Short communication

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Stimulating luteinizing hormone

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Low molecular weight luteinizing hormone (LMWLH) receptor agonists could be of interest as a potential doping substance for athletes. These orally active compounds induce the production of endogenous hormones such as testosterone in a similar way to LH. A method for the detection of these compounds needs to be direct as their effect – the excess production of endogenous hormones – cannot be proven by analysis techniques which test for endogenous hormones. In order to detect a broad range of potential LMWLH receptor agonists, a precursor ion monitoring liquid chromatography tandem mass spectrometry method was developed. The method was tested against a selection of urine samples to ascertain potential problems with background analytes interfering with the compounds of interests. Selected compounds were extracted with an established methodology from urine to determine suitability of implementing into general screening methodologies. The two available LMWLH receptor agonists could be detected at concentrations of 100 ng/ml in urine samples. This establishes a basic precursor ion monitoring method suitable for screening purposes for the detection of LMWLH receptor agonists in urine samples. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: low molecular weight luteinizing hormone receptor agonists; LMWLH

Introduction

Luteinizing hormone (LH) is a peptide hormone which could be abused by athletes to either induce the production of endogenous hormones such as testosterone or used to hide the use of anabolic agent use which would normally suppress the production of LH.[1] There are two pharmaceutical products available which contain LH. One is sold under different trade names, such as Pergonal, and is prepared through purification of a urine extract from post-menopausal women while the other is a recombinant product, Luveris, sold by Merck Serono.[2] LH products can be used by both men and women for the treatment of infertility but are primarily used by women.[2] Anti-doping laboratories are becoming capable of testing for and uniquely identifying a range peptide hormones, which have been difficult to detect in the past and thus were doping agents of choice for athletes.[3–5] This then leads to an increased interest in other substances which may not currently be tested for by anti-doping laboratories.

There have been several publications recently of highly effective low molecular weight luteinizing hormone (LMWLH) receptor agonists.[6–8] LMWLH receptor agonists induce the production of endogenous hormones such as testosterone in a similar way to LH. These compounds are banned in the World Anti-Doping Agency (WADA) Prohibited List (S2. Hormones and related substances) as they have biological effect similar to the use of LH.[9]

Two different compound groups of LMWLH receptor agonists have been described in the literature and are generally referred to as Pyrazoles and Org series (Table 1). Ten pyrazole compounds with varying activity have been discussed with the most active form denoted as Pyrazole 10.[6] Pyrazole 10 (1H-Pyrazole-5-pentanamide, N-[(1S)-2-amino-1-[(4-hydroxyphenyl)methyl]-2-oxoethyl]-1-[4-(1,1-dimethylethyl)phenyl]-3-(3-pyridinyl)) was administered to male rats that received diethylstilboestrol subcutaneously to suppress testosterone levels. The highest dose given to the rats resulted in a five-fold increase of serum testosterone concentration to a mean value of 2.0 ng/ml from a level of about 0.4 ng/ml.[6] The required dose for this increase in testosterone concentration was 32 mg/kg. This is a large dose to administer for a single substance but one of the attractions of these LMWLH receptor agonists is that they are orally active.

The Org series of LMWLH receptor agonists (Table 1) has had six different compounds described.[7] The most active of these, Org 43553 (3H-5-amino-2-methylsulfanyl-4-[3-(2-morpholin-4-ylacetylamino)-phenyl]-thieno[2,3-*d*]pyrimidine-6-carboxylic acid tert-butylamide), has been the focus of the majority of the continued development studies conducted.[7,8,10] In a recent study, Org 43553 was given to male rats (n = 3) and an increase in testosterone levels was observed similar to the use of human chorionic gonadotrophin (hCG).[8] The testosterone concentration increased from about 5.8 ng/ml in blood to 32 ng/ml when administered with Org 43553. Three different levels of oral doses of Org 43553 were administered to the rats: 10, 50, and 250 mg/kg. It was the two highest doses – 50 and 250 mg/kg – that showed a five-fold increase in the concentration of testosterone while the lower dose (10 mg/kg) doubled the concentration levels. In the same study, hCG (1000 IU/kg) was administered subcutaneously for comparison purposes to rats and gave a similar testosterone concentration elevation to the Org 43553 administration of 50 and 250 mg/kg oral doses. An important difference for Org 43553 is that it is orally active while hCG needs to be injected. This oral activity has been clinically tested on a human and has been shown to be effective for ovulation induction after a single dose.[8]

A method of detection for these compounds for the purpose of anti-doping needs to be direct, as their effect – the excess production of endogenous hormones – cannot be proven by analysis techniques which test for the endogenous hormones directly. Consideration also needs be given to the fact that LMWLH receptor agonists are in the development stages and as such it is currently unknown which compound will be released for human use. A general analysis technique which can detect the entire group of

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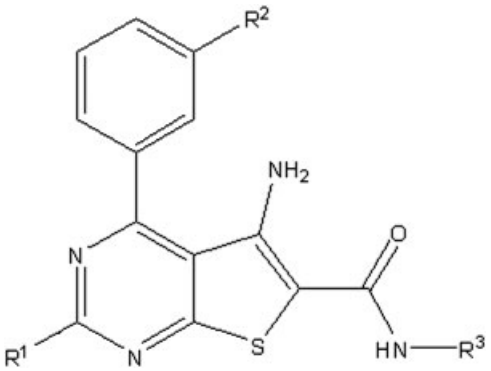
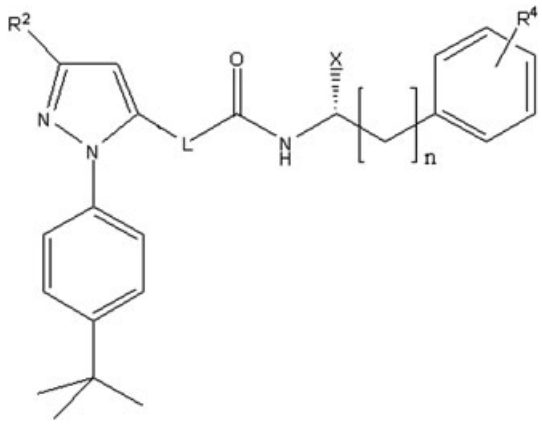
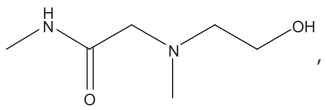
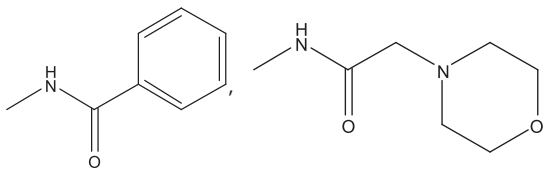
Table 1. Chemical structures of LMWLH receptor agonists for the Org series and Pyrazoles.[6,7]

Org series	Pyrazoles
	
R[1]: SMe or Ph	**R[2]**: 2-, 3- or 4-Pyridine
R[2]: OMe,  ,	**R[4]**: 3-OH, 4-OH or 4-O'Bu
	L: $(CH_2)_2$, $(CH_2)_4$, $(CH_2)_5$, $HN(CH_2)_3$ or 1,3-Ph
	**X**: $CONH_2$, CH_2CONH_2 or H
R3: *t*Bu or *i*Pr	**n**: chain length (1 or 0)

compounds would be the most advantageous to develop and implement for WADA laboratories.

Experimental

Chemicals

1, 3, 5-pyrazoles series had the most active form produced, Pyrazole 10, where R_2 is 3-Pyridine, L is $(CH_2)_4$, X is $CONH_2$, R^4 is 4-OH and n = 1 (Table 1) and the Org series had 43553 synthesised, 5-amino-2-methylsulfanyl-4-[3-(2-morpholin-4-yl-acetylamino)-phenyl]-thieno[2,3-d]pyrimidine-6-carboxylic acid tert-butylamide (BDG Synthesis, Wellington, New Zealand).

All reagents used were of analytical grade and all solvents were of high performance liquid chromatography (HPLC) grade. Water was obtained using a Millipore purification system (Bedford, MA, USA).

Sample preparation

The extraction protocol with NEXUS (Varian Inc., Sydney, Australia) cartridge as published by Goebel *et al.* was followed for the extraction of urine samples as part of the validation.[11]

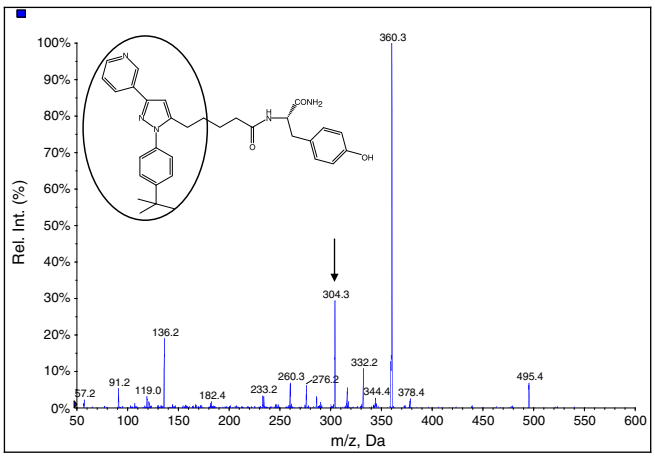


Figure 1. Product ion mass spectrum for precursor ion *m/z* 540 of Pyrazole 10 (CE: 55 V).

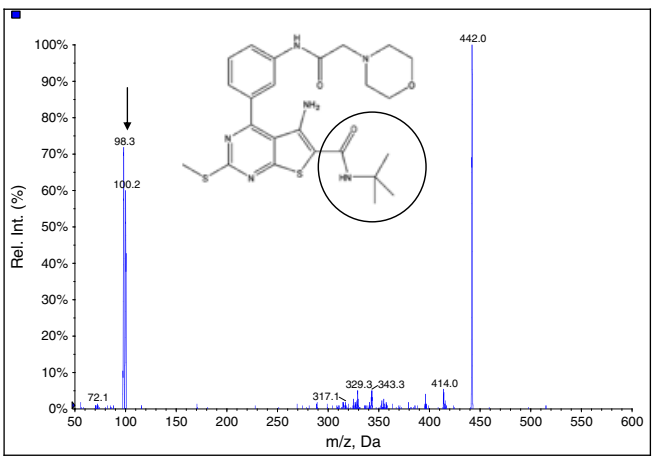


Figure 2. Product ion mass spectrum for precursor ion *m/z* 515 of Org 43553 (CE: 45 V).

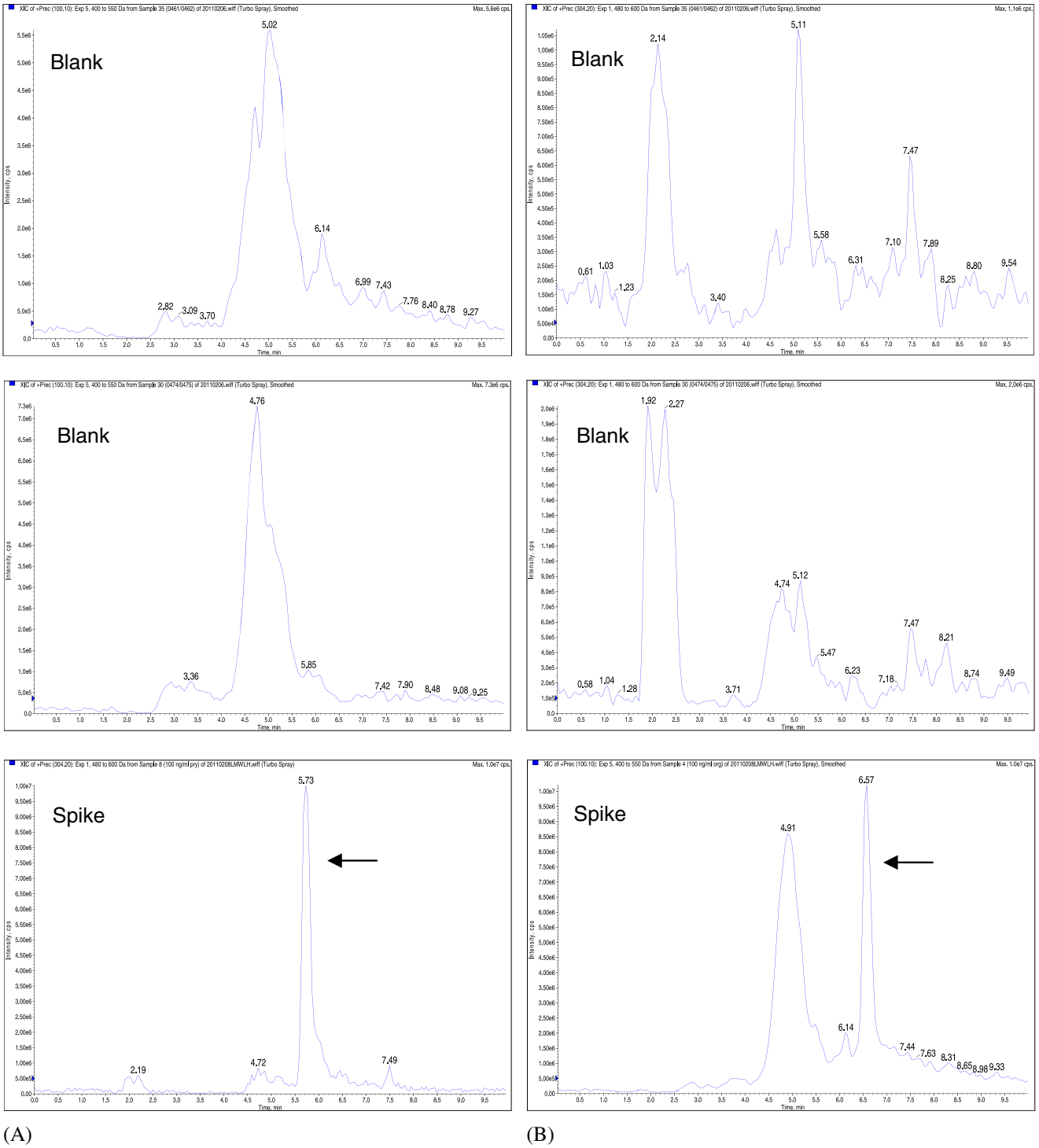


Figure 3. (A) Extracted ion chromatogram from the precursor ion scan of *m/z* 400 to 550 for the Org series. (B) Extracted ion chromatogram from the precursor ion scan of *m/z* 480 to 600 for the Pyrazole series.

Analytical methods

Standard were analyzed by direct infusion on a QTRAP 5500 (AB SCIEX, Foster City, CA, USA) and extracted samples were analyzed with a gradient separation (UPLC Acquity, Waters Corporation, Milford, MA, USA) using an Acquity UPLC BEH C18 (1.7 μm) column (1 mm ID × 100 mm) connected to a QTRAP 5500. The gradient separation used 0.2% aqueous formic acid (A) and 0.2% formic acid in 90% acetonitrole 10% water (B). The gradient separation at a flow rate of 50 μl/min started at 90% A for 0.2 min, reduced linearly to 50% A by 1.5 min, reduced linearly to 30% A by 7.0 min, reduced linearly to 10% A by 8.0 min and held at 10% A till 8.2 min before returning to 90% A by 9 min for equilibration for next sample injection.

A sample volume of 10 µl was injected and the column temperature was 35 °C.

Results and discussion

In an effort to develop a generalized screening methodology for the LMWLH receptor agonist, it is proposed to set up a precursor ion monitoring method.[12] A general product ion for each group was selected which would in theory be detected from the analysis of all the Pyrazoles and Org series. This would then result in a method that could detect not just the compounds for which standards are available, but also other similar compounds from the same group.

The synthesized standards for Pyrazole 10 and Org 43553 were direct infused into the QTRAP 5500 to optimize ionization conditions for the precursor ion of each compound. The declustering potential (DP) for Pyrazole 10 was optimized at 40 V with an ion observed at *m/z* 540 while for Org 43553 it was optimized at 25 V with an ion observed at *m/z* 515. Each compound was optimized for the product ion collision energy setting. The spectrum of the product ions for Pyrazole 10 is shown in Figure 1. In order to select an ion which would detect other Pyrazoles, the product ion needs to be selected considering the structure of the Pyrazoles as a group. The ion at *m/z* 304 is for the section of the structure as highlighted in Figure 1. The use of this ion would allow the detection of nine of the ten published Pyrazole structures (Table 1) and cover all of the more active forms.[6]

The product ion spectrum of Org 43553 for collision energy of 45 V is shown in Figure 2. The ion of *m/z* 100 represents a product ion for the part of the molecule as highlighted in Figure 2. This area of the Org series compounds always has the same substitution for the most active forms and would cover five of the six published structures.[7] Moore *et al*. described slightly different substitution to the Heitman *et al*. publication but still, of the 23 published structures, the ten most active would in theory be detected with the precursor ion monitoring using ion of *m/z* 100.[7,13]

The published compounds for the Pyrazole series have a molecular weight range of 496 to 596 Da. The precursor ion chromatogram of *m/z* 304 can then be extracted to cover the ions which would most likely relate to the Pyrazoles (*m/z* 480 to 600). In a similar manner the Org series, while monitoring the precursor ion of *m/z* 100, can extract ions of *m/z* 400 to 550 to identify any of the most active published compounds in molecular weight range 402 to 514 Da. A selection of 20 urine samples from low-risk sports was analyzed with the precursor monitoring method to ascertain the background reading. The chromatograms for two of these samples are represented in Figure 3 for each group of compounds. Precursor ion monitoring results, when selecting a range of *m/z* to review, have background peaks which can interfere with the identification of a suspect sample during the screening process. The selection of 20 urine samples from low-risk sports were spiked at 100 ng/ml and extracted before analysis as described in the experimental. A representative chromatogram from one of the extracted spiked urine samples is shown in Figure 3. The peak for Pyrazole 10 and Org 43553 can be distinguished in comparison to peaks present naturally in the background of urine samples.

Currently, excretion studies conducted on human subjects are not available for these compounds. The identification of possible metabolism products needs to be conducted through the use of an *in vitro* assay to ensure the best coverage for the analysis technique.[14] The use of precursor ion monitoring partially negates the need to monitor for each separate metabolite as they should have the same product ion depending on their metabolism. Confirmation of the identification of a compound detected from screening analysis would require more extensive analytical analysis including high resolution mass spectrometry.

Conclusion

The doses for the orally active LMWLH receptor agonists range from 10 to 250 mg/kg for the studies which have been conducted in animals.[6,8] The screening method as described can detect down to at least 100 ng/ml of the parent compound in urine samples which was shown using the synthesized LMWLH receptor agonists Pyrazole 10 and Org 43553. The analytical method as it has been described here is capable of detecting the most active of the Pyrazoles and Org series by precursor ion monitoring.

Acknowledgement

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