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Microwave-assisted Derivatization: Application to the Analysis of Diuretics and Corticosteroids
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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 ($\mu g\ l^{-1}$) 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

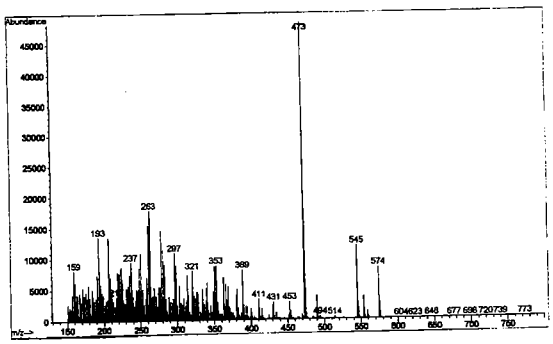
Derivatization of glucocorticoids has been possible by both the use of a specific derivatization mixture and by a combination of traditional thermal incubation and MW assisted energy transfer (Table 2). The use of the derivatizating mixture N-trimethylsilylimidazole (TMSIm):N,O-bis(trimethylsylil)acetamide (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$_{max}$ x 100) in different operating conditions.

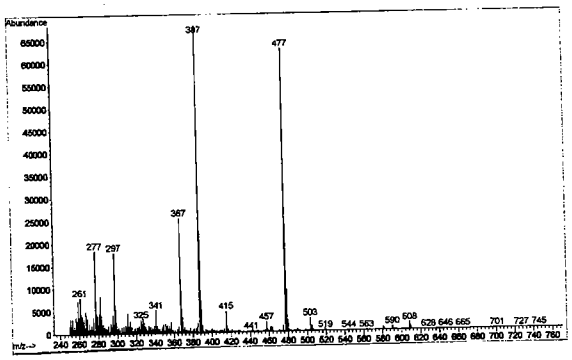
Compound	MW 900 W				MW 900 W + heating at T=70 °C			MW 900 W	
	10 min	20 min	30 min	40 min	40 + 60 min	40 + 90 min	40 + 180 min	50 min	60 min
Amcinonide	10	15	30	40	70	100	90	35	30
Betamethasone	15	30	35	50	70	100	90	35	35
Cortisol	10	10	20	25	100	100	70	20	20
Cortisone	25	25	35	40	70	100	80	30	30
Desoximethasone	15	20	30	45	60	100	100	30	30
Dexamethasone	0	5	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	30
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	30
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	50
Tetrahydrocortisone	35	35	45	50	70	100	90	40	40
Tetrahydrodeoxycortico	40	40	50	50	80	100	85	40	40
Tetrahydro-S	35	35	45	50	75	100	100	45	45
Triamcinolone	5	15	20	40	50	100	95	30	15
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.

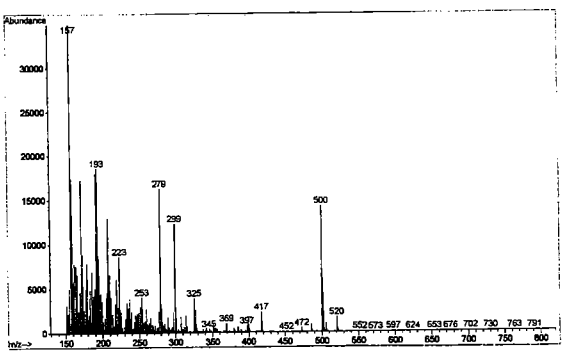
AMCINONIDE-TMS	
Relative Rt	2.98
Molecular ion (m/z)	574
100% ion (m/z)	**473**
Diagnostic ions (m/z)	**574**, 554, 545, 389, 263



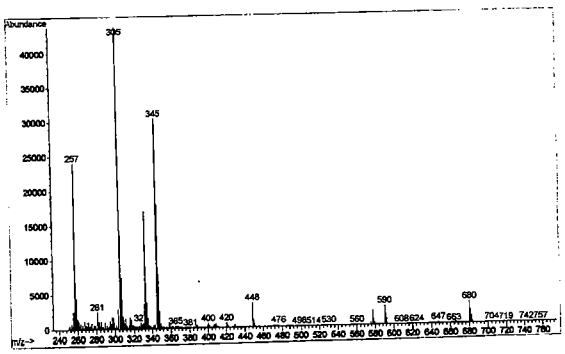
BETAMETHASONE-4TMS	
Relative Rt	1.79
Molecular ion (m/z)	680
100% ion (m/z)	**387**
Diagnostic ions (m/z)	608, **477**, 457, 367, 297



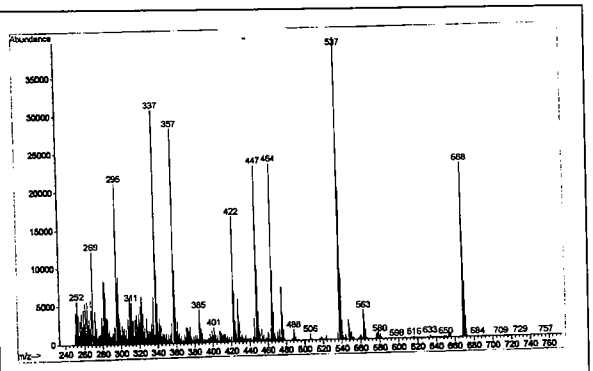
DESOXIMETHASONE-2TMS	
Relative Rt	1.12
Molecular ion (m/z)	520
100% ion (m/z)	157
Diagnostic ions (m/z)	520, **500**, **299**, 279, 193



DEXAMETHASONE-4TMS	
Relative Rt	2.31
Molecular ion (m/z)	680
100% ion (m/z)	305
Diagnostic ions (m/z)	**680**, 590, **345**, 332, 305



FLUDROCORTISONE-4TMS	
Relative Rt	1.63
Molecular ion (m/z)	668
100% ion (m/z)	**537**
Diagnostic ions (m/z)	**668**, 464, 447, 357, 337



FLUMETHASONE-4TMS	
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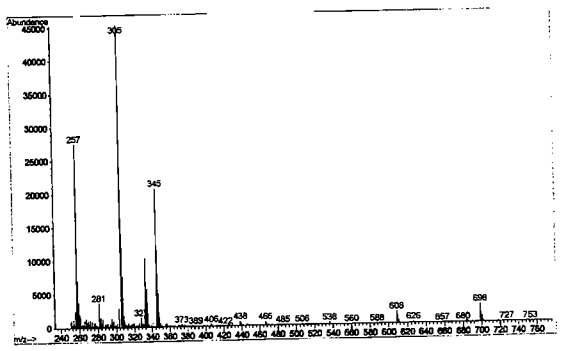
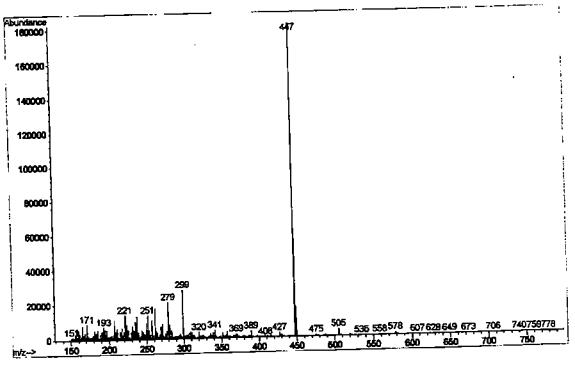
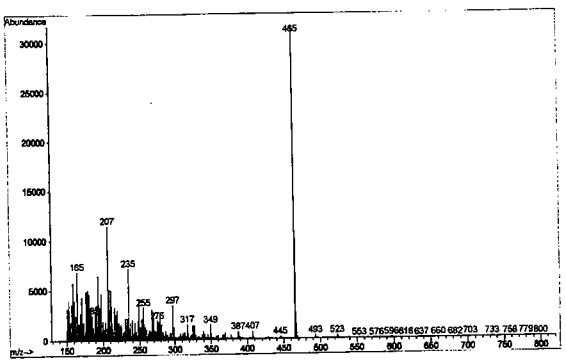
100% ion (m/z)	305
Diagnostic ions (m/z)	**698**, 608, **345**, 332, 305



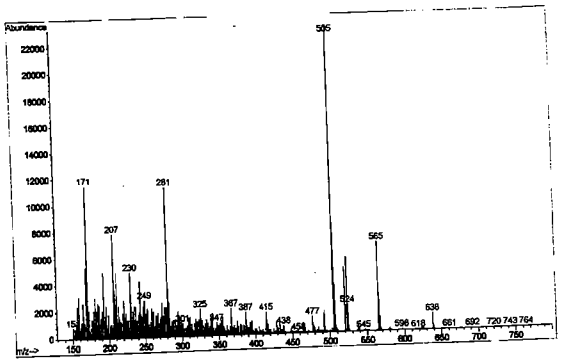
FLUNISOLIDE-2TMS	
Relative Rt	2.00
Molecular ion (m/z)	578
100% ion (m/z)	**447**
Diagnostic ions (m/z)	578, 505, 447, 299, **279**



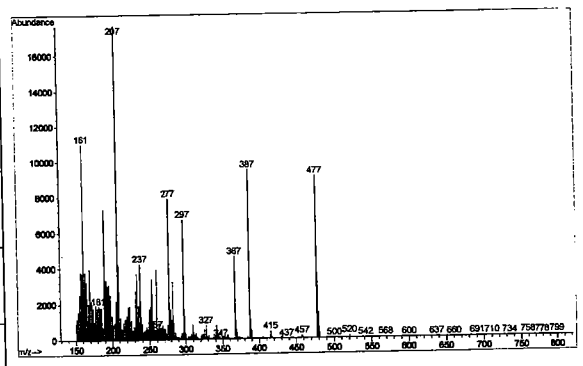
FLUOCINOLONE ACETONIDE-2TMS	
Relative Rt	2.12
Molecular ion (m/z)	596
100% ion (m/z)	**465**
Diagnostic ions (m/z)	407, 387, 297, 235, 207



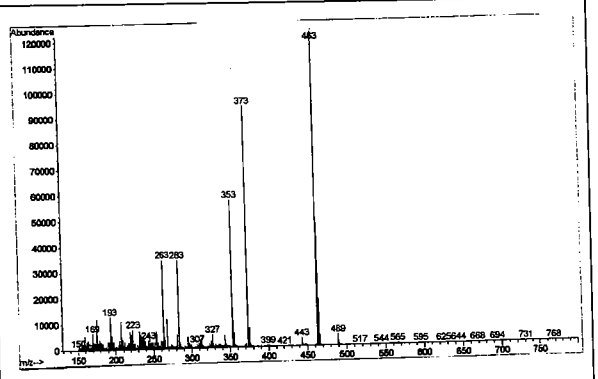
FLUOCINONIDE-2TMS	
Relative Rt	2.47
Molecular ion (m/z)	638
100% ion (m/z)	**505**
Diagnostic ions (m/z)	638, 565, **523**, 477, 281



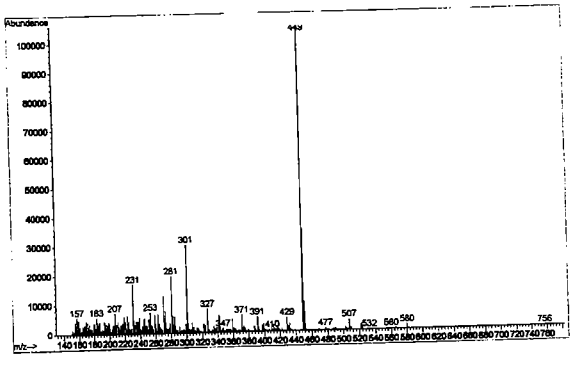
FLUOROMETHOLONE-3TMS	
Relative Rt	1.46
Molecular ion (m/z)	592
100% ion (m/z)	207
Diagnostic ions (m/z)	**477, 387**, 367, 297, 277



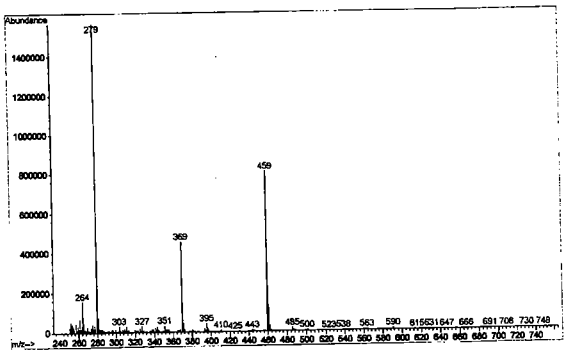
FLUPREDNISOLONE-4TMS	
Relative Rt	1.80
Molecular ion (m/z)	666
100% ion (m/z)	**463**
Diagnostic ions (m/z)	489, 373, **353**, 283, 263



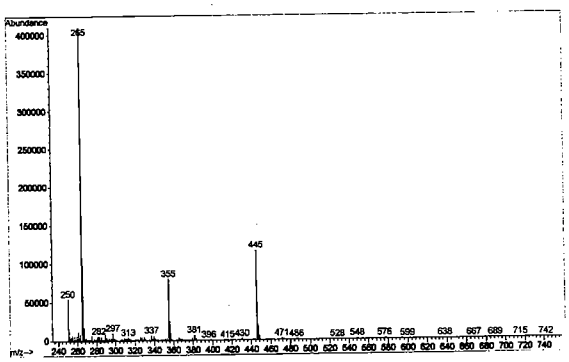
FLURANDRENOLIDE-2TMS	
Relative Rt	1.93
Molecular ion (m/z)	580
100% ion (m/z)	**449**
Diagnostic ions (m/z)	580, 507, **429**, 391, 301



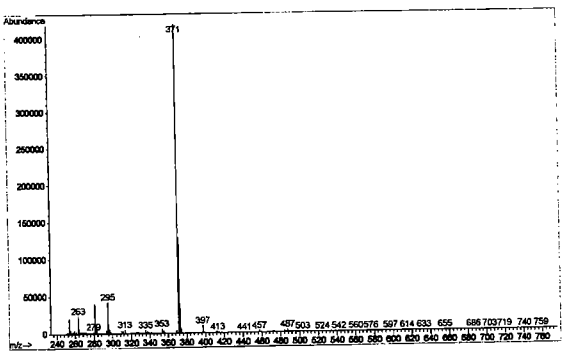
METHYLPREDNISOLONE-3TMS	
Relative Rt	1.77
Molecular ion (m/z)	590
100% ion (m/z)	**279**
Diagnostic ions (m/z)	**459**, 395, 369, 264



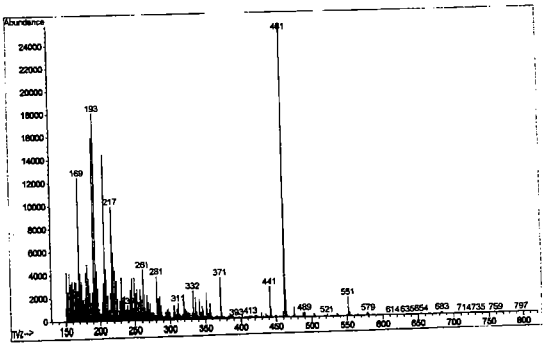
PREDNISOLONE-3TMS	
Relative Rt	1.70
Molecular ion (m/z)	576
100% ion (m/z)	**265**
Diagnostic ions (m/z)	445, **355**, 250, 223



PREDNISONE-2TMS	
Relative Rt	1.62
Molecular ion (m/z)	502
100% ion (m/z)	**371**
Diagnostic ions (m/z)	397, 295, 281, 263, 239



TRIAMCINOLONE-4TMS	
Relative Rt	2.03
Molecular ion (m/z)	682
100% ion (m/z)	**461**
Diagnostic ions (m/z)	551, 441, **371**, 281, 193



TRIAMCINOLONE ACETONIDE-2TMS	
Relative Rt	2.08
Molecular ion (m/z)	578
100% ion (m/z)	**447**
Diagnostic ions (m/z)	578, 389, 369, 351, 279

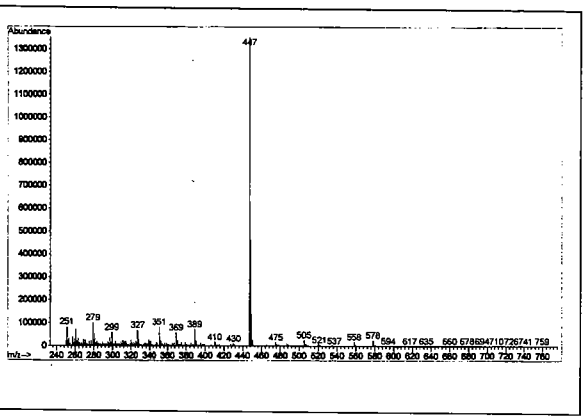


Table 4 Limits of detection (LOD) of synthetic glucocorticoids in human urine.

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^n-NCI.

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