INTRODUCTION

The Purpose of this study was to perform excretion study of Tamoxifen and to Detect its main active metabolite 3-hydroxy-4-methoxy Tamoxifen by GC-MS. Tamoxifen (C$_{29}$H$_{28}$NO with mol mass-371.515, fig-1) works against the effect of estrogen on cell (an “anti-estrogen”) slows the growth of cancer cell and prevent original breast cancer from returning. It also has beneficial effect of menopausal estrogen replacement therapy such as lowering of blood cholesterol and slowing of osteoporosis.

In recent year, athletic competition has intensified to the point that increasing number of athletes are striving to upgrade their performance with the desire to be recognized as the best. Hence a sports person needs to keep himself vigorous, energetic, full of vitality and endurance. To achieve all this, some sports persons resort to pills and injections as shortcuts to success, as they don’t understand that good genes, scientific coaching, proper nutrition and dedication to the task are what ultimately bring them success. The use of performance enhancing drugs is banned in sports.
Doping is the administration of or use by a competing athlete of any substance foreign to the body of any physiological substance in abnormal quantities or taken by an abnormal route of entry into the body with the sole purpose of increasing an artificial and unfair manner his/her performance in competition. To check such practice, the International Olympic committee (IOC)/WADA has devised a list of substance banned for use by sports persons. Tamoxifen recently included anti-estrogenic substance in the WADA list of prohibited substance. This Drug is working as a selective Estrogen Receptor Modulator that activate/block the estrogen receptor by occupying the estrogen receptor site 

Usage of Tamoxifen is prohibited in males because in male the anti-estrogen substance may cause an increase in the endogenous production of androgen and fights against increase in estrogen level in the body, breast swellings, gynecomastia. The objective of this study is to identify the activity of metabolite 3-hydroxy-4-methoxy Tamoxifen which is secondary metabolite from the primary major metabolites. After administration of single dose tamoxifen (20mg), the urine samples were collected at different hrs from the 1st -5th day. Tamoxifen is rapidly absorbed and attains steady-state serum level within 5 days. The Drug is extensively metabolized to N-demethyl-4-hydroxymetabolites (major metabolite) and 4-hydroxytamoxifen (minor metabolite) by the main metabolite. This primary metabolite further hydrolyzed in the form of secondary metabolites. Only the main major secondary metabolite 3-hydroxy-4-methoxylamoxifen (C,H,N) gives two different neutral loss m/z value at 58 & 72. 58 m/z value selected for the presence of metabolite in urine because it shows more abundance than 72m/z value 

The analytical method for analysis of the drug metabolism is gas Chromatography Mass spectrometry (GCMS), because it is increasingly employed in the metabolic study of the drug with accurate mass measurement in order to predict unknown metabolites of Drug.

**MATERIAL AND METHOD**

Methanol (HPLC grade), potassium carbonate & Anhydrous Sodium Sulphate were procured from Merck. Derivatizing agents Tertiary butyl methyl ether (TBME) & N-Methyl-N-(trimethylsilyl) trifluoroacetamide [MSTFA] were procured from Across Organics and Sigma Aldrich. B-glucuronidase was procured from Roche diagnostics. 17α- methyl testosterone & chlorotestosterone (clostebol) acetate & deuterated testosterone & Epitestosterone were procured from Sigma Aldrich.

**Preparation of Reagents**

1. **Amberlite XAD2**

Mix 500g of XAD-2 in 500-600ml of acetone vigorously. Allow the mixture to stand for 10-15 min., aspirate the acetone & floating XAD-2 resin. Repeat this process two times. Place 500g of XAD-2 in a 1000ml glass bottle. Add 500-600ml of distilled water & mix vigorously. Allow the mixture to stand for 10-15 min. Discard water layer and floating particles. Repeat this process five times with methanol. Keep at ambient temperature.

2. **Amberlite XAD2 columns**

Take clean Pasteur pipette. Put one glass bead. To pack the column, pour XAD-2 slurry slowly with the help of another Pasteur pipette carefully up to the height of approximately 3 inch & there should not be any air bubble inside. Wash the column with distilled water. Similarly prepare requisite number of XAD-2 column prior to analysis.

3. **MSTFA/Iode-TMS/DTE (1000/2/2)**

Place 5ml of MSTFA in an amber colour glass bottle using a 5ml micropipette, add 10mg of dithioerythritol & mix well. Add 10ul of iodo –TMS using a micropipette. Keep at room temperature.

**Preparation of internal standard:-**

Solution 1:- Weigh 1.0mg of 17α-methyl testosterone on the electronic balance; add 1.0ml ethanol to make 1mg/ml as stock solution.

Solution 2:- Weigh 1.3mg of chlorotestosterone acetate on the electronic balance, add 1.0ml ethanol to make 1mg/ml as stock solution of chlorotestosterone (clostebol). Take 100µl of solution 2 & add 900µl of ethanol to make 0.1mg/ml solution.

Solution 3:- Prepare 1mg/ml solution of d3- testosterone & d3- epistosterone separately in ethanol.

Take 200µl stock solution 1 in clean dry glass test tube & add 10µl of working solution -2 (0.1mg/ml solution), add 16 & 4µl from solution 3 respectively [d3-testosterone ; d3-epistosterone(4:1)] evaporate the ethanol. Reconstitute the dry residue with 10ml ethanol to make working Internal STD & keep in the refrigerator below 0°C.

**Sample collection Protocol**

After oral administration of tamoxifen (20mg), urine sample were collected at different interval for 5 days. A pinch of sodium azide was added in urine sample container as a preservative. The entire sample stored at 20°C after measuring pH and gravity.

**Sample Extraction**

1. **Applied into XAD-2 column**

The sample extraction procedure for given drug involves solid phase extraction. 2-4 ml of urine sample based on specific gravity was taken & 50µl of internal standard was added in XAD-2 column. XAD-2 column was first washed with distilled water & then it was eluted 5 times with 0.5ml methanol each. Squeeze the XAD-2 column properly to remove remaining methanol in the screw test tube. The hydrolysis process was carried out at 60°C for 1 hour. The solution was cooled to room temp. & the pH was adjusted to 9-10 adding 250µl of K₂CO₃. The mixture was extracted with 5ml of tertiary- butyl methyl ether; after horizontal shaking for 10 min & centrifugation (5 min, 3000rpm), the organic layer was separated by adding 1gm Na₂SO₄. The separated organic layer was taken in derivatizing tube which was dried up under N₂ evaporator at 60°C.

2. **Derivatization**
The sample was derivatized by adding 50 µl MSTFA/iodo TMS/dithiocrythritol (1000/2/2). Derivatization was carried out at 60°C for 30 minutes. Derivatizing tube was then transferred into 200 µl conical glass vial & then 2 µl was injected into GCMS.

3. Instrumentation

The analyses were performed in a Hewlett Packard GC–MSD system. The GC was carried out using a ultra-1 dimethylpolymer polysiloxane fused silica capillary column from Agilent Technologies (ultra1, 17-m × 0.22-mm id, 0.11-µm), operated in SIM and scan modes. Two micro liters was injected into the GC–MSD in the split mode (1:1). The GC–MSD parameters included a column flow of 0.2mL/min of Helium. The injector temperature was 280°C in the split mode. The oven temperature was programmed from 180°C, 1 min hold to 229°C at a rate of 3°C/min and then from 229°C to 300°C at rate of 40°C/min. The final Run time was 23.1 min. It was operated in the SIM mode using a post run macro for anabolic steroids including ions m/z 58, 72, and 489 at the retention time of Tamoxifen metabolite of tamoxifen. (Figure 4).

RESULTS

After oral administration of single dose (20mg) tamoxifen to a healthy volunteer, urine samples were collected and studied by the GC-MS. The mass spectrum of tamoxifen in product ion sim mode was studied carefully, which was chemically characterized and observed that Tamoxifen firstly gives seven major metabolite (fig-2), these metabolite further hydrolyzed in to the secondary metabolite in urine. In the resulted secondary metabolite it seems that the only 3-hydroxy-4-methoxy tamoxifen (Secondary metabolite) shows the main activity than other metabolite. A metabolite was obtained by the accurate extraction of the m/z 390.2060 (Fig.3). On the basis of accurate mass results, M6 had additional two oxygen atoms and a reduced carbon atom than unchanged tamoxifen, which showed the existence of two hydroxyl groups. Product ion at m/z 58.0655 (C3H7N, masscalc = 58.0651) in targeted MS/MS experiment indicated the presence of N-desmethylated side chain.

Figure 2: The chemical structures of tamoxifen and its new metabolites in human urine².
By the GC-MS Chromatography study it observed that only 58 m/z value shows more abundance than 72 m/z value in the Chromatogram. Therefore 58 m/z value selected as a marker m/z value to check the presence of tamoxifen metabolite in the urine sample(Fig-4b,4c). All urine samples collected at the different time intervals were examined by GC-MSD. A blank was also injected in GC-MS (Fig-4a) to observe the chromatogram and mass spectrum. It was observed that excretion of 3-hydroxy-4-methoxy tamoxifen which is a metabolite of tamoxifen started appearing in urine from the 1 hr. The highest peak concentration of this metabolite was observed at 8.5 hrs after the administration of tamoxifen. No further excretion of metabolite was found after 75 hrs in urine as demonstrated with decrease in abundance from 8.5 hours as shown in figure no. 5 and Table no. 1

Table 1: Abundance of m/z 58 of 3-Hydroxy-4-Methoxy Tamoxifen Metabolite at Different hours:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Hours</th>
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GC-MS Chromatograms showing Abundance of m/z 58 of 3-Hydroxy-4-Methoxy Tamoxifen.

Figure 4a: The extraction ion chromatogram and mass spectrum of blank in targeted GCMS SIM mode.
Figure 4b: The extraction ion chromatogram and mass spectrum of 3-hydroxyl-4- methoxy tamoxifen at 1hr in targeted GCMS SIM mode.

Figure 4c: The extraction ion chromatogram and mass spectrum of 3-hydroxyl-4- methoxy tamoxifen at 8.5hr in targeted GCMS SIM mode.
DISCUSSION

In sports athletes may be encouraged to treat the adverse effect of extensive abuse of anabolic androgenic steroid (suppuration of androgen and gynecomastia) by using anti-estrogen drug. Tamoxifen is prohibited in males because in male the anti-estrogen substance may cause and increase the endogenous production of androgen. Although Tamoxifen is beneficial in Breast Cancer therapy, it is a banned category drug in sports by the World Anti Doping Agency due to its adverse effect.

CONCLUSION

Tamoxifen is an anti-estrogenic substance, which basically binds to estrogen receptor for blocking unwanted effect of estrogen. These drugs are able to reduce gynecomastia and enhance testosterone production. Tamoxifen is quickly metabolized after oral administration into compound that binds to the estrogen receptor but do not activate it. Because of this competitive antagonism, tamoxifen act like a key broken off in the lock that prevents any other key (drug or metabolite) from being inserted, thereby preventing estrogen from binding to its receptor site.

From the present study it is concluded that tamoxifen metabolite started excreting from 1st hr of oral administration and it remains in the body for a maximum period of 5 days which will help in its detection even after the administration of drug is stopped up to 5 days.

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REFERENCES: