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Excretion study of Clomiphene and its correlation with unusual findings in the routine doping control samples

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Abstract

Clomiphene (2-4-(2-chloro-1,2-diphenylethenyl)phenoxy)-*N*,*N*-diethyl-ethanamine) is used for the treatment of infertility and is listed as a doping substance on the World Anti-Doping Agency (WADA) prohibited list due to its selective estrogen receptor modulator capabilities [1]. Clomiphene is extensively metabolized by both phase I and phase II pathways of N-deethylation, hydroxylation, methoxylation and N-oxidation, sulfation and glucuronidation. Based on the metabolism of clomiphene, its metabolites are potentially targeted for doping control analysis. However, in the year 2013 and 2014, the National Dope Testing Laboratory (NDTL), India has encountered nine cases in routine doping control wherein ther presence of clomiphene parent (<10 ng/mL) and absence of its major metabolites 4-OH clomiphene and 3-methoxy 4-hydroxy clomiphene was observed. This finding was unusual when compared to reported excretion study literature on clomiphene [2-4]. Hence, the aim of this study was to perform an excretion study with clomiphene in human volunteers and to correlate the results with the unusual finding in the routine doping control samples.
The excretion study samples of four human volunteers upon administration of a single oral dose of clomiphene citrate (50 mg) revealed that lomiphene parent and its metabolites (4-OH clomiphene and 3-methoxy 4-hydroxy clomiphene) could be detected up to 100 hours of collection of samples. The presence of clomiphene parent and one of its metabolite 3-methoxy-4-OH clomiphene was detectable after 10 days of drug administration, whereas its metabolite 4-OH clomiphene couldn't be detected. The peak concentration of clomiphene parent (< 10 ng/mL) was found between 24-36 hours. Therefore, it is essential to target clomiphene parent along with its major metabolites in screening procedure to report its abuse in sports.

Introduction

Clomiphene is available in India as a mixture of two stereiomers en clomiphene and zu clomiphene which are metabolized by both phase I and II pathways. The ratio of the two isomers is 70:30, which may vary depending upon the brand [2,5]. En clomiphene is absorbed and eliminated faster than its stereoisomer and its metabolites 4-hydroxy-en-clomiphene and 3-methoxy-4-hydroxy-en-clomiphene are major markers for the screening of clomiphene in routine doping control samples [6]. Recently NDTL India has encountered nine routine samples with the presence of clomiphene parent (<10 ng/mL) and absence of 4-OH clomiphene and 3-methoxy-4-hydroxy clomiphene. The aim of this study was to correlate the results with unusual findings in routine samples with excretion study samples in human volunteers.

Experimental

Reagents and Chemicals

The certified reference material of clomiphene was obtained from Sigma Aldrich, USA. All reagents and chemicals used were of analytical grade.

Excretion study samples

Single oral dose of clomiphene citrate (clomid 50 mg, Cadilla Pharmaceuticals Ltd, India) was administered to four healthy human volunteers (age 25±3 years). The study was duly approved by the Ethics Committee of NDTL, India. The urine samples were collected at different time intervals (3, 6, 12, 16, 24, 30, 36, 42, 48, 60, 72, 84 and 100 hours). One spot urine

 ISBN 978-3-86884-040-7

sample of each volunteer was collected after 10 days to examine the long term excretion profile. The samples were stored at -20°C immediately after collection. Clomiphene parent was quantitated in the samples whereas peak area of two metabolites was monitored due to non-availability of reference standard.

Doping control samples

Nine old routine doping control samples with unusual findings were reanalyzed for the presence of clomiphene and its major metabolites 4-OH clomiphene and 3-methoxy 4-hydroxy clomiphene.

Sample preparation

The samples were hydrolyzed in different batches using by β-glucuronidase from *E.coli* and *Helix Pomatia* both for deconjugation of phase II glucuronides and sulfates respectively followed by liquid-liquid extraction and analysis on LC-MS/MS system [7].

Instrumentation

Agilent 1100 series high performance liquid chromatograph coupled to API 3200 mass spectrometer (LC-MS/MS) was used for analysis. The details of the method are enlisted in Table 1.

HPLC	**Agilent 1100 series HPLC**
Column	C-18 (Inertsil-ODS-3), 4.6mmX 50mm, 5µ
Flow	0.7 ml/min
Run Time	11 min
Injection volume	10 µl
Solvent-A	1% formic acid
Solvent-B	Acetonitrile
Mass spectrometer	**AB Sciex, API 3200 triple quad mass spectrometer**
Ionization	Positive
Collision Gas	Nitrogen
Source temperature	550°C
MRM Transitions	clomiphene parent- 406-297, 100, 72.1, 58.1, 205 4-OH clomiphene- 422-100.1, 72, 85, 298, 229.3 3-methoxy 4 OH clomiphene- 452-100, 72, 86, 315, 343

Table 1: Instrument parameter and MRM transitions

Results and Discussion

Excretion study samples

The excretion study samples of four human volunteers upon administration of single oral dose of clomiphene citrate (50 mg) revealed that clomiphene parent and its metabolites (4-OH clomiphene and 3-methoxy-4-hydroxy clomiphene) could be detected up to 100 hours. The highest concentration of clomiphene parent (< 10 ng /ml) was found between 24-36 hours (Figure 1a). Excretion profile of the clomiphene metabolites (obtained by area abundance; counts of the main ion (*m/z* 100) of clomiphene) shows that the highest concentration of 4-OH clomiphene and 3-methoxy-4-hydroxy clomiphene between 3-12 hours (Figure 1b and 1 c).

ISBN 978-3-86884-040-7

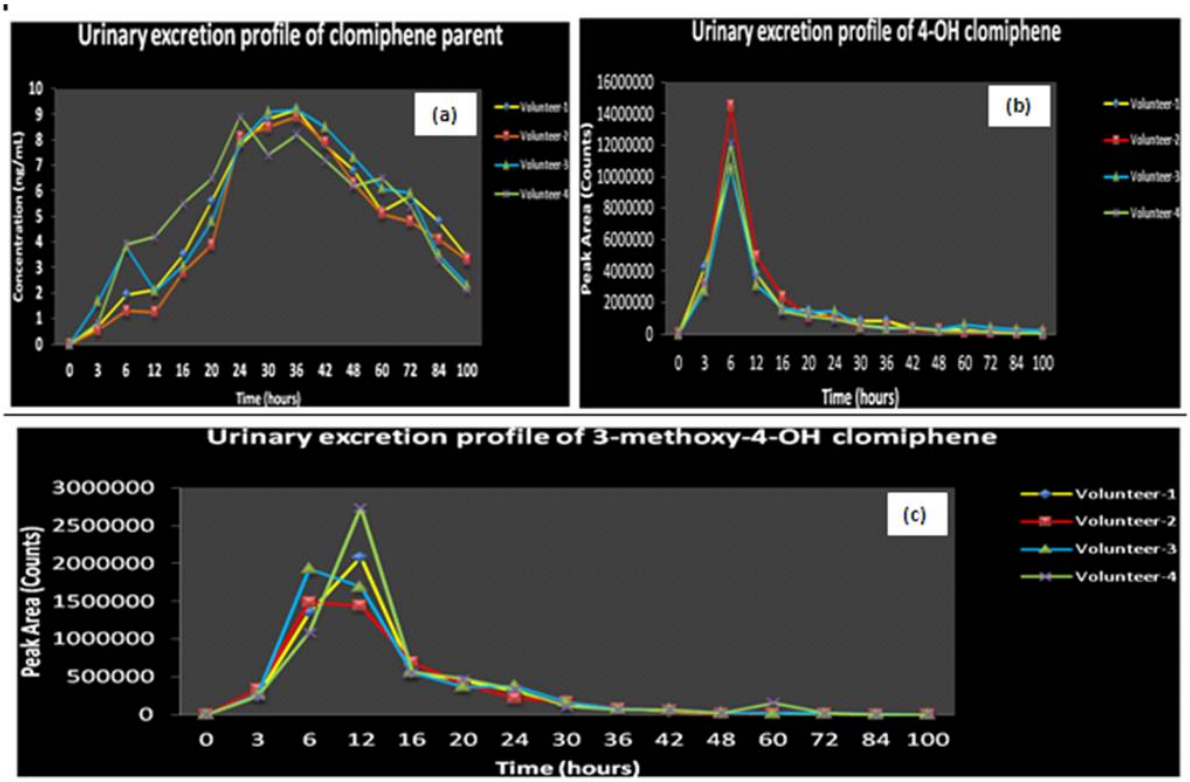

Figure 1: Urinary excretion profile of clomiphene parent (a), 4-OH clomiphene (b) & 3-methoxy-4-OH clomiphene (c) after single oral dose of clomiphene citrate (50 mg).

The spot urine sample of all the four volunteers collected after 10 days of administration of a single oral dose of clomiphene citrate (50 mg) revealed the presence of clomiphene parent (< 5 ng/mL) and 3-methoxy-4-hydroxy clomiphene but did not show the presence of its marker metabolite 4-OH clomiphene. The unusual finding of the presence of clomiphene parent as most abundant form of clomiphene in early urine samples has already been reported by Cholbinski et al [6]. However, in the present study clomiphene parent is detected even after 10 days of drug administration. Extended excretion studies should be performed to get the extended excretion profile of clomiphene parent and its metabolites to conclude its adverse analytical finding (AAF) reporting criteria.

Reanalysis of routine doping control samples

Reanalysis of the old nine urine samples confirmed the initial findings *i.e.* presence of only clomiphene parent at concentrations below10 ng/mL. It was noticed that the majority of these samples (7 out of 9) belonged to in-competition (IC) test menu (Table 2). These samples did not show the presence of any steroid or abnormal steroid profile. The doping control forms of these samples indicated the use of various supplements and multi-vitamins.

The results of the preliminary findings suggest that the use of a contaminated supplement consumed before the competition may be a contributing factor. Further work is in progress with the excretion study of en-clomiphene to find difference in excretory profile, if any.

ISBN 978-3-86884-040-7

Routine Sample	Gender	Test menu	Discipline	Concentration of clomiphene parent (ng/ml)
Sample-1	M	out of competition	Kabaddi	3.5
Sample-2	F	out of competition	Kabaddi	6.5
Sample-3	F	competition	Weightlifting	9.1
Sample-4	M	competition	Kabaddi	4.1
Sample-5	M	competition	Bowling	3.8
Sample-6	M	competition	Athletics	9.9
Sample-7	F	competition	Wrestling	6.1
Sample-8	M	competition	Wrestling	6.3
Sample-9	M	competition	Kabaddi	2.1

Table 2: In-competetion test menu

Conclusions

The presence of clomiphene parent with the absence of its major metabolites in screening procedure sample may not be overlooked /ignored. It is essential to target clomiphene parent along with its major metabolites in screening procedure to report its abuse in sports. However, further work is required to perform an excretion study for a drug preparation of en clomiphene to study differences in excretion profiles.

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Acknowledgements

The financial support of Ministry of Youth Affairs and Sports, Government of India to conduct the study is duly acknowledged.