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Short Communication

Microwave-assisted derivatization: Application to steroid profiling by gas chromatography/mass spectrometry

Gregori Casals [a,*], Josep Marcos [b,c], Oscar J. Pozo [b], José Alcaraz [a], María Jesús Martínez de Osaba [a], Wladimiro Jiménez [a]

[a] Biochemistry and Molecular Genetics, Hospital Clínic of Barcelona, University of Barcelona, Spain
[b] Bioanalysis Research Group, IMIM (Institut Hospital del Mar d'Investigacions Mèdiques), Barcelona, Spain
[c] Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain

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ABSTRACT

Gas chromatography–mass spectrometry (GC–MS) remains as the gold-standard technique for the study of the steroid metabolome. A main limitation is the need of performing a derivatization step since incubation with strong silylations agents for long periods of time (usually 16 h) is required for the derivatization of hindered hydroxyls present in some steroids of interest. In the present work, a rapid, simple and reproducible microwave-assisted derivatization method was developed. In the method, 36 steroids already treated with methoxyamine (2% in pyridine) were silylated with 50 μl of *N*-trimethylsilylimidazole by using microwave irradiation, and the formed methyloxime-trimethylsilyl derivatives were analyzed by GC–MS. Microwave power and derivatization time silylation conditions were optimized being the optimum conditions 600 W and 3 min respectively. In order to evaluate the usefulness of this technique, the urine steroid profiles for 20 healthy individuals were analyzed. The results of a comparison of microwave irradiation with the classical heating protocol showed similar derivatization yields, thus suggesting that microwave-assisted silylation is a valid tool for the rapid steroid metabolome study.

1. Introduction

The measurements of urinary metabolites excreted over a 24 h period provide the most comprehensive picture of the steroidal hormonal status. Thus, steroid profiling in humans is a tool of paramount importance for the establishment of the biochemical phenotype of many pathologies [1,2].

Despite all the progress in steroid biosynthesis research brought about through molecular biology techniques [3,4] and by the introduction of liquid chromatography tandem mass spectrometers (LC–MS/MS) in clinical laboratories [5–8], gas chromatography coupled to mass spectrometry (GC–MS) remains as the reference technique in clinical steroid investigations [9].

The first methods for the determination of the steroid profile by GC–MS were developed by Horning et al. back in 1969 [10]. Later on, several methodological improvements were introduced in the analytical strategy [11] which eventually led to a reference GC–MS profiling method [12] that, with slights modifications, has been employed worldwide in many investigations [13–17]. The targeted compounds are the main metabolites of selected steroid hormones and their precursors in the steroid biosynthetic pathway (Fig. 1).

Steroidal metabolites typically contain a combination of hydroxyl and ketonic groups. Ketones pose no difficulty to GC–MS analysis, but in order to improve the volatility and thermal stability of the steroids having hydroxyls in their structure, a derivatization step, typically a silylation, is also necessary.

Different silylation protocols are used depending on the set of steroids included in the panel or profile. Thus, various trimethylsilyl (TMS) reagents such as *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) or N-O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) with or without the presence of a catalyst are employed [18–20]. The use of N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) in order to form more stable tert-butyldimethylsilyl derivatives has also been reported [21].

Nevertheless, when dealing with the comprehensive clinical steroid profiling, the reference protocol is the classical

* Corresponding author at: Service of Biochemistry and Molecular Genetics, Hospital Clínic Universitari, Villarroel 170, Barcelona 08036, Spain.
Tel.: +34 93 2275400x2199; fax: +34 93 22775697.
E-mail address: casals@clinic.cat (G. Casals).

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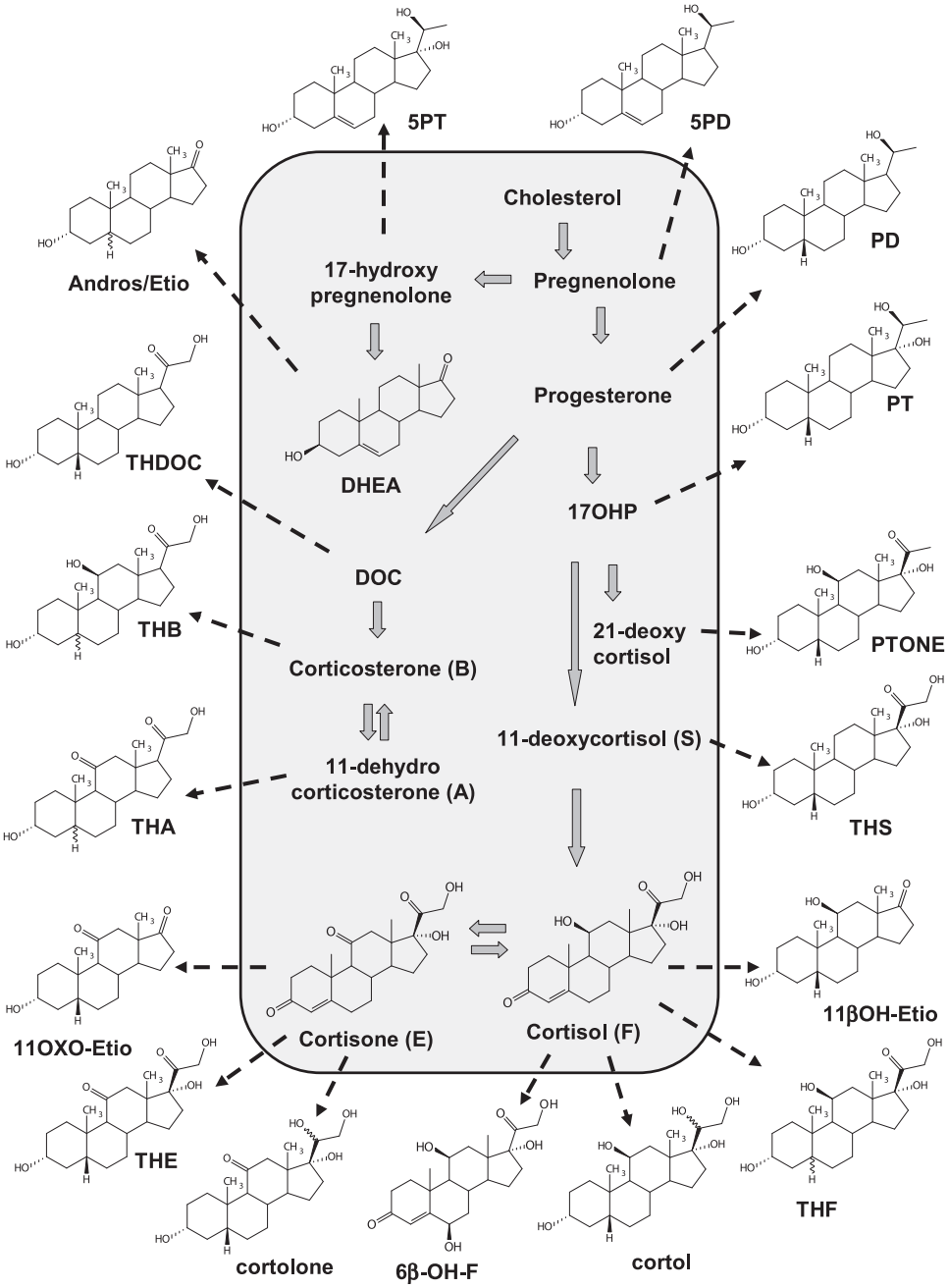

Fig. 1. Main reactions in the human steroid biosynthesis and catabolism. The central gray area represents hormones present in circulation. The final urinary metabolites are those illustrated outside that area. For abbreviations see Table 2.

two-steps procedure that leads to the formation of methyloxime-trimethylsilyl ethers (MO-TMS). In a first step, ketone groups are converted to methoximes by the addition of a methoxyamine hydrochloride (2% in pyridine) solution. This step derivatizes all ketone functions except for those in position C11. In a second step, hydroxyls are converted to TMS groups by the use of *N*-trimethylsilylimidazole (TMSIm) which is considered the strongest silylation reagent due to its ability to react with hindered hydroxyls [22].

To ensure a complete conversion of all hydroxyls, it is recommended to incubate the methoxaminated urinary extracts with TMSIm at 100 °C for 16 h or at 120 °C for 4 h [1].

In an effort to take advantage of the profiling capabilities of GC–MS for steroid analysis, the search for more efficient derivatization protocols is focused not only in obtaining better yields and stabilities, but also in the reduction of the incubation time. Among the strategies that have been employed to enhance this process, few have the potential of microwave-accelerated derivatization (MAD) [23].

In the present study, the use of MAD for the silylation with TMSIm was evaluated and compared to traditional thermal derivatization methods [1] in terms of yield, reproducibility and overall analysis time. The usefulness of this alternative approach in the determination of steroid profiles was tested by analyzing twenty human urine samples and comparing the derivatization yields of 28 endogenous metabolites and 8 internal standards with those obtained with the classical heating method.