6 min. This condition was maintained for 1 min, after which the column was re-equilibrated to initial conditions for an additional 9 min. E_1 and E_1S were detected using a Waters 2996 photo diode array at a wavelength of 200 nm.

2.2.3. Sulfatase hydrolysis

To further ensure the reference concentration, the E_1S standard was subjected to enzymatic sulfatase hydrolysis prior to GC-MS E_1 quantification. For this purpose, 100 µL of a 28.54 nmol/L E_1S solution was incubated at 37°C for 1 hour in the presence of 20 units of human sulfatase (Sigma) in a final volume of 500 µL of a sodium acetate acetic acid buffer (pH 5). The digestion products were then frozen and kept at -80°C until GC-MS quantification. For this purpose, digestion products sustained E_1 derivatization with Pyridine (Pierce) / anhydrous ethyl acetate (500 µL; 1/99; v/v), then pentafluorobenzoyl-Cl (Sigma Aldrich) / anhydrous ethyl acetate, and the final extracts were reconstituted in 50 µL of isooctane and transferred to conical vials for injection into the GC-MS (see 2.2.6.).

2.2.4. LC-MS/MS assay (method I)

Briefly, as previously published [19], plasma samples (100 µL) were diluted in PBS (2 mL) containing a deuterated internal E_1S standard, then solid-phase extracted using Oasis HLB SPE columns. E_1S analytes were eluted in 4 mL of methanol then evaporated at 35°C and reconstituted in 125 µL of methanol:water (50:50, v:v). The chromatographic system consisted of an Alliance 2690 Waters (Milford, MA, USA). Analytes were separated with a 100 x 4.6 mm, 3.5 µm particle size C_{18} Phenomenex Luna column (Torrance, CA, USA). In all analyses, solvent A corresponded to water 0.1 % ammonium hydroxide, and solvent B to methanol 0.1 % ammonium hydroxide. E_1S was eluted at a flow rate of 1.0 mL/min with a split ratio of 1:4. The initial conditions were 40 % A and 60 % B, followed by a linear gradient to 85 B in 3 min. This condition was maintained for 2 min, and then the column was flushed with 95 % B for 2 min and re-equilibrated to initial conditions for an additional 2 min. E_1S was detected using a Sciex Api 3000 triple quadrupole mass spectrometer equipped with

TurboIonspray^{TM} source operating in negative ion mode (Applied Biosystems Inc., Foster City, CA, USA), and the MRM measurement through *m/z* 349 at 269 and *m/z* 353.2 at 273 transitions for E_1S and E_1S-d_4, respectively [19].

2.2.5. GC-MS assay (method II)

The plasma of 100 male subjects was assayed simultaneously with other human samples in 10 runs.

2.2.5.1 Separation of non-conjugated steroids from conjugated steroids in plasma samples

500 µL of plasma samples, controls, and standards were added to a methanol solution (50 µL) containing a mixture of deuterated unconjugated steroid internal standards and 2.69 pmol of deuterated E_1S. The tubes were vortexed for 1 min. Three mL of 1-chlorobutane was added to each tube and mixed. After centrifugation, the aqueous lower phase was frozen and the upper organic phase collected for possible analysis of non-conjugated steroids.

2.2.5.2. Deproteinization of aqueous lower-phase extract obtained from plasma and standard samples

The aqueous lower phases (containing the conjugated steroids of the plasma samples) and the E_1S standard samples were added to pure methanol (3 mL) vortexed for 1 min, and left to stand overnight at -20°C. They were then centrifuged for 30 min at 4°C (3300 t/min). The supernatant was collected and evaporated at 60°C until dryness with a TurboVap® LV concentration workstation (Caliper Life Sciences, Roissy-Charles de Gaulle, France).

2.2.5.3. Acid solvolysis

The dried residues were redissolved in NaCl 9 gr/L (0.5 mL), H_2SO_4 (2N) (0.1 mL), and ethyl acetate (3 mL). After vortexing, the tubes were covered with aluminum paper and let stand at 37°C overnight in a water bath. Ethyl acetate was then added to complete the initial volume, then vortexed for 1 min and centrifuged for 10 min at 2500 t/min. The organic upper phase was collected in a new tube and evaporated to dryness. The dry residue was neutralized with 0.5 mL $NaHCO_3$ (50 mM) [14].

2.2.6. GC-MS measurement of the free E_1 produced by solvolysis of E_1S. (derived from Labrie, et al., [19])

2.2.6.1. Extraction of free E_1 produced by E_1S solvolysis

1-chlorobutane (2.5 mL) was added to all tubes, which were then vortexed for 2 min. After centrifugation (3300 rpm), the 1-chlorobutane extracts were collected and purified on conditioned Varian LC-Si SPE columns. The columns and adsorbed material were then washed with ethyl acetate / hexane (6 mL; 1/9, v/v). Free E_1 was eluted using ethyl acetate / hexane (4 mL; 1/1, v/v), then evaporated at 60°C.

2.2.6.2. Derivatization of E_1

Pyridine (Pierce) / anhydrous ethyl acetate (500 µL; 1/99; v/v), then pentafluorobenzoyl-Cl (Sigma Aldrich) / anhydrous ethyl acetate (50 µL; 1/10; w/v) were added to the dried residue of free steroids and incubated 30 min at 60°C. After evaporation, a solution of $NaHCO_3$ (0.5 M; 1 mL) was added to the tubes, which were then let stand for 10 min at room temperature. Hexane (2.5 mL) was then added to them and the mixture vortexed for 2 min. The hexane phases were transferred to new tubes and evaporated at 50°C. The final extracts were reconstituted in 50 µL of isooctane and transferred to conical vials for injection into the GC-MS.

2.2.6.3. GC-MS analysis

The 6890N GC system (Agilent Technologies, Palaiseau, France) for E_1 analysis uses a 50 % phenyl – 50 % methylpolysiloxane Varian VF-17MS capillary column (id: 20 m x 0.15 mm, film thickness 0.15 µm) in splitless mode with helium as the carrier gas. E_1 and deuterated E_1 were detected using an HP 5973N quadrupole mass spectrometer (Agilent Technologies) equipped with a chemical ionization source and operating in single ion monitoring (SIM) mode. The linearity of E_1 measurement was confirmed by plotting the ratio of the E_1 peak response / E_1-d_4 peak response to the concentration of E_1S for each calibration standard.

The injection port and transfer line temperatures were respectively 310 and 300°C. The mass spectrometer source and quadrupole temperatures were respectively 200 and 110°C. The oven temperature was linearly ramped from 110 to 308°C at 22°C/min and held at 308°C for 4.85 min. The two target ions were m/z 464 for E_1 and 468 for E_1-d_4.

2.2.7. RIA with chromatographic separation (method III)

As previously reported [13], after monitored extraction of E_1S, separation of E_1S from DHEAS was carried out by chromatography on C_{18} Hypersil phase minicolumns (Thermo Fisher**,** Villebon-sur-Yvette, 91963 Courtaboeuf, France). The chromatographic step was carried out using the Visiprep Vacuum Manifold (Supelco), in order to help the entry of the solvents through the hypersil phase. All the solvents used in the chromatography step were a mixture of methanol/H20, 25/75, v/v.

The minicolumns kept in pure methanol, were regenerated by successively adding 6 ml of water, then 12 ml methanol/H2O, 25/75, v/v, then passed through the phase. The vacuum was

stopped in order to transfer the 0.5 ml of extract, containing the conjutated plasma steroids into the minicolumns.

After introducing the 0.5 ml of re-dissoved extract, 2.5 ml of solvent was immediately added to the minicolumns, but the eluates were not kept. Release the depression and add another volume of 2.5 ml of solvent. Now, this chromatographic eluate was kept, and evaporated to dryness.

After re-dissolving, the purified eluate subjected to RIA, using an E_1S-6-carboxymethyloxime/BSA antibody and tritiated E_1S. After overnight incubation at 25°C, we added dextran-charcoal, incubated 15 min and centrifuged. The supernatants were decanted into the scintillation liquid and counted in a Tri-Card 2300 TR Liquid Scintillation Analyzer (Packard).

2.2.8. Direct RIA method using DSL-5400 kit (method IV)

A direct RIA (DSL-5400 kit, Webster TX, USA) was used according to manufacturer's instructions. Briefly, 0.1 mL of plasma, controls, and standards were added to conical polystyrene tubes, followed by E_1S ^{125}I reagent (100 µL) and E_1S anti-serum (100 µL). The mixture was incubated 180 min while stirring at 25°C. A goat anti-rabbit globulin gamma + polyethylene glycol precipitating reagent was then added. After 10 min of incubation, the mixture was centrifuged, the supernatant aspirated, and the tubes counted in a Wallac Wizard 1470 Automatic Gamma Counter (PerkinElmer, Courtaboeuf, France).

2.2.9. DHEAS RIA assay

DHEAS was assayed with a commercial RIA kit (Ref. IM 0729, Immunotech Beckman Coulter, 13009 Marseille, France).

2.3. Statistical analysis

E1S plasma levels comparison using the reported four methods were assessed using the non-parametric Wilcoxon matched pairs test and the non-parametric concordance test of Kendall.

3. Results

3.1. Additional quality controls for the analytical standards

HPLC-UV analysis of the E_1S standard purchased from Research Plus (USA) revealed that it did not contain detectable concentration of unconjugated E_1. Furthermore, no significant differences were observed between the mean ± SD (n = 5) E_1 concentration obtained after sulfatase hydrolysis of E_1S (29.22 ± 1.92 nmol/L) and the calculated concentration expected (28.51 nmol/L). This observation further ensured the purity of the E_1S analytical standard.

3.2. Analytical qualities of the four methods

3.2.1. Linearity

The curve-response ratios (varying amounts of E_1 / constant quantities of deuterated E_1) / measured concentration of LC-MS/MS and GC-MS) were linear. For LC-MS/MS, the obtained coefficient of determination r^2 in 10 consecutive runs was > 0.999; for GC-MS the r^2 was higher than 0.997.

The concentration ranges of the four methods were the following (in nmol/L): 0.214 to 28.57 for method I, 0.134 to 16.11 for method II, 0.252 to 16.11 for method III, and 0.134 to 67.13 for method IV.

3.2.2. LLOQ

The Lower Limit of Quantification (LLOQ) for E_1S, defined as the lowest concentration of the analyte that could be determined with a signal-to-noise ratio of 10 or greater, were 0.214 nmol/L ± 0.022 (n = 10) for LC-MS/MS, and 0.134 nmol/L ± 0.016 (n = 10) for GC-MS. The low detectable dose of E_1S was 0.094 nmol/L ± 0.014 for RIA ^3H, and 0.027 nmol/L for the DSL-5400 kit (table 1).

3.2.3. Precision and accuracy

E_1S inter-day precision and accuracy measured with the methods studied are reported in table 1. On the other hand, inter assay CVs were 4.93, 2.81 and 1.86%, and intra assay CVs were 2.97, 1.65 and 1.15% for LC-MS/MS (method I), when assayed with low (0.64 nmol/L), intermediate (14.28 nmol/L) and high (22.55 nmol/L) quality controls. With GC-MS (method II), inter- and intra-assay CVs (%) were 6.79, 3.19, 2.68, and 4.11, 1.93, 1.63, for quality control samples containing 1.34 nmol/L, 4.03 nmol/L, and 8.06 nmol/L, respectively. With the RIA ^3H (method III), inter- and intra-assay CVs (%) were 7.41, 1.96, 2.71, and 6.10, 1.95, 2.67, for quality control samples containing 1.34 nmol/L, 4.03 nmol/L, and 8.06 nmol/L, respectively. The interassay CVs using the DSL-5400 kit (method IV) were < 10 % for low (0.21 nmol/L), medium (1.31 nmol/L), and high concentrations (30.34 nmol/L) of quality control samples (DSL-5400 datasheet; revision date: October 17, 2005).

3.2.4. Recovery

The percentage of recovery after adding two quantities of exogenous E_1S (2.68 and 13.4 pmol) to different male plasma samples (n = 5) were between 97 and 98 % for LC-MS/MS and GC-MS, and between 80 and 115 % for RIA ^3H and the DSL-5400 kit.

3.2.5. Specificity

The specificity of the RIA methods depends on the cross-reactivity of the antibody with or without a chromatographic purification step prior to immunoassay. With method III, DHEAS (the principal steroid in plasma, the level of which was highest) cross-reactivity with the anti-E_1S antibody was 0.002 %. Moreover, a pre-chromatographic separation of DHEAS from E_1S on C_{18} Hypersil phase minicolumns was carried out. For method IV (DSL-5400 kit), the specificity of this direct method was based only on anti-E_1S specificity. The cross-reactivity of the DHEAS was reported to be < 0.1 %. In methods I and II (LC-MS/MS and GC-MS), DHEAS and DHEA are totally separated from E_1S and E_1 respectively, as shown in fig. 1 and 2 and the discrimination of the mass spectrometry was ensure by using the detection at 349.2 m/z for LC-MS/MS and 464.4 m/z for GC-MS.

3.3. E1S plasma levels comparison using the reported four methods

E_1S values (mean, SD, and concentration ranges in nmol/L) obtained by the four methods are reported in table 2. E_1S mean values obtained with the DSL kit were respectively 3.56-, 3.45-, and 3.37- fold higher than those obtained with LC-MS/MS, GC-MS, and RIA ^3H. The mean ratios of the results of GC-MS / LC-MS/MS, RIA ^3H / LC-MS/MS, and RIA ^3H / GC-MS were respectively 1.03, 1.06, and 1.03.

The Wilcoxon paired test showed no significant difference between E_1S results obtained by GC-MS and LC-MS/MS, whereas significant differences were found between E_1S levels measured by RIA ^3H and GC-MS, RIA ^3H, and LC-MS/MS. E_1S levels measured with the DSL-5400 kit were significantly higher than those obtained with RIA ^3H, LC-MS/MS, and GC-MS.

The regression equations and correlations between the methods studied were the following: DSL-5400 kit = 1.588 LC-MS/MS + 1.125 (r = 0.914); GC-MS = 1.005 LC-MS/MS + 0.01 (r = 0.991); RIA ^{3}H = 1.089 LC-MS/MS - 0.021 (r = 0.973) (fig. 3).

The non-parametric Kendall concordance coefficients were the following: GC-MS and LC-MS/MS: 0.9129; LC-MS/MS and RIA ^{3}H: 0.9078; DSL-5400 kit and LC-MS/MS: 0.7286.

The much higher E_1S results measured using the DSL-5400 kit could be due to a lack of specificity and/or to a different standardization. We first sought possible interference by DHEAS, whose plasma level is 1000- to 10,000-fold higher than that of E_1S.

For this purpose, 0.05 mL of a 5.12 µmol/L concentration DHEAS solution (physiologic concentration level in man) were added to 0.1 mL of the DSL-5400 E_1S kit standards with theoretical concentrations of 0 - 0.134 - 0.54 - 2.69 - 6.71 - 13.43 nmol/L. All these standards were assayed using the DSL-5400 kit antibody. The results are reported in table 3. The assayed E_1S concentrations in DHEAS overloaded standards were much higher than the E_1S in the same standards overloaded with the absence of E_1S, indicating a cross-reaction of DHEAS with the anti-E_1S antibody of the DSL-5400 kit. The interference was higher as the DHEAS concentration increases. Indeed, the different E_1S levels assayed with the kit minus E_1S assayed with LC-MS/MS is significantly correlated to the plasma DHEAS levels (r = 0.810).

Comparatively, the DHEAS in the three other methods do not interfere in the E_1S measurement, because DHEA is well separated from E_1 (after solvolysis in the GC-MS

method) and DHEAS is clearly discriminated from E_1S in the RIA ^3H and LC-MS/MS methods.

In addition to the lack of specificity of antibody used, a second source of false determination of E_1S could be the amount of E_1S in the standard of the DSL-5400 kit. Indeed, measurement of standards using LC-MS/MS employed in the establishment of the DSL-5400 kit standard curve gave the following values: 0.129 - 0.35 - 1.89 - 8.57 and 42.94 nmol/L, instead of the following theoretical values: 0.134 - 0.54 - 2.69 - 13.43 and 67.13 nmol/L. Consequently, insufficient specificity and non-equivalent E_1S standards were associated in yielding falsely elevated results obtained with the DSL-5400 kit.

4. Discussion

Our data reveal that direct E_1S assay with the DSL-5400 kit led to significant three-fold higher plasma levels than those obtained by mass spectrometry or RIA after chromatographic purification. Two reasons could likely explain this discrepancy: lack of specificity of the kit anti-E_1S antibody against DHEAS (of which there is a high level in human male plasma) and the inaccurate quantity of E_1S in the kit standards.

Brind *et al*. reported in 1989 and 1990 [15-16] that the high levels of DHEAS, which were 1,000- to 10,000-fold higher than E_1S in human adult plasma, and the probable high cross-reactivity of DHEAS with all anti-E_1S antibodies, may lead to false estimations of E_1S levels, and that DHEAS interference may have accounted for the up to fourfold differences in reported mean normal male values [16]. Our group previously showed [13] that when using an anti-E_1S antibody with cross-reactivity of approximately 0.002 %, omission of the chromatographic step to separate E_1S from DHEAS yielded and overestimation of E_1S in

human plasma. The precise cross-reactivity of the anti-E_1S antibody in the DSL-5400 kit is not reported. A direct E_1S RIA method, similar to those employed in the DSL-5400 kit, was reported previously [20] but the interference of DHEAS in E_1S determination is not provided in this paper. Interference of DHEAS in many direct immunoassays for testosterone in male and female plasma has also been reported previously, due to the considerably higher plasma levels of DHEAS than testosterone, associated with insufficient specificity of the anti-testosterone antibody employed [21]. A very recent paper [22] comparing LC-MS/MS with the DSL-5400 kit also reported higher E_1S using the kit and suggested the interference of DHEAS, but do not proved it.

A comparative study of the DLS-5400 kit and the GC-MS/MS method was recently reported [23]. In 32 plasma samples from eight menopausal women, a good correlation was seen between the two methods ($r = 0.96$). A lower correlation ($r = 0.906$) was obtained in the present study with male sera. The discrepancy between these two studies could be due to the low DHEAS blood concentrations in post-menopausal women, which can be almost 5- to 10-fold lower than in adult men [24].

The cross-reactivity of DHEAS in the E_1S assay with the kit explains the overestimation positively correlated with DHEAS ($r = 0.81$, $n = 100$ samples), and that plasma E_1S level measured with the DSL-5400 kit is much more correlated to DHEAS than E_1S measured with one of the other specific methods.

Moreover, contrary to the validated E_1S reference standards used for standardization of methods I, II, and III, the kit's E_1S standards contained smaller quantities of E_1S than expected, resulting in higher levels of assayed E_1S.

Overall, for measuring E_1S in plasma, LC-MS/MS has the advantages of specificity and practicability, GC-MS has a similar specificity, but requires a solvolysis before E_1 measurement, and RIA ^3H is cumbersome, but not so expensive.

5. Conclusion

We report on three plasma E_1S assays, two using mass spectrometry and a more classical RIA after chromatographic purification, which yielded similar for plasma E_1S levels, although RIA ^3H gave slightly higher E_1S levels than those obtained with the two other mass spectrometry methods. GC-MS requires solvolysis prior to E_1 assay, whereas LC-MS/MS [19] is more practicable and RIA after chromatographic purification requires a less expensive apparatus, but is cumbersome. According to the material available, these methods could allow accurate plasma E_1S level determination, which is very important and useful in oncology and endocrinology [12, 25] since it is a precursor of unconjugated estrogens.

According to our results, the direct E_1S method of the DSL-5400 kit has led to spurious E_1S results in men ‘plasma samples.

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List of abbreviations

E_1S: Estrone Sulphate, $E_{1:}$ Estrone, DHEAS: Dehydroepiandrosterone Sulphate, LC-MS/MS: Liquid Chromatography/Mass Spectrometry/Mass Spectrometry, GC-MS: Gas Chromatography/Mass Spectrometry, RIA: Radio Immuno Assay.

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Table 1

E_1S inter-day precision and accuracy measured with the methods studied.

	LLOQ ± SD (nmol/L)	RE (%)	Level 1 ± SD (nmol/L)	RE (%)	Level 2 ± SD (nmol/L)	RE (%)	Level 3 ± SD (nmol/L)	RE (%)
Method I (n = 10) *Theoretical concentration*	0.214 ± 0.022 *0.214*	0	22.49 ± 0.42 *22.55*	-0.3	14.26 ± 0.40 *14.28*	-0.2	0.63 ± 0.03 *0.64*	-1.6
Method II (n = 10) *Theoretical concentration*	0.134 ± 0.016 *0.134*	0	7.99 ± 0.23 *8.06*	-0.9	4.02 ± 0.14 *4.03*	-0.3	1.33 ± 0.09 *1.34*	-0.8
Method III (n = 10) *Theoretical concentration*	0.094 ± 0.014 *0.091*	3.3	8.12 ± 0.22 *8.06*	0.8	4.08 ± 0.08 *4.03*	1.2	1.35 ± 0.10 *1.34*	0.8
*Method IV (n = 8)	0.027	#	30.34 ± 1.66	#	1.32 ± 0.08	#	0.215 ± 0.03	#

(RE = relative error: mean percentage deviation from theoretical value)

*(* : DSL-5400 datasheet; revision date: October 17, 2005)*

Table 2

E_1S means, SD, (nmol/L) in the one hundred plasma samples measured with the four methods studied.

	LC-MS/MS	GC-MS	RIA (^3H)	DSL-5400 kit
Mean ± SD (nmol/L)	1.53 ± 0.83	1.58 ± 0.86	1.62 ± 0.95	5.46 ± 1.47
Min-Max (nmol/L)	*0.16-4.81	0.16-4.80	0.19-6.15	2.70-10.88

*(* : One plasma sample was measured below the reported LLOQ of the LC-MS/MS method)*

Table 3

E_1S concentrations of the E_1S kit standards overloaded with physiological concentrations of DHEAS, compared with overloads with the 0 nmol/L E_1S kit standard.

E_1S kit standard theoretical concentrations (nmol/L)	E_1S assayed concentrations in DHEAS overloaded standards (nmol/L)	E_1S assayed concentrations in standard 0 overloaded standards (nmol/L)
0	0.79	0
0.134	1.13	0.161
0.54	1.75	0.52
2.69	4.90	2.72
6.71	9.81	7.01
13.43	16.95	13.64

Fig. 1. E_1S and DHEAS retention times (min) in LC-MS/MS method.

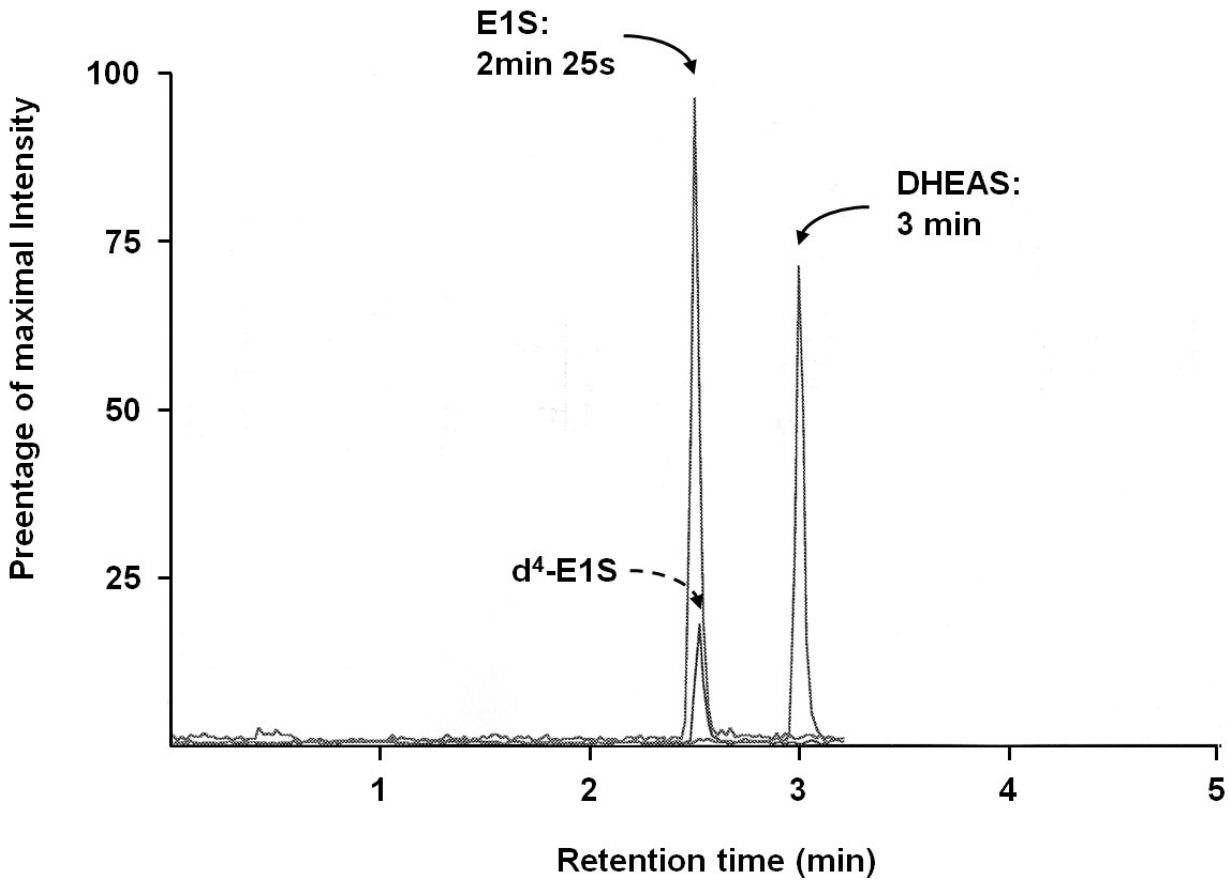

Fig. 2. E_1 and DHEA retention times (min) in GC-MS method after acid solvolysis of E_1S and DHEAS.

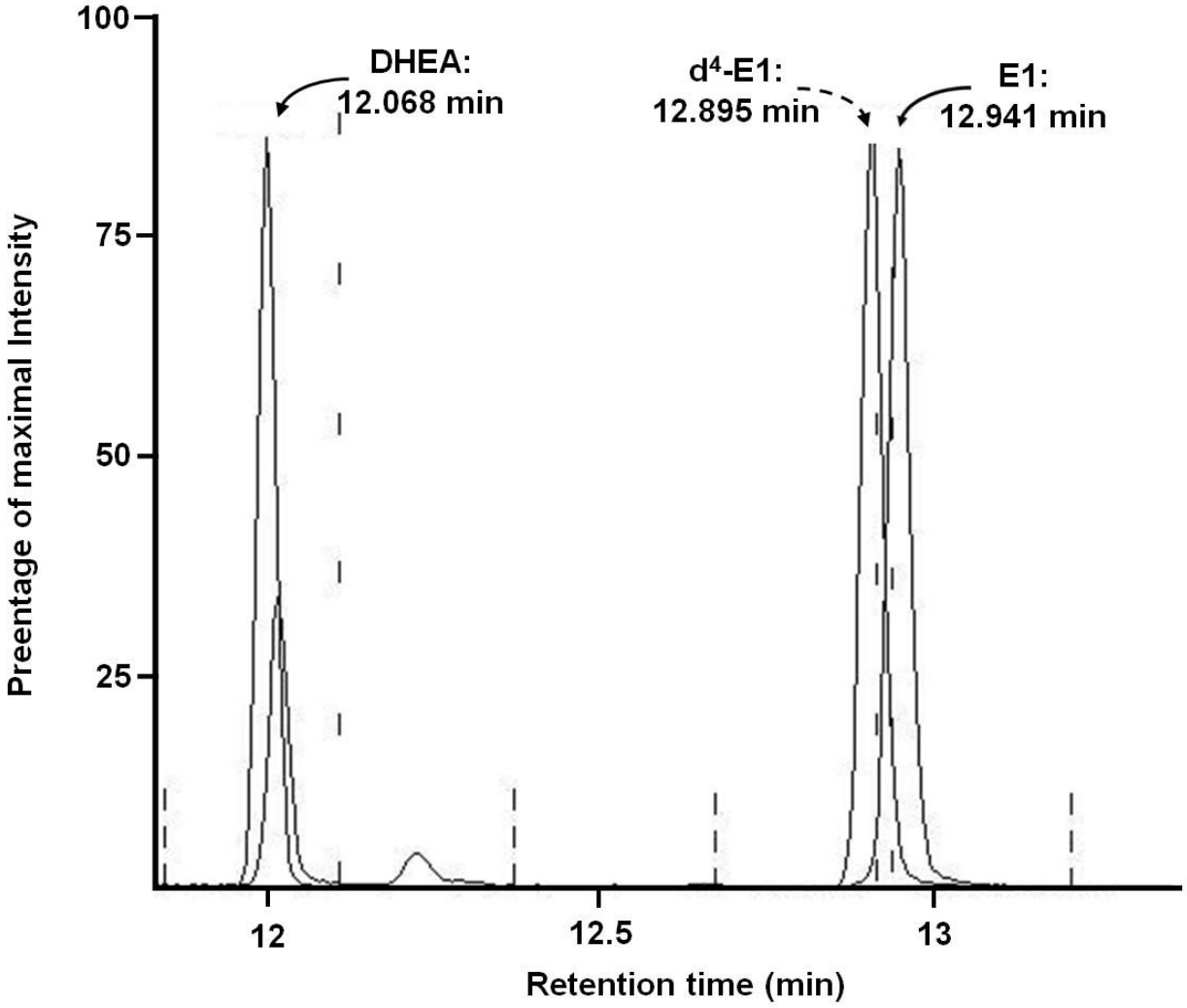

Fig. 3. Correlation plot between E_1S concentrations obtained by LC-MS/MS *versus* GC-MS, RIA after C_{18} purification step, and direct DSL-5400 commercial kit.