# Reprint from

# RECENT ADVANCES IN DOPING ANALYSIS (10)

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

Sport und Buch Strauß, Köln, 2002

L. AMENDOLA, C. COLAMONICI, F. GARRIBBA, F. BOTRÈ: Microwave-assisted Derivatization: Application to the Analysis of Diuretics and Corticosteroids

In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (10). Sport und Buch Strauß, Köln, (2002) 107-116

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# Microwave-assisted derivatization: application to the analysis of diuretics and corticosteroids

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#### Introduction

This work describes the potential use of microwave (MW) assisted organic synthesis to derivatization reactions for analytical toxicology. The performance of MW assisted derivatization has been evaluated for the analysis of diuretics and glucocorticoids.

A GC-MS screening procedure for diuretics ("screening V", GC-MS-EI analysis of methyl derivatives) which requires a time consuming derivatization procedure (3 h at T=70 °C) is compared with an alternative one, where the energy transfer for the derivatization stage is ensured by microwave (MW) irradiation, thus reducing the overall derivatization time to 10 min. In addition to this, a preliminary investigation on the possibility of obtaining TMS - derivatives suitable for GC-MS analysis of endogenous and synthetic (including fluorinated) glucocorticoids was also carried out. The effects of time, power, temperature and different derivatizing reagents were evaluated. It has been demonstrated that a general screening analysis of corticosteroids is possible by GC-MS. The time required for the overall experimental procedure is comparable to that of other screening procedures commonly followed by an antidoping laboratory.

#### **EXPERIMENTAL SECTION**

# **Materials**

All reagents (analytical grade) were supplied by Carlo Erba (Milano, Italy) and by Fluka Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Indometacine (used as internal standard for the diuretics analysis) and the 18 diuretics considered in the present study were supplied by Sigma Chemical Co. Methyltestosterone (used as internal standard for the glucocorticoids

analysis) and the 23 glucocorticoids considered in the present study were supplied by Sigma Chemical Co., apart from fluocinonide and flurandrenolide that were supplied by Steraloids-Chebios Italia (Rome, Italy). Stock standard solutions were prepared dissolving the reference standard in methanol (1 mg/mL); all stock solutions were stored in the dark screwed cap vials at T=-20 °C. Working standard solutions were daily prepared, at the appropriate dilution, from the correspondent stock solution. Twice distilled/deionized water was used for the preparation of all reagents and solutions. Spiked urine samples were prepared by diluting the corresponding methanol working standard solution with blank reference urine to the final desired concentration. C18 cartridges (Sep-Pak) were supplied by Waters (Waters Spa, Milano, Italy). The microwave oven used for the MW assisted derivatization of was a Whirlpool MWO105 (Whirlpool Italia) Urine pretreatment: diuretics

The pretreatment of urine samples is identical to the one generally followed by the antidoping laboratories using GC-MS of the methyl-derivatives for the screening analysis of diuretics (reviewed in Ventura and Segura, 1996). In the reference procedure the residue is taken up in 200 µL of acetone/methyl-iodide 1/9, and 50 mg of potassium carbonate are added. The tubes are then warmed up to 70°C for 3 hours. In the alternative procedure the energy transfer was obtained introducing the tubes in a commercial microwave oven. In this case, all materials were preliminarily tested to verify their resistance to the operating conditions. The reaction tubes were stoppered by silicon septa and placed in a water bath to maintain the temperature ≤100 °C during the microwave irradiation. Maximum continuous incubation time was 10 min.

## Urine pretreatment: glucocorticoids

The pretreatment of urine samples is identical to the one generally followed by the antidoping laboratories for the screening analysis by GC-MS of the TMS-derivatives of steroids (see Buiarelli et al., 2001, for the procedure followed in our laboratory) up to derivatization stage. Derivatization yields obtained by different derivatizing reagents under thermal and/or microwave assisted energy transfer were compared. Incubation times longer than 10 min were obtained by consecutive 10-min steps, after replacing the water in the outer bath at the end of each step.

#### RESULTS AND DISCUSSION

The use of MW irradiation to support the energy transfer required for the formation of the

desired derivatives for GC-MS analysis can represent a very effective choice whenever it is necessary:

- 1. to speed up the derivatization process;
- 2. to obtain specific derivatives from poorly reactive residues.

In the present study both the possibilities were evaluated. MW assisted derivatization of diuretics showed a general improvement of the limits of detection for all the considered compounds (Table 1), reducing the time required for the derivatization step from 3 h to 10 min.

Table 1

Limit of detection (LODs) by GC-MS SIM (3 ions), following derivatization by microwave irradiation, and comparison with data obtained following derivatization with thermal incubation.

Drug	Limit of detection (µg l <sup>-1</sup> ) after MW derivatization	LOD ratio Thermal/MW
Acetazolamide	50	1.1
Althiazide	140	1.5
Bendroflumethiazide	60	3.5
Bumetanide	50	2.4
Canrenone/ Spironolactone	250	1.0
Chlorothiazide	70	3.3
Chlorthalidone	50	3.7
Clopamide	50	2.4
Diclofenamide	80	1.3
Ethacrynic Acid	20	1.1
Furosemide	40	2.6
Hydrochlorothiazide	100	2.0
Hydroflumethiazide	50	2.1
Indapamide	40	2.5
Probenecid	10	1.1
Triamterene	130	3.3
Trichlormethiazide	100	3.0

Derivatization of glucocorticoids has been possible by both the use of a specific derivatization mixture and by a combination of traditional thermal incubation and MW assisted energy transfer (Table 2). The use of the derivatizating mixture N-trimethylsilylimidazole (TMSIm):N,O-bis(trimethylsylil)acetamide (BSA):Trimethylchlorosilane (TMCS) 3:3:2, with a two-stage derivatization procedure (40° at 900 W followed by 90° at T=70 °C) allowed to obtain diagnostic MS-EI spectra for all synthetic glucocorticoids considered in this study (Table 3), with limits of detection in the range 3-25 ng/mL (Table 4).

Table 2 Relative derivatization yields (as yield/yield<sub>max</sub> x 100) in different operating conditions.

Compound		M 000 W	₩ W		1 006 MW	MW 900 W MW 900 W + heating at T	t T=70 °C	WW	WW 900 W
Compound	10 min	20 min	30 min	40 min	40 + 60 min	40 + 60 min 40 + 90 min 40	0 + 180 min	50 min	60 min
Ameinonide	10	15	30	40	70	100	90	35	30
Betamethasone	15	30	35	50	70	100	90	35	35
Cortisol	10	10	20	25	100	100	70	20	20
Cortisone	25	25	35	40	70	100	80	30	30
Desoximethasone	15	20	30	45	60	100	100	30	30
Dexamethasone	0	51	15	35	50	90	100	45	45
Fludrocortisone	10	15	30	40	60	100	80	30	35
Flumethasone	0	4	12	30	55	85	100	40	40
Flunisolide	25	25	40	40	80	100	80	30	30
Fluocinonide	25	30	35	45	70	100	85	35	33
Fluorometenolone	15	25	30	45	65	100	95	35	35
Fluocinolone Acetonide	10	15	25	40	70	100	100	30	30
Fluprednisolone	30	30	35	40	70	100	80	30	32
Flurandrenolide	25	25	35	40	90	100	80	40	40
Metilprednisolone	30	25	35	45	75	100	95	30	30
Prednisolone	25	25	30	40	70	100	80	30	30
Prednisone	40	40	50	50	80	100	85	30	25
Tetrahydrocortisolo	35	35	45	55	70	100	100	50	8 8
Tetrahydrocortisone	35	35	45	50	70	100	90	40	3 6
Tetrahydrodeoxycortico	40	40	50	50	80	100	85	40	4
Tetrahydro-S	35	35	45	50	75	100	100	45	45
Triamcinolone	5	15	20	40	50	100	95	30	ਤ ਹ
Triamcinolone Acetonide	10	15	25	40	65	100	90	30	30

Table 3 Summary of GC-MS-EI data for the most significant TMS derivative of each synthetic glucocorticoids considered in this study. Fragments used for the screening analysis in SIM are indicated in boldface.

considered in this stu	idy. Fragments used for the so	creening analysis in SIM are indicated in boldrace.
AMCINONIDE-TMS		40000 40000
Relative Rt	2.98	35000 50000- 25000-
Molecular ion (m/z)	574	20000 253 545 10000 193 277 277 353 389 574
100% ion (m/z)	473	5000 do
Diagnostic ions (m/z)	<b>574</b> , 554, 545, 389, 263	
BETAMETHASONE-4T	MS	Prioritation 347 es000 477 e0000 55000
Relative Rt	1.79	4000 4000 4000 3006
Molecular ion (m/z)	680	3000   25000   277 297
100% ion (m/z)	387	16000 10000 261 5000 305 1000 411 415 500 540 560 600 646 665 701 727 745 411 1 519 544 560 560 600 646 665 701 727 745
Diagnostic ions (m/z)	608, 477, 457, 367, 297	240 260 260 300 300 300 400 400 400 400 400 500 500 500 500 5
<b>D</b> ESOXIMETHASONE-	2TMS	Abundarea 157
Relative Rt	1.12	25000
Molecular ion (m/z)	520	15000       299   500
100% ion (m/z)	157	5000 253 325 417 520 522573 597 624 653 676 712 730 763 781
Diagnostic ions (m/z)	520, <b>500, 299,</b> 279, 193	1972-> 150 200 250 300 360 400 400 au
DEXAMETHASONE-4	TMS	ADD   ADD
Relative Rt	2.31	3000 345
Molecular ion (m/z)	680	15000
100% ion (m/z)	305	10000 5000 281 333 365-96 400 420 476 499514530 500 1000000 4917650 70-1713 742737 1000000 4917650 70-1713 742737 10000000 4917650 70-1713 742737 10000000 4917650 70-1713 742737 100000000000000000000000000000000000
Diagnostic ions (m/z	<b>680</b> , 590, <b>345</b> , 332, 305	7/c > 2/0 260 280 300 320 340 360 400 420 440 480 480 500 500 500 580 580 580 580 580 580 5

FLUDROCORTISONE-4	TMS	537 35000-
Relative Rt	1.63	3577   28000
Molecular ion (m/z)	668	15000-250
100% ion (m/z)	537	5000 257 1 361 306 40 40 40 40 40 40 40 40 40 40 40 40 40
Diagnostic ions (m/z)	<b>668</b> , 464, 447, 357, 337	
FLUMETHASONE-4TM	AS	40000 306 40000
Relative Rt	2.35	3000 257 2800
Molecular ion (m/z)	698	20000   340   15000
100% ion (m/z)	305	10000 281 22 37389 40647 48 485 500 530 560 660 67 660 777 753
Diagnostic ions (m/z)	<b>698</b> , 608, <b>345</b> , 332, 305	200 300 300 300 300 300 300 300 400 400 4
FLUNISOLIDE-2TMS		Physiciance 180000 447
Relative Rt	2.00	120000
Molecular ion (m/z)	578	ecoco
100% ion (m/z)	447	40000 250 250 250 270 271 192 24 251 361 362 369 40 677 705 740759778 271 192 141 141 141 141 141 141 141 141 141 14
Diagnostic ions (m/z)	578, 505, 447, 299, <b>279</b>	153 32 154 154 154 154 155 155 155 155 155 155
FLUOCINOLONE ACI	ETONIDE-2TMS	Section
Relative Rt	2.12	2000-
Molecular ion (m/z)	596	18000 - 207 - 10000 -
100% ion (m/z)	465	165 235 5000 75 317 349 387457 445 493 523 553 576596916/37 660 682 703 733 756 779800
Diagnostic ions (m/z	) 407, 387, 297, 235, 207	01

		Fluridance 555
FLUOCINONIDE-2TMS	5	2000 2000
Relative Rt	2.47	19000 19000 14000 12000 171 281
Molecular ion (m/z)	638	10000 8000 207 565 8000 207
100% ion (m/z)	505	2000 15 25 367 257 415 477 153 455 550 650 650 650 650 750 750 750 750 750 750 750 750 750 7
Diagnostic ions (m/z)	638, 565, <b>523</b> , 477, 281	11.7
FLUOROMETHOLONE	-3TMS	Auurdance 247 18000 - 14000 -
Relative Rt	1.46	12000 161 161 10000 387 477
Molecular ion (m/z)	592	8000 277 257 8000 347
100% ion (m/z)	207	2000 118 327 415 477 500550 542 568 600 537 660 591710 734 758778739
Diagnostic ions (m/z)	<b>477, 387</b> , 367, 297, 277	150 260 280 300 360 400 460 660 660 660 660 700 750 660
FLUPREDNISOLONE-	4TMS	Abundance 120000 463 110000
Relative Rt	1.80	80000 80000 - 70000
Molecular ion (m/z)	666	60000 353 60000 263263
100% ion (m/z)	463	30000 20000 1000 1000 1000 1000 1000 100
Diagnostic ions (m/z	489, 373, <b>353</b> , 283, 263	01 200 200 300 300 300 450 600 500 600 650 700 750 100 100 100 100 100 100 100 100 100 1
FLURANDRENOLIDE	2-2TMS	Poundence 4455 1000000- 200000-
Relative Rt	1.93	00000-   70000-   00000-
Molecular ion (m/z)	580	50000-   40000-   30000   1
100% ion (m/z)	449	20000 231 251 10000 157 183 207 253 377 371 391 429 477 507 50 500 500 755 153 100 100 100 100 100 100 100 100 100 10
Diagnostic ions (m/	z) 580, 507, <b>429</b> , 391, 301	m/z> 140 180 180 200 220 240 280 280 300 320 340 380 400 420 480 480 480 480

METHYLPREDNISOLO	NE-3TMS	(Sbundance) 219
Relative Rt	1.77	1200000- 1000000- 8000000-
Molecular ion (m/z)	590	600000 369
100% ion (m/z)	279	200000 264 303 377 351 395 410425 443 45500 52568 553 590 616531647 666 691706 730 748 778-2 240 260 260 303 305 305 305 405 405 440 440 440 500 500 500 640 500 540 640 640 640 740 720 740
Diagnostic ions (m/z)	<b>459</b> , 395, 369, 264	
PREDNISOLONE-3TM	S	Abundance 400000 245 550000
Relative Rt	1.70	300000 250000
Molecular ion (m/z)	576	140000 140000
100% ion (m/z)	265	305 5000 5
Diagnostic ions (m/z)	445, <b>355</b> , 250, 223	72 25 31 32 32 33 38 445 50 471 485 528 548 775 598 538 597 699 715 742 1742 1742 1742 1742 1742 1742 1742
PREDNISONE-2TMS		Abinderes 37; 400000 350000
Relative Rt	1.62	20000
Molecular ion (m/z)	502	1 160000
100% ion (m/z)	371	100000
Diagnostic ions (m/z	397, 295, 281, 263, 239	240 280 280 300 300 300 300 380 400 400 400 400 400 600 600 600 500 600 600 600 600 700 740 740 760
TRIAMCINOLONE-4	гмѕ	Plantitions 481 2400 22000
Relative Rt	2.03	2000 1800 1800 1800 1400
Molecular ion (m/z)	682	12000 150 10000 1517 10000 1517
100% ion (m/z)	461	200 201 332 371 441 200 591 591 200 800 800 800 800 800 714735 789 197
Diagnostic ions (m/z	551, 441, <b>371</b> , 281, 193	NX-> 150 250 250 350 350 350 350 350 350 350 350 350 3

TRIAMCINOLONE AC	ETONIDE-2TMS	750-ciance 447 1200000 1700000 11000000 1
Relative Rt	2.08	1000000   800000   800000
Molecular ion (m/z)	578	7000000   600000   5000000
100% ion (m/z)	447	200000 200000 251 279 299 327 351 359 369 410 435 475 505 521 527 558 578 564 617 635 560 678654710726741 759
Diagnostic ions (m/z)	578, 389, 369, 351, 279	htts-> 240 280 380 380 380 380 400 600 400 440 480 800 500 540 580 880 880 880 880 880 780 720 780 780

Table 4 Limits of detection (LOD) of synthetic glucocorticoids in human urine.

Compound	LOD (ng/ml)
Amcinonide	15
Betamethasone	12
Desamethasone	25
Desoximethasone	5
Fludrocortisone	5
Flumethasone	15
Flunisolide	6
Fluocinonide	15
Fluoromethenolone	6
Fluocinolone Acetonide	3
Fluprednisolone	10
Flurandrenolide	5
Methylprednisolone	3
Prednisolone	4
Prednisone	6
Triamcinolone	25
Triamcinolone Acetonide	3

Currently in progress are additional experiments to evaluate the suitability of the proposed approach for the confirmation analysis of glucocorticoids and their metabolites by GC-MS<sup>n</sup>-NCI.

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