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Review

The evolution of methods for urinary steroid metabolomics in clinical investigations particularly in childhood

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ABSTRACT

The metabolites of cortisol, and the intermediates in the pathways from cholesterol to cortisol and the adrenal sex steroids can be analysed in a single separation of steroids by gas chromatography (GC) coupled to MS to give a urinary steroid profile (USP). Steroids individually and in profile are now commonly measured in plasma by liquid chromatography (LC) coupled with MS/MS. The steroid conjugates in urine can be determined after hydrolysis and derivative formation and for the first time without hydrolysis using GC–MS, GC–MS/MS and liquid chromatography with mass spectrometry (LC–MS/MS). The evolution of the technology, practicalities and clinical applications are examined in this review. The patterns and quantities of steroids changes through childhood. Information can be obtained on production rates, from which children with steroid excess and deficiency states can be recognised when presenting with obesity, adrenarche, adrenal suppression, hypertension, adrenal tumours, intersex condition and early puberty, as examples. Genetic defects in steroid production and action can be detected by abnormalities from the GC–MS of steroids in urine. New mechanisms of steroid synthesis and metabolism have been recognised through steroid profiling. GC with tandem mass spectrometry (GC–MS/MS) has been used for the tentative identification of unknown steroids in urine from newborn infants with congenital adrenal hyperplasia. Suggestions are made as to areas for future research and for future applications of steroid profiling. As routine hospital laboratories become more familiar with the problems of chromatographic and MS analysis they can consider steroid profiling in their test repertoire although with LC–MS/MS of urinary steroids this is unlikely to become a routine test because of the availability, cost and purity of the internal standards and the complexity of data interpretation. Steroid profiling with quantitative analysis by mass spectrometry (MS) after chromatography now provides the most versatile of tests of adrenal function in childhood.

1. Introduction to urinary steroid profiling (USP)

In endocrine investigations, many steroids including gonadal and adrenocortical hormones, as well as aldosterone and its precursors need to be determined. The methods for determining several steroids in one analysis (steroid profiling) have been available for urine samples for many years. The structures of these compounds include molecules of 18–21 carbon atoms based on cyclopentanoperhydrophenanthrene nucleus, aromatic and non-aromatic rings, zero to three carbonyl groups and zero to five hydroxyl groups. The present paper arises from the experience and quantitative data from urinary steroid profiling by GC–MS performed as a clinical service at the Middlesex Hospital, London and University College London Hospitals (UCLH). In clinical practice urinary steroid profiling (USP) has beeen most useful in children because most of the steroid disorders present symptoms in childhood. The complexity of the analysis and interpretation of data has rather limited its uptake until recently [1]. In the last five years the title metabolomics has been adopted to summarise the investigation of metabolites. Such investigations complement proteomics and genomics for phenotypic and disease identities.

The metabolism of endogenous androgens, as well as exogenous androgens used in the context of doping in sport, was elegantly presented by Schanzer [2] with detail that is largely applicable to all steroids. Phase 1 metabolism is mainly through chemical reduction of the steroids. Hormonal steroids with 3-keto-4-ene structure are chemically reduced to 3α − hydroxy with 5α- and 5β-dihydro steroids hence 3α − hydroxy androstane or pregnane steroids. There are further reductions at C-20 (α and β isomers) and at C-11 so there are many more steroids in urine than the hormones in blood. Phase 2 metabolism involves conjugation of steroids to assist clearance from the body.

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Glucuronides and sulphates are the main metabolites.

The excretion rates of steroids in urine from children were first determined by paper chromatography and colorimetric visualisation of the steroids [3–6]. Fractionation was needed to separate the androgens from the corticosteroids before paper chromatography in by-gone years [4–6]. The chemical methods for steroid groups determination such as 17-oxosteroids, ketogenic steroids and 17-hydroxycorticosteroids are not now used globally because the determination of individual steroids and profiles has greater diagnostic power. Gas chromatography (GC) of steroids was pioneered by the Horning's [7] and this has been the mainstay of urinary steroid profiling (USP) until recently. The GC was coupled with mass spectrometer (MS) by Ryhage in 1964 [8] with a system (separator) to reduce carrier gas flow to the MS. The introduction of capillary columns overcame this interface and the column is linked directly into the MS. USP with GC–MS has most commonly in clinical laboratories used methyloxime-trimethysilyl ether (MO-TMS) derivatives and quantitation of the peak height or area in total ion chromatograms [9–12] or specific selected ion monitoring (SIM) in GC–MS [13,14]. The use of SIM increases the sensitivity over the scanning mode. The determination of individual free steroid concentrations in urine after HPLC separation was achieved with immunoassay [15] of fractions collected of the eluates from the HPLC. This was clearly only a research tool, too lengthy for routine hospital laboratory use. Fluorescent detection of cortisol, cortisone and tetrahydro- metabolites in biological fluids required formation of derivatives such as 9-anthroyl-nitrile before HPLC [16,17].

Profiling of steroids is possible for biological matrices other than urine. Analysis of steroid hormones from biological samples extracted into an organic solvent and separated by HPLC is possible with ultraviolet detection but limited to steroids with 3-keto-4-ene structures at concentrations above 100 nmol/L. The method was used for diagnostic work of blood samples from patients with adrenal enzyme disorders [18]. Conjugated steroids in plasma were hydrolysed with a Helix pomatia enzyme preparation and enol derivatives were prepared for GC–MS analysis [19,20]. Plasma concentrations of oestrogens and androgens are higher for age in girls than boys consistent with earlier appearance of pubic hair (adrenarche) rather than the effects of puberty. Steroid analysis by liquid chromatography (LC) before MS analysis is now the preferred method for steroid measurements in plasma because of high specificity [21–24]. Circulating concentrations of sex steroids were determined by LC–MS/MS for accuracy in prepubertal and pubertal boys and girls [21]. LC–MS/MS and GC–MS/MS are now in use for steroids from urine [25–28]. The use of high resolution MS [29] would not normally be considered in a routine hospital laboratory setting because of the high instrument costs. In a research setting committed to metabolomic research however this will become an essential resource.

The USP test is not routinely available in hospital laboratories but research in centres of excellence globally provide services with variable turnaround times. A few purely research laboratories have used steroid profiling in their projects. As with any laboratory test, reference ranges of steroid concentrations or ratios are important when interpreting results, although few laboratories provide comprehensive reference ranges for urinary steroids in children. Recruitment of normal subjects is difficult, so papers where reference ranges have been reported are often based on small numbers of subjects. The urine of newborn infants has a particularly complicated mixture of steroids derived from a fetal zone of the adrenal cortex which disappears over the first six months after birth. In the first 5 years of childhood the urinary excretion rates of the androgen metabolites are lower than the cortisol metabolites and both increase slowly with age [3–5,28,30]. At 5–9 years of age a new zona reticularis appears in the adrenal cortex, just outside the adrenal medulla. Like the fetal adrenal zone, the major steroid from the zona reticularis is dehydroepiandrosterone sulphate (DHEAS) which after metabolism is excreted as androgen metabolites such as 16-hydroxy DHEA, androsterone and aetiocholanolone. The excretion rates of those steroids increase significantly during this period of childhood called adrenarche [30,31]. In laboratories offering an interpretative steroid profiling service there has been a move to simplify the reading of the USP data by using a number of reference ranges for metabolite ratios of intermediates to cortisol metabolites that can separate the metabolome in the steroid enzyme disorders where specific ratios are high [11,13,32–34].

The monograph of Vestergaard [3] and the reviews by Shackleton are essential reading for an analyst studying USP [35,36]. The current review does not include a systematic description of the synthesis of adrenocortical and gonadal steroids through a series of hydroxylation, oxidation and reduction steps, but these are summarised in review articles [37,38]. Many developments in the methods of analysis of androgens and corticosteroids in urine have been researched and developed for the control of drugs in sport, these will not be discussed in detail except when relevant to clinical applications. The interested reader will benefit from the review by the leading scientists at the Cologne anti-doping laboratory [39].

The present review looks at the evolution of the methods for steroid profiling and includes GC–MS, GC–MS/MS and LC–MS/MS to determine USP. Each step in a USP analysis is discussed in some detail and appropriate references. In order to produce a USP by GC–MS the steroids need extraction from the urine, hydrolysis of conjugates, derivative formation, GC separation, selective MS detection and data processing. GC–MS/MS is shown to provide more structural information to enable tentative identification of unusual steroids but reference steroids are not available and difficult to synthesise hampering progress in this area. LC–MS/MS ideally obviates the need for derivative formation in keeping with clinical laboratory needs for rapid analysis. Steroid metabolites in urine, as free and intact conjugated steroids, can now also be measured by LC–MS/MS. In addition to glucuronide and sulphate conjugates double conjugates have been found for pregnanediols and pregnanetriols including n-acetylglucosamine [40]. Glutationes [41,42] and fatty acid esters are also found [43]. The significance of these conjugates has yet to be elucidated. USP will be seen to offer a range of clinical and metabolomic applications. The review offers suggestions to areas for future research, development and for further applications of steroid profiling.

2. Urine collection and preservation

Urine is a useful biological fluid because sample collection is painless. The salt content of urine is high and protein concentrations are low in contrast to plasma. The timing of a sample is important because excretion rates of steroids vary during the day and night. The highest concentrations of cortisol metabolites in urine are between 1000 and 1800 h [44] presumably because hepatic metabolising genes are then best expressed within the 24 h period. A spot urine sample after awakening has been used for many investigations [45] with recommendations to take the sample mid-stream probably to avoid particulates collected in the bladder overnight. A random urine from a newborn infant is acceptable when testing for excess metabolites in a child with steroid metabolic defect [46]. The urine of females can be contaminated with intestinal bacteria from the perineum if hygiene is poor. Degradation of steroids in urine has been documented [47]. In forensics and doping control, the samples are not subjected to preservation techniques so the impact of bacterial degradation is rarely accounted for. For children and adults the urine can be collected directly into a container but neonates pose additional challenges. In-dwelling catheters provide for reliable collection of urine, adhesive bags are available and diapers can be processed to yield representative urine samples [48].

The collection of urine for 24 h is difficult to achieve without forgetting to collect all voidings. The determination of creatinine concentration has been used to assess compliance. This is reliable between samples from an individual but very poor between individuals as