

## Confirmation of Triamcinolone Acetonide Use By LC-MS/MS

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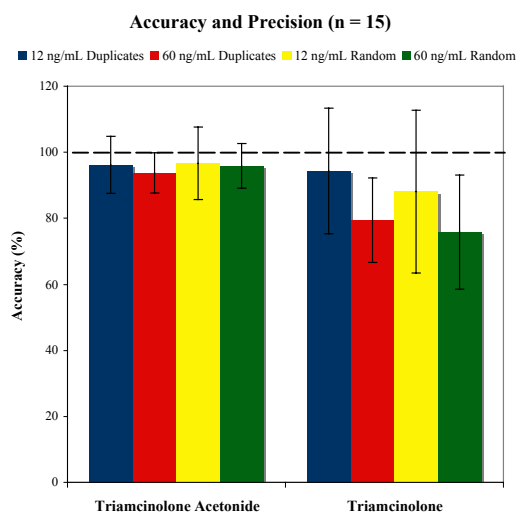
Triamcinolone acetonide (TA) is a commonly prescribed synthetic glucocorticosteroid used in the treatment of minor, acute and chronic inflammatory conditions such as eczema, sports injuries, arthritis and asthma. For doping control purposes, WADA restricts administration to dermatological preparations or with appropriate therapeutic use exemption, local forms such as inhalation and intra-articular injection. To enable the confirmation of synthetic corticosteroid use, the excretion and metabolism of TA was investigated.

### Quantification of Triamcinolone Acetonide

A quantitative ESI-LC-MS/MS assay was developed for the analysis of TA in urine utilizing a stable isotopically-labelled processed internal standard or surrogate (Figure 3). After addition of d<sub>6</sub>-TA surrogate and enzymatic hydrolysis, 2.5 mL urine aliquots were prepared by liquid-liquid extraction with ethyl acetate. Fludrocortisone was added as internal standard, and then the extract was dried and reconstituted in 200 uL of mobile phase ready for analysis. HPLC separation was performed on a Waters Alliance 2795 Separations Module, using a C18 column and a gradient elution program (2% Formic Acid/H<sub>2</sub>O:Acetonitrile). Mass spectrometric detection was performed on a Waters Micromass Quattro Micro, with electrospray ionisation.

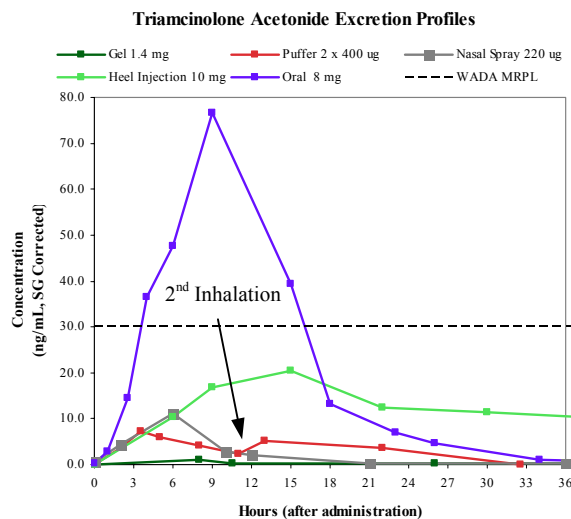
Calibration standards (0 - 100 ng/mL) were accurately prepared from stock solutions in methanol, evaporated and reconstituted in 200 uL of mobile phase for LC-MS/MS analysis. Calibration curves ( $y = ax^2 + bx + c$ ) were created from the calibration standards, by plotting the analyte/surrogate peak area ratio (y) vs. the nominal concentration of the standards (x). Method validation was undertaken and the results from accuracy and precision testing are shown below (Figure 1, accuracy and precision are represented as percentages).<sup>1</sup> A total of

60 samples were analysed, including replicate and random blank urine aliquots (SG 1.006 - 1.028) spiked with multiple analytes at two concentrations (12 and 60 ng/mL).



**Figure 1: Method Validation Results**

The related analyte triamcinolone (Figure 3) was quantified without the use of a stable isotopically-labelled surrogate (i.e. with internal standard, fludrocortisone). A marked decrease in the accuracy and precision of the quantification can be observed with comparison to triamcinolone acetonide. The use of appropriate surrogates (such as stable isotopically-labelled analogues) is extremely important in LC-MS/MS quantitation of corticosteroids. This is due to the pronounced matrix effects observed to influence absolute analyte response in biological matrices (such as human urine).<sup>2</sup>

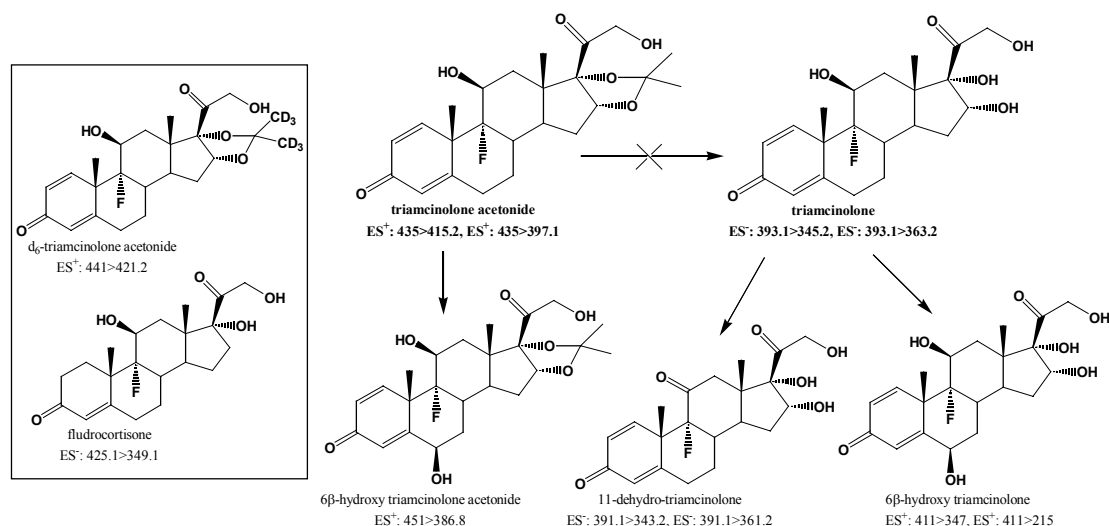


**Figure 2: TA Excretion Profiles**

The validated method was then applied to five TA excretion studies from WADA permitted and prohibited modes of administration (Figure 2).<sup>3</sup> Results from four different forms of topical/local administration showed maximum urinary concentrations below the WADA specified (laboratory) minimum required performance limit (MRPL) of 30 ng/mL. An oral administration of TA (8 mg) produced a maximum urinary concentration of 77 ng/mL. For all studies, excretion was complete in 24-36 hours, except for the heel injection for which a prolonged excretion was observed (> 3 days).

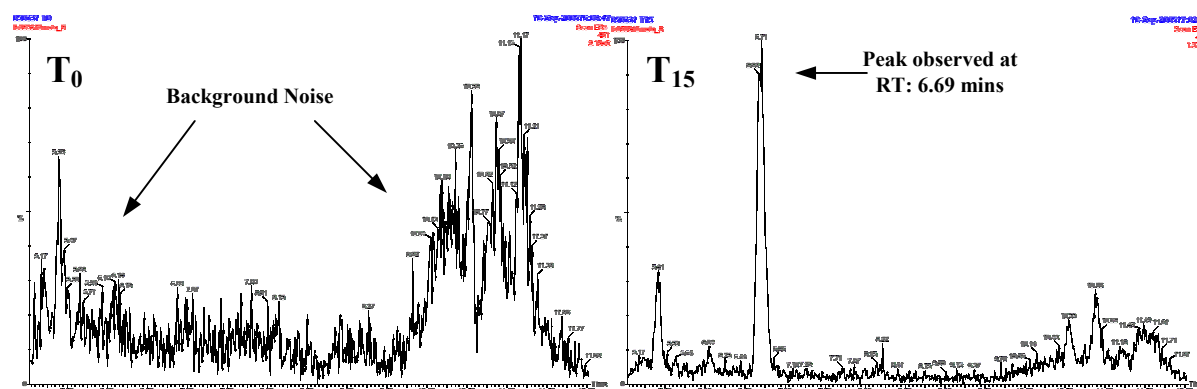
### Metabolism of Triamcinolone Acetonide

In humans, TA is predominantly metabolised to 6 $\beta$ -hydroxy TA (Figure 3).<sup>4</sup> 6 $\beta$ -hydroxylation is a common metabolic route for steroid hormones, both endogenous and synthetic.<sup>5,6</sup>



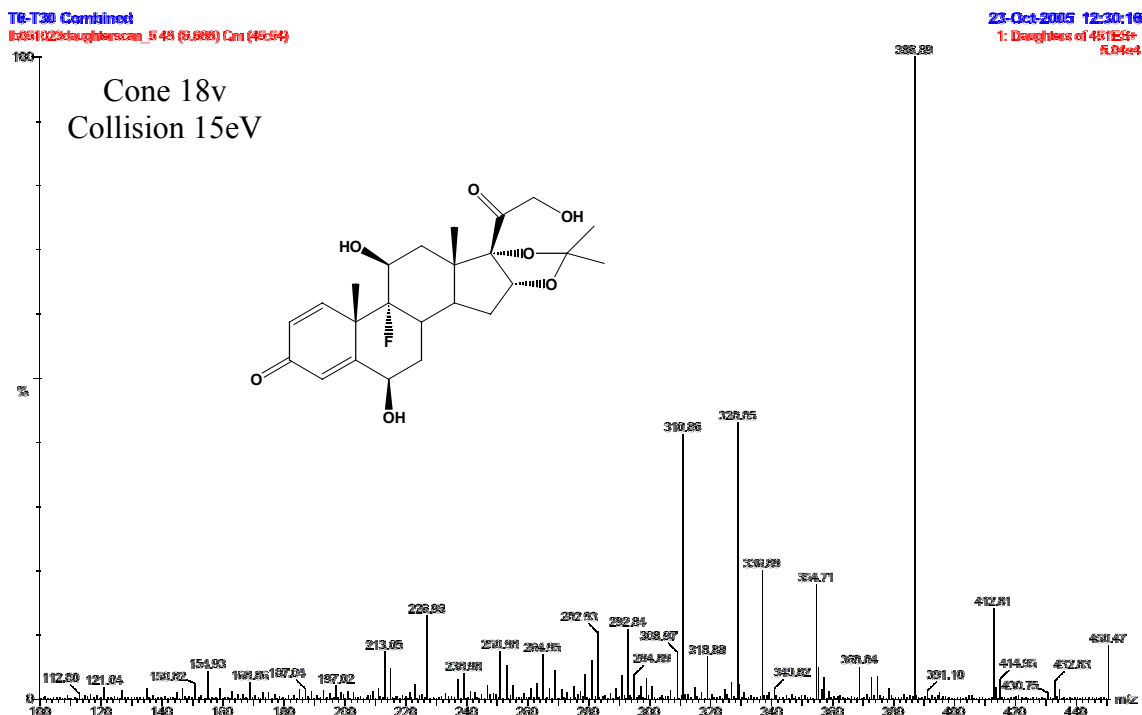
**Figure 3: Corticosteroid Surrogate, Internal Standard, Analytes and Proposed Metabolites**

For metabolite investigation, full scan LC-MS/MS analysis was performed on TA excretion study extracts. Extracted ion chromatograms corresponding to the proposed TA metabolite ( $[M+H]^+ = 451$ ), from before and after administration are shown in Figure 4.



**Figure 4: EIC of  $m/z = 451$  from TA excretion study extracts ( $T_0$  and  $T_{15}$ )**

The cone voltage was then optimised for the chromatographic peak located at the retention time of 6.69 minutes. Daughter scan MS/MS analysis was undertaken to obtain a mass spectrum of the proposed TA metabolite (Figure 5, Cone 18V, Collision 15eV). Using this data, a multiple reaction monitoring (MRM) window was incorporated into the ASDTL LC-MS/MS screening method for corticosteroids. In order to confirm the identity of this metabolite, work has begun on the synthesis and certification of an appropriate reference material.<sup>7,8</sup> 6β-hydroxy triamcinolone acetonide has been synthesized, isolated and partially characterized - its MS/MS spectrum is consistent with the proposed TA metabolite (Figure 5). Using the above LC-MS/MS methodology, MRM transitions have also been developed for two possible triamcinolone metabolites (Figure 3). As also observed with the TA metabolite, excretion of these metabolites follows a similar concentration-time profile to the parent compound (Figure 6).



### Triamcinolone and Metabolites

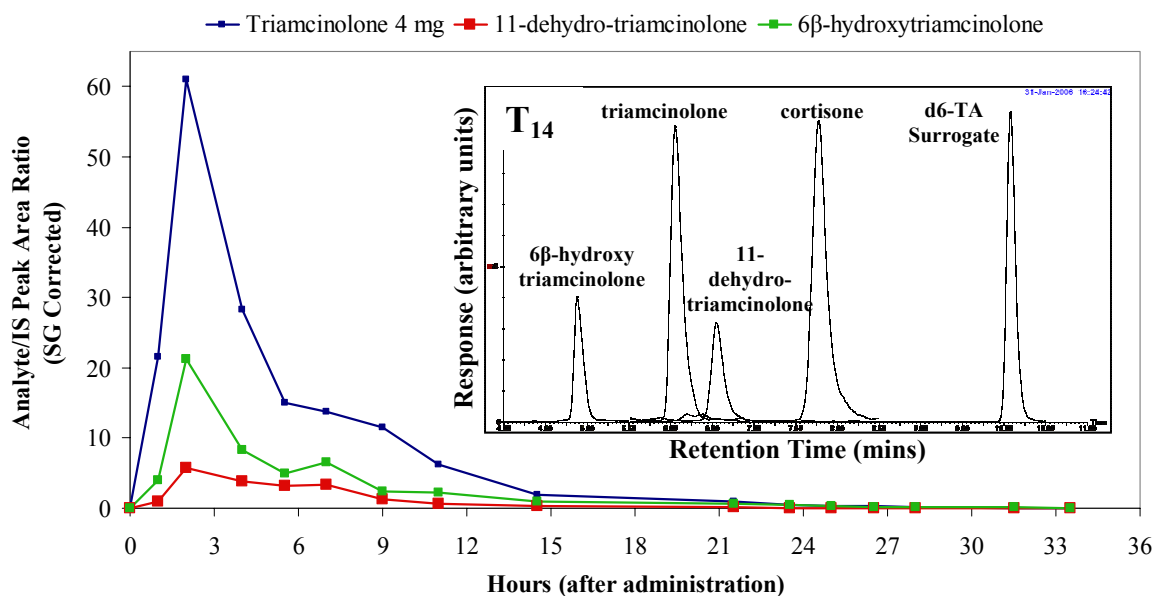


Figure 6: Triamcinolone and Metabolites Excretion Profiles and HPLC Chromatogram (T<sub>14</sub>)

### References

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