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W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

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H. M. G. PEREIRA. M. A. S. MARQUES, J. N. CARDOSO, F. R. AQUINO NETO:
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Henrique M. G. Pereira, Marlice A. S. Marques, Jarí N. Cardoso and Francisco R. Aquino Neto

Derivatization Study on Endogenous and Synthetic Corticosteroids by Gas Chromatography Mass Spectrometry

Doping Control Laboratory, Ladetec, Rio de Janeiro, Brazil.

Introduction

Glucocorticosteroids (CT) show a powerful anti-inflammatory effect. In view of this, they are extensively used in sports medicine, but their misuse (systemic administration) can lead to severe injuries on athletes and has been restricted by the IOC[1] in recent years. For GC-MS analysis, a step of derivatization is necessary in order to convert them to a stable form preventing thermal breakdown with the loss of the dihydroxy acetone side chain. Derivatization studies for several CT were carried out to obtain methoxime–trimethylsilyl (MO–TMS) and trimethylsilyl ethers (TMS–Enol–TMS) for evaluation in screening for CT in doping control.

Experimental

Chemicals: All CT standards were purchased from steraloids (USA). N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), ammonium iodide, 2-mercaptoethanol and methoxime hydrochloride were purchased from Sigma (USA). Pyridine was purchased from Vetec (Brazil). Retention index standard solution was purchased from Sigma (USA).

GC/MS analysis: Analyses were carried out on an Agilent 6890 Series GC System equipped with a 7683 automatic injector with electronic pressure control and interfaced to an Agilent 5973 mass selective detector. MS operating temperatures were as follows: transfer line, 280°C; ion source, 230 °C; and quadrupole, 150 °C. Detection was done by selected ion monitoring (SIM) with a dwell time of 20 ms. The ionisation was done by electron impact at 70eV. GC operation conditions were as follows: injector, 280 °C; column, 180 °C (initial temperature, 0 min); followed by a gradient of 3.0 °C/min to 229 °C/min (0 min) and 40 °C/min to a final temperature of 310 °C (5.0 min); total flow, 18.4 ml/min; pressure, 16.0 psi; average linear velocity, 38 cm/s;

1 µl samples were injected in the split mode (ratio 1:10). An HP-1 fused-silica capillary column (17.0 m x 0.2 mm x 0.11 µm film thickness) was used.

Derivatization Conditions: The formation of TMS – Enol – TMS derivatives was evaluated using MSTFA – NH_4I – 2 – mercaptoethanol solution at 60 °C for 20 min[2]. The MO – TMS derivatives were formed by reaction of CT with a methoxime – pyridine solution at 60 °C for 30 minutes. Pyridine was removed under N_2 flow and silylation was carried out at 70 °C for 15 minutes[3,4].

Results and Discussion

The procedure used to obtain MO–TMS derivatives shows two disadvantages such as the formation of syn and anti isomeric forms (see figure 1) and the need for two steps[5]. Also, compounds which present C-17 hydroxy and C-16 methyl groups (e.g. dexamethasone and betamethasone) or C-17 and C-16 hydroxy (e.g. triamcinolone) in the neighbourhood of the C-20 keto function need longer reaction periods (9 hours) and more severe conditions[6] due to steric hindrance (ring D). The TMS–Enol–TMS procedure is more simple, produce just one derivative for each CT and is not as time consuming as the formation of MO – TMS. The OH group at C-17 resulting is eliminated as of H_2O (table 1 and figure 2), but the shapes of the peaks are very symmetric (figure 2) and the results very reproducible. Another possibility is the elimination of TMSOH ($M^{+\cdot}$-90) as was reported by Hartmann and Steinhart[7]. Experiments are underway to confront those possibilities.

The relative response factor (R.F.) for all compounds was evaluated both for MO–TMS and TMS–Enol–TMS derivatives at the level of 2.86 ng (n=3) injected into GC–MS. The 9α-fluoro-17α-methyl-4-androsten-3α, 6β, 11β, 17β-tetrol (Fluoxy – M_1) was used as a standard reference (table 1 and 2). The response factors of TMS–Enol–TMS of endogenous and synthetic CT were greater than the MO-TMS derivatives, except for tetrahydrocortisone and isoflupredone. The formation of anti and syn isomers reduces both sensitivity (splitting of substances in two different peaks reduces the S/N ratio) and reproducibility. The chromatographic behavior was evaluated by confrontation of Kovats indices[8] (K.I.) for MO–TMS and TMS–Enol–TMS derivatives (table 1 and 2). The K.I. resulting from MO-TMS derivatives show co-elution among exogenous and synthetic CT, like prednisolone and one isomer of cortisol, and 6β-hydroxycortisol (a metabolite of cortisol) and isoflupredone. Since the endogenous CT are excreted in higher amounts than the

synthetic ones, it will be difficult obtain an unequivocal confirmation of presence or absence of these synthetic CT. In comparison just the epimers of dexamethasone and betamethasone as the TMS–Enol–TMS derivatives show a co-elution. No co-elution was observed among TMS–Enol–TMS derivatives of synthetic and endogenous CT.

Conclusions

The formation of MO–TMS derivatives for compounds which show steric hindrance at ring D is only possible with long reaction times and severe conditions. So, their use is not suitable for screening purposes. In addition, some exogenous CT show co-elutions with endogenous ones (excreted in high amounts), making an evaluation of endogenous profiles and the diagnosis of exogenous CT abuse difficult. The derivatization procedure involving MSTFA–NH_4I–2 mercaptoethanol was efficient for all CT considered and co-elution was present only for dexamethasone and betamethasone (epimers). The obtained TMS–Enol–TMS derivatives showed better response factors and less co-elutions compared to the methoxime–trimethylsilyl counterparts. Further study in urine specimen will be done to evaluate if this procedure could be used to screen for low amounts of CT in doping control.

Reference

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Table 1. Chromatography Data and GC-MS Response Factor (RF) of TMS-Enol-TMS Derivatives.

Corticosteroids	Derivatives	Characteristic Ions m/z (rel. int. %)	R.F.	t_R (min)	K.I.
Tetrahydrocortisone	Tetra – TMS (- 18)	**634** (100);619 (41); 529 (56)	0.62	18.13	3075.73
Tetrahydrocortisol	Tetra – TMS (- 18)	**636** (100); 546 (6); 282 (5)	4.01	18.33	3126.71
Prednisone	Tetra – TMS (- 18)	**628** (100); 613 (6); 557 (2)	1.12	18.40	3152.82
11-Desoxycortisol	Tris – TMS (- 18)	**544** (100); 529 (7); 272 (4)	4.49	18.43	3161.72
Desoxycorticosterone	Tris – TMS (- 18)	**546** (100); 301(18); 230 (23)	4.18	18.48	3171.51
Cortisone	Tetra – TMS (- 18)	630 (42); **615** (100); 147 (32)	1.02	18.66	3219.83
Beclomethasone	Tetra – TMS (- 18)	**570** (100); 555 (11); 296 (50)	0.56	18.68	3233.80
Betamethasone	Tetra – TMS (- 18)	**662** (100); 456 (16); 206 (32)	1.91	18.77	3251.12
Dexamethasone	Tetra – TMS (- 18)	**662**(100); 456(18); 206(32)	2.25	18.77	3251.12
Prednisolone	Tetra – TMS (- 18)	**630** (100); 615 (16); 191 (82)	0.70	18.90	3295.25
Corticosterone	Tetra – TMS (- 18)	**634** (100); 283 (8); 230 (29)	3.55	18.95	3310.38
Cortisol	Tetra – TMS (- 18)	**632** (100); 316 (8); 193 (13)	3.42	18.96	3313.52
Isoflupredone	Tetra – TMS (- 18)	**648** (100); 442 (12); 206 (23)	0.20	18.99	3322.96
6α-methyprednisolone	Tetra – TMS (- 18)	**644** (24); 424 (8); 147 (100)	1.18	19.02	3332.39
Triamcinolone	Penta – TMS (- 18)	738 (42); **736** (100); 147 (74)	0.13	19.18	3382.70
6β-hydroxycortisol	Penta – TMS (- 18)	**720** (100); 360 (5); 317 (8)	2.97	19.38	3418.98

The ions in bold were used for calculation of Response Factors. Standard reference: Fluoxy – M_1.

Table 2. Chromatography Data and GC-MS Response Factor (RF) of MO -TMS Derivatives.

Corticosteroids	Derivatives	Characteristic Ions m/z (rel. int. %)	R.F.	t_R (min)	K.I.
Tetrahydrocortisone	MO – Tris TMS	609 (23); **578** (100); 488 (64)	1.33	17.70	2665.85
Tetrahydrocortisol	MO – Tetra TMS	**653** (100); 562 (64); 472 (31)	0.89	17.98	2724.62
Desoxycorticosterone	Bis MO - TMS	**460** (93); 429 (100); 286 (61)	0.35	18.07	2748.97
11-Desoxycortisol[1]	Bis MO – Bis TMS	548 (17); **517** (100); 427 (18)	0.48	18.24	2792.05
11-Desoxycortisol[2]	Bis MO – Bis TMS	548 16); **517** (100); 427 (22)	1.12	18.27	2800.00
Prednisone[1]	Bis MO – Bis TMS	**560** (26); 529 (37); 309 (100)	0.06	18.56	2885.93
Prednisone[2]	Bis MO – Bis TMS	**560** (20); 529 (31); 309 (100)	0.07	18.58	2893.11
Cortisone[1]	Bis MO – Bis TMS	562 (30); **531** (100); 441 (38)	0.15	18.60	2897.90
Cortisone[2]	Bis MO – Bis TMS	562 (31); **531** (100); 441 (38)	0.49	18.64	2910.03
Corticosterone[1]	Bis MO – Bis TMS	**548** (100); 517 (89); 427 (52)	0.22	18.67	2920.06
Corticosterone[2]	Bis MO – Bis TMS	**548** (100); 517 (89); 427 (49)	-*	18.70	2927.59
Prednisolone	Bis MO – Tris TMS	634 (24); **603** (100); 262 (61)	0.35	18.83	2969.91
Cortisol[1]	Bis MO – Tris TMS	636 (19); **605** (100); 515 (36)	0.48	18.83	2969.91
6α-methyprednisolone[1]	Bis MO – Tris TMS	648 (18); **617** (100); 276 (18)	0.20	18.84	2972.41
Cortisol[2]	Bis MO – Tris TMS	636 (19); **605** (100); 515 (30)	0.72	18.86	2979.94
6β-hydroxycortisol[1]	Bis MO – Tetra TMS	725 (23); **694** (100); 603 (53)	0.08	18.88	2984.95
6α-methyprednisolone[2]	Bis MO – Tri TMS	648 (13); **617** (100); 276 (91)	0.21	18.92	2997.49
6β-hydroxycortisol[2]	Bis MO – Tetra TMS	725 (28); **694** (100); 603 (71)	0.19	18.98	3000.00
Isoflupredone	Bis MO – Tris TMS	652 (4); 621 (8); **350 (100)**	0.79	19.04	3000.00

The ions in bold were used for calculation of Response Factors. 1 and 2 represent the isomeric forms, anti and syn, of the MO – TMS derivatives. *Integrated as one peak. Standard reference: Fluoxy – M_1.

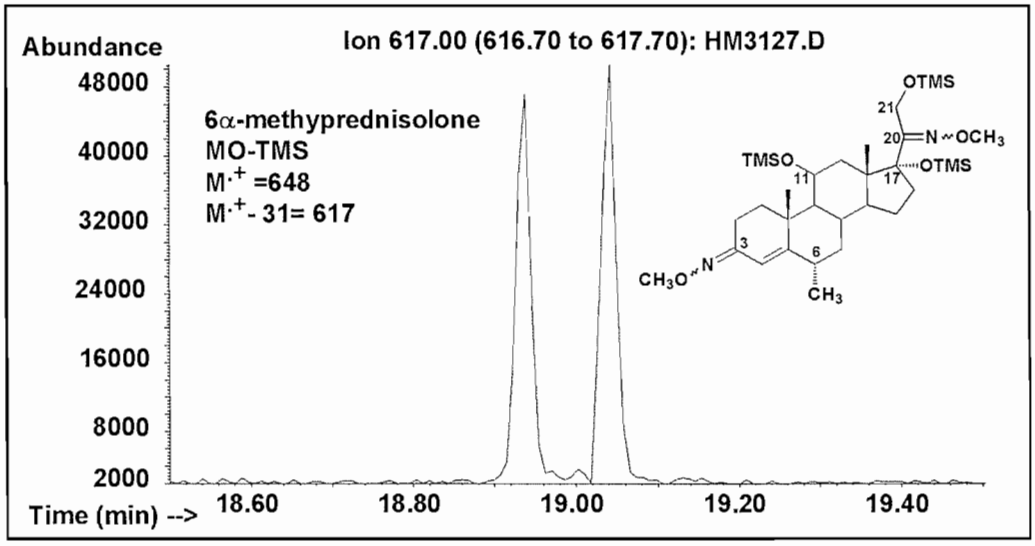

Figure 1. Anti and syn isomers of MO-TMS 6α-methylprednisolone

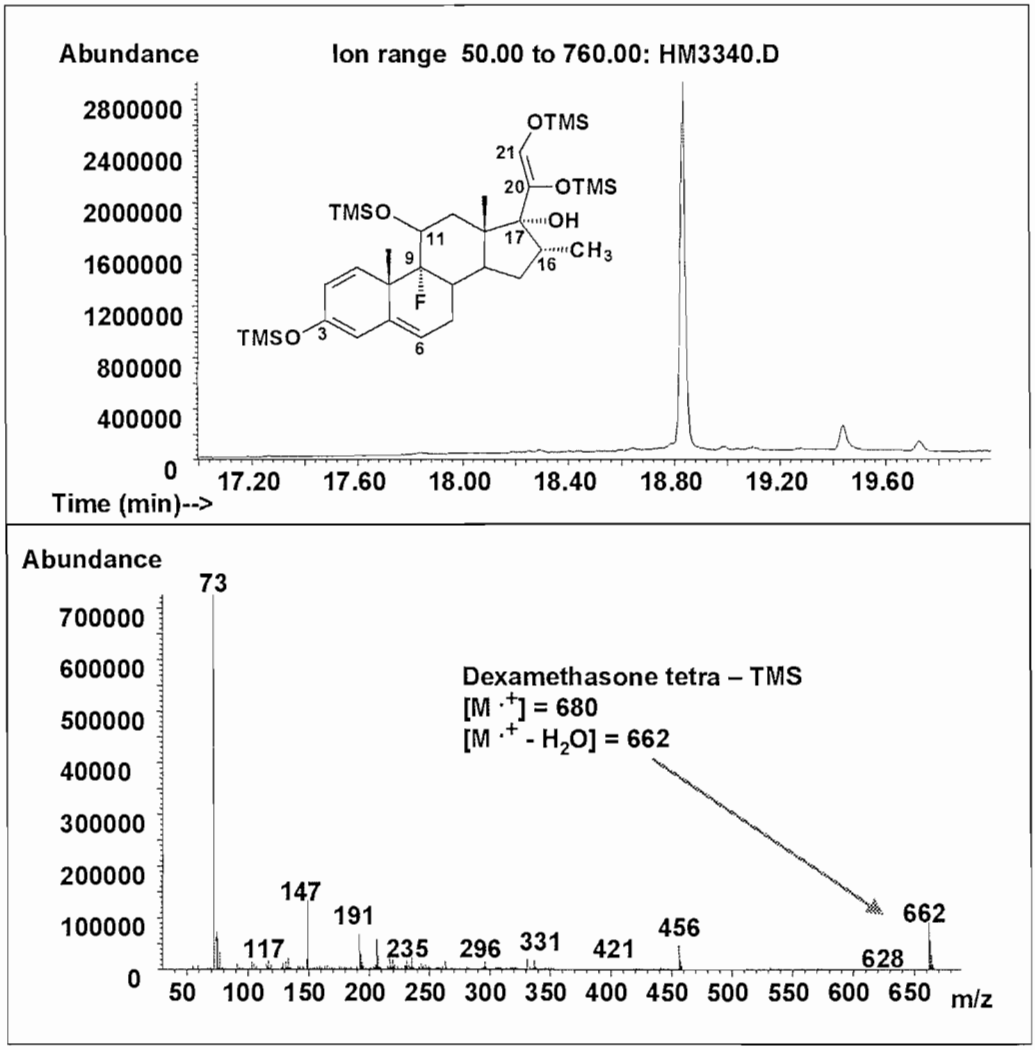

Figure 2. (a) TIC and (b) MS of Dexamethasone-TETRA-TMS.