Urinary steroid profiling: an example of the fundamental importance of MS in the clinical biochemistry laboratory

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Clinical biochemists are hospital based scientists who provide an interpretative advice service to doctors, nurses and other healthcare workers regarding the results of biochemistry tests performed in the laboratory. Clinical biochemistry is a sub-specialism of blood sciences and involves the interpretation of biochemistry results for bodily fluids, most often blood samples, but also urine, cerebrospinal fluid (CSF), vitreous fluid (VF), saliva, and other various fluid samples on occasion. The biochemical analyses performed on these samples will depend on the clinical situation and may include analysis of electrolytes, hormones, drugs, trace elements and specific proteins.

Common analytical techniques for biochemical analysis include immunoassay, spectrophotometry, chemiluminescent assays and chromatographic techniques such as HPLC. There is a growing trend for assays to move to MS platforms such as LC-MS/MS, GC-MS/MS, ICP-MS or standalone MS analysis due to the advantages conferred by these methods such as the increased sensitivity and specificity of assays. This increase in analytical sensitivity is key to the detection and quantification of analytes that are present in the body only in minute quantities such as many of the trace metals, vitamins, or the metabolic intermediates used to diagnose a variety of inherited metabolic diseases. Increased analytical specificity is desirable in the assays for which the analytes exhibit high levels of structural homogeneity with other endogenous or exogenous compounds, e.g. the steroid hormones and the opioid drugs. There are of course barriers to the use of MS in the clinical laboratory and MS based methods will not be appropriate for all analyses. The choice of method for a clinical assay must consider available expertise, analysis time, likely batch sizes, and cost per test, weighing up the clinical need for sensitivity and specificity versus the speed and cost of analysis. Assays which require a rapid turnaround time at a low cost are the least likely to make use of MS methodologies.

One clinical example which illustrates the importance of assay specificity is in the investigation of causes of congenital adrenal hyperplasia (CAH), a rare condition which may occur secondary to the failure of the adrenal glands to produce adequate amounts of cortisol. Cortisol is a steroid hormone that acts as a potent glucocorticoid, regulating blood glucose levels via a variety of physiological mechanisms. There are a number of enzymes in the cortisol production pathway, the deficiency of any of which will hinder cortisol production. Depending on the nature of the adrenal enzyme deficiency present there may also be a decrease in production of aldosterone; a mineralocorticoid which is essential for sodium retention and subsequently blood pressure and volume. Deficiency of aldosterone can therefore lead to life threatening salt wasting crises in early infancy as excessive amounts of sodium are lost into the urine. As cortisol and potentially aldosterone production are blocked in CAH, the pathway intermediates usually end up as androgens instead; thus many forms of CAH present with ambiguous genitalia at birth, and other types of CAH present with virilisation and infertility later in life due to this excess production of androgens. These can obviously be distressing conditions for sufferers and for the parents of sexually ambiguous infants and so an accurate diagnosis of the specific cause of the condition is important.

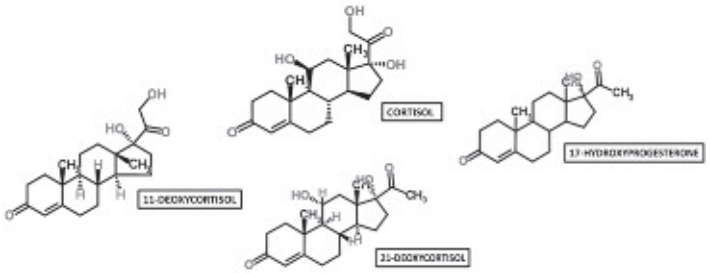

Figure 1: Cortisol and some of the structurally similar precursor compounds which cross-react in the cortisol immunoassay. Specificity of epitope recognition in immunoassay for individual steroid hormones is limited due to the conserved cyclopentanoperhydrophenanthrene ring system.

Traditional immunoassays do not have the ability to distinguish the cortisol in a sample from many of the highly structurally similar cortisol precursors and metabolites that will also be present in the blood or urine. In health this is not of great significance as the levels of these precursors and metabolites will be proportional to cortisol; however, when there is a block in the cortisol production pathway this lack of specificity is problematic for diagnosis. Cortisol concentrations measured by immunoassay may deceptively appear to be normal or even slightly high in cases of CAH as the precursors to the enzyme block cause positive interference in the assay. This may lead to missed diagnosis of CAH, delayed treatment and potentially serious clinical consequences for the patient. GC-MS and GC-MS/MS analysis of urinary steroids can overcome this specificity issue by producing a detailed profile of all of the different species present in the sample; a 'urinary steroid profile' (USP). USPs provide qualitative and semi-quantitative data on the excretion of steroid hormones, their precursors and metabolites, and by analysis of the relative concentrations of each intermediate in the pathway it is therefore possible to assess where any block may be present and to diagnose the specific enzyme deficiency accordingly. Analysis can be performed on random or 24 hour urine collections with samples prepared for analysis with extraction, hydrolysis and derivatisation steps. Extraction is performed using reversed phase solid-liquid partition chromatography, during which organic compounds including the non-polar steroids are absorbed onto the solid phase before being eluted with methanol as a purified and concentrated extract. Enzymatic hydrolysis is then performed to cleave the sulphate and glucuronic acid groups that are commonly conjugated to the steroid

species. Finally, derivatisation with trimethylsilylimidazole and methoxyamine hydrochloride to methyl oxime trimethylsilane ethers (MO-TMS) renders compounds more stable, more volatile, and less prone to ionic interactions, with MO-TMS also contributing to the ease of pattern interpretation. Addition of internal standards at specific points during these processes accounts for the recovery, success of derivatisation, and quantification of the compounds measured in the assay.

Figure 2 shows the USPs that would be expected to be seen in health, in 21-hydroxylase deficiency, and in 11β-hydroxylase deficiency. 21-hydroxylase deficiency is the most common enzyme deficiency seen in CAH, accounting for around 90-95% of cases, and other enzyme deficiencies such as 11β-hydroxylase deficiency (around 5% of CAH cases) are frequently misdiagnosed as this. The USPs for each of these enzyme deficiencies are clearly able to differentiate between the different enzyme deficiencies with the profiles showing a predominance of metabolites of the compound closest to the enzyme block.

In a similar fashion to USPs, methods for the analysis of panels of steroid hormones in blood by LC-MS/MS are also beginning to be developed in clinical laboratories. These can allow valuable insights into the function of the adrenal, with many conditions such as adrenal tumours, adrenal and gonadal hyperplasia, polycystic ovarian syndrome (PCOS), and precocious puberty and menarche all frequently demonstrating abnormalities of adrenal steroid production. Mass spectrometry is fundamental to these steroid profiling techniques, as it is fast becoming in many other aspects of the clinical biochemistry service.

Acknowledgements
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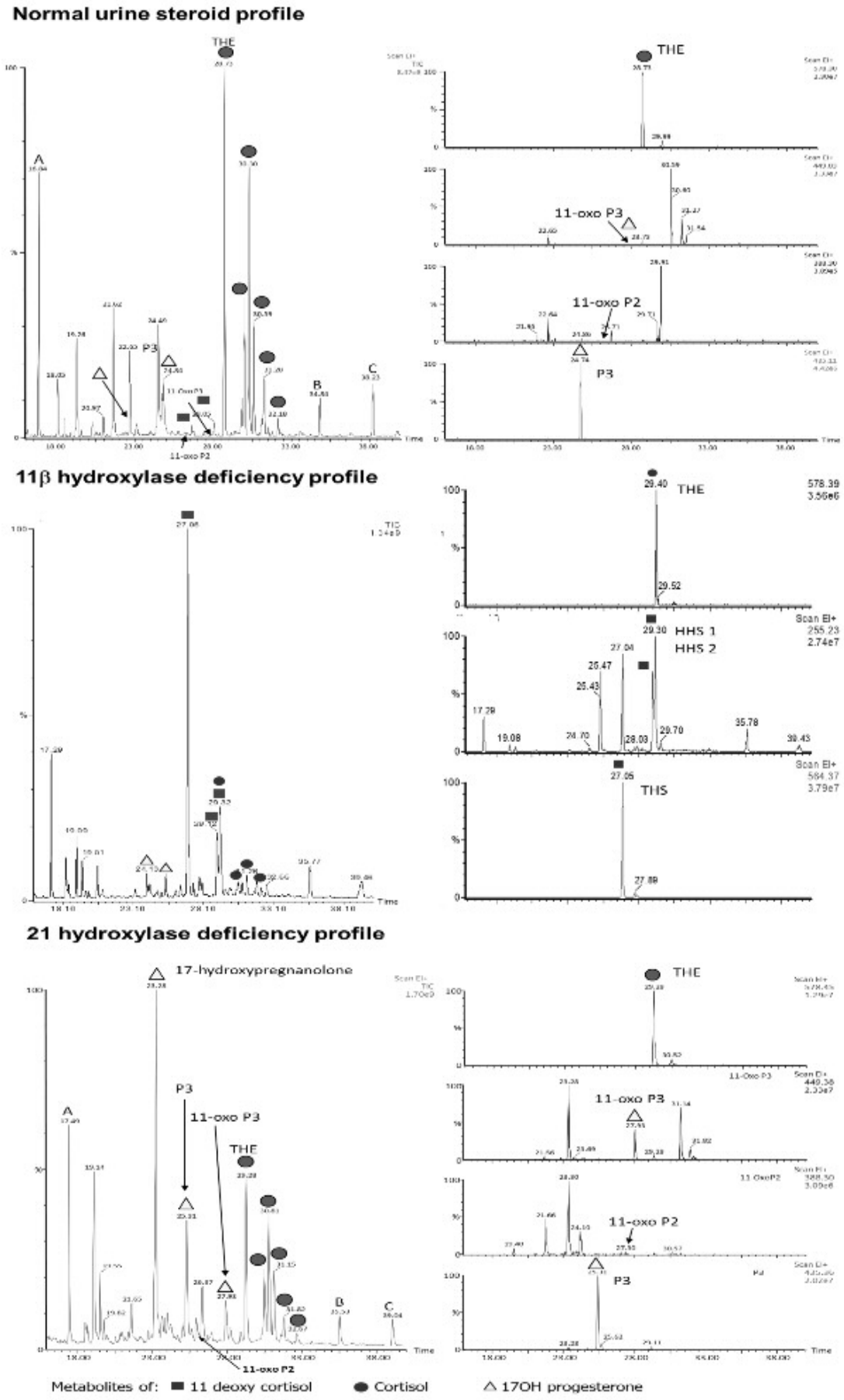

Figure 2: Example urinary steroid profiles for 'normal', 11β-hydroxylase deficiency, and 21-hydroxylase deficiency individuals.