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Mass spectrometry techniques in the survey of steroid metabolites as potential disease biomarkers: A review

Maria João Gouveia[a,b], Paul J. Brindley[c], Lúcio Lara Santos[d], José Manuel Correia da Costa[a,b], Paula Gomes[e], Nuno Vale[e,*]

[a] Center for the Study of Animal Science, ICETA, University of Porto
[b] INSA, Rua Alexandre Herculano, 321, 4000-055 Porto, Portugal
[c] George Washington University School of Medicine & Health Sciences-Department of Microbiology, Immunology and Tropical Medicine, Ross Hall, 20037, Washington, DC, USA
[d] Experimental Therapeutics and Pathology Research Group-IPO-Porto, Portuguese Institute of Oncology Francisco Gentil, Rua Dr António Bernardino de Almeida, 4200-072 Porto, Portugal
[e] CIQUP, Chemistry and Biochemistry Department, Faculty of Sciences, University of Porto, Rua Campo Alegre, 687, 4169-007 Porto, Portugal

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ABSTRACT

Mass spectrometric approaches have been fundamental to the identification of metabolites associated with steroid hormones, yet this topic has not been reviewed in depth in recent years. To this end, and given the increasing relevance of liquid chromatography–mass spectrometry (LC–MS) studies on steroid hormones and their metabolites, the present review addresses this subject. This review provides a timely summary of the use of various mass spectrometry-based analytical techniques during the evaluation of steroidal biomarkers in a range of human disease settings. The sensitivity and specificity of these technologies are clearly providing valuable new insights into breast cancer and cardiovascular disease.
We aim to contribute to an enhanced understanding of steroid metabolism and how it can be profiled by LC–MS techniques.
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1. Mass spectrometry overview

Mass spectrometry has an important history in the identification of drug metabolites and has recently emerged as the foremost technology in endogenous metabolite research [1], given its proven success in drug metabolite analysis and pharmacokinetic studies [2–12]. In fact, the recent rise of the «metabolomics era» stems from the enhanced ability to

Abbreviations: APCI–MS, Atmospheric-pressure chemical ionization mass spectrometry; APPI–MS, Atmospheric-pressure photoionization mass spectrometry; CA, Cholic acid; CAT, 1,2-Dihydroxybenzene (benzene catechol); CAT-Q, 1,2-Dihydroxybenzene-Quinone; CID, Collision-induced dissociation; COMT, Catechol-O-methyltransferase; DCA, Deoxycholic acid; ESI–MS, Electrospray ionization; FIA, Flow injection analysis; FTMS, Fourier transform mass spectrometry; GC–MS, Gas chromatography–mass spectrometry; GP, Girard P; HPLC–EDC, High performance liquid chromatography–electro-chemical detection; LC–MS, Liquid chromatography–mass spectrometry; MALDI-TOF, Matrix-assisted laser desorption/ionization-time-of-flight; *N*-AcCys, *N*-acetylcysteine; NADA, *N*-acetyldopamine; NADA-Q, *N*-acetyldopamine-quinone; NQO-2, NRH quinone oxidoreductase 2; Resv, Resveratrol; SPE, Solid phase extration; SLOS, Smith–Lemli–Opitz syndrome; SRM, Selected reaction monitoring; TOF, Time-of-flight; UPLC–MS/MS, Ultra-performance liquid chromatography-tandem mass spectrometry.

* *Corresponding author*. CIQUP, Chemistry and Biochemistry Department, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 687, P-4169-007 Porto, Portugal. Tel.: +351 220402567; fax: + 351 220402563.
E-mail address: nuno.vale@fc.up.pt (N. Vale).

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perform faster, more accurate and comprehensive metabolite analyses, along with the need to understand intracellular biochemical events towards identification of both disease and pharmaceutical biomarkers [1].

Metabolite analyses have been typically carried out by means of liquid or gas chromatography with mass spectrometry (LC–MS or GC–MS, respectively), or inclusively high field proton nuclear magnetic resonance (NMR). The primary advantage of NMR in metabolite analysis is its ability to measure analytes in biofluids quickly and accurately, without the need of initial processing or separation [13–15]. Over recent years, improvements have included higher spectral resolution, lower instrument cost, and the addition of stop-flow chromatography on fractions of samples. Yet, the major weakness of NMR is that it has a poor dynamic range (100–1000) that results in only the major components being observed [1]. High-resolution capillary GC–MS has also been a landmark in metabolite research and disease diagnosis, as it enables identification of key small molecules, such as fatty acids, amino acids and organic acids, in biofluids, particularly in urine and blood [16–18]. This technique has been influential in providing diagnostic information for many inherited diseases, such as numerous metabolic disorders, disorders of the metabolism of amino acids [19–22], bile acids [23,24] and steroids [25–27]. Nevertheless, GC–MS techniques have limited applicability to metabolite profiling, as they usually require (i) convoluted sample preparation including metabolite extraction and subsequent derivatization to volatile adducts, (ii) long analysis times, and (iii) ideal size and type of molecules to be analyzable; in other words, non-volatile, highly polar and/or large molecules cannot be studied by GC–MS [1]. In this context, LC–MS techniques present several advantages over NMR or GC–MS techniques in metabolite profiling, namely greater sensitivity and dynamic range. Therefore, LC–MS techniques will be overviewed in greater detail.

2. Competing MS technologies

LC–MS with an electrospray ionization interface (LC–ESI–MS) has become a popular choice for metabolite analysis and studies for new biomarkers [18,28]. This technique is advantageous over GC–MS in that sample preparation and analysis are relatively simple, providing access to metabolites of higher structural diversity. ESI offers many advantages over other ionization techniques, for example, the ability to analyze low- and high-molecular weight compounds, excellent quantitative capabilities and reproducibility, high sensitivity, simple sample preparation, amenability to automation, soft ionization and absence of matrix [29]. The utility of ESI lies in its ability to generate gas-phase ions directly from the liquid phase, which establishes the technique as a convenient mass-analysis platform for both LC and direct flow injection analysis (FIA), especially when combined with tandem mass spectrometry (MS/MS) [1]. While previous LC separation of the diverse molecules present in biofluids can reduce ESI ion suppression [30–32], making LC–MS especially attractive in the initial stages of metabolite research, it also delays data acquisition and analysis. Therefore, for ESI–MS quantitation of a known biomarker, extraction combined with flow injection analysis (FIA) is the method of choice, as the extracted sample is directly injected into the mass spectrometer, without prior chromatographic separation [33]. Altogether, ESI–MS techniques result in a selective approach that allows for both qualitative and quantitative metabolite analysis, while sensitivities in the pg/mL range can be readily achieved [34]. Still, a challenge in metabolite profiling is that potential biomarkers may be present in the biofluid in even lower abundances, thus requiring especially sensitive techniques, like nano-LC–ESI–MS; this technique is performed at flow rates (~200 $nL \cdot min^{-1}$) much lower than those in standard LC–ESI–MS (~300 $\mu L \cdot min^{-1}$), which produces ions with less evaporation, thus enabling detection of highly diluted species. This improves the sensitivity and ultimately offers a greater dynamic range in metabolite discovery [30–36].

Finally, though atmospheric-pressure chemical or photo-ionization mass spectrometry (APCI–MS or APPI–MS, respectively) is not widely used in metabolite-profiling studies, these techniques have been employed in the analysis of more easily ionizable molecules, such as phospholipids, to produce molecular and fragment ions complementary to those obtained by ESI with collision-induced dissociation (CID). APCI–MS provides a dynamic range higher than ESI–MS and is considered robust, easy to operate and relatively tolerant to higher buffer concentrations. Yet, it is a mass-sensitive rather than concentration-sensitive technique, so no sensitivity gain can be reached with smaller columns or lower flow rates.

A summary of the main characteristics of the techniques outlined above is provided in Table 1.

Another critical parameter in MS-based metabolite studies is the mass analyzer, a central piece in the performance of any mass spectrometer. Among the most commonly used are the quadrupole, the quadrupole ion trap, the time-of-flight (TOF) reflectron, and the Fourier transform ion cyclotron resonance (FTMS) analyzer.

Quadrupole is presently the most common type of mass analyzers; quadrupoles tolerate relatively high pressures, have the capability of analyzing up to an m/z of 4000 and are relatively low cost instruments. Yet, a triple-quadrupole is required if tandem mass analysis is to be performed; the three quadrupoles are placed in series, and each of them has a separate function: the first (Q1) is used to scan across the full m/z range and select an ion of interest; the second (Q2), also known as the collision cell, focuses and transmits the ions while introducing a collision gas (argon or helium) into the flight path of the selected ion; the third (Q3) serves to analyze the fragment ions generated in the collision cell (Q2) [29].

Quadrupole ion trap analyzers are also useful in tandem MS analysis, as a single ion species can be isolated by ejecting all others from the trap, enabling the isolated species to be further fragmented by collisional activation (CID); a key advantage of quadrupole ion traps is that multiple CID experiments can be performed quickly without requiring multiple analyzers. Other advantages include their ability to trap and accumulate ions to provide a better signal-to-noise ratio and their mass range up to ~4000 m/z. Yet, quadrupole ion traps are unable to perform high-sensitivity triple quadrupole-type precursor-ion scanning and neutral loss scanning experiments. Also, the upper limit on the ratio between precursor m/z and the lowest trapped fragment ion is

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