A comparison of Orbitrap™ and QTRAP® technologies to study the *in vitro* metabolism of stanozolol in the equine

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Overview

The use of Thermo Scientific LTQ Orbitrap™ and Applied Biosystems Sciex QTRAP® 5500 instruments were compared to study the *in vitro* metabolism of stanozolol in the equine. Several major stanozolol metabolites were identified on both instruments, but the use of an elongated HPLC gradient and product ion scanning on the QTRAP® 5500 (not subject to the MS/MS low-mass cut-off of the LTQ Orbitrap™) allowed several additional isomers to be detected and their stereochemistry to be postulated.

Introduction

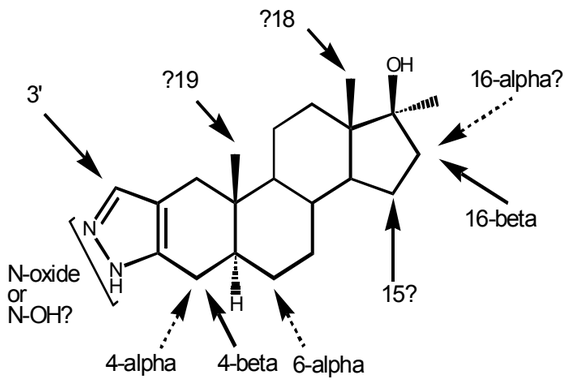
During the last decade, *in vitro* techniques to study drug metabolism have become routine in the pharmaceutical industry [1]. In addition, *in vitro* metabolism studies are also expected to play an increasingly important role in supporting routine sports surveillance [2,3].

In vitro incubations are able to produce metabolites that can be used to confirm the presence of a metabolite where no reference standard or *in vivo* post administration sample is available. In addition, *in vitro* studies can be used to compliment *in vivo* metabolite identification studies as experiments can be performed over much shorter time scales than animal administrations and they produce a cleaner, more concentrated extract for analysis.

Triple-quadrupole mass spectrometry has been established as a reliable analysis tool in the drug surveillance industry; however the use of a high resolution, accurate mass instrument was assessed to determine the potential benefits of such a system.

The metabolism of the synthetic anabolic steroid; stanozolol (see Figure 1) was investigated in this study as an illustration of the future applicability of this technique.

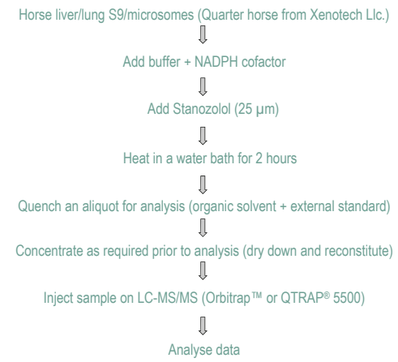
Figure 1. Structure of stanozolol, with arrows indicating potential sites of hydroxylation that could either be confirmed by comparing to reference standards or suggested (shown by a ?) based on a pattern of fragmentation



Methods

In vitro assays were performed with the anabolic steroid stanozolol in order to produce phase I metabolites (see Figure 2).

Figure 2. Schematic of sample preparation method for the incubation of stanozolol, with liver/lung S9 and microsomes



Concentrated incubates were analysed by LC-MS/MS using a high resolution, accurate mass Thermo Scientific LTQ Orbitrap™ and a triple-quadruple linear ion trap Applied Biosystems Sciex QTRAP® 5500. Chromatography was carried out on a 2.1 x 100 mm, 3 u, Atlantis T3 column using a gradient of increasing acetonitrile in acidified aqueous solution.

Urine sample extracts from an *in vivo* equine stanozolol administration study were also prepared by solid phase extraction and analysed on the Orbitrap™.

Results

Data were analysed to compare drug metabolism by the equine liver and lung S9 and liver microsome fractions with results obtained from the literature and *in vivo* post-administration samples.

A large number of hydroxy-stanozolol metabolites were identified (*m/z* 345.2537) in both liver microsomes and liver S9 incubates following analysis on the Orbitrap™ (see Figure 3) .

The liver microsomes produced a more concentrated extract of hydroxy-stanozolol metabolites relative to the liver S9. This can be explained by the fact that CYP enzymes likely to be responsible for the metabolism of stanozolol are known to be located in the microsomal fraction. As observed in many species, metabolic activity was low in the lung S9 relative to the liver (see Figure 3).

Initial results for the anabolic steroid stanozolol indicate that all metabolites observed in *in vivo* samples were also produced by the *in vitro* incubations (see Figure 4). In addition, two keto- and one hydroxy-keto-stanozolol metabolites were identified in the microsome incubation fraction, by both full scan analysis on the Orbitrap™ and product ion scans on the QTRAP® 5500, which have not previously been published in the equine. Retrospective analysis of the Orbitrap™ full scan data was also possible for further metabolite detection.

Analysis of equine liver microsomal fractions on a QTRAP® 5500 using an elongated gradient relative to the Orbitrap™ identified several additional hydroxy metabolites not detected on the Orbitrap™ (see Figure 4).

Figure 3. Comparison of hydroxy-stanozolol (*m/z* 345.2537) chromatograms on the Orbitrap™ following 25 µM stanozolol incubations with different equine tissues *in vitro*; A) liver microsomes, B) liver S9 and C) lung S9.

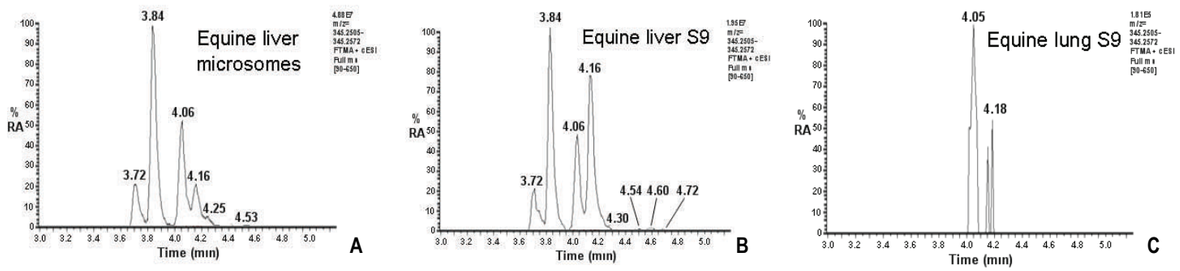


Figure 4. comparison of hydroxy-stanozolol (*m/z* 345.2537) chromatograms following 25 µM stanozolol incubations for A) an equine *in vivo* post-administration sample on the Orbitrap™ (full scan MS), B) using equine liver microsomes on the Orbitrap™ (full scan MS) and C) using equine liver microsomes on the QTRAP® 5500 (product ion scan of 345.25 using an shallower LC gradient relative to the Orbitrap™ method).

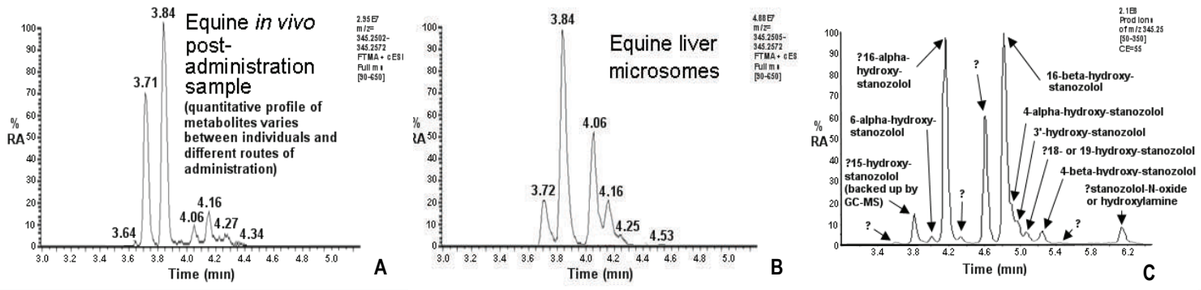
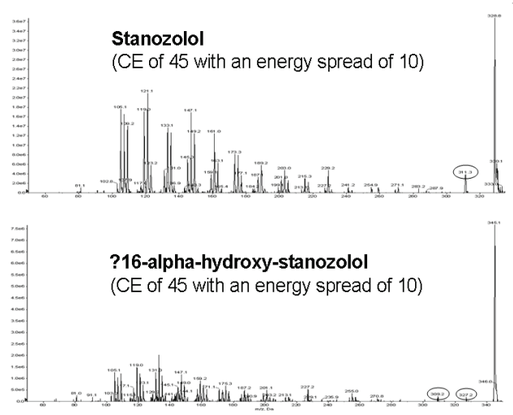


Figure 5. Comparison of the product ion spectra of stanozolol and a putitative 16-alpha-hydroxy-stanozolol obtained on the QTRAP® 5500 following incubation with equine liver microsomes. The loses of one or two H_2O water molecules from stanozolol and ?16-hydroxy-stanozolol respectively distinguish the two molecules. An MRM experiment targeted against the *m/z* 271 fragment of hydroxy-stanozolol led to postulation that the hydroxylation for this metabolite is in the 16 position (in common with 16-beta-hydroxy-stanozolol, but with a different retention time).



Conclusions

Full scan, high resolution accurate mass technology on the Orbitrap™ produced high throughput, automatable results. However, it was not possible to assign positional- or stereo-isomer information to all the hydroxy metabolites detected due to the low-mass cut-off encountered when performing MS/MS on the ion trap, although a higher specification Orbitrap™ with higher collision decomposition (HCD) may be able to achieve this fragmentation. Enhanced resolution product ion scans and selected reaction monitoring analysis on the QTRAP® 5500 allowed assignment of the hydroxy functions on the A and D ring or elsewhere on the molecule from the additional fragmentation patterns observed (see Figure 5).

The targeted nature of the analysis carried out on the Qtrap® 5500 using an extended LC gradient allowed the detection of several very minor hydroxy-stanozolol isomers that were not detected using a full scan generic screening method on the Orbitrap™. However, the Orbitrap™ provided rapid, broad coverage, information rich data with the added certainty of accurate mass for the elemental composition of metabolites in the equine and the use of the full scan mode on the Orbitrap™ is considered suitable for the needs of a sports drug surveillance programme.

Acknowledgements

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References

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