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Discrepancy between radioimmunoassay and high performance liquid chromatography tandem-mass spectrometry for the analysis of androstenedione

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ABSTRACT

The discrepancy of results for the quantification of androstenedione in human serum between a radioimmunoassay (RIA) method and high performance liquid chromatography tandem-mass spectrometry (LC–MS/MS) was investigated. RIA overestimated concentrations compared to LC–MS/MS on 59 clinical samples (RIA = 1.79 × LC–MS/MS + 0.94). RIA kit and LC–MS/MS calibrants were also determined by both methods. The RIA performed with improved accuracy on the calibrants (RIA = 1.35 × LC–MS/MS − 0.28). Lipid, protein, electrolyte content, and pH of the two sets of calibrants were further investigated. The RIA calibrants contained little lipid material, while the LC–MS/MS calibrant material contained the same levels expected in normal serum/plasma. The pH and sex hormone binding globulin (SHBG) values were different between the RIA calibrants and the LC–MS/MS calibrant material (SHBG, 31 ± 2 and 38 ± 2 nmol/l; pH, 8.27 ± 0.18 and 8.66 ± 0.03, respectively). No correlation was observed between androstenedione RIA and LC–MS/MS discrepancy and lipid or protein. LC–MS/MS sample preparation was tested for the removal of protein-bound material and recovery determined (99–108%). The corresponding RIA results overestimated androstenedione by 52–174% compared to LC–MS/MS. The results here demonstrate that LC–MS/MS is the more accurate method.

Quantitative analyses of steroid hormones within clinical laboratories is important for the diagnosis of endocrine-related disorders [1,2]. These clinically important markers are synthesized from cholesterol in the mitochondria and smooth endoplasmic reticulum of cells located in the adrenal cortex, the gonads, and the placenta. Androstenedione is an immediate precursor of estrone and testosterone, and is one of the steroid hormones routinely targeted for quantitative measurement in clinical laboratories. Until recently, steroid hormones have generally been routinely quantified by immunoassay (IA)[1]-based methods, and often more specifically by radioimmunoassay (RIA). However, since the relatively recent introduction of bench-top LC–MS/MS-based technology, many laboratories have switched from the traditional IA methods to the arguably more specific and robust LC–MS/MS-based approaches for routine quantification [3–11].

Although standardized materials and kits are available and can be used for specific markers in targeted LC–MS/MS-based analysis [11], many clinical LC–MS/MS quantitative procedures rely on in-house development and validation [12], thus proving within the laboratory that the specific method(s) are robust enough to be routinely applied, and also outlining the limitations of each individual method (e.g., limits of detection) [9,13]. Although commercially available kits (for IA, RIA, and also LC–MS/MS-based methods) can be further evaluated/validated in-house, and should certainly be deemed accurate/reproducible enough to be applied routinely, the extent of the validation work is normally less compared to in-house developed methods. It can also be that certain matrices can sometimes cause problems analytically and reasons as to why an analysis should "fail" are not always apparent. There have already been a number of publications that have highlighted performance characteristics and specific interference issues for IA and RIA methods for steroid hormone analysis including androstenedione [14,15]. There are also a number of studies that have compared different types of IAs including RIA with LC–MS/MS methods [8,11,16,17], highlighting significant quantitative discrepancies for androstenedione and other steroid hormones between methods. However, these discrepancies have not yet been explored

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[1] *Abbreviations used:* IA, immunoassay; LC–MS/MS, high performance liquid chromatography tandem-mass spectrometry; RIA, radioimmunoassay; SHBG, sex hormone binding globulin.

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to the degree that would allow reasoning as to why they exist and to help realize which method is the more suitable/reliable. Here we present data from an androstenedione targeted RIA commercial kit-based method and an in-house LC–MS/MS method, in an attempt to further understand and explore the observed discrepancies between the two methods, and to show which method is the more specific/robust.

Materials and methods

Chemicals and reagents

Androstenedione (VETRANAL analytical standard), ammonium hydroxide, phosphoric acid (85% wt in H_2O, 99.99% trace metal basis), LC–MS grade methanol, and isopropanol were all purchased from Sigma–Aldrich (St. Louis, MO, USA). The labeled internal standard [7-^2H] androstenedione was purchased from CDN Isotopes (Quebec, Canada). Charcoal dextran stripped human serum was from Innovative Research Inc. (Novi, MI, USA), ultrapure water used throughout was from a Synergy UV ultrapure water system with a 0.22 μm Millipore filter (Millipore, MA, USA).

Samples, calibrants, and internal standards

The serum samples used within this work were taken anonymously from the clinical chemistry sample storage facility at CHUV (Lausanne, Switzerland). The LC–MS/MS calibrants were made by spiking charcoal dextran stripped human serum with a prepre-pared androstenedione solution in methanol (4000 nmol/l) to create LC–MS/MS calibrant C7 (final concentration = 40 nmol/l). Subsequent serial dilutions with the same human stripped serum were made from C7, 1:1 for each lower level calibrant, that gave the final calibrant androstenedione levels: C7 (40 nmol/l), C6 (20 nmol/l), C5 (10 nmol/l), C4 (5 nmol/l), C3 (2.5 nmol/l), C2 (1.25 nmol/l), and C1 (0.63 nmol/l). A stock of the [7-^2H]androstenedione internal standard solution was made in methanol (120 nmol/l), aliquoted (2 ml) into tubes, and kept at −80 °C until required.

LC–MS/MS sample preparation

Samples were thawed at room temperature, briefly vortex mixed, and pipetted (100 μl) into Eppendorf tubes (1.5 ml). A [7-^2H]androstenedione stock aliquot was left at room temperature for ∼10 min, and pipetted into a separate vessel (1.7 ml) to which was added ultrapure water (14.3 ml) and phosphoric acid (1.0 ml); this solution (100 μl) was added to each thawed sample (no observed sample precipitation). The samples then underwent a quick vortex followed by centrifugation (33,000*g*, 5 min, 4 °C). A solid-phase extraction (SPE) Oasis MCX 96 well plate (Waters, MA, USA) was prepared by passing through each well: methanol (200 μl) followed by ultrapure water (200 μl) under positive pressure (N_2) using a Positive Pressure-96 system (Waters, MA, USA). The samples (200 μl) were then added to individual wells on the MCX plate, which were then passed through the plate under positive pressure. This was followed by two washing steps: 5% NH_4OH solution (aq) (200 μl) followed by a 10% methanol solution (aq) (200 μl). Finally a 96-well collection plate was placed under the MCX plate, and the analytes were eluted through the MCX plate under positive pressure using isopropanol (2 × 60 μl). The final contents of the collection plate were evaporated under N_2 using a TurboVap 96 system (Biotage, Uppsala, Sweden), after which they were reconstituted in 50% methanol solution (aq) (80 μl) in the same collection plate (equivalent to the initial mobile phase conditions) and were ready for LC–MS/MS analysis.

LC–MS/MS analysis

The LC–MS/MS system comprised an auto sampler (CTC-PAL Analytics, Zwingen, Switzerland), an ultrahigh pressure transcend pump Janeiro CNS system (Thermo Scientific, Bremen, Germany), and a Finnigan TSQ Quantum Discovery Max triple-quadrupolar mass spectrometer (Thermo Scientific, Bremen, Germany) operating with positive electrospray ionization (ESI) in the SRM mode with Xcalibur software version 2.1. Samples were injected (20 μl) onto a C18 Symmetry column (2.1 × 100 mm) (Waters, MA, USA). The flow rate was 300 μl/min throughout, and the column was placed in a column oven (40 °C). The mobile phase comprised ultrapure water (A) and methanol (B). The starting mobile phase conditions were A = 50% and B = 50% which was held for 6.0 min, after which gradients were employed. At 9.0 min A = 47% and B = 53%, and at 15.0 min A = 30% and B = 70%. At 17.0 min A = 5% and B = 95% and at 17.5 min A = 0% and B = 100% which was held for 2.5 min. At 20 min the initial conditions were reinstated and column reequilibration was achieved after a further 5 min (total run time = 25 min). The spray voltage was set to 4500 V, sheath gas (N_2) pressure = 50 (arb), aux gas (N_2) pressure = 20 (arb), capillary temperature = 350 °C, with a skimmer offset = 10 V and a Q2 gas (Ar) pressure = 1.5 (arb). A scan time of 0.05 s was applied, with transitions 287.2 → 97.0 for androstenedione and 294.2 → 100.0 for [7-^2H]androstenedione used for quantification in the SRM mode. All LC–MS/MS quantification was performed using Xcalibur 2.1 with final quantification results exported as Microsoft Excel short reports.

RIA androstenedione analysis

The quantitative measurement of androstenedione by RIA was realized using a commercially available antibody coated tube Coat-A-Count kit TKAN1 (Siemens, Zurich, Switzerland), used according to the suppliers instructions. Samples were analyzed on a Wizard 1470 automatic gamma counter (Perkin Elmer, Schwerzenbach, Switzerland). All samples (including calibrants) were prepared in duplicate. The kit contained 6 calibrants, A–F, at concentrations (nmol/l) of 0.00 (A), 0.59 (B), 1.82 (C), 5.13 (D), 12.57 (E), and 32.12 (F).

Androstenedione incubation experiment

Human pooled serum samples were made by pooling 30 human serum samples, which underwent a vortex and centrifugation (6100*g*, 15 min, 4 °C) before use. Pooled serum samples were then spiked at two separate levels with androstenedione in methanol, with an additional fraction kept with no spiking. The first pooled serum spiking level = +2.5 nmol/l. The second pooled serum spiking level = +20 nmol/l. Aliquots (1 ml) of nonspiked pooled serum (n = 3), pooled serum + 2.5 nmol/l (n = 3), and pooled serum + 20 nmol/l (n = 3) were pipetted into Eppendorf tubes (1.5 ml), and subsequently placed in a water bath (37 °C, 60 min). After which aliquots were taken and analyzed by both RIA and LC–MS/MS.

Lipid, electrolyte, protein, and pH measurements

Sodium, potassium, chloride, total calcium, total magnesium, phosphate, bicarbonate, cholesterol, HDL-cholesterol, triglycerides, total protein, albumin, and transferrin were all determined on a COBAS 8000 system (Roche, Basel, Switzerland). Protein fractions (alpha-1, alpha-2, beta, and gamma) were measured by agarose electrophoresis (Sebia, Lisses, France). Sex hormone binding globulin (SHBG) was measured using an Immulite 2000 automat (Siemens, Zurich, Switzerland), and pH measurements were made