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## Clomiphene - How about including the parent compound in screening procedures?

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### Abstract

Clomiphene has been in use as the first line infertility drug to induce ovulation for almost four decades. It is also used off-label in the treatment of hypogonadism in men and to restore testosterone production that has been compromised by abuse of anabolic-androgenic steroids (AAS). In addition, it is used by male athletes to overcome other side effects of extensive AAS use such as gynecomastia. Therefore, the World Anti-Doping Agency prohibited the use of clomiphene in sport at all times.

Clomiphene is extensively metabolized, and based on available literature, metabolites of clomiphene are the preferable targets for the detection of its misuse. However, our recent data shows that in some cases, the parent compound may be the most abundant form of clomiphene found in urine. The reason for such a rather unexpected excretion profile is unknown. Nevertheless, our results clearly show that including the parent compound in routine screening procedures for doping agents in urine should considerably improve detection capabilities for clomiphene abuse.

### Introduction

Clomiphene is marketed as a mixture of isomers, namely zuclomiphene and enclomiphene (Figure 1), and its use in sport is prohibited by World Anti-Doping Agency (WADA) at all times [1]. Both isomers are extensively metabolized and undergo phase I metabolic modifications such as, e.g., hydroxylation and methoxylation at different positions [2-6]. However, formation of phase I metabolites seem to follow different kinetics for the isomers as plasma levels of unchanged zuclomiphene are much higher than those observed for enclomiphene [7]. The stereospecificity of metabolic enzymes seems also to underlie different fates of the formed phase I metabolites; those of zuclomiphene may preferably undergo sulfation whereas metabolites of enclomiphene seem to be rather glucuronated or excreted in the free form [4,6]. The data presented here show that even though clomiphene undergoes extensive metabolism, in some cases, the parent compound may be the most abundant form of clomiphene found in urine samples. Hence, including the parent compound in routine screening procedures for doping agents in urine should considerably improve detection capabilities for clomiphene abuse.

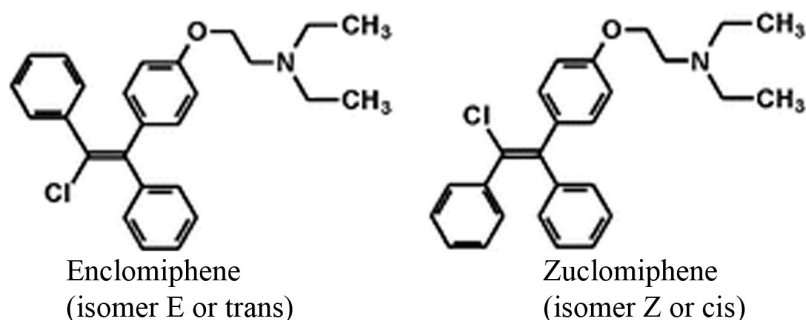


Figure 1. Clomiphene is a nonsteroidal, selective modulator of estrogen receptor marketed as a mixture of two isomers.

## Experimental

### *Sample preparation*

6 mL of urine was spiked with 10  $\mu$ L of the internal standard solution (methyltestosterone at the concentration of 100  $\mu$ g/mL), and subsequently 2 mL of 0.8M phosphate buffer and 100  $\mu$ L of  $\beta$ -glucuronidase (*E. coli*) were added. Samples were incubated for 1h at 50°C, cooled down, and then 1 mL of  $K_2CO_3/KHCO_3$  and 6 mL of MTBE were added. Following extraction, the organic phase was collected, evaporated to dryness, and samples were reconstituted in 80  $\mu$ L of MeOH/H<sub>2</sub>O mixture (v/v, 1/1).

### *Instrumental analysis*

Chromatographic separation was carried out on a Waters Acquity UPLC system with an Acquity UPLC column (BEH C18 100 mm  $\times$  2.1 mm, 1.7  $\mu$ m). The mobile phase consisted of 0.1% formic acid in methanol (A) and 0.1% formic acid in water (B) and a step-wise LC gradient was employed at a constant flow rate of 300  $\mu$ L/min at 40°C. The initial concentration of A was 40% which subsequently increased linearly to 80% in 8 min. Next, it was further increased linearly to 100% in 0.5 min, and then held for additional 0.5 min. The column was re-equilibrated for 3 min with 40% of solution A. Samples were stored at 4°C in the autosampler prior to analysis and the injection volume was fixed at 10  $\mu$ L.

Clomiphene and its metabolites were traced with a Micromass Quattro Premier XE mass spectrometer (Waters, USA) equipped in an ESI source. The desolvation gas flow was set at 800 L/h at the temperature of 300°C and the source temperature was 120°C. The cone and collision gas flows were set at 50 L/h and 0.35 mL/min, respectively. The capillary voltage applied was 2.8 kV. The cone voltage and collision energies were the same for every MRM, and were set at 45 V and 25 V, respectively.

## Results and Discussion

In the course of routine testing for doping agents in urine, a sample (Figure 2, sample 1) was found suspected for 4-hydroxyclophene what suggested a possible clomiphene abuse by the athlete. Both the metabolite and unchanged clomiphene were targeted in the confirmatory analysis, and their presence in the sample was confirmed. Interestingly, the peak area of the parent compound was more than 10 times larger than that of 4-hydroxyclophene what was in a striking contrast with the published studies on clomiphene metabolism [2-6], and the data obtained for a control, administration sample provided by WADA (Figure 2). Further analysis of the sample revealed that peak areas obtained for other clomiphene metabolites were smaller than the peak area of the parent compound even though some of them were reported previously to be even more abundant than 4-hydroxyclophene [5]. Based on this data, the parent compound was included in the screening procedure leading to detection of clomiphene in 3 additional urine samples (Figure 2).

The reason for such a rather unexpected excretion profile is unknown; however, a sudden increase in AAF cases may suggest that a new, clomiphene-contaminated supplement has been introduced to the market. The predominance of in-competition samples among those deemed positive indicates that this supplement would rather be taken just before a competition than during an out-of-competition period. This in turn, may explain the observed excretion profile as samples would be collected shortly after clomiphene intake when the blood level of unchanged drug is high when compared with the levels of its metabolites [7]. At this point, it is likely that a fraction of the administered dose would be eliminated in urine unchanged and/or in glucuronide-conjugated form, both of which are targeted in the analysis. In fact, the detection of unchanged clomiphene in urine has already been reported [6].

## Conclusions

In conclusion, our data clearly shows that targeting the parent compound in routine screening procedures for doping agents in urine should considerably improve detection capabilities for clomiphene abuse.

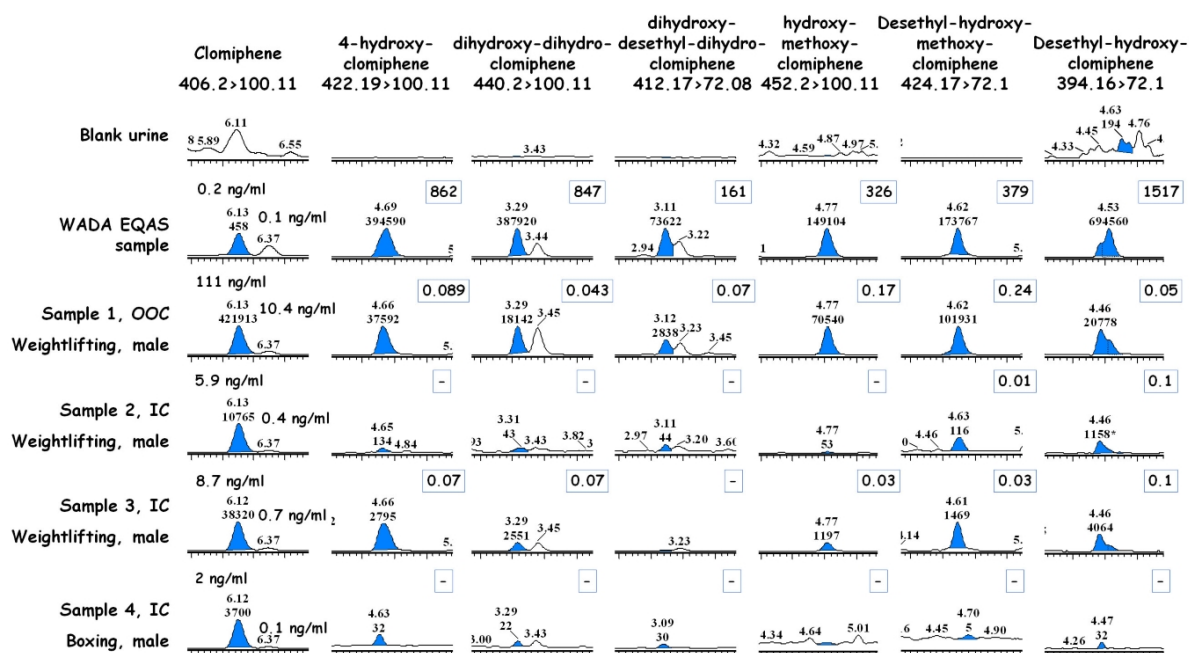


Figure 2. Chromatograms of clomiphene and its metabolites obtained for urine samples reported as AAFs. MRMs used for detection are given under the names of corresponding substances. A ratio of the peak area of a respective metabolite to the peak area of the parent compound in a given sample is provided in the upper right-hand corner of each chromatogram. The estimated clomiphene concentration is given next to the clomiphene peak. Interestingly, even though clomiphene is marketed as a mixture of isomers containing 40%-70% of enclomiphene, the parent compound is found in urine with one peak strongly predominating the other. This phenomenon may reflect different metabolic fates of both isomers as, e.g., zuclomiphene is excreted as a sulfate conjugate which is not hydrolyzed and therefore, not targeted in the analysis.

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