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Determination of xenobiotic glucocorticoids in urine by gas chromatography–mass spectrometry.

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Introduction

Glucocorticoids are potent anti-inflammatory and immunosuppressive agents used to treat a variety of medical conditions^[1]. In sport, the systemic administration of these steroids is forbidden by the World Anti-doping Agency (WADA)^[2].

Detection and quantitative determination of glucocorticoids in routine analysis of biological matrices, including human urine, has been accomplished by a variety of methods, including immunological, electrochemical and chromatographic–spectrometric techniques^[3-15]. Of these methods, methyl oxime–trimethylsilyl (MO-TMS) derivatisation with gas chromatography–mass spectrometry (GC-MS) is well established and is still used in many laboratories for profiling endogenous steroids for clinical purposes^[13-16]. By contrast, there are very few papers describing the urinalysis of xenobiotic glucocorticoids as MO-TMS derivatives by GC or GC-MS; a restricted number of analytes were targeted (usually betamethasone, dexamethasone and prednisolone), with very different derivatisation and analytical conditions being used. For example, with respect to betamethasone, methoximation has been carried out with conditions varying from 10 µL of a 10 % (w/v) solution of methoxylamine hydrochloride in pyridine to 100 µL of an 8 % (w/v) solution, with reaction times and temperatures varying from 30 min at 80 °C to overnight at 57 °C. The silylating conditions used have been equally varied, with 10 µL to 50 µL of trimethylsilylimidazole (TMSIm) and reaction times and temperatures from 2 h at 80 °C to 6 h at 110 °C^[18-25]. There is consensus that glucocorticoids with a methyl substituent on the C-16 position are much harder to derivatise, i.e. dexamethasone and betamethasone (16 α and 16 β methyl

respectively) and require harsher conditions than prednisolone or 6 α -methylprednisolone. With the exception of one publication [25], details on assay sensitivity are not provided.

Recently, it has become apparent that there is a clinical need to extend the analysis to a larger number of xenobiotic glucocorticoids. Such a need is primarily to elucidate cases of patients presenting with adverse symptoms associated with glucocorticoid excess, where the pathology shows no obvious intrinsic cause but there is a history of use of non-pharmaceutically approved medications, e.g. herbal products or lotions that may be surreptitiously prepared with steroids. In addition, the development of a comprehensive GC-MS assay could serve as a useful ancillary procedure to those employing LC-MS/MS in WADA accredited laboratories.

Here we present a method that can detect xenobiotic glucocorticoids (betamethasone, budesonide, desonide, dexamethasone, fludrocortisone, flunisolide, prednisone, 6 α -methylprednisolone, prednisolone and triamcinolone) in human urine at a concentration of 30 ng/mL or better, using MO-TMS derivatisation and GC-MS detection. In the development of the assay, particular attention was focused on optimisation of derivatisation conditions and subsequent removal of the chromatographically harmful and involatile derivatisation agents prior to injection.

Experimental

All reagents were analytical grade. Methanol, glacial acetic acid, sodium acetate trihydrate, potassium carbonate, diethyl ether, anhydrous sodium sulphate and cyclohexane were supplied by Fisher (Loughborough, UK). Dodecane was supplied by Aldrich (Gillingham, UK); pyridine and methoxylamine hydrochloride were supplied by Fluka (Gillingham, UK); IST C8 solid phase extraction cartridges were supplied by Kinesis (St Neots, UK); β -glucuronidase from *Helix pomatia*, N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), 5 α -cholestane, N-trimethylsilylimidazole (TMSIm), beclomethasone, betamethasone, budesonide, dexamethasone, flumethasone, flunisolide, prednisone, 6 α -methylprednisolone, prednisolone and triamcinolone were supplied by Sigma (Poole, UK). 9, 11, 12, 12-d₄-cortisol (used as internal standard) was supplied by Cambridge Isotopes Ltd (Cambridge, MA, USA), desonide and fludrocortisone were supplied by Steraloids (Newport, RI, USA).

Stock standard solutions were prepared by dissolving the reference standard in methanol (1 mg/mL); all stock solutions were stored in screwed cap vials at -20 °C. Working

standard solutions were prepared at the appropriate dilution, from the corresponding stock solution, and stored at 4 °C. Spiked urine samples were prepared by diluting the methanolic working standard solutions with 'blank' reference urine to give the desired concentration.

Quality controls were prepared by spiking thirteen glucocorticoids into blank urine to give final concentrations of 10 and 30 ng/mL.

Methoximation Following optimisation experiments using 2 %, 4 % and 8 % methoxylamine hydrochloride (data not presented here), methoximation was carried out using 50 µL of methoxylamine hydrochloride 8 % (w/v) in pyridine and heating at 60 °C overnight. The excess pyridine was then evaporated at 60 °C under nitrogen.

Optimisation of Silylation Tubes were prepared containing 500 ng (0.1 mL of 5 µg/mL) of dexamethasone or betamethasone in methanol, the solvent was evaporated to dryness under nitrogen and methoximation was carried out as described above. The silylation step was carried out by adding 50 µL of TMSIm (100 %) or 20, 40, 60 and 80 % (v/v) TMSIm in MSTFA and the tubes were heated at 100 °C for either 1, 2, 3, 4, 5, 6, 7 or 24 h (n = 3 tubes for each compound at each time point). Excess reagent was removed following derivatisation and the samples were prepared for GC-MS analysis as described below.

Reagent Removal After cooling 1 mL of cyclohexane containing 2 % (v/v) dodecane and 100 ng/mL of 5α-cholestane (external standard) was added, the contents were vortex mixed and after adding 1 mL of water, vortex mixed again and centrifuged (1320 g for 5 min). The aqueous layer (lower) was removed with a pipette and discarded. Approximately 250 mg of anhydrous sodium sulphate was added and allowed to stand for 5 min. The organic phase was decanted to a clean tube; the sodium sulphate was washed with 1 mL of cyclohexane and decanted to the same tube as above. The solvent was evaporated at 60 °C under nitrogen for 10-12 min and the residual dodecane was transferred to an autosampler vial for GC-MS analysis.

Urinalysis The extraction method for urine samples is analogous to the one we use for the screening of endogenous and synthetic anabolic steroids analysis by GC-MS. To 2 mL of urine, 50 µL of internal standard (cortisol-d₄ 2 µg/mL in methanol) was added and the mixture was passed through a C8 solid phase extraction cartridge (200 mg) that had been previously conditioned with 3 mL of methanol and 3 mL of water. The cartridges were

washed with 3 mL of water and then the retentate was eluted with 3 mL of methanol. The eluant was evaporated to dryness at 60 °C under nitrogen, and the residue was taken up in 0.5 mL of acetate buffer (0.1 M, pH 5.2) to be incubated with 0.5 mL of β -glucuronidase from *H. pomatia* at 50 °C for 2 hours. After adjustment of pH to 9.6 with potassium carbonate the hydrolysate was extracted using 5 mL of diethyl ether. Anhydrous sodium sulphate was added to absorb the aqueous layer. The diethyl ether was decanted off to a clean tube; the sodium sulphate was washed with 2 mL of diethyl ether. This was decanted to the same tube and the combined ether fractions were evaporated to dryness at 35 °C under nitrogen. Following methoximation (as described earlier), silylation was carried out using 50 μ L of a solution of 20 % (v/v) TMSIm in MSTFA and heating at 100 °C for 6 h. The derivatised samples were then analysed by GC-MS as described below.

Instrumentation and GC-MS parameters The GC-MS system was an Agilent Technologies 6890N gas chromatograph coupled to a 5973N mass spectrometer equipped with a methylsilicone column, length 15 m, 0.2 mm internal diameter, 0.11 μ m film thickness (HP1, Agilent Technologies). The carrier gas was helium at constant pressure with an average linear velocity of 39 cm/s. Injection mode: splitless; injection volume: 1 μ L; injector temperature: 280 °C; GC temperature program: 180 °C for 1 minute, 10 °C/min to 320 °C, held for 5 minutes; transfer line: 290 °C.

Results

Heating dexamethasone with TMSIm for 6 h at 100 °C gave the di-MO, tri-TMS derivative as the main product but also significant amounts of the di-MO, di-TMS derivative (Figure 1). In addition, a pair of unidentified components was observed in the chromatogram with similar mass spectra. These components are labelled “M-88” in Figure 1. These artefacts were diminished significantly by reducing the TMSIm content of the silylating reagent, such that when a mixture containing 20 % TMSIm in MSTFA was used, negligible amounts were produced (Figures 2 and 3). Investigation of the optimisation of silylation showed that 6 h at 100 °C with 20 % TMSIm in MSTFA (following MO derivatisation of the oxo groups at C-3 and C-20) was sufficient for complete silylation of dexamethasone to form the di-MO, tri-TMS derivative (Figure 4).

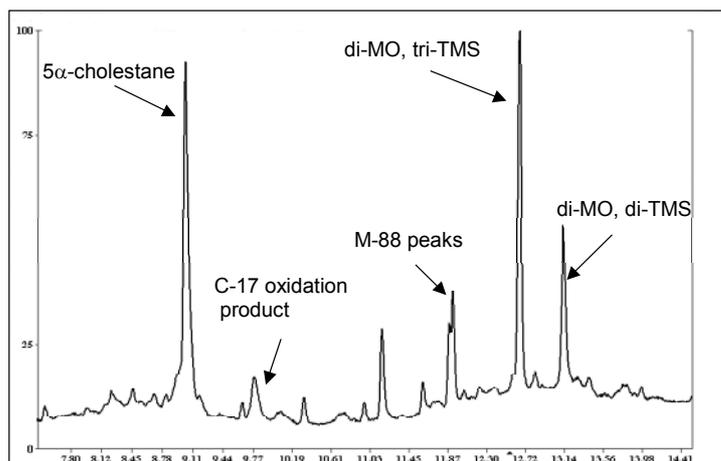


Figure 1: Total ion chromatogram of dexamethasone di-MO, silylated with TMSIm at 100 °C for 6 h. Peaks were characterised using full scan mass spectrometry.

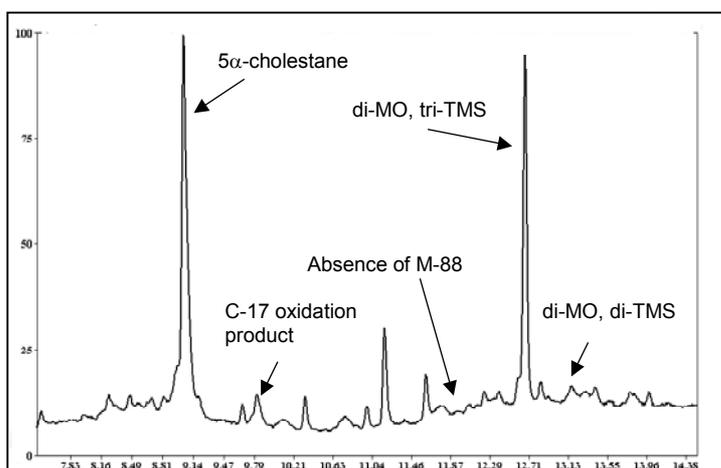


Figure 2: Total ion chromatogram of dexamethasone di-MO, silylated with 20 % TMSIm in MSTFA at 100 °C for 6 h.

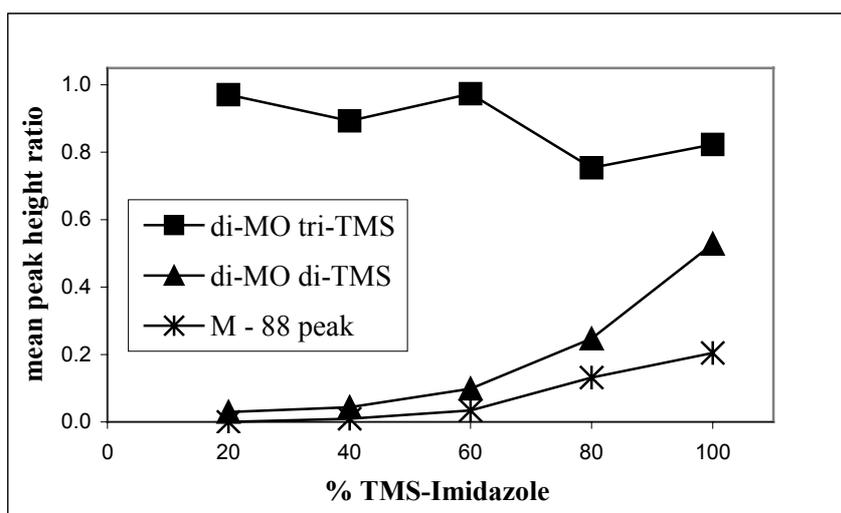


Figure 3: The effect of reducing the amount of TMSIm in a mixture with MSTFA on the formation of dexamethasone derivatives. The ratio is the peak height of the product (common base peak; m/z 364,) over that of the external standard, 5α-cholestane(m/z 372) (results expressed as the mean).

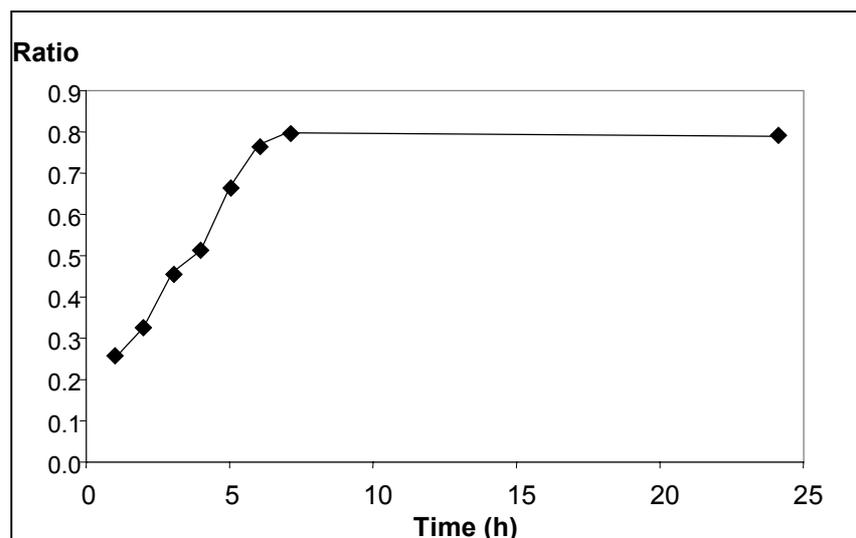


Figure 4: Rate of silylation of dexamethasone di-methyl oxime to form the tri-TMS derivatives using 20 % TMSIm: MSTFA. The ratio is the peak height of the product over that of the external standard, 5 α -cholestane (results expressed as the mean).

Table 1, on the following page, lists the main derivatised products, the m/z value, retention times, methylene units and the signal to noise ratio for glucocorticoids added to blank urine (30 ng/mL) and for a few metabolites from elimination studies.. The methylene units were calculated using a cubic spline interpolation algorithm calibrated against a series of straight chain hydrocarbons from C₂₄ to C₃₈.

Discussion

In order to optimise the formation of MO-TMS derivatives of the target analytes, we initially reviewed the literature and adopted the derivatisation approach described by Houghton *et al.* [19]. There these investigators targeted prednisolone, betamethasone and dexamethasone. Of these, we chose dexamethasone as the model analyte because it was reasoned that if complete silylation of the 17 α -hydroxyl group could be achieved, despite steric hindrance from the 16 α -methyl substituent, then other target analytes could also be satisfactorily derivatised. We found that using 8 % methoxylamine hydrochloride for 30 min at 80 °C followed by TMSIm at 80 °C for 2 h did not yield the di-MO tri-TMS derivative as the main product. Instead the di-MO, di-TMS derivative was the primary product, with the C-17 oxidation product next largest and much smaller amounts of di-MO, tri-TMS and MO, tetra-TMS derivatives (silylation of the 20-hydroxyl group, presumed to have formed by enolisation after incomplete methoximation of the 20-oxo group).

Table 1: , Retention times, methylene units (MU) and signal to noise ratio of derivatised glucocorticoids (30 ng/mL) following urinary extraction

Compound	Derivative	m/z	Retention Time (min)	Methylene Units	Signal to noise at 30 ng/mL in urine
Cholestane	None	372	9.007	2810	N/A
Prednisone	di-MO, di-TMS	309	11.560	3194	> 5
d4-Cortisol	di-MO, tri-TMS	609	12.018	3267	N/A
	di-MO, tri-TMS	609	12.059	3274	N/A
6 α -Methylprednisolone	di-MO, tri-TMS	617	12.030	3269	> 10
	di-MO, tri-TMS	617	12.184	3294	> 10
Prednisolone	di-MO, tri-TMS	603	12.054	3273	> 10
20-dihydroprednisolone ²	MO, tetra-TMS	205	12.320	3317	N/A
Fludrocortisone	di-MO, tri-TMS	379	12.459	3340	> 10
Dexamethasone	di-MO, tri-TMS	364	12.650	3371	> 10
6-Hydroxydexamethasone ²	di-MO, tetra-TMS	237	12.680	3376	N/A
Flunisolide	di-MO, di-TMS	587	12.707	3381	
Desonide	MO, di-TMS	558	12.732	3385	> 10
Betamethasone	di-MO, tri-TMS	364	12.793	3395	> 10
6-Hydroxydexamethasone ²	di-MO, tetra-TMS	237	12.810	3398	N/A
Budesonide ¹	di-MO, di-TMS	601	13.114	3450	> 10
Triamcinolone	di-MO, tetra-TMS	709	13.374	3495	> 5
Budesonide ¹	di-MO, di-TMS	601	13.738	3559	> 10

Where two peaks are tabulated together this denotes separation of syn and anti isomers. N/A = not applicable. ¹ Budesonide has R and S diastereoisomers. ² denotes metabolite data from elimination urines included here as supplementary information – the concentration of these metabolites in urine was not known.

It is difficult to give an explanation for this difference in findings but the reaction conditions for silylation are relatively mild with respect to the incubation time and temperature compared with those described by Thenot *et al.* [18], Midgley *et al.* [25] and Rodchenkov *et al.* [24]. The silylation conditions recommended by Thenot *et al.* [18] for the formation of a single MO-TMS derivative of dexamethasone were thus investigated, i.e. 50 μ L TMSIm at 100 °C for 6 h. Further, to ensure complete methoximation, rather than incubating with methoxylamine hydrochloride for 30 min at 80 °C, the reaction was

performed overnight (~16 h) at 60 °C^[22]. This indeed resulted in the di-MO, tri-TMS being the main product but the chromatogram also contained significant amounts of the di-MO, di-TMS derivative and a pair of unidentified peaks ('M - 88' in Figure 1). Full scan spectrometry of these showed an apparent molecular ion at m/z 578, 88 amu less than the molecular ion of the di-MO, tri-TMS derivative of dexamethasone, which is m/z 666. Other significant ions were at m/z 547, 527 and 364, consistent with the loss of a methoxy group (31 amu), hydrogen fluoride (20 amu) and fragmentation of the D-ring (214 amu). To the best of our knowledge, these peaks have not been reported in the literature but a subsequent personal communication with Dr Rodchenkov (WADA accredited laboratory in Moscow) revealed that he too had observed them using similar derivatising conditions. The formation of the di-MO, di-TMS and the artefacts is undesirable, and they together represent approximately 40 % of the total peak area of the common base peak of m/z 364 (areas were normalised against the cholestane external standard; m/z 372), which will have an adverse effect on the assay sensitivity for the fully derivatised target analyte.

We reasoned that the formation of these artefacts was due to the harsher conditions used by Thenot *et al.*^[18]. We investigated therefore whether diluting the TMSIm with a milder silylating reagent (MSTFA)^[26] could achieve full silylation of the MO-derivatised steroid without the formation of the M-88 artefacts, using the same temperature (100 °C). Incubation with 20 % TMSIm in MSTFA (v/v) for 6 h was sufficient for full silylation and the M-88 artefacts were greatly diminished (see Figure 2). The formation of the M-88 artefacts thus appears to be due to harsher silylating conditions causing some chemical breakdown of dexamethasone but the underlying mechanism is not obvious. By contrast, using undiluted TMSIm at a lower temperature (80 °C) and shorter incubation time (2 h), as described by Houghton *et al.*^[19] did not produce these artefacts but was not sufficient for complete derivatisation. The silylating conditions that we have developed thus appear to strike a balance between the methods described by Thenot *et al.*^[18] and Houghton *et al.*^[19]. As an adjunct, silylation of betamethasone di-MO was complete in a shorter period (3 h) under these conditions, probably because the steric hindrance of the 17 α -hydroxyl by a 16 β -methyl substituent is less than that from a 16 α . Betamethasone did not give rise to corresponding artefacts when incubated for 6 h at 100 °C (data not shown for brevity).

We standardised on a 6 h incubation time for silylation, and found the derivatisation to be effective for ten glucocorticoids (Table 1). The method developed could also detect the topical steroid flumethasone but multiple peaks were observed in the chromatogram, while

beclomethasone (unesterified) could not be detected. Further work is required to evaluate whether these two compounds do not derivatise well or whether there is another reason, such as instability of these steroids when prepared in methanolic solutions.

Thenot *et al.* showed that the presence of pyridine hydrochloride and methoxylamine hydrochloride reagents are detrimental to GC columns and promotes degradation of the analytes [17]. The GC column also can be damaged by TMSIm and because it is very involatile it is not practical to try to remove it by evaporation. Lipidex [27] or Sephadex LH-20 [19] column chromatography is often employed to remove these harmful reagents prior to injection but this approach is time consuming. A much faster and simpler approach is to perform liquid-liquid extraction using dichloromethane [28] and washing with an aqueous solution. We chose to use cyclohexane instead of dichloromethane, as the use of chlorinated solvents is discouraged for environmental reasons. Cyclohexane is very non-polar and the steroid derivatives appear to be shielded by the solvent, as there was no evidence of hydrolysed products. Dodecane (2 %) was added to the cyclohexane and this remained following the evaporation of the cyclohexane. The advantage of this approach is that the derivatised steroids are kept in solution, preventing possible loss of silyl groups, that can otherwise occur if the organic solvent is evaporated to dryness and then re-dissolving the analytes in a small amount of solvent for injection.

In conclusion, the method developed could detect the target analytes at a concentration in urine of at least 30 ng/mL. The assay provides a useful alternative to LC-MS/MS when problems are encountered with sports samples that chromatograph poorly by LC or if there is LC-MS/MS downtime in a laboratory. For clinical laboratories that mainly use GC-MS for the analysis of endogenous corticosteroids for pathological purposes, we hope this method will be useful where there is a suggestion that the patient's condition has arisen from exposure to xenobiotic glucocorticoids.

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