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Systematic Study of Glucocorticosteroids O-TMS derivatives

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Introduction: Glucocorticosteroids (CT) are on the current Prohibited List of Substances for In-Competition Testing[1]. Despite of the "state of the art" of the detection of the exogenous CT being the use of LC-MS^n, endogenous CT are excreted in high amounts and most of then can be perfectly detected by GC-MS. CT such as cortisol and tetrahydrocortisol used to be monitored in steroid profile screening after formation of trimethylsilyl-derivatives (O-TMS). However, there is some controversy in the literature about the structure of these derivatives, mainly concerning the hydroxy group at position 17. Our goal was to perform a systematic derivatization study involving the synthesis of O-TMS derivatives of CT using the technique of mass spectrometry in order to allow for structure elucidation.

Experimental: Sample Preparation: The O-TMS endogenous CT were derivatised with 100 ml of MSTFA:NH_4I:2-mercaptoethanol (1000:2:3, v:w:v) within 20 min at 60 ^oC. Apparatus: A Hewlett Packard (Palo Alto, CA, USA) gas chromatograph (GC) model 6890 equipped with a 7673 HP auto sampler coupled with a quadrupole mass spectrometer (MS 5973 Network). For the CI spectra a High Resolution Mass Spectrometry AUTOSPEC Micromass - Waters (Manchester, UK) were used. Methane 3.7 was used as a ionization gas. The experiment was performed with a scan of the magnet from m/z 100 to m/z 800.

Results: The systematic study was performed comparing the behavior of the polar groups in different endogenous CT against the silylation mixture. The evaluation was performed in order of supposed derivatization difficulty from the tris-, tetrakis-, pentakis- derivatization sites compounds, up to the hindered $C_{17}\alpha$ hydroxy one. The desoxycorticosterone presents derivatizations sites in positions 3, 20 (ketone) and 21 (hydroxyl). The extracted ion chromatogram shows only one peak, related to the tris-OTMS derivative. This fact evidences the effectiveness of the silylation procedure for α, β-unsaturated in ketones in C_3, ketones in C_{20} and OH in C_{21}. This behaviour is observed in all CT without OH groups in C_{17} α.

Corticosterone shows the same structure of desoxycorticosterone with an additional OH group in C_{11} (C ring). The analysis of its mass spectrum shows the formation of a tetrakis-OTMS derivative, resulting in a molecular-ion at m/z 634. Then, besides the effectiveness of the silylation procedure in C_3- α,β-unsaturated ketones, ketones in C_{20} and OH in C_{21}, the position 11 is accessible to the silylation procedure as well. However, the majority of the CT presents an OH group in $C_{17}\alpha$. The behaviour of this group against the silylation mixture can be illustrated through the study of cortisol. The analysis of the extracted ion chromatogram of cortisol obtained through same the derivatization shows the formation of two peaks (Figure 1). The peak with low intensity presents the fragment at m/z 722 (Figure 1B). This m/z corresponds to the molecular ion of pentakis-OTMS cortisol. The main peak shows as highest mass in the spectrum at m/z 632 (Figure 1A). This can be explained through two hypotheses. The first one is based on the formation of an isomer of pentakis-OTMS cortisol, therefore, pertrimethylsilylated[2]. Through a peculiar spectrometric behavior, the molecular ion $M^{+\cdot}$ 722 Da would suffer an exothermic dissociation, in order not to allow the observation of the molecular ion. In such a case, the fragment at m/z 632 would be the result of the loss of an O-TMS group ($M^{+\cdot}$ - 90) from the molecular ion ($M^{+\cdot}$ 722). This hypothesis should be considered despite of this behavior not be, *in thesis*, expected for trimethylsilylated derivatives, characterized by a good thermal stability. The second hypothesis is based on the possibility of an incomplete derivatization reaction. So, the polar groups in C_{17} would be partially silylated during the derivatization. By this way, the fragment at m/z 632 would result from the loss of a free hydroxy group from the molecular ion at m/z 650 Da (632 + 18), not observable. A comparison with the results obtained with the experiment done with corticosterone and desoxicorticosterone allow to conclude that C_3- α,β-unsaturated ketones, ketone in C_{20} and OH in C_{11} and C_{21} suffer total silylation, since only one peak was observed in both cases. On the other hand, all CT with OH group in C_{17} α position shows the formation of two products. Facing this, and aiming the perspective of use O-TMS derivatives in order to monitor the endogenous CT in screening 4B or 4A (from the best of our knowledge, the profile of phase II metabolites of CT are still open) experiments were done with the goal to elucidate the structure of the molecular ion of the main peak. Considering the possibility of the incomplete derivatization of the C_{17} α due to steric hindrance, more drastic conditions were used, including the rising of the temperature (from 60 ^{o}C to 80 ^{o}C) and time of incubation (from 20 min to 4 hours). It would be expected a reduction of the area of the main

peak and a progressive increase of the secondary peak, referring to the pertrimethylsilylated compound. This result would confirm the second hypothesis. However, it was not observed any significant change in the ratio between these products. The possibility of the thermal degradation in the injector was discarded as well, since the product of the derivatization reaction was introduced in the GC-MS system through the cold on-column technique. If the loss of the OH group happens as a result of the chromatographic process, the molecular ion $M^{+\cdot}$ 650 would be detected. This result would confirm hypothesis 2. However, the $M^{+\cdot}$ 650 was not detected and no change between the areas of the peaks was observed. In both hypotheses, the lack of observation of the molecular ion could be associated to a process of exothermic dissociation, where this species would not be observed by mass spectrometry in EI ionization mode. Therefore, another experiment was developed using CI mode to analyze cortisol after the derivatization step. Figures 2A and 2B show the spectra of the first and second OTMS cortisol peaks, respectively. The CI spectrum shows the formation of the same cortisol pentakis-OTMS (Figure 2 B). The m/z 751 would be the $M^{+\cdot}$ 722 plus 29 Da (adduct with the ionization gas). Figure 2A shows as highest mass at m/z 661. This artifact could be explained by the formation of the m/z 632 (m/z 650 – H_2O) plus 29 Da. So, even though using a soft ionization technique the expected $M^{+\cdot}$ 650 was not found. On the other hand, this result discarded the hypothesis 1 since it is not easily justified a possible difference of stability between two pentakis-OTMS cortisol. This observation opens some new perspectives. In the first one, a new CI experiment using ammonia or isobutene should be performed. If the lack observation of the $M^{+\cdot}$ 650 persists, an alternative structure can be proposed, including a different position in the enolization ($C_{19} - C_{20}$) or an elimination of the hydroxy group in the derivatization step. Ultimate NMR and APCI experiments are being planned to allow the elucidation of the structure.

Conclusion: Despite of the detection of the exogenous glucocorticosteroids by $LC\text{-}MS^n$, the endogenous hormones can the detected in the already established steroid screening. In this way the structure of the O-TMS derivatives for the C_{17} α OH compounds need to be elucidated. MS experiments with endogenous CT shows that the OH group in C_{17} has a main role in the chromatographic and spectrometric behaviour. A CI mode mass spectrometry experiment was performed but no unequivocal elucidation was reached. New experiments including ammonia as CI gas ionisation, APCI mass spectrometry and NMR are being undertaken.

References

1. World Anti-Doping Agency. The 2005 Prohibited List. Montreal 2005.

2. Hartmann, S.; Steinhart, H. J. Chromatogr. B, 704 (1997), 105 – 117.

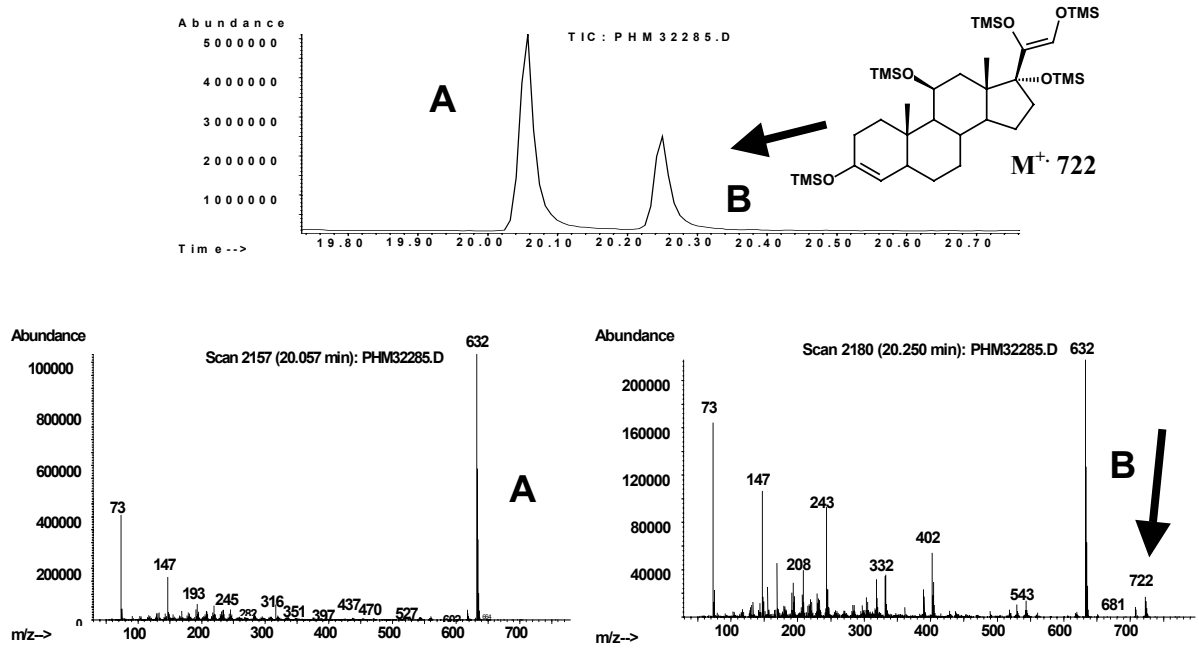

Figure 1. Mass spectra obtained from the first peak (A) and from the second peak (B) in the electron impact (70eV) mode.

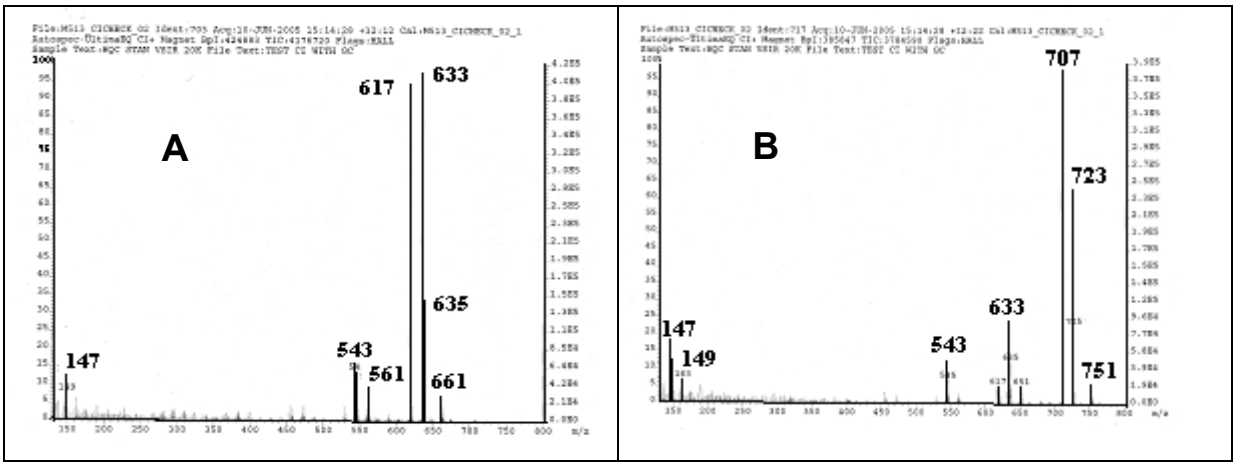

Figure 2. Spectrum of cortisol TMS-enol-TMS obtained from CI ionization mode. The main peaks of the spectra were highlighted due to the quality of the original picture.