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Improved detectability of sex steroids from frozen sections of breast cancer tissue using GC-triple quadrupole-MS

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ABSTRACT

Sex steroids in clinical endocrinology have been mainly investigated with peripheral blood and urine samples, while there is limited information regarding the local levels within tissues. To improve analytical properties of sex steroids from trace amounts of tissue samples, two-phase extractive ethoxycarbonlyation and subsequent pentafluoropropionyl derivatization coupled to gas chromatography–tandem mass spectrometry (GC–MS/MS) was developed. The optimized analytical conditions led to excellent chromatographic separation of 15 estrogens, 6 androgens, and 2 progestins. The quantitative results were calculated based on *in-house* control samples as the steroid-free tissues, and the precision and accuracy were 4.2%–26.8% and 90.8%–116.4%, respectively. The on-column limit of quantification was from 180 fg to 0.5 pg for androgens and estrogens, and 1.25 pg for progestins, which were found to be linear ($r^2 > 0.990$). The validated method was then applied to quantify 7 sex steroids from three 100-μm-thick frozen breast tissue slices from postmenopausal patients with breast cancer. This is the first report on the improved GC–MS/MS method for the detection of androgens and pregnenolone from breast cancer tissues, and it can be a useful technique to measure the local levels of sex steroids, thus, enhancing our understanding of the pathophysiological significances of steroidogenesis.

1. Introduction

Sex steroids play important biochemical roles in hormone-related physiological processes, such as cancer biology and reproductive endocrinology [1]. They are generally produced in the reproductive organs (e.g. ovary, or testes) but are also produced locally by peripheral conversion in target tissues [2]. In contrast to the examination of blood and urine samples as a surrogate measure for the estrogen action in diseases, the importance of *in situ* production of steroids from circulating precursors in breast cancer is becoming increasingly evident [3–7]. However, little is known about steroid levels in local tissues, because conventional techniques require amount of biopsy samples, and thus they could not be routinely measured.

Comprehensive methods with an acceptable analytical sensitivity for quantifying sex steroids are necessary to investigate the diversity of *in situ* sex steroid actions. However, measuring steroids in local tissue samples encounters a number of technical difficulties, such as high tissue lipid contents, high spatial heterogeneity, and small volumes inherent in sampling [8]. Immunoaffinity-based measurements are simple and sensitive, but their specificity and accuracy are questionable due to cross-reactivity [9]. Mass spectrometry coupled to chromatographic separation has been extensively used in biological and clinical applications due to its better selectivity with an excellent reproducibility in quantification [10]. However, techniques of conventional mass spectrometry combined with gas chromatography and liquid chromatography (GC–MS and LC–MS) have inadequate sensitivity for screening analysis of sex steroids from limited amounts of biological specimens.

To improve the analytical properties, a variety of chemical derivatization techniques for GC–MS and LC–MS analysis of steroids have been developed [11,12]. Although LC–MS-based assays are capable of quantifying estrogens and androgens at low pg/mL concentrations [13] and it was successfully applied to measure sex steroids in 8-μm breast cancer tissue sections [14], the complex derivatization steps and a long analytical run have been required. In GC–MS, sex steroids including androgens, estrogens, and progestins are generally derivatized with trimethylsilylation and pentafluorobenzoylation [11]. However, these derivatization methods are not sensitive enough to detect trace amounts in clinical samples. For technical advances in GC–MS-based steroid analysis, tandem mass spectrometry (MS/MS) coupled to electron impact and chemical ionizations (EI and CI) have been conducted with

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selective derivatization techniques [15,16].

Hydroxylated estrogens at C2, C4 or C-16 and their methoxy derivatives were detected in 1 g of normal and malignant breast tissue, and higher 16α-OH-E1 and lower 2-OH-E1:16α-OH-E1 ratio could associate with a prolonged survival of breast cancer patients [17,18]. Androstenediol and 5α-androstane-3β, 17β-diol produced by steroid sulfatase from DHEA-sulfate via DHEA have been also reported to bind to estrogen receptor, and decreased androstenediol might be effective for suppressing ER-α positive breast cancer with a lower intratumoral E2 concentration [19]. In our previous studies, estrogen metabolites in biological specimens were successfully analyzed using a combination of ethoxycarbonylation (EOC) with subsequent pentafluoropropionylation (PFP), prior to GC separation with a high-temperature GC (HTGC) column. The method resulted in enhanced detectability, thermo-stability, and good chromatographic resolution [20,21]. The direct EOC derivatization of polar compounds in aqueous solution is completed in a short time (< 1 min) and the EOC derivatives are extractable by non-polar organic solvents, which can effectively reduce matrix interference [20–22].

To continue challenging the limits of steroid profiling, the present study was undertaken to further develop the derivatization procedures. Here, we present a highly sensitive and selective GC–MS/MS method combined with selective two-step derivatization for quantifying 23 sex steroids, including 6 androgens, 2 progestins, and 15 estrogens, from clinically prepared breast cancer tissue slices using GC-tandem mass spectrometry (GC–MS/MS) with a triple quadrupole analyzer.

2. Materials and methods

2.1. Chemicals

Endogenous steroids examined in this study were as follows (Table 1): testosterone (T), androstenedione (A-dione), dehydroepiandrosterone (DHEA), 5α-androstane-3α,17β-diol (ααβ-diol), 5α-androstane-3β,17β-diol (αββ-diol), progesterone (Prog), pregnenolone (Preg), estrone (E1), 17β-estradiol (17β-E2), estriol (E3), 2-hydroxyestrone (2-OH-E1), 2-hydroxy-17β-estradiol (2-OH-E2), 4-hydroxyestrone (4-OH-E1), 4-hydroxy-17β-estradiol (4-OH-E2), 2-methoxyestrone (2-MeO-E1), 2-methoxy-17β-estradiol (2-MeO-E2), 4-methoxyestrone (4-MeO-E1), 4-methoxy-17β-estradiol (4-MeO-E2), 17-epiestriol (17-epi-E3), 16-epiestriol (16-epi-E3), 16α-hydroxyestrone (16α-OH-E1), and 16-keto-17β-estradiol (16-keto-E2). All steroids listed above were obtained from Steraloids (Newport, RI). The internal standards (ISs), 16,16,17-d_3-testosterone for the six androgens, 2,2,4,6,6,17α,21,21,21-d_9-progesterone for the two progestins, and 2,4,16,16-d_4-17β-estradiol for the fifteen estrogens, were purchased from NARL (Pumble, Australia) and C/D/N Isotopes (Pointe-Claire, Canada).

Sodium acetate (reagent grade), acetic acid (glacial, ≥99.99%) and L-ascorbic acid (reagent grade) were acquired from Sigma (St. Louis, MO), and anhydrous potassium carbonate anhydrous (K_2CO_3) was supplied by J. T. Baker (Phillipsburg, NJ). For two-step derivatization, triethylamine (TEA), ethylchlorofomate (ECF) and pentafluropropionic anhydride (PFPA) were obtained from Sigma-Aldrich. All organic solvents were analytical or HPLC grade, and were purchased from Burdick & Jackson (Muskegan, MI). Deionized water was prepared using a Milli-Q purification system (Millipore; Billerica, MA). Pulverization of tissue slices was performed using a TissueLyser (Qiagen; Hilden, Germany)

Table 1
GC–MS/MS information for the quantitative analysis of 23 sex steroids from tissue slices.

Compounds (trivial name)	Abbreviation	Retention time (min)	Characteristic ions (*m/z*)[a]	Transition (*m/z*)[b]		Collision energy[b]
				Precursor ion	Product ion	
Androgens						
5α-androstane-3α,17β-diol	ααβ-diol	2.31	584, 569, 420, 405, 257	420	241	18
Testosterone	T	2.49	580, 565, 417, 270	580	565	18
5α-androstane-3β,17β-diol	αββ-diol	2.58	584, 569, 420, 405, 257	420	241	18
Androstenedione	A-dione	2.75	432, 417	432	119	18
Dehydroepitestosterone	DHEA	2.77	270, 255	270	121	10
Dihydrotestosterone	DHT	3.00	436, 421, 364	364	185	18
Progestins						
Progesterone	Prog	3.43	460, 445	460	445	14
Pregnenolone	Preg	3.45	298, 283, 213	298	213	8
Estrogens						
Estriol	E3	4.09	652, 608, 580	580	183	30
17β-estradiol	17β-E2	4.29	490, 446, 418	418	172	16
17-epiestriol	17-epi-E3	4.41	652, 608, 580	580	172	16
16α-hydroxyestrone	16α-OH-E1	4.77	504, 460, 432	432	240	10
4-methoxy-17β-estradiol	4-MeO-E2	4.79	520, 476, 448	448	202	16
16-epiestriol	16-epi-E3	4.80	652, 608, 580	580	172	16
Estrone	E1	4.81	342, 298, 270	270	185	10
2-methoxy-17β-estradiol	2-MeO-E2	5.06	520, 476, 448	448	202	18
16-keto-17β-estradiol	16-keto-E2	5.14	504, 460, 432	432	172	16
4-methoxyestrone	4-MeO-E1	5.35	372, 328, 300	300	215	10
2-methoxyestrone	2-MeO-E1	5.60	372, 328, 300	300	215	12
4-hydroxy-17β-estradiol	4-OH-E2	6.16	578, 534, 506, 490, 434	434	188	18
2-hydroxy-17β-estradiol	2-OH-E2	6.39	578, 534, 506, 490, 434	434	188	18
4-hydroxyestrone	4-OH-E1	6.69	430, 386, 358, 342, 286	286	201	12
2-hydroxyestrone	2-OH-E1	6.90	430, 386, 358, 342, 286	286	201	12

[a] All steroids were derivatized with the acylation agent, pentafluropropionic anhydride (PFPA), for the hydroxy group of androgens, progestins, or estrogens, and/or 3-oxo-4-ene group of androgens or progestins, after ethoxycarbonylation (EOC) with ethyl chloromate for phenolic hydroxy groups of estrogens in aqueous buffer. All ions in full scan at *m/z* 100–600 are given as within 30% of the base peak.

[b] Transitions of the 23 sex steroid-related compounds were used for SRM method optimization.