Mass spectrometry and its evolving role in assessing tissue specific steroid metabolism

MASS SPECTROMETRY AND ITS EVOLVING ROLE IN ASSESSING TISSUE SPECIFIC STEROID METABOLISM

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Abstract

Glucocorticoid hormones play vital roles in regulating diverse biological process in health and disease. Tissue levels are regulated by enzymes which activate and inactivate hormones. The enzyme, 11β-hydroxysteroid dehydrogenase type 1, in particular, has been identified as a potential drug target; inhibiting this enzyme attenuates glucocorticoid action by lowering local levels of active hormone.

A variety of mass spectrometric approaches have been developed to characterise the enzyme *in vivo*. Endogenous glucocorticoids and their metabolites can be profiled in urine by gas chromatography mass spectrometry and circulating steroids are now more commonly quantified by liquid chromatography tandem mass spectrometry. Tracer dilution studies have allowed rates of generation of glucocorticoids by the enzyme to be distinguished from hormone generated directly by the adrenal glands and, in combination with arterio-venous sampling, rates of production by specific tissues have been quantified. This has allowed the contribution of liver, adipose, muscle and brain to cortisol production in metabolic disease and hence drug targets prioritised. Most recently mass spectrometry imaging in combination with on-tissue derivatisation has been developed to profile keto-steroids in tissue sections, allowing molecular maps to be generated across complex tissues, where regional functions are important.

The review provides a synopsis of how measurement of steroids by mass spectrometry has evolved to provide insight into the dynamic turnover of glucocorticoids *in vivo*, highlighting the milestones that have advanced the field and identifying the remaining challenges for researchers, in terms of analytical chemistry and endocrine physiology and biochemistry.

Glucocorticoids: tissue specific metabolism and actions

Glucocorticoid hormones regulate diverse physiological functions, including immune, metabolic and stress responses, through their actions on nuclear hormone receptors (mainly glucocorticoid and mineralocorticoid receptors). Glucocorticoid biosynthesis happens principally in the adrenal gland through a steroidogenic cascade, triggered by activation of the hypothalamic-pituitary-adrenal (HPA) axis. This negative feedback loop tightly regulates concentrations of circulating steroids, but active hormone concentrations are also controlled within tissues, where distinct enzymes inactivate and activate steroids, e.g. 11β-hydroxysteroid dehydrogenases (11βHSDs), 5α and 5β-reductases, 3αHSD and CYP3A4 (catalysing 6-hydroxylation) [1] (Figure 1).

There are two isozymes of 11βHSDs [2]. Type 2 inactivates 11β-hydroxy-glucocorticoids forming inert 11-ketosteroids (cortisol and cortisone respectively in humans) and is present mainly in kidney [3;4]. It also plays a vital role in the placenta where it protects the foetus from exposure to endogenous, maternal glucocorticoids [4], although is a less efficient barrier to exogenous steroids, such as dexamethasone [5;6].

In contrast 11βHSD1 regenerates active 11-hydroxy from 11-keto-glucocorticoids [7], and has become an attractive therapeutic target to attenuate glucocorticoid action in metabolic disease and cognitive decline [2]. In defining 11βHSD1 as a therapeutic target, it is important to understand the contribution made by the enzyme to active hormone levels in distinct tissues (e.g. liver, brain, adipose), where dysregulation may arise. Approaches to measure glucocorticoid production rates and tissue specific metabolism by 11βHSD1 have evolved, driven largely by advancing mass spectrometric (MS) tools. MS offers superior analytical specificity to immunoassays and indeed a recent Endocrine Society statement has indicated the need for MS analysis of steroid hormones for acceptance for publication [8]. This mini-review summarises how understanding of glucocorticoid physiology and pathophysiology has been illuminated by the use of MS.

A. Mass spectrometry applied to steroid biochemistry

For steroids to be detected by MS, they must be ionised, typically forming singly charged ions, and subsequently detected by their mass to charge ratios (*m/z*). For quantitative MS, the mass analyser is commonly a quadrupole, ideal for its fast scanning rate and wide dynamic range, allowing multiple species of low and high abundance to be measured concomitantly. Initially single quadrupole systems were used, monitoring the most abundant and discriminatory ions from the molecule's mass spectrum, but more recently tandem MS has become the method choice. By measuring the transition of a precursor to product ion, following fragmentation in the collision cell, specificity and sensitivity are improved, since background noise is minimised. Accurate mass systems are now coming on-line for quantitation, with some researchers asserting similar quantitative performance, with improved specificity [9]. Accurate mass systems enable the potential for non-targeted analysis in conjunction with targeted quantitation.

Conventionally liquid extracts of samples are introduced into the MS via a gas or liquid chromatograph (GC or LC). GC-MS has underpinned many pivotal studies of glucocorticoid metabolism, with Shackleton leading the field [10;11]. Analytes are deposited onto a column lined with a chemically defined stationary phase. The column is heated and sample components volatilise at their boiling point into the carrier gas at distinct temperatures. They are detected by the MS, generating a mass chromatogram. GC-MS therefore allows

identification of steroids by their retention times, *m/z* ratios and fragmentation patterns. To minimise polar interactions with the stationary phase, compounds are commonly derivatised to disguise hydrogen bonding sites. Methoxime-trimethyl silyl derivatives are the most common, offering characteristic fragment ions with loss of *m/z* 31 and 90. Peaks elute rapidly from GC columns over narrow time windows, well suited to resolve steroid isomers. GC-MS and now MS/MS is an excellent analytical approach to define complex steroid mixtures as has been exemplified in the "steroidomic" approach characterising adrenal malignancies [12].

GC instruments were interfaced to MS before LC, due to facile removal of the carrier gas eluting from the column. The interface between LC and MS took longer to develop, through difficulties in desolvating the mobile phase. However ion transfer into the LC-MS/MS systems is now efficient, and routinely used for steroid analysis e.g. in Clinical Biochemistry [13]. Although small molecular weight drugs may be relatively easily analysed, steroids prove more challenging due to their resistance to ionisation and susceptibility to matrix interference, so called "ion suppression". GC commonly employs "electron impact" ionisation, whereby a beam of highly energised electrons break covalent bonds readily generating ions. In contrast softer "electrospray" with LC-MS/MS, relies on spontaneous ionisation. Glucocorticoids lack readily ionisable groups and therefore ionisation efficiency is poor. Alternative ionisation modes such as atmospheric pressure chemical ionisation (APCI) can be helpful but reduced steroids (e.g. A-ring reduced metabolites) still respond poorly. LC-MS/MS is mainly used for measuring selected plasma steroids, whereas reduced metabolites are still preferentially determined by GC-MS(MS). Derivatisation, in conjunction with LC, can enhance ion abundance throughout introduction of charged or chargeable groups improving sensitivity and broadening scope; for example use of Girard reagents for testosterone analysis [14], an approach which has also been applied for oxysterol profiling [15]. Sensitivity of analysis of estrogens benefits similarly following derivatisation [16].

B: How MS can be applied to measure tissue-specific glucocorticoid metabolism

Analysis by both GC-MS(MS) and LC-MS/MS have underpinned studies characterising glucocorticoid metabolism in health and disease. Features of glucocorticoid excess in Cushing's Syndrome, resemble those of idiopathic Metabolic Syndrome (central obesity, type 2 diabetes, hypertension, dyslipidaemia), leading to the hypothesis that attenuating glucocorticoid action by inhibiting 11βHSD1 may be beneficial in metabolic disease [2]. Urinary glucocorticoid profiling by MS is widely used to assess integrated whole body daily glucocorticoid production rates. In humans, cortisol and ~10 of its metabolites [10] are detected and the approach can be extended to include androgens, progestogens and mineralocorticoids, offering insights into the balance of synthetic and metabolic pathways of adrenal hormones.

The ratio of the main urinary cortisol metabolites (αTHF and βTHF) to those of cortisone (βTHE) is commonly reported as an index of the balance of whole body activities of the types 1 and 2 isozymes of 11βHSD [17]; changes in the activities or either enzyme can alter the ratio, seen when 11βHSD1 or 2 are disrupted genetically or by pharmacological/xenobiotic inhibitors [18-21]. 5α and 5β-Reduced metabolites of cortisol but only 5β-reduced metabolites of cortisone are used in the calculation, and thus the ratio may be skewed by differential dysregulation of either 5α or 5β-reductases, e.g. in obesity [22;23] and liver disease [24;25]. Therefore, although this index is reported widely, confounders in the measure have contributed to conflicting and inconsistent data e.g. in obesity [23;26;27] .

Therefore, with 11βHSD1 becoming a potential pharmaceutical target, it became important to devise ways to measure 11βHSD1 independently of other enzymes. The following approaches reported by Rask et al [26;28] yielded early novel insights into tissue-specific dysregulation of 11βHSD1 in liver and adipose in obesity, but caveats persisted over the interpretation of the data. The velocity of 11βHSD1 in adipose biopsies *ex vivo* may be assessed in conjunction with transcript levels. These data reflect protein and transcript amounts, but the actual rate of generation of cortisol *in vivo* is dependent on available substrate concentration; circulating cortisone concentrations are ~100 nM [29], considerably lower than the low micromolar Km for reduction. The activity of the *hepatic enzyme* was assessed by formation of cortisol by first pass hepatic metabolism of oral cortisone. This may be confounded by variable gastrointestinal absorption, and changes the activities of other hepatic metabolic enzymes [22]. For example, A-Ring reductases operate within similar kinetic ranges [30;31], and therefore compete with 11βHSD1 for substrates. Cyp3A4, generating 6β-hydroxortisol, is highly variable in its activity between people, modified by genetics, nutrition and prescription drugs [32].

A major step forward in the field came with *in vivo* tracer dilution studies of regeneration of cortisol by 11βHSD1. Tracer dilution [33] has been used commonly to assess rates of fuel production and utilisation e.g. glucose and amino acids/proteins. The endogenous pool of bioanalyte, the "tracee", is enriched to a small degree with stable-isotope labelled tracer administered, usually as a fixed rate infusion. At steady state, concentrations of compounds in the blood are proportional to their rates of production, assuming equivalent clearance. Endogenous tracee production rates may be calculated from the relative abundance of the tracee and tracer measured by their distinct masses by MS in blood or other biomatrices.

Early developments in the field of tracer kinetics relied on radioactively labelled tracers [34] and revealed that important information could be gleaned by careful consideration of the site of the radiolabel within the molecule. Tritiated tracers labelled at the 11α position were converted to unlabelled cortisone upon exposure to 11βHSD2 and recycled by addition of a proton to unlabelled cortisol. In contrast, cortisol tracers labelled elsewhere form labelled cortisone, which is recycled back to the original labelled cortisol, under-estimating clearance [34]. Measuring differential clearance between these types of tracer gave the first insight into potential approaches to achieve a specific measure of 11βHSD1 activity. Aside from safety reasons, radiotracers are not ideal because quantitation achieved by the abundance of radionuclide, may be confounded by labelled metabolites.

Safer stable-isotope labelled tracers have now been developed to quantify cortisol kinetics [29;35]. In particular "D4-cortisol" or 9,11α,12,12 [$^{2}H_{4}$]-cortisol has allowed investigation of the contribution of 11βHSD1 to cortisol production [29]. When administered *in vivo* D4-cortisol loses its 11α-deuterium through metabolism by 11βHSD2 generating 9,12,12 [$^{2}H_{3}$]-cortisone, a substrate for 11βHSD1 (Figure 2) [35]. Reduction incorporating a proton generates 9,12,12 [$^{2}H_{3}$]-cortisol (D3-cortisol), which can be distinguished by mass from cortisol and the original tracer. Similar to 11α-tritiated cortisol, the rate of clearance of D4-cortisol is more rapid than that of cortisol or other tracers [29], since D4-cortisol cannot be regenerated by 11βHSD1. The rate of generation of D3-cortisol by 11βHSD1 can be assessed by comparing its steady state levels to those of infused D4-cortisol.

This approach measures both cortisol and D3-cortisol production rates. Cortisol production rates incorporate steroid generated by adrenal synthesis and by 11βHSD1. Indeed 11βHSD1

makes a considerable contribution to the overall total [36]. D3-Cortisol generation reflects 11βHSD1 alone and the rates may be adjusted for D3-cortisone substrate concentrations, commonly below the Km of the enzyme. In typical experimental protocols the endogenous pool of cortisol is enriched to ~10-20% with D4-cortisol to allow easy analytical detection. Lower enrichments would be desirable to limit suppression of the HPA axis and disruption of the endogenous pool [33] and this may become possible as new instrumentation with improved sensitivity becomes available.

Using the D4-cortisol tracer has opened doors to measures of whole body generation of cortisol and D3-cortisol first and foremost in metabolic disease. Whole-body generation of cortisol by 11βHSD1 does not appear increased in obesity, at least until it is more severe [37-39]. Given previous interest in tissue-specific dysregulation of the enzyme, the tracer approach was soon applied in combination with arterio-venous (AV) sampling to assess the contribution of 11βHSD1 in specific tissues; the splanchnic bed [40;41], liver [40], sub-cutaneous and visceral adipose [36;39;40;42;43], heart [44], muscle [43] and brain [45]. The vast majority of whole body cortisol generation may be accounted for by the splanchnic bed [36;39;40;42], but there is discernable production across the sub-cutaneous adipose beds and muscle [36;43]. Measurement across the splanchnic bed reflects not only the liver but also visceral adipose tissue. Portal sampling, applied in conjunction with tracers, in humans [36] and dogs [42] suggests that most if not all of the splanchnic production is by liver. A less invasive approach to discriminate hepatic metabolism has been to combine AV tracers with the oral cortisone "First pass metabolism" test [46]. By assessing rate of cortisol production from cortisone by tracer dilution [36;39], it is possible to overcome a previous critique that the test is influenced by differential clearance [39]. These techniques have been applied to the study of nutrition and weight loss [47-49] and insulin sensitising drugs [50] on cortisol production by 11βHSD1 and helped to quantify the enzyme target for pharmacotherapy.

Finally, combining D4-cortisol infusions with a further D2-cortisone tracer allows concomitant quantitation of dehydrogenation of cortisol [43]. This is largely believed to be through 11βHSD2, however debate exists over whether 11βHSD1 operates in a reversible capacity *in vivo* [43;51] and whether this might be manipulated by altering co-factor availability. An example of use of the combined tracer approach was in the demonstration of impaired clearance of cortisol in subjects with critical illness [52]. However the enzyme involved in dehydrogenation could not be confirmed by these types of tracer dilution studies alone.

C: What MS holds for the future

The techniques described have greatly advanced our understanding of 11βHSD1 biology but questions still remain about how the enzyme influences receptors within sub-regions of complex tissues; functional regions of the brain and kidney or within cell sub-populations within tissues, e.g. inflammatory cells within adipose. Within the placenta, discrimination of steroid metabolism in the foetal and maternal zones is necessary. 11βHSD1 inhibitors are being developed to treat Alzheimer's disease [53], and investigators need to understand whether drugs reach the target brain region (e.g. hippocampus) and reduce local active glucocorticoid levels in these specific sites. AV sampling across the tissue will not reveal the hormonal milieu in sub regions and analysis of tissue homogenates loses regional information [54], although dissection of larger sub-regions within tissues is possible as achieved with human placenta [55;56] in combination with LC-MS/MS. MS imaging (MSI) [57] has recently joined the analytical armamentarium to address these complex questions. Here

steroids are measured in intact tissue sections, rather than fluids or tissue homogenates. Laser beams are rastered across a tissue surface collecting ionised species from distinct subzones. Collation of the species detected allows tissue maps of analytes to be reconstructed.

Cobice et al [58] recently applied this approach to visualise glucocorticoids in brain and address whether an 11βHSD1 inhibitor could suppress the active steroid levels within the hippocampus (Figure 3). Measuring steroids in sections in their native state was not possible due to poor ionisation and extensive ion suppression by the matrix, but the steroid signal could be distinguished from background following derivatisation with Girard T reagent. This technique is exciting, although not yet mainstream, as it requires highly specialised accurate mass MS instruments. Great care must be taken to prepare and store the tissue at low temperatures to prevent tissue degradation (compounding ion suppression problems) or analyte diffusion, delocalising the signal. Consideration must be taken to align the size of the region required to provide histologically meaningful data. Laser size is limited to ~50μm; indeed sampling smaller areas may generate insufficient ions for robust detection. For a thorough review of the technical challenges see [57]. MSI provides relative as opposed to absolute quantitation, but an internal standard can be applied to the tissue surface to normalise the signal. Imaging could be combined with tracer infusions to allow comparison of rates of regional regeneration of glucocorticoids.

One caveat of MSI is that in the absence of chromatography, the data generated by MSI cannot distinguish isomeric or isobaric species. Although this has not restricted the use of MSI as yet in studying glucocorticoid biology, extrapolation to other steroid or sterol classes presents greater challenges. Girard T derivatives may be useful in the study of testosterone in tissues but a natural isomer DHEA forms an isobaric derivative; DHEA is of greater abundance in humans but believed to be biologically inert. The future may lie in ion mobility as an alternative separation approach. Some success has been reported for the separation of estrogen derivatives using ion mobility, but not as yet in conjunction with imaging.[59].

Conclusion

MS has been pivotal in understanding the complex biochemical pathways relating to steroid hormone biology and its application in conjunction with *in vivo* physiology techniques has supported insightful studies of dynamic glucocorticoid activation and inactivation within tissues in health and disease. With continued research and development of MS instrumentation, the endocrine field will continue to benefit from the advancing technology and in the next decade we expect more specific analysis with greater application of accurate mass resolution. Instruments that permit introduction of biological samples in their intact state as opposed to extracts (e.g. liquid extraction surface analysis, DESI [57;58]) are becoming commercially available. However it is vital that new technologies retain the specific isomeric resolution afforded by GC and LC–MS (MS) to be aligned to the challenges of understanding vital contributions of individual steroids in endocrine research.

Figure 1
Glucocorticoids (cortisol in humans) are synthesised by the adrenal glands and released into the circulation under the stimulus of the hpothalamic-pituitary-adrenal axis. Within tissues glucocorticoids are activated and inactivated enzymatically. CRH = corticotrophin releasing hormone, ACTH = adrneocorticopic hormone; HSD = hydroxysteroid dehydrogenase; CYP = cyotchrome P450.

Figure 2
D4-cortisol is metabolised by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), losing 11α-deuterium and forming D3-cortisone. D3-cortisone is reactivated to D3-cortisol by reduction by 11βHSD1 and NADPH as a co-factor. All steroids have distinct mass transitions when assessed by liquid chromatography tandem mass spectrometry.

Figure 3
Mass spectrometry imaging of Girard T derivatives of active (corticosterone) and inactive (11-dehydrocorticosterone) glucocorticoids in rodent brain. Heat map showing intensity of signal collected by matrix assisted laser desorption ionisation with a spatial resolution of 150μm. Data were collected using a Fourier Transform Ion Cyclotron Resonance Mass Spectrometer. Adapted from Cobice et al [58].

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