Analysis of sex steroids in human tears using LC-MS and GC-MS: considerations and developments to improve method sensitivity and accuracy.

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Abstract

Sex steroids play a role in regulation of tear film function and may exert their action locally at the ocular surface. However, measurement of sex steroids in tears is difficult due to small-volume tear samples and very low concentrations of the hormones. This short communication highlights what has been achieved to date in the analysis of tear sex steroids using ultra-performance LC-MS (UPLC-MS) as previously published, and reports further and more recent investigations toward optimising mass spectrometry method sensitivity and accuracy. The published UPLC-MS method successfully measured progesterone, androsterone glucuronide and 5α-androstane-3α,17β-diol in pooled basal tears of postmenopausal women, and fourteen sex steroid standards in methanol. Limitations included sub-optimal limits of detection (LOD) and lower limits of quantification (LLOQ) for some analytes (particularly oestrogens), exclusion of sample matrix effects and no use of internal standards. This update reports on further experiments carried out to improve sensitivity and accuracy. Sample matrix effects, internal standard spiking, and derivatisation with dansyl chloride and oximes were investigated. Dansylation significantly improved the LOD and LLOQ of oestrogens and their metabolites, by a factor of 10 for oestradiol and a factor of 5 for oestrone, but sensitivity of this updated method is not sufficient however for analysis of these oestrogens in human tears. Using gas chromatography-mass spectrometry (GC-MS) as an alternative technique to LC-MS, improved sensitivity for derivatised oestradiol is reported. This work demonstrates the need to develop higher sensitivity methods and points researchers towards specific MS ionisation techniques for future analysis of sex steroids in tears, in order to progress current understanding of the role of sex steroids in tear function and dry eye.

Key words: sex steroids, LC-MS, GC-MS, tear film, serum

The importance of measuring sex steroids in tears

Sex steroids play a role in regulation of tear film function and dry eye. Women have a higher prevalence of dry eye and experience more severe symptoms than men (Matossian et al., 2019; Schaumberg et al., 2013; Stapleton et al., 2017). Dry eye is more common in women with age, with use of oral contraceptives, when undergoing hormone replacement therapy or oestrogen-blocking treatment for breast cancer (Boga et al., 2019; Gibson et al., 2020; Sullivan et al., 2017).

The influence of systemic sex steroids (androgens and oestrogens) on the aetiology of dry eye and ocular surface functions has been comprehensively reviewed (Gibson et al., 2017; Sullivan et al., 2017; Truong et al., 2014), confirming the contribution of androgen deficiency to the onset of dry eye, and highlighting the lack of clarity regarding the impact of oestrogens. Serum sex steroids make up only a small portion of sex steroids produced in the body, especially in women after menopause. Most androgens and all oestrogens are, in fact, locally made and metabolised in peripheral tissues after menopause (Labrie, 2010; Labrie et al., 1997; Rothman et al., 2011), and this is likely to include the ocular tissues (Gibson et al., 2017). However, the importance of local sex steroid levels to the clinical signs and symptoms of dry eye remains poorly understood. To date, little is known about the presence or levels of sex steroids in human ocular surface tissues, and the relationship with circulating sex steroids is not established.

Substantial evidence exists for sex steroid action on the ocular surface; ocular surface tissues contain all machinery required to make and utilise sex steroids. mRNAs of steroidogenic enzymes are found in human tear-producing tissues including meibomian glands and lacrimal glands (Butovich et al., 2019; Rocha et al., 2000; Schirra et al., 2006). Receptors for androgen, oestrogen, and progesterone are present in the ocular surface tissues (Auw-Haedrich and Feltgen, 2003; Esmaeli et al., 2000; Fuchsjäger-Mayrl et al., 2002; Schröder et al., 2016; Suzuki et al., 2001), suggesting sex steroids influence the structure, functions, and pathology of these tissues (Rocha et al., 2000). *In vitro* human studies show that 5α-dihydrotestosterone (DHT) enhances genes for lipogenesis in meibomian gland

epithelial cells and upregulates genes involved in wound healing and regeneration in corneal epithelial cells (Khandelwal et al., 2012). *In vitro* mice studies also show that testosterone and oestrogens alter expression of thousands of genes in meibomian glands and lacrimal glands (Sullivan et al., 2009).

Establishing the pathophysiological profile and ocular surface levels of sex steroids is a key step in improving current understanding of their role in dry eye and tear film regulation. At the ocular surface, tears and meibum are the biological fluids closest to the sites of probable local hormone synthesis, and thus are feasible candidates for non-invasive measurement of sex hormone levels in the ocular surface tissues. The collectable volume of tears is limited however (approximately 5-20 μL per individual per collection), adding to the difficulty of quantifying sex steroids present at low concentrations. Collection of meibum volume is also low, and the collection process is more uncomfortable for the patient. Detection or quantification of sex steroids in meibum has not previously been attempted although the tear-analysis techniques discussed below could be adapted to analysis of meibum.

Liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) are both advanced analytical techniques with good sensitivity, which have been commonly used to quantify sex steroids and their metabolites in serum. However, only two LC-MS methods are reported to date to be successful for analysis of tear sex steroids (Gibson et al., 2019; Pieragostino et al., 2017), and a GC-MS method has not yet been published.

This short communication perspective aims to highlight what has been achieved to date in the analysis of tear sex steroids using ultra-performance LC-MS (UPLC-MS) as previously published (Gibson et al., 2019), and to report more recent experimental investigations toward optimising LC-MS method sensitivity and accuracy. Limits of detection (LOD) and lower limits of quantification (LLOQ) of the UPLC-MS method previously reported in Gibson et al. (2019), were recalculated and reported as picograms (pg) on column to compare with those of the optimised and revised methods

presented in this update (**Table 1**) to better visualise the sensitivity improvements achieved. LOD and LLOQ for sex steroids previously reported for tears and for serum were also converted to pg on column to facilitate comparison (**Table 2**). The outcomes of a previously unpublished GC-MS method developed to analyse sex steroids are also reported. Finally, the merits of specific ionisation and MS techniques that offer promise for analysis of sex steroids in tears are advocated as candidates for future experimental evaluation.

Analysis of sex steroids in tears using UPLC-MS: what has been achieved to date and method modifications to optimise sensitivity and accuracy

Key outcomes and limitations of the previously published UPLC-MS method

The UPLC-MS method developed by Gibson et al. (2019) successfully and simultaneously measured fourteen sex steroid standards in methanol, including precursors (progesterone, 4-androstene-3,17-dione (4-dione), dehydroepiandrosterone sulphate (DHEAS)), active androgens (testosterone, 5α-dihydrotestosterone (DHT), androsterone (ADT)) and oestrogens (17β-oestradiol (E2), oestrone (E1)), and their metabolites (5α-androstane-3α,17β-diol (3α-diol-G), androsterone glucuronide (ADT-G), 2-hydroxy-17β-oestradiol (2-OH-E2), 2-hydroxyoestrone (2-OH-E1), 16α-hydroxyoestrone (16-OH-E1) and oestriol (E3)) that are involved in and synthesised during intracrine steroidogenesis (**Table 1**). The androgen and oestrogen metabolites selected are often measured in other publications because measuring the levels of these metabolites provides a more accurate estimation of the amount of active androgens and oestrogens in human peripheral tissues (Labrie et al., 2003; Gao et al., 2015). The UPLC-MS analysis was performed on a Dionex 3000 UPLC system (Thermo Fisher Scientific, Waltham, MA) coupled with Thermo Scientific™ QExactive™ Plus quadrupole-orbitrap MS (Thermo Fisher Scientific, Waltham, MA) in alternating positive and negative atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) modes with method details published previously (Gibson et al., 2019). Chromatograms and method of LOD/LLOQ calculations for the sex steroids analysed were detailed in Gibson et al. (2019). In brief, LODs and LLOQs were estimated from the

signal to noise (S/N) of the chromatograms, which was defined as the ratio of the chromatographic peak height and half of the peak-to-peak baseline noise after appropriate smoothing. S/N was plotted against concentration (regression) in order to determine LODs and LLOQs at S/N values of 3:1 and 10:1 respectively. These LOD and LLOQ concentrations (pg/mL) were then converted into the on-column LOD and LLOQ values (pg) and are reported in **Table 1**.

The main outcomes of this UPLC-MS method for tear sex steroid analysis are summarised as follows. Better signal intensity and reduced matrix effects were achieved when using positive APCI (APCI+) than ESI mode (Gibson et al., 2019), consistent with previous reports that steroid ESI is highly susceptible to ion suppression in the presence of biomatrices (Keski-Rahkonen et al., 2013; McCulloch and Robb, 2017). Effects of tear sample preparation techniques were evaluated in the publication (Gibson et al., 2019). Protein precipitation performed best for oestrogens, progesterone, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEAS) (Gibson et al., 2019); Schirmer strip extraction was preferable for their metabolites; and both extraction methods worked similarly for testosterone (Gibson et al., 2019). The on-column limit of detection (LOD) and lower limit of quantification (LLOQ) of our published UPLC-MS method were in line with those reported for the same sex steroids analysed in tears by Pieragostino et al. (2017). For example, the LLOQ for testosterone, 4-dione and progesterone was 6.9 pg, 3.1 pg and 2.9 pg respectively (**Table 1**) (Gibson et al., 2019), which are very similar to the values reported by Pieragostino et al. (2017): 4 pg, 4 pg and 2.5 pg respectively (**Table 2**).

The UPLC-MS method published previously by Gibson et al. (2019) has some limitations. The method sensitivity was only sufficient to estimate the levels for three of the selected fourteen analytes in pooled basal tears of post-menopausal women, including progesterone (0.1 pg/μL), androsterone glucuronide (ADT-G, 30.9 pg/μL) and 5α-androstane-3α,17β-diol (3αDiol-G, 9.8 pg/μL) (Gibson et al., 2019). Challenges remained with detection and quantification of the other 11 sex steroids in tears, especially oestrogens and their metabolites. This was attributed largely to their established weak LC-

MS ionisation (Harwood and Handelsman, 2009). The UPLC-MS method was least sensitive to oestrogens, with LOD on-column 263 pg for 17β-oestradiol (E2) (Gibson et al., 2019).

Historically, there have been issues in quantifying sex steroids in tears with any methodology, due to the very limited tear volumes available, and very low analyte concentrations. LC-MS analysis of sex steroids in tears collected on Schirmer strips (Pieragostino et al., 2017) did not report concentrations but only the percentage of each analyte in tears of dry eye patients relative to that in normal tears. Only DHEA level in normal tears (0.07-2.1 pg/μL), determined by enzyme immunoassays, has previously been reported in a conference abstract (Banbury and Morris, 2007).

Method modifications to optimise sensitivity and accuracy

Derivatising sex steroids

Noting the limitations of the published UPLC-MS method, further experiments were subsequently conducted towards improving its sensitivity and accuracy. It is common practice to use derivatising reagents to chemically modify analytes and thereby make them more amenable to either LC-MS or GC-MS analysis. Derivatisation generally enhances LC-MS sensitivity for oestrogens (Abdel-Khalik et al., 2013). In this update of new work, two different derivatising protocols were tested with LC-MS analysis. Both dansylation with dansyl chloride for oestrone (E1) and E2, and oximation for all analytes excluding E2 were evaluated. Dansylation and oximation of sex hormone standards followed published methods (Keski-Rahkonen et al., 2011; Nelson et al., 2004; Regal et al., 2009). The pseudomolecular ions formed in both ESI and APCI LC-MS for the underivatised steroid hormones and oxime derivatives were the $[M+H]^+$ (positive ion mode) singly protonated species and $[M-H]^-$ (negative ion mode) singly deprotonated species. Dansylation was more effective than oximation in improving signal intensity of the analytes under APCI LC-MS mode. It has been reported by other authors that oximation improved sensitivity for androgens and progestogens analysed in

ESI mode (Keski-Rahkonen et al., 2011; Regal et al., 2009), but this improvement was not observed with APCI mode in the present study.

In our experiments using APCI+, dansylation improved the response of E1 and E2 by factors of 5 and 10 respectively (**Table 1**), with minimal impact on the responses of other hormones without a phenol group. This derivatising technique significantly improved the LOD and LLOQ of oestrogens and their metabolites ionised with APCI. Dansylated E2 were 20 and 30 times more detectable and quantifiable respectively than underivatised E2. Note that only the 17β isomer of E2 was measured in this study, and there is the possibility that the response of E2 obtained with this UPLC-MS method might derive from both 17α-E2 and 17β-E2 isomers as they may not be chromatographically separated. The LLOQ of 2-hydroxyoestrone (2-OH-E1) and 2-hydroxy-17β-oestradiol (2-OH-E2) was improved by 10 and 4 times, respectively. Dansylation, however, did not greatly alter the detection and quantification of androgens and precursors (DHEA, DHEAS) in APCI compared to the underivatised compounds. For example, the LLOQ of dansylated testosterone and dihydrotestosterone (DHT) was 3-4 times higher than that of their underivatised compounds. The improved sensitivity of the derivatised oestrogens in the current study is in line with previous reports (Boggs et al., 2016; Nelson et al., 2004). Nelson and co-authors found that dansyl chloride derivatisation significantly improved LOD for E2 ionised with APCI+ on a triple quadrupole MS/MS (Nelson et al., 2004). Similarly, Boggs and co-authors established that dansylation improved chromatographic separation and enhanced E1 and E2 responses analysed with ESI+ on a triple quadrupole MS/MS by two- to eight-fold (Boggs et al., 2016).

Alternative derivatising agents, such as 1-methylimidazole-2-sulfonyl chloride (MISC) (Li and Franke, 2015); 1,2-dimethylimidazole-5-sulfonyl chloride (DMIS) (Keski-Rahkonen et al., 2015); 3-bromomethyl-propyphenazone (Khedr and Alahdal, 2016); and 1-(2, 4-dinitrophenyl)-4,4-di-methylpiperazinium (MPPZ) (Denver et al., 2019), have variously been shown to improve sensitivity of oestrogens (**Table 2**). Li and Franke found that, compared to dansylation, derivatisation of

oestrogens and their metabolites with MISC, achieved much better sensitivity with ESI+ on quadrupole orbitrap MS (Li and Franke, 2015). The observed improvement was greatest for analytes having two phenolic groups (e.g. 2-OH-E1, 2-OH-E2). Reported LODs and LLOQs of the major oestrogens (E1, E2, and oestriol (E3)) derivatised with MISC were 2-10 times lower than that with dansyl chloride (**Table 2**) (Li and Franke, 2015). Additional benefits of MISC derivatisation include shorter LC run time and reduced use of organic solvents (Li and Franke, 2015). The targeted SIM (tSIM) acquisition mode provided greater sensitivity than the MS mode for both dansyl chloride and MISC derivatives analysed by the Orbitrap (Li and Franke, 2015). Future studies should explore the use of derivatising agents other than dansyl chloride to improve sensitivity for analysis of oestrogens in tears.

In the new work reported in this update, attempts to analyse serum samples using dansylation by UPLC-MS were not successful, although dansylation improved sensitivity for some sex steroid standards. An unacceptably high intra-assay coefficient of variance (CV) for some of the analytes (CV > 20%) indicated that the method showed poor reproducibility. Another observed issue was the increased back pressure due to partial column blockage after repeated injection of serum extracts onto a 1-mm diameter UPLC column. A column of small diameter had been selected for our previously published work because it had shown improved sensitivity for underivatised sex steroids standards due to reduced flow rate into the ion source (Gibson et al., 2019). The same column was used in the study reported herein. In the available literature, larger diameter columns, for example 2.1 mm, have been more commonly used for serum analysis of sex steroids (Ferreira et al., 2017; Johänning et al., 2015; Ke et al., 2014; Keski-Rahkonen et al., 2015; Yuan et al., 2019). Due to the unexpectedly high intra-assay variability and the column blockage issue, the UPLC-MS derivatised method was not further investigated for serum analysis.

Spiking with stable isotope internal standards

In this update, the sensitivity of the UPLC-MS method without derivatisation (Gibson et al., 2019) was also reassessed compensating for sample matrix effects using stable isotope internal standard spiking to improve quantitative accuracy. The LOD and LLOQ (pg/μL tears) reported by Gibson et al. (2019) were estimated based upon the instrument LOD/LLOQ for standard solutions prepared in organic solvents, which were then used to calculate the LOD/LLOQ for tears considering the amount of tear sample on column. In addition, Gibson et al. (2019) did not use internal standards in sample preparation to compensate for any loss of the analytes during sample processing and ionisation, and did not consider the influence of the sample matrix upon ionization efficiency. The LOD and LLOQ for tears were redetermined in this study, utilising tear-like-fluid (TLF, a manufactured fluid containing proteins, salts and lipids at concentrations analogous to human basal tears) as the sample matrix. The TLF spiked with sex steroid standards and internal standards went through the same extraction process as pooled tear samples prior to column injection. Stable deuterium isotope labelled sex steroids, including oestradiol-d_4, oestrone-d_4, testosterone-d_2, dihydrotestosterone (DHT)-d_3, progesterone-d_9 and pregnenolone-d_4, were used as internal standards to improve the accuracy of LOD/LLOQ calculations and quantification of sex steroids. Note that testosterone-d2 has the same mass with DHT but these compounds were chromatographically separated thanks to their different retention time (9.46 min for DHT and 9.05 min for testosterone-d2).

When comparing the LODs (**Table 1**) determined with- *vs*. without- sample matrix and internal standards spiking, the sensitivity of the method appears to have reduced with the inclusion of sample matrix and internal standards, which was possibly due to sample matrix effects. Of note, these LODs were determined on two different occasions, and therefore the sensitivity could depend upon how clean the MS ion source was at the time of analysis. If these conditions could be optimised and consistent throughout the analysis, it is likely that the sensitivity of the updated method could be improved upon. Nevertheless, it would still likely fall short of that needed for routine analysis of a broad panel of sex steroids in tears.

LC-MS methods with great sensitivity have been reported by other authors for sex steroids in serum (Desai et al., 2019; Keski-Rahkonen et al., 2013; Li and Franke, 2015; Wang et al., 2015). The most common MS/MS used for the analysis of serum sex steroids is the tandem (triple) quadruple, which achieves higher sensitivity compared to the quadrupole orbitrap MS when using selected reaction monitoring (SRM) (also called multiple reaction monitoring (MRM) mode). Tandem quadrupole MS/MS exhibits superior duty cycle advantages in SRM mode, relative to the Orbitrap (Conklin and Knezevic, 2020; Pitt, 2009). This gives, excellent sensitivity for the analysis of target compounds. It is therefore often the preferred choice for the analysis of targeted sex steroids in serum. In contrast, quadrupole orbitrap MS, known for its extraordinary mass accuracy, is superior for the analysis of unknown compounds that have proximate mass and time of elution (Zubarev and Makarov, 2013). As mentioned previously, controversy exists as to which sex steroids are present in tears. The selection of sex steroid analyte targets for tears in our study was based upon the pathway of local sex hormone synthesis in peripheral tissues (Gibson et al., 2019; Gibson et al., 2017; Sullivan et al., 2017; Truong et al., 2014). Some of the analytes were near co-eluting and could suffer from ion suppression. This partly explains why the quadrupole orbitrap MS was originally chosen for analysing tear sex steroids in our studies. This MS technique with SIM and tSIM scans has also previously been successfully applied by other authors for the ESI+ analysis of derivatised oestrogens and their metabolites in serum (Li and Franke 2015). Great sensitivity was achieved, for example LOD for E2 derivatives: 7-103 fg on column with SIM, and 0.5-267 fg on column with tSIM (**Table 2**) (Li and Franke, 2015). The use of derivatising agents (other than dansyl chloride) likely contributed to the increased sensitivity observed with a quadrupole-Orbitrap in the study.

Analysis of sex steroids in tears using GC-MS: outcomes and challenges

We also report here the outcomes of a previously unpublished GC-MS method developed to analyse sex steroids. GC-MS has been used by multiple investigators to measure sex steroids in serum (Caron et al., 2015; Courant et al., 2010). Highly sensitive analysis of sex steroids by GC-MS can be achieved

with derivatisation of the analytes (Abdel-Khalik et al., 2013). Since our derivatised UPLC-MS method was not successful in analysing oestrogens in serum, GC-MS was evaluated with the aim of improving the LLOQ's for oestrogens. The method used and the outcomes observed are briefly described below. Complete experimental details are available in a published PhD thesis (Gibson 2018).

The GC-MS analyses that are reported here for the first time, were carried out on a Thermo TSQ XLS tandem quadrupole instrument coupled to a Trace GC Ultra system (Thermo Fisher Scientific, Waltham, MA) equipped with a Thermo Trace TR-50MS GC column (60 m x 0.25 mm ID, 0.25 µm df), using SIM and SRM modes. For GC-MS sample preparation, tear samples were initially treated with β-glucuronidase (at 55^{o}C, overnight) from *Helix pomatia (H. pomatia)* (type H-3, Sigma-Aldrich, St Louis, MO) in order to hydrolyse steroid sulfates and glucuronides. Thus conjugated steroids are converted into their free form and GC-MS values represent a total of both forms of steroid. Numerous derivatisation methods were explored and optimised using electron capture negative ionisation (ECNI) or positive electron ionisation (EI) modes for GC-MS. Fluorinating agents such as pentafluorobenzylhydroxylamine (PFB-NH2), pentafluorobenzylbromide (PFB-Br) and heptafluorobutyric anhydride (HFB) (which produce electrophilic steroid derivatives that are suited to ECNI); and trimethylsilyl (TMS) agents such as N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), Hexamethyldisilzane (HMDS), Trimethylchlorosilane (TMCS), N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and 1-trimethylsilylimidazole (TMSI) were chosen for derivatisation (Caron et al., 2015; Courant et al., 2010). Fluorinated derivatives were analysed using ECNI with methane as reagent gas. ECNI is a lower energetic ionization technique compared to EI and results in much less fragmentation and more eficient formation of specific higher mass steroid derivative ions. Depending on the derivative formed, the dominant ion species included [M-H]-, [M-HF]-or [M-PFB]-, where PFB represents a pentafluorobenzyl derivative group, and HF represents hydrogen fluoride. Specific ions and their m/z value are detailed in Gibson, 2018.

A three-stage derivatisation was examined using a combination of PFB-NH_2 followed by PFB-Br and TMS agent for ECNI (Courant et al., 2010). This combination of derivatising agents selectively derivatised different polar funtional groups of the steroid and resulted in the best sensitivity and chromatography of all the options trialled. Unfortunately, further experiments demonstrated that the limits of detection of this derivatising method, with only 5 of the standards (ADT, DHT, E2, testosterone, and E3) being detectable in SIM at a concentration of 0.1 ng on column, were not low enough for sex steroids in tears. The same outcome was seen for a two-step derivatisation (PFB-NH_2 followed by TMS agent) with EI. A single stage TMS derivatisation of the hydroxyl-containing analytes, including all the target hormones except 4-Dione and progesterone, was further explored. A comparison of six TMS agents was performed using EI, with derivatisation time, temperature and catalysts optimised for each TMS reagent. The TMS reagent which yielded the highest peak areas for the hormones was Tri-Sil HTP mixture (HDMS: TMCS: pyridine 2:1:10), heated at 80°C for one hour.

The LODs and LLOQs were estimated from the signal to noise of the chromatograms for both SIM (MS) and SRM (MS/MS) (**Table 1**). SIM resulted in better sensitivity than SRM, indicating that collision induced dissociation (CID) of steroid derivatives resulted in inefficient parent ion fragmentation and formed product ion with diminished abundance and insufficient signal/noise to enhance sensitivity. Although sensitivity with Tri-Sil HTP was much better than for the other silytating reagents, the LLOQs still did not reach the levels required for the analysis of the analytes in biological samples, being two orders of magnitude above hormone levels in serum. A similar GC-MS method with a better LOD (0.49 pg/μL) for derivatised E2 (Courant et al., 2010) failed to detect oestrogens in serum of pre-pubertal children. The sensitivity of the GC-MS assays trialled in the current study would not be sufficient to detect sex steroids in tear samples.

Future directions: developing higher sensitivity methods for analysis of sex steroids in tears:

Several possible future directions could be explored to improve sensitivity and reproducibility for analysis of sex steroids in tears. One of the most promising options is nano-ultra high-performance

liquid chromatography coupled with electro spray ionisation and tandem mass spectrometry (nano-UHPLC-ESI-MS/MS). This technique provides higher sensitivity due to the analytes being concentrated into nanolitre volumes when they elute from the column at very low flow rates (Juraschek et al., 1999; Wilm and Mann, 1996). Compared to traditional ESI emitters, much smaller and finer spray droplets are produced from nanospray emitters with small internal diameters, resulting in enhanced charge to volume ratio, increased surface area, reduced specific heat capacity, and consequently fewer desolvation cycles and increased ionisation efficiency (Juraschek et al., 1999; Wilm and Mann, 1996). As the chromatographic system is miniaturised, resulting in lower solvent flows, sensitivity increases dramatically in a way not seen with APCI. This effect and the reduction in column dilution, with nano technology, are synergistic. This method is a promising approach for tear steroid analysis, given its advantage of nano flow that requires a considerably lower sample volume. The application of direct nanoflow UPLC coupled with ESI-MS has been suggested to improve the detection of poorly ESI-ionised oestrogen derivatives in extracts of tissues or biofluids (Chetwynd et al., 2014). The sensitivity of a Waters 2695 system at 0.2 mL/min was compared with that of a Waters nanoAquity UPLC system at 2 μL/min using ESI+ (Wang et al., 2015). An increase in signal-to-noise by 2.5-fold was observed at the lower flow rate (2 μL/min) (Wang et al., 2015). The LLOQ for E2 derivatised with pre-ionized N-methyl pyridinium-3-sulfonyl and analysed with nanoflow UPLC-ESI-MS was 1 fg on column (**Table 2**), which appears to be the best sensitivity for this compound reported to date (Wang et al., 2015). This method required only 100 μL of human serum but enabled simultaneous quantification of six unconjugated and conjugated oestrogens within a short time (20 min) (Wang et al., 2015). The potential downsides of nano UHPLC-MS/MS are the intricate nature of the mobile phase plumbing and production of columns, plus the availability of a nano-flow chromatograph (Juraschek et al., 1999; Wilm and Mann, 1996).

In terms of conventional-scale chromatography solutions, atmospheric pressure photoionisation (APPI) has been successfully utilised for ionisation of sex steroids in serum and tissues. The steroid

sensitivity of APPI and the impact of matrix interferences on this ionising technique have been compared by other investigators, with ESI and APCI in both polarities on triple quadrupole MS/MS for the analysis of E2 in human serum and endometrial tissue (Keski-Rahkonen et al., 2013). APPI was the most effective ionising technique and was applied successfully in both positive and negative mode for E2 analysis in serum and the endometrial tissue, with the lowest LLOQ 0.27 pg on column achieved in APPI- (**Table 2**) (Keski-Rahkonen et al., 2013). APPI+ resulted in stronger signal response but higher background, thus sensitivity was similar to ESI- and APCI- (Keski-Rahkonen et al., 2013). Unlike ESI+ suffering from severe ion suppression, ionisation efficiency of APPI and APCI in both polarities was not affected by biomatrix background (Keski-Rahkonen et al., 2013). When using APPI+ combined with DMIS derivatisation, a significantly better sensitivity was achieved for E2 with LLOQ 0.05 pg on column (**Table 2**) (Keski-Rahkonen et al., 2015). This method enabled quantification of serum oestrogens in healthy postmenopausal women, 98% of the cases for E2 and 100% for E1, and notably also in aromatase inhibitor-treated postmenopausal women, 61% for E2 and 93% for E1 (Handelsman et al., 2020). Another MS method with alternating positive and negative APPI was later successfully developed to simultaneously detect 18 steroids including both androgens and oestrogens in human and mouse serum without derivatisation (Desai et al., 2019). The superior sensitivity of APPI over ESI and APCI in serum oestrogens analysis was also confirmed in another study (McCulloch and Robb, 2017). Orthogonal geometry field-free APPI (FF-APPI) was utilised in this study and provided higher sensitivity for all analytes selected including E1, E2, testosterone, progesterone, pregnanediol and androsterone, with order-of-magnitude improvements for E1 and E2 (**Table 2)** (McCulloch and Robb, 2017). Matrix suppression effects were also found to be minimal with FF-APPI and APCI, which otherwise were highest with ESI (McCulloch and Robb, 2017). FF-APPI, unlike the commercial open-geometry APPI, features an extended enclosed reaction region and unique flight tube geometry, which improve the confinement of the ion beam and ion transmission, positively impacting sensitivity (McCulloch and Robb, 2017). Future studies should explore the utility

of APPI for the analysis of sex steroids in tears and ocular surface tissues. The APPI technique is currently unavailable in our laboratory.

Conclusion

In conclusion, according to the results obtained, UPLC-MS plus derivatisation was more suitable than GC-MS for analysing the 14 sex steroids selected. The UPLC-MS method performed on quadrupole Orbitrap MS in APCI+ mode with dansyl chloride derivatisation improved sensitivity for oestrogens. However, this method, unlike the commonly used triple quadrupole MS with ESI for serum sex steroids reported elsewhere, was unsuitable for use with serum samples due to its observed high intra-assay variability on the orbitrap MS. The UPLC-MS method without derivatisation was able to measure only progesterone, androsterone glucuronide and 5α-androstane-3α,17β-diol in pooled basal tears of post-menopausal women (Gibson et al., 2019).

The analysis of sex steroids in tears is very challenging due to the extremely limited sample volumes and low analyte concentrations. Most sex steroids are not ionized efficiently using regular LC-MS ion sources such as ESI and APCI. The literature suggests that either nanoflow ESI or full-flow APPI ion sources coupled with a tandem (triple) quadrupole MS, are the most promising potential techniques for analysing sex steroids in tears. With nanoflow ESI, the effects of derivatization would likely yield greater sensitivity gains than seen here with APCI, and these gains would be synergistic with the overall greater sensitivity of nanoflow ESI compared to conventional-scale UPLC or HPLC. Whilst further work on electron-capturing GC-MS derivatives might yield a sensitive detection method for a sub-group of the sex steroids, indications from this work and literature review suggest that it will likely not lead to a successful broad-spectrum quantification assay for all sex steroids in tears. We provide this information to inform and guide other researchers in their future experiments.

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545

546 **Table 1.** Experimentally determined limit of detection (LOD, pg on column) and limit of

547 quantification (LOQ, pg on column) of the selected sex steroids analysed by UPLC-APCI-MS

548 with/without derivatisation and EI+ GC-MS or EI+ MS/MS.

Compound	Method	**UPLC-MS (QE+, APCI+/-)**						**GC-MS (ECNI, SIM)**	**GC-MS (EI+, SIM)**		**GC-MS/MS (EI+, SRM)**	
	Derivatisation	No		No		Dansyl chloride		$PFB\text{-}NH_2$ → PFB-Br → TMS	Tri-Sil HTP		Tri-Sil HTP	
	Sample matrix	Tear-fluid-like		Methanol		Methanol		Methanol	Methanol		Methanol	
	Internal standards	Yes		No		Yes		No	No		No	
	Sensitivity (pg on column)	LOD	LLOQ	LOD	LLOQ	LOD	LLOQ	LOD	LOD	LLOQ	LOD	LLOQ
Progesterone		14.1	47.1	0.9	2.9	6.6	21.8		37.1	123.7	-	-
Pregnenolone		13.8	46.1	-	-	13.9	46.1		-	-	-	-
E1		42.4	141.2	6.6	18.8	4.2	13.9		6.5	21.7	9.1	30.4
E2		3680.3	8934.3	263.0	564.7	3.2	8.7	100	3.4	11.4	2.7	8.8
E3		119.6	398.6	9.9	33.2	-	-	100	9.6	31.9	-	-
2-OH-E1		-	-	45.9	154.6	1.5	5		18.1	60.3	-	-
16-OH-E1		-	-	3.8	21.3	-	-		12.2	40.8	-	-
2-OH-E2		2762.8	9222.7	58.6	138.6	3.8	12.6		15.1	50.3	-	-
Testosterone		53.6	178.6	2.0	6.9	2.4	8.1	100	5.25	17.5	15.9	53.1
DHT		138.1	460.3	2.3	7.9	2.7	8.8	100	19.9	66.3	6.3	21.1
ADT		603.4	2011.2	27.7	95.8	5	16.8	100	3.3	11.1	6.8	22.6
DHEA		28.7	95.8	-	-	27.9	92.9		2.8	9.3	1.0	3.5
4-dione		46.7	155.7	0.8	3.1	3.1	10.3		-	-	-	-
DHEAS		28.7	95.8	10.5	39.7	40.3	134.4		-	-	-	-
ADT-G		736.4	2454.7	28.8	96.1	19.3	64.1		-	-	-	-
3α-diol-G		425.5	1418.4	27.1	90.4	12.1	40.2		-	-	-	-

549 E1: oestrone; E2: 17β-oestradiol; E3: oestriol; 2-OH-E1: 2-hydroxyestrone; 16-OH-E1: 16α-hydroxyestrone; 2-OH-E2: 2-hydroxy-17β-
550 oestradiol; DHT: dihydrotestosterone; ADT: Androsterone; DHEA: dehydroepiandrosterone; 4-dione: 4-androstene-3,17-dione; DHEAS:
551 dehydroepiandrosterone sulphate; ADT-G: : Androsterone glucuronide; 3α-diol-G: 5α-androstane-3α,17β-diol; $PFB\text{-}NH_2$:
552 pentafluorobenzylhydroxylamine; PFB-Br: pentafluorobenzylbromide; TMS: trimethylsilyl.

553

554 **Table 2.** LC-MS method information and LOD/LLOQ (pg on column) for the selected sex steroids in serum and tears reported by others cited in this paper

Compound	Sample matrix	Human serum						Human tears (collected using Schirmer strips)		Human serum and plasma			Human and mouse serum		Activated-charcoal stripped FBS		Human serum	Human serum											Human plasma		Human plasma		Human serum		Human serum	
	Sample volume (mL)	0.15						0.09*		0.6			0.2		2		0.5	0.1											0.25		0.5		0.1		0.2	
	Column type and dimension	Agilent Poroshell 120 SB-C18 (2.1 × 50 mm, 2.7 µm)						Luna®C8 (3 x 50 mm, 3 mm)		Phenomenex, Luna C18, (2 x 100 mm, 3 µm)			Restek Raptor biphenyl column (2.1 mm x 100 cm, 2.7 µm)		Restek Ultra Biphenyl (4.6x250 mm, 5.0 µm		Phenomenex Synergi 4mMax-RP (2.0x150 mm)	Ascentis Express C18 (3.0x150 mm, 2.7 mm)											Zorbax Extend C18 (4.6 × 150 mm, 5 µm)		ACE Excel C18-PFP (2.1 x 150 mm, 2 µm)		Waters BEH130 C18 (0.15 x 100 mm, 1.7 mm)		Kinetex Phenyl-Hexyl (2.1x100 mm; 1.7 µm)	
	Injection volume (uL)	20						50		10			50		5		15	25											10		30		1		25	
	Mobile phases	ESI+: water (A) and MeOH (B) containing 0.1% formic acid ESI-: water (A) and MeOH (B) containing NH4OH 2.5 mM APCI and APPI: water (A) and MeOH (B)						A: 2% methanol in water B: 100% methanol Both containing 0.025% solvent additive.		Isocratic solvent: MeOH: water 90:10, v/v containing 0.05% formic acid			A: water B: MeOH C: toluen (dopant)		A: 0.1% formic acid in methanol B: 0.1% formic acid in acetonitrile		A: 5% acetonitrile containing 0.1% formic acid B: 95% acetonitrile containing 0.1% formic acid	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile											A: water B: ACN both containing 0.1% formic acid		A: water B: ACN both containing 0.1% formic acid		A: water/acetonitrile (99.5:0.5, v/v) B: acetonitrile/water (98:2, v/v) both containing 0.1% formic acid		A: water B: methanol	
	Method	HPLC-tqMS, MRM						HPLC-qMS-ESI+, MRM		HPLC-tqMS			UHPLC-tqMS-APPI+/-, MRM		HPLC-tqMS-ESI+, MRM		HPLC-tqMS-APCI+, MRM	UHPLC-orbMS-ESI+, SIM						UHPLC-orbMS-ESI+, tSIM						HPLC-tqMS-ESI+		UHPLC-tqMS-ESI+, MRM		nanoUPLC-tqMS-ESI+, SRM		HPLC-tqMS-APPI+, MRM	
		ESI+	ESI-	APCI+	APCI-	APPI+	APPI-			ESI+	APCI+	FF-APPI+																									
	Derivatisation	No						No		No			No		No	DC	DC	DC	PA	PS	IS	DC	IS	DC	PA	PS	IS	DC	IS	BP		MPPZ		PS + IM		DMIS	
	Sensitivity (pg on column)	LLOQ						LOD	LLOQ	LOD			LOD	LLOQ	LOD	LOD	LOD	LOD				LLOQ		LOD				LLOQ		LOD	LLOQ	LOD	LLOQ	LLOQ		LLOQ	
Progesterone								2.5	2.5	0.9	1	0.2	6.67	13.33	30.8			0.433	3.333	0.183	0.075			0.250		0.110	0.183										
Pregnenolone													6.67	13.33	16.5	1.08																				
E1										8.3	5.3	0.7	0.33	0.67		5.75	0.26	0.047	0.088	0.032	0.012	0.093	0.020	0.073	0.050	0.073	0.002	0.107	0.007	0.010	0.036	0.210	0.430				

E2	13.62	0.54	1.09	0.54	0.54	0.27			8.1	3.8	1	0.33	0.67	27.3	10	0.21	0.033	0.083	0.103	0.007	0.042	0.015	0.010	0.267	0.183	0.0005	0.028	0.007	0.010	0.036	0.210	0.430	0.001	0.050
E3															10.5		0.060	0.105	0.048	0.032	0.063	0.047	0.038	0.083	0.003	0.013	0.072	0.020	0.010	0.036				
2-OH-E1																	0.333	0.217	0.450	0.020	1.667	0.053	0.025	0.183	0.067	0.0003	0.048	0.012						
16-OH-E1																	0.100	1.300	0.217	0.033	0.133	0.078	0.052	0.383	0.015	0.007	0.082	0.010	0.040	0.111	0.430	2.170		
2-OH-E2																													0.100	0.300			0.010	
Testosterone							1	4	0.6	1.1	0.2	1.33	3.33	20			0.217	1.083	0.050	0.107			0.367		0.167	0.200								2.50
DHT												6.67	13.33																					10.00
ADT							2.5	4	35	49	6.2	6.67	13.33																					
DHEA												6.67	13.33	3.2																				
4-dione												3.33	6.67	26.3																				
References	Keski-Rahkonen et al. (2013)						Pieragostino et al. (2017)		McCulloch and Robb (2017)			Desai et al. (2019)		Boggs et al. (2016)		Nelson et al. (2004)	Li and Franke (2015)													Khedr and Alahdal (2016)		Denver et al. (2019)		Wang et al. (2015)	Keski-Rahkonen et al. (2015)

555 *Tears were collected using Schirmer strips, after which sex steroids were extracted and reconstituted in 90 µL of 40% methanol (Pieragostino et al., 2017).

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