standards (IS), for example, for cortisol [31].The hydrolysis is itself a process that requires careful validation, since enzymatic hydrolysis varies in efficiency depending upon the chosen enzyme (e.g., b–d–glucuronidase), with regards to the cleavage of glucuronide and sulfate con– jugates.Urine steroid profiling by GC–MS is primarily concerned with the analysis of polar steroid metabolites and their sulfate/glucuronide conjugates, such as pregnanetriol, androsterone and tetrahydrocortisol, and their excretion patterns and ratios.The common ionization method for GC–MS, electron impact ioniza– tion, is a 'hard' technique that causes exten– sive molecular fragmentation, generating mass spectra that are 'fingerprints' providing detailed structural information.Use of instruments with high mass resolution may enhance the distinction of targeted steroids that are isobars for nonsteroidal compounds at nominal mass, but offers no distinction of steroid isobars unless unique secondary fragment ions can be identified.Concentrations of endogenous steroids rela– tive to these interfering compounds vary in dif–ferent matrices, so that matrix–specific validation is required before applying one analytical tech– nique to an alternative matrix [41].Endogenous steroids are transported around the body extensively (>95%) bound to nonspecific plasma proteins (e.g., albu– min) and to specific steroid–binding proteins 2554 Bioanalysis (2011) 3(22) future science group Challenges & benefits of endogenous steroid analysis by LC–MS/MS lSampling A number of factors must be considered prior to sampling, including age, sex, body mass, fasting/ nonfasting, pubertal stage, menstrual cycling, pregnancy and diurnal/circadian rhythm [41,48], as these will heavily influence the reference inter– vals and interpretation of any findings.When used in MS/MS mode, an ion–trap instrument enables the distinction of hundreds of newly recognized components in urine from newborns with congenital adrenal hyperplasia (CAH) [35], leading to the establishment of the best diagnostic markers [36].Analyses have been developed to measure free hormone concen– trations (typically using equilibrium dialysis [42] or ultrafiltration [43]), although the majority of analytical methods measure total serum concen– trations after disruption of any protein binding.Whilst common isotopically labeled analytes are now available at reasonable prices (e.g., in the UK, 1 mg trideuterated testoster– one costs ~UKGBP114 [~US$185 at time of press]; LGC Standards, Teddington, UK), some stable isotope–labeled IS are either very expensive (as they require custom synthesis) or, for some minor metabolites, not easily available.A reliable method for the analysis of estrogens in serum from males and postmeno– pausal females, for example, should have a LOQ of the order of 1 pg/ml (~3 pmol/l, 1 ppt).In an example from vitamin D analysis, Bunch et al. used postcolumn infu– sion of isotopically labeled analyte as a surro– gate to quantify matrix effects in the analysis of 25–hydroxyvitamin D metabolites [62].For example, cortisol [31] has been used clinically in the diagnosis and moni– toring of endogenous steroid disorders in serum [32,33] and has been used in reference centers for more than 30 years for urinary steroid profiling.There is little dif– ference when using plasma or serum for steroid analysis, although gel separation tubes may cause problems for some hydrophobic/lipophilic steroids such as progesterone.It has been used clinically in the diagnosis and monitoring of endogenous steroid disorders using serum [32,33] and has been in use in refer– ence centers for more than 30 years for urinary steroid profiling.A relatively cheap benchtop system with a single–mass fil– ter (quadrupole or ion–trap) enables the routine distinction of approximately 100 steroid compo– nents.GC–MS of urinary steroid metabolites requires extensive sample preparation (extraction

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from the matrix, conjugate hydrolysis, clean-up and two-step derivatization). Isobaric steroids are, however, difficult to distin- guish since they show similar secondary fragmen- tation, thus chromatographic resolution is essen- tial. Samples for steroid analysis are relatively stable once col- lected [41], although tubes used for collection and during extraction should be evaluated to ensure steroids do not adsorb to their surfaces [49,50]. future science group For LC-MS/MS, matrix effects (either ion suppression and/or ion enhancement) caused by coextracted interfering compounds in the MS source are well-documented [53-57], and should always be evaluated when implementing new methods [58-61]. Sample preparation for such analyses therefore requires a hydrolysis step (enzymatic or acidic) to cleave the conju- gated moieties, prior to chemical derivatization. Precision of quan- tification is also relatively poor, almost certainly related to sample instability within the GC injector, but this can be overcome if isotopically- labeled IS are included. The ions observed are influenced by eluent ?? and buffer composition [39]. Challenges

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