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Do Metabolites Account for Higher Serum Steroid Hormone Levels Measured by RIA Compared to Mass Spectrometry?

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Abstract

Higher circulating estradiol levels are generally obtained using conventional radioimmunoassays (RIA) compared to liquid chromatography/tandem mass spectrometry (LC-MS/MS), and this has been attributed to the presence of estradiol metabolites that cross-react with the antibody used in the RIA. This study aimed to determine which estradiol metabolites may contribute to this effect. LC-MS/MS analysis was performed on 70 serum samples from premenopausal women, after purification by extraction and Celite column partition chromatography as would be used prior to conventional RIA for estradiol. The metabolites estrone and 2-methoxyestradiol accounted for 6.92% and 2.15% of the estradiol fractions in the purified samples overall, but the extent of contamination with these metabolites was greater in the samples containing <50 pg/mL estradiol (14.6% and 3.83% respectively) than in those containing >50 pg/mL estradiol. However, since these metabolites have a <1% cross-reactivity to the antibody in the estradiol RIA, this level of contamination is too small to account for the differences between RIA and LC-MS/MS measurements of estradiol.

Keywords

Radioimmunoassay; mass spectrometry; estradiol; metabolite; hormones

Introduction

During the past 50 years or so the predominant methodologies used to measure circulating levels of steroid hormones have been immunoassays and mass spectrometry (MS) assays. In the 1960s, Sjovall and Vihko set the standard for steroid hormone analysis using gas chromatography-mass spectrometry (GC-MS) [1]. Horning et al, produced the first

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comprehensive urinary steroid profile by GC-MS in 1966 [2]. A few years later the development of the first radioimmunoassay (RIA) for a steroid hormone (estradiol) in serum was reported by Abraham [3]. The estradiol RIA method involved separation of estradiol from interfering metabolites in serum by organic solvent liquid-liquid extraction and Celite or Sephadex column chromatography prior to its quantitation by RIA (often referred to as conventional RIA), Soon afterwards, the RIA method was applied successfully to other steroid hormones, such as testosterone and progesterone. Because conventional RIAs are time-consuming and unsuitable for automation, direct assays such as chemiluminescent immunoassays, with no purification steps, were developed on automated platforms and became commonly used in clinical diagnostic laboratories. As for GC-MS assays, due to the technical complexity and high cost of these assays compared to RIAs, the latter assays became widely used in research and diagnostic laboratories but use of GC-MS was limited to a small number of specialty labs.

Impact of RIAs

The immediate impact of conventional RIAs was that they allowed measurement of an immensely wide range of clinically and biologically important compounds found at very low concentrations in the peripheral circulation. The long-term impact of the RIA method was that its use in numerous studies enriched the field of endocrinology with new knowledge on the physiologic and pathophysiologic roles of steroid hormones in a variety of endocrine applications. Its use in diagnostic testing provided physicians with valuable information for diagnosing and treating a countless number of patients. In addition, the RIA method opened the door for epidemiologic studies that permitted us to better understand the role of steroid hormones in the etiology of a number of diseases, notably the hormone-dependent breast and prostate cancers.

Advances in mass spectrometry assays

As mentioned earlier, due to the complexity of the GC-MS assay and high cost of GC-MS instrumentation and operation, use of this methodology was restricted to a relatively small number of laboratories. However, the advent of liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays during the past 2 decades resulted in dramatic improvements in the accuracy and semi-automation of serum steroid hormone measurements [4]. Because of the high specificity and throughput of these assays, use of this methodology for quantifying steroid hormones grew dramatically in both clinical and research laboratories. In some larger clinical diagnostic laboratories, these assays have replaced conventional RIAs, which are cumbersome and time-consuming, and direct immunoassays, which lack specificity and/or sensitivity. Although the high cost of MS instrumentation, related operating costs, and equipment requiring high technical expertise have prohibited smaller laboratories from using this instrumentation for high-throughput routine testing of steroid hormones, this situation is changing and MS assays are becoming much more widely used.

Comparison between conventional RIAs and MS assays

Because of the widespread use of MS assays for quantifying steroids in diagnostic and research laboratories, an important question posed in recent years is: how do the steroid

hormone reference ranges obtained by MS assays compare with corresponding ranges determined by RIA methodology? This is especially important since a substantial amount of our knowledge about the role of steroid hormones in samples from the general population of women and men, as well as in samples from patients with different endocrine diseases, is based on data obtained by conventional RIAs in the 1970s and 1980s. It appears that there are no major differences in reference intervals between conventional RIA and LC-MS/MS assay methods, with the exception of serum estradiol levels in postmenopausal women, men and prepubertal children, and testosterone levels in women and prepubertal children [5]. However, higher circulating steroid hormone levels are generally obtained with the conventional RIA method [6].

Although higher estradiol values are obtained by the RIA method, serum estradiol measured by RIA appears to correlate better with certain biologic parameters than corresponding levels determined by MS [7]. Estradiol levels were measured in serum samples from 40 postmenopausal women by conventional RIA and by GC-MS/MS; the median estradiol levels obtained by the respective methods were 11.0 pg/mL and 3.8 pg/mL. Although the median RIA values were approximately 3 times higher than the corresponding median MS value, the RIA values correlated highly with the MS values (r=0.91) [7]. More important, however, is that the estradiol levels measured by RIA had an overall better correlation with the study subjects' weight, BMI, and bone density than the corresponding levels determined by MS (r=0.66 vs. 0.60, 0.65 vs. 0.65, 0.38 vs. 0.27, respectively) [7, and unpublished data].

The most frequently cited explanation for higher estradiol levels measured in serum by conventional RIA compared to corresponding levels determined by MS is cross-reactivity of estradiol metabolites witb tbe antibody in tbe RIA. Estradiol is readily converted to estrone and the 2 compounds give rise to a total of over 100 metabolites in the circulation. The metabolites include unconjugated and water-soluble conjugated (sulfated and glucuronidated) estrogens. Approximately two-thirds of these metabolites are conjugated and are removed by organic solvent extraction in the conventional RIA. The subsequent purification step in the RIA involves separation of estradiol from other unconjugated estrogen metabolites by Celite column partition chromatography. Additional specificity in the RIA is achieved by use of a highly specific estradiol antibody. The study reported here was designed to investigate which metabolites of estradiol may contribute to higher levels of this estrogen when measured by conventional RIA.

Methods

Seventy serum samples with known estradiol levels, ranging from 20 to 600 pg/mL, were selected from residual specimens obtained from premenopausal female patients during clinical testing. Identification markings were removed from all specimen tubes. All serum samples were extracted with hexane:ethyl acetate (3:2), and following evaporation of the solvents the extracts were subjected to Celite column partition chromatography. Estradiol was eluted with 40% ethyl acetate in isooctane. The procedural losses after purification are, on average, approximately 20%. These purification steps are the same as those used prior to quantifying estradiol by the conventional RIA used routinely in Dr. Stanczyk's laboratory at the University of Southern California [7,8]. In the present study, instead of quantifying

estradiol by RIA in the eluted fractions after chromatography, the eluates were dried and the residues were reconstituted in methanol, and subsequently sent to Dr. Xia's lab at the National Cancer institute for analysis by LC-MS/MS.

Details of the method for measuring estrogens by LC-MS/MS, including sample preparation and assay conditions, have been published previously [9,10], In the current study, an updated LC-MS/MS instrument and additional stable isotope-labeled estrogens were employed. Briefly, LC-MS/MS analysis was performed using a Thermo TSQ™ Vantage triple quadrupole mass spectrometer (Thermo Fisher, San Jose, CA) coupled with a Prominence UFLC system (Shimadzu Scientific Instruments, Columbia, MD). Both the LC and mass spectrometer were controlled by Xcalibur™ software (Thermo Fisher, San Jose, CA). Twelve stable isotopically-labeled estrogens were used to account for losses during sample preparation (such as dansylation) and analysis, which included estriol-d_3, (C/D/N Isotopes, Inc., Pointe-Claire, Quebec, Canada); 16-epiestriol-d_3 (Medical Isotopes, Inc., Pelham, NH); and $^{13}C_6$-labeled estrone, estradiol, 2-hydroxyestrone, 2-methoxyestrone, 2-hydroxyestradiol, 2-methoxyestradiol, 2-hydroxy estrone-3-methyl ether, 4-hydroxyestrone, 4-methoxyestrone, and 4-methoxyestradiol (Cambridge Isotope Laboratories, Andover, MA). The current method showed that the intraassay precision was <6% and interassay precision was <15%. The lower limit of quantification was 50 fg for estrone, estradiol, and 2-hydroxyestradiol.

Results

The median estradiol level in the 70 samples was 84.7 pg/mL by LC-MS/MS. In addition, 5.6 pg/mL of estrone and 1.7 pg/mL of 2-methoxyestradiol were detected in the estradiol fractions (Table 1). This accounted for 6.92% and 2.15% of estrone and 2-methoxyestradiol, respectively, in the estradiol fractions.

The concentrations of the 2 contaminating metabolites in the estradiol fractions varied between the low, medium, and high estradiol groups (Table 1). The contaminants were lowest in the highest estradiol groups (E2 > 100 pg/mL); they were 4.16% and 1.76% for estrone and 2-methoxyestradiol, respectively. In the 50-100 pg/mL E2 group, there was only a small increase in the estrone contaminant compared to the highest estradiol group (from 4.16% to 6.58%). However, the 2 contaminants were highest in the low estradiol group (<50 pg/mL); they were 14.6% and 3.83% for estrone and 2-methoxyestradiol, respectively.

Another explanation given for higher estradiol levels obtained by conventional RIA is that an iodinated (^{125}I) derivative of estradiol (estradiol-carboxymethyl oxime) is used in the assay instead of a marker that has a chemical structure close to estradiol, such as ^{3}H-estradiol. We compared estradiol levels in 28 serum samples measured by our conventional estradiol RIA, which utilizes an iodinated marker, to a similar RIA in which the iodinated marker was substituted with ^{3}H-estradiol. No significant difference was observed in the estradiol levels between the 2 assays. The mean (± SD) estradiol concentrations obtained with the ^{125}I and ^{3}H RIAs were 120 ± 55 pg/mL and 115 ± 53 pg/mL, respectively. Therefore, use of an iodinated marker in the estradiol RIA does not contribute to higher measured values of estradiol.

Conclusions

Two estradiol metabolic contaminants, namely estrone and 2-methoxyestradiol, were identified by LC-MS/MS in the estradiol fraction obtained after the column chromatography used pre-analytically for conventional RIA. Estrone, which differs in chemical structure from estradiol by only 2 hydrogens, is the second highest unconjugated estrogen (next to estradiol) present in the circulation in premenopausal women, whereas 2-methoxyestradiol levels are very low (<10 pg/mL) [9]. The levels of the contaminants measured would not account for the substantially greater estradiol levels obtained by RIA versus MS. Furthermore, the cross-reactions of estrone and 2-methoxyestradiol with the antibody in the estradiol RIA are <1%. Therefore, the 2 contaminants identified here would add insignificant amounts to the measured estradiol values and do not account for the quantitative differences between RIA and LC/MS-MS measurements.

Compared to LC-MS/MS and GC-MS/MS assays, higher steroid hormone values are generally obtained with the RIA method. The reason for this difference is not yet known. Cross-reaction of metabolites of compounds being measured with the antisera in RIAs to account for the higher values has not yet been demonstrated. A possible explanation is that the higher RIA values are characteristic of a biological method that utilizes the antibody/antigen principle, in contrast to MS assays, which are purely chemical methods.

Because published studies have shown a better correlation between certain biologic parameters and the RIA method than the MS method further studies are warranted to understand the methodological differences.

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Table 1.

Means and medians of serum estrogens and ratios, by levels of estradiol (E_2)

	Mean	s.d.[1]	Median	Q1-Q3[2]
All samples (n=70)				
E_2 (pg/mL)	143	148	84.7	32.8 - 204
E_1 (pg/mL)	7.98	8.70	5.59	3.47 - 8.14
E_1/E_2 (%)	12.8	20	6.92	4.24 - 13.9
$2ME_2$ (pg/mL)	3.15	3.66	1.70	0.76 - 4.00
$2ME_2/E_2$ (%)	3.75	6.04	2.15	1.44 - 3.64
E_2 <50 pg/mL (n=23)				
E_2 (pg/mL)	22.9	14.4	19.7	9.74 - 32.8
E_1 (pg/mL)	3.28	1.16	3.06	2.36 - 3.96
E_1/E_2 (%)	26.0	30.4	14.6	9.59 - 24.8
$2ME_2$ (pg/mL)	0.89	0.53	0.81	0.45 - 1.26
$2ME_2/E_2$ (%)	6.79	9.40	3.83	2.72 - 6.39
E_2 50-100 pg/mL (n=18)				
E_2 (pg/mL)	76.4	13.7	72.4	63.5 - 91.2
E_1 (pg/mL)	5.47	2.75	4.65	4.30 - 6.71
E_1/E_2 (%)	7.38	4.42	6.58	5.33 - 7.48
$2ME_2$ (pg/mL)	1.82	2.09	1.19	0.66 - 2.16
$2ME_2/E_2$ (%)	2.51	3.30	1.70	0.81 - 2.80
E_2 >100 pg/mL (n=29)				
E_2 (pg/mL)	280	140	251	158 - 370
E_1 (pg/mL)	13.3	11.4	8.43	6.84 - 15.2
E_1/E_2 (%)	5.72	5.93	4.16	2.97 - 6.05
$2ME_2$ (pg/mL)	5.77	4.21	4.46	2.71 - 8.63
$2ME_2/E_2$ (%)	2.10	1.59	1.76	1.35 - 2.29

[1] s.d.=standard deviation.

[2] Q1-Q3=interquartile range