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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 three-fold 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 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

Abbreviations: E_1S, Estrone sulfate; E_1, Estrone; DHEAS, Dehydroepiandrosterone sulfate; LC-MS/MS, Liquid chromatography/mass spectrometry/mass spectrometry; GC–MS, Gas chromatography/mass spectrometry; RIA, Radio immunoassay.

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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 was 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 75×4.6 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 h 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 100×4.6 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 Turbolonspray™ 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 was 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. 3 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 re-dissolved in NaCl 9 gr/L (0.5 mL), H_2SO_4 (2 N) (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×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